DEVELOPMENT OF HOMOLOGOUS TRANSFORMATION SYSTEMS FOR THE FILAMENTOUS FUNGI 'CEPHALOSPORIUM ACREMONIUM' AND 'PENICILLIUM CHRYSOGENUM'

Michael Phillip Whitehead

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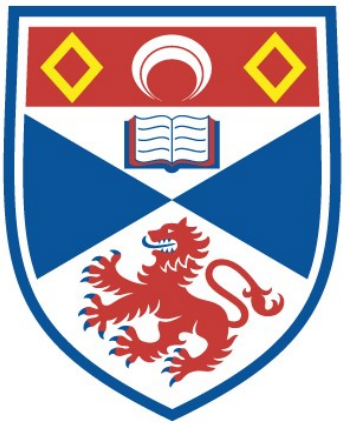

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DEVELOPMENT OF HOMOLOGOUS TRANSFORMATION SYSTEMS FOR THE FILAMENTOUS FUNGI *CEPHALOSPORIUM ACREMONIUM* AND *PENICILLIUM CHRYSOGENUM.*

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DECLARATION

I, Michael Phillip Whitehead, hereby certify that this thesis has been composed by myself, that it is a record of my own work and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed.....Date.22/5/90.

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance No. 12 on 1st October, 1986 and as a candidate for the degree of Ph.D. on 1st November, 1987.

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I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D.

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ABBREVIATIONS.

A Adenosine

Ac Acetate

ATP Adenosine triphosphate

bp Base pairs

°C Degrees Celsius

C Cytosine

CTP Cytosine triphosphate

cm Centimetres

DNA Deoxyribonucleic acid

DNase Deoxyribonuclease

EDTA Ethylenediamine tetra-acetic acid

EGTA Ethyleneglycol-bis-D-aminoethylether N',N', N', N'-tetra-acetic acid

et al *et alia*

EtBr Ethidium bromide

FAD Flavine adenine dinucleotide

g Gram

G Guanine

GTP Guanine triphosphate

hrs Hours

IPTG Isopropyl-β-D-thiogalactopyranoside

kb Kilobase

Kdal Kilodaltons

Kg Kilogram

λ Lambda

l Litre

M Molar

mM Millimolar

mg Milligram

mins Minutes

ml Millilitre

µCi Microcurie

µg Microgram

µl Microlitre

µM Micromolar

MOPS Morpholinopropanesulfonic acid

NADPH Nicotinamide adenine dinucleotide phosphate (reduced form)

ng Nanogram

nl Nanolitre

nM Nanomolar

OD Optical density

PEG Polyethylene glycol

pers. comm. Personal communication

RNA Ribonucleic acid

RNase Ribonuclease

rpm Revolutions per minute

mRNA Messenger RNA

rRNA Ribosomal RNA

SDS Sodium dodecyl sulphate

T Thymine

Tris Tris (hydroxymethyl) aminomethane

TTP Thymine triphosphate

'Tween 80 Polyethylene sorbitan mono-oleate

UV Ultraviolet

v/v Volume per volume

w/v Weight per volume

X-gal 5′-Bromo-4′-chloro-3′-indolyl-B-D-galactopyranoside

AMINO ACID ABBREVIATIONS

A Alanine

C Cysteine

D Aspartic acid

E Glutamic acid

F Phenylalanine

G Glycine

H Histidine

K Lysine

L Leucine

M Methionine

N Asparagine

P Proline

Q Glutamine

R Arginine

S Serine

T Threonine

V Valine

W Tryptophan

Y Tyrosine

* Stop codon

SUMMARY

Spontaneous chlorate resistant mutants of *Penicillium chrysogenum* and *Cephalosporium acremonium* have been isolated. Putative genotypes of the *P. chrysogenum* mutants were identified using the phenotypic characterization of Cove (1979). This analysis was also attempted with *C. acremonium*, however it was found that the wild type organism could not grow on minimal media containing glucose and hypoxanthine and thus *cnx* and *nia*D mutants could not be differentiated using this method. Following the distinction of *cnx* and *nia*D mutants using cytochrome C reductase and purine hydroxylase I assays, a simple plate test using minimal media containing quinic acid as carbon source and inosine as nitrogen source was developed to analyse *cnx* and *nia*D mutants. Non-reverting (at less than 1 in 10^8) *nia*D mutants were isolated for later experiments.

*P. chrysogenum nia*D mutant *nia*D19 was transformed to nitrate utilization at a frequency of up to 20 transformants/µg DNA using the *Aspergillus nidulans nia*D gene, up to nine/µg DNA with the *A. niger nia*D gene and up to three/µg DNA using the *A. oryzae nia*D gene. Vector constructs carrying the *A. nidulans ans*-1 sequence with the *A niger nia*D gene did not show increased transformation efficiency. Linearization of the *A. niger nia*D containing plasmid resulted in a two to three times increase in transformation frequency. Southern blot hybridization analysis demonstrated that

vector sequences had integrated into the recipient genome. The control of heterologous *niaD* gene expression generally agreed with that found in the wild type strain, that is, induction by nitrate and repression in the presence of ammonium. The *A. nidulans*, *A. niger* and *A. oryzae* genes failed to transform *C. acremonium niaD* mutants.

A DNA fragment containing the *C. acremonium niaD* gene was isolated by cross hybridization to the *A. nidulans niaD* gene. This fragment was sub-cloned into pUC18 (designated pSTA700), and showed back hybridization to *C. acremonium* wild type DNA exhibiting the expected hybridization pattern. When partially sequenced, it showed nucleotide and determined amino acid homology to the *A. nidulans niaD* gene and protein.

The clone pSTA700 transformed a *C. acremonium niaD* mutant, CSG116 and *P. chrysogenum* STP19 to nitrate utilization at a frequency of up to 47 and six transformants/µg DNA, respectively. When heat shock was applied to *C. acremonium* protoplasts 10mins prior to the addition of DNA, transformation frequencies of up to 137/µg DNA were obtained. Southern analysis of transformants revealed multiple integration of vector sequences, with no detectable preference for the homologous *niaD* site. Some *C. acremonium* transformants showed a greatly increased nitrate reductase activity (up to 10 times wild type activity) when induced with nitrate. The *niaD* transformation system was successfully used to introduce unselected markers into

C. acremonium such as hygromycin B and benomyl resistance. The co-transformation frequency was up to 25% when equal molar ratios of plasmids were used.

Antibiotic biosynthetic genes isolated from *C. acremonium* (*pcb*C and *cef*EF) and *A. nidulans* (*pcb*C and *pen*E) were introduced into *C. acremonium* CSG116 by co-transformation with the *C. acremonium nia*D gene or the construction of vectors containing both the *C. acremonium nia*D gene and antibiotic biosynthetic genes. Isolates transformed with the *C. acremonium pcb*C and *cef*EF genes exhibited an increase in antibacterial activity (greater than 20% compared to wild type) at frequencies of up to 10% and six percent of transformants, respectively. When *A. nidulans* constructs containing the *pcb*C and *pen*E genes, but not the *pcb*C gene alone, were co-transformed into *C. acremonium*, up to 18% of transformants exhibited an increase in antibacterial activity. The presence of penicillin antibiotics could not be found in these transformants and thus the exact cause of this increase in antibacterial activity could not be determined.

1 INTRODUCTION

1.1 *CEPHALOSPORIUM ACREMONIUM* AND MOLECULAR GENETICS

Cephalosporium acremonium reproduces by vegetative spores (conidia) and therefore belongs to the class Hyphomycetes of the Deuteromycotina (Fungi Imperfecti). Gams (1971) reclassified *Cephalosporium* as *Acremonium* due to a nomenclatural priority and suggested that *C. acremonium* be renamed *Acremonium strictum* (from Onions and Brady, 1987). However, the organism is still referred to as *C. acremonium* in most publications and by research laboratories and industrial companies and for ease of reference the organism will be termed *C. acremonium* within this thesis. It should also be noted that some publications refer to the organism as *A. chrysogenum*.

C. acremonium was first found to possess antibacterial activity when Brotzu (1948) set up an isolation and screening programme specifically to find antibiotic producing organisms. He isolated the organism from a sewage outlet in Sardinia in 1945 and it is from this culture that all industrial strains are derived. However, interest in the organism did not develop until the new antibiotic cephalosporin C was isolated (Abraham and Newton, 1961) and found to be partially β-lactamase resistant. Once a method had been found to remove the side chain from the cephalosporin C structure to form 7-amino cephalosporanic acid (Morin *et al*, 1962) the possibility of semi-synthetic

cephalosporin antibiotics became apparent. Since then *C. acremonium* has become an organism of major industrial importance. The strain *C. acremonium* M8650, used as wild type in these studies, is a mutant isolated from the Brotzu culture with increased biosynthesis of cephalosporin C.

Since *C. acremonium* is an imperfect fungus, genetic studies must be based on parasexual techniques. Due to this, knowledge of the genetics of this organism are sparse. Any information that has been produced is from the application of protoplast fusion techniques (Peberdy *et al*, 1986). Extensive studies using these techniques have resulted in the assignment of eight linkage groups (with the possibility that two more may exist; Perez-Martinez and Peberdy, 1987). However the genetics of *C. acremonium*, and in particular the antibiotic biosynthetic pathway, are still largely unknown. These techniques have not, as yet, proved productive in the development of strains with increased cephalosporin C biosynthetic capability (Harford, pers. comm.).

The advent of molecular genetical techniques in filamentous fungi holds great promise for *C. acremonium*. Its application has already resulted in an increase in information about this organism and has helped confirm data from parasexual analysis. Recently Skatrud and Queener (1989) have developed an electrophoretic molecular karyotype for *C. acremonium* and have confirmed the presence of eight chromosomes.

The size ranged from approximately 1700kb to 4000kb, with a total genomic content of at least 22500kb for that strain. This size of genome being of approximately the same size as other Ascomycetes previously investigated (from Gurr *et al*, 1987).

More importantly, three genes involved in the cephalosporin C biosynthetic pathway have been cloned from *C. acremonium*, these being the *pcb*C gene coding for isopenicillin N synthetase (Samson *et al*, 1985), the *cef*EF gene coding for the bifunctional enzyme deacetoxycephalosporin C synthase/deacetylcephalosporin C synthase (Samson *et al*, 1987a; see section 1.5) and the *cef*D gene coding for isopenicillin N epimerase (Queener, 1989). Other genes to have been cloned from *C. acremonium* include the actin gene (Patino *et al*, 1987), the LEU2 gene coding for β-isopropylmalate dehydrogenase (Friedlin and Nuesch, 1984) and the 5.8S, 16.18S and 23.28S rRNA genes (Carr *et al*, 1987; Jarai *et al*, 1987). In addition, a *C. acremonium* mitochondrial DNA fragment that functions as an autonomous replication sequence (ARS) in yeast has been cloned (Tudzinsky and Esser, 1982; Skatrud and Queener, 1984). Also the mitochondrial DNA has been separated and the position of mitochondrial genes mapped (Minuth *et al*, 1982). The LEU2 gene had been isolated to act as a selectable marker in transformation experiments but to date no recipient mutant in this gene has been found.

Prior to this thesis, two-gene mediated transformation systems had been described for *C. acremonium*, based on the antibiotic resistance genes conferring resistance to G418 (Penalva *et al*, 1985) and hygromycin B (Queener *et al*, 1985). Both systems have resulted in transformation at extremely low efficiencies. Recently Skatrud *et al* (1987) has improved the hygromycin B based system, achieving transformation efficiencies of up to 20 transformants/µg DNA. Skatrud *et al* (1987) has also attempted to improve transformation frequencies by including either the rRNA genes or the mitochondrial ARS fragment in transformation vectors. Neither sequences resulted in improvements in transformation. The level of efficiency obtained by Skatrud *et al* (1987) allowed the antibiotic biosynthetic genes *pcb*C and *cef*EF to be re-introduced to wild type and industrial strains of *C. acremonium* (Skatrud *et al*, 1986; Skatrud *et al*, 1989). They found only the *cef*EF gene produced transformants with an increased biosynthetic capability.

It was our aim to develop a homologous gene mediated transformation system for *C. acremonium* such that transformation can be achieved at high efficiency and antibiotic biosynthetic genes can be reintroduced into the organism. With a homologous system, any problems resulting from the introduction of heterologous DNA into an industrial organism should be avoided.

1.2 PENICILLIUM CHRYSOGENUM AND MOLECULAR GENETICS

Penicillium chrysogenum is an imperfect fungus and is also classified within the class Hyphomycetes. The organism has many synonyms, amongst them *P. notatum*, in which Fleming first reported penicillin.

Following the exploitation of the antibacterial activity of penicillin during the Second World War, extensive research has been carried out into *P. chrysogenum* and its production of penicillins (for review see Bycroft and Shute, 1987). Since *P. chrysogenum* lacks a perfect (sexual) stage, the genetic studies of this organism are also in their infancy. Using parasexual analysis via protoplast fusion detailed studies have been undertaken. Up to six linkage groups have so far been identified using this analysis, with detailed mapping of loci thought to be involved in antibiotic production (Saunders and Holt, 1987). However, like *C. acremonium* these studies have not been fruitful in the isolation of improved strains for industrial use (Harford, pers. comm). It is therefore hoped that the use of molecular techniques will help in the manipulation and investigation of this organism.

P. chrysogenum has proved more amenable as a research organism than *C. acremonium*. During the course of this research a number of gene transformation systems for *P. chrysogenum* have been reported. The heterologous systems are based on transformation using

the *Neurospora crassa pyr*-4 gene, coding for orotidine-5'-monophosphate decarboxylase (Cantoral *et al*, 1987), the *N. crassa* mutated β-tubulin gene (*tub*-2) conferring resistance to benomyl (Chiang, 1988), the bacterial Tn5 phleomycin resistance gene (Kolar *et al*, 1988) and the *Aspergillus nidulans amd*S gene (Beri and Turner, 1987) coding for acetamidase. The latter system has recently been used as the selection system for the construction of a *P. chrysogenum* cosmid gene bank (Smith *et al*, 1989c). The two homologous systems are based on the *trp*C gene, which codes for tryptophan synthase (Sanchez *et al*, 1987; Picknett *et al*, 1987) and oligomycin C resistance (Bull *et al*, 1988), using a mutated ATPase subunit nine gene.

A number of genes have also been cloned from *P. chrysogenum*, the more important of these being the *pcb*C gene (Carr *et al*, 1986) and the *pen*E gene coding for acyltransferase (van Solingen *et al*, 1989; Diez *et al*, 1989). Smith *et al* (1989a) have also isolated a clone which transforms the non β-lactam antibiotic producing fungi *N. crassa* and *A. niger* to antibiotic producing organisms. This clone contains all the genes required for antibiotic production and must therefore contain the *pcb*AB gene coding for L-alpha-aminoadipyl-L-cysteinyl-D-valine synthase.

Additionally, some useful molecular techniques and experiments have been performed in *P. chrysogenum* revealing information about its genetics and the antibiotic biosynthetic pathway. Kolar *et al* (1988)

developed a system for the introduction of the *E. coli lacZ* gene fused to a fungal promoter for use in studying promoter activity in *P. chrysogenum*. Kolar *et al* (1989) have used such systems to study the *pcbC* promoter in both *A. nidulans* and *P. chrysogenum*. Beckman *et al* (1989) have introduced the *Streptomyces clavuligerus* deacetoxycephalosporin C synthetase gene into *P. chrysogenum* with the transformants being found to possess this enzyme activity.

The strains V992, X50 and 26211 used in this study are ancestral strains supplied by Glaxochem. (Harford, pers. comm.) and will be referred to as wild type organisms. It was our intention to develop a homologous transformation system for *P. chrysogenum*. During the course of research for this thesis we have published details on the development of a heterologous transformation system based on the *A. niger* and *A. nidulans niaD* genes (Whitehead *et al*, 1989). Details of this paper are presented in this report.

1.3 TRANSFORMATION IN FILAMENTOUS FUNGI

Although Mishra and Tatum (1973) first described the transformation of a filamentous fungus (*N. crassa*) it did not become a recognised technique until Case *et al* (1979) and Tilburn *et al* (1983) transformed *N. crassa* and *A. nidulans*, respectively. These methods involved the creation of protoplasts from the fungi by the use of enzymes (such as glucanases) which digest the cell walls of the fungi. This method is now used

for the majority of fungal transformations, with most protocols employing a commercial preparation of enzyme, Novozyme 234, to digest the cell wall. However groups have developed the transformation of whole cells via the use of a high concentration of lithium ions (Dhawale *et al*, 1984; Binninger *et al*, 1987).

A variety of cell types have been used for the source of protoplasts, including germinating macroconidia, microconidia or young mycelia for Ascomycetes and basidiospores or mycelia for Basidiomycetes (for review see Fincham 1989). Once protoplasts are formed they are maintained within an osmotically-stabilized solution, which varies for different fungi, and are separated from the non-protoplast material by centrifugation or filtration.

The conditions for uptake of DNA into the protoplasts primarily involve the incubation of the protoplasts (approximately 10^7-10^9) with the transforming DNA in the presence of PEG and $CaCl_2$. The transformation frequencies of different fungi have been optimised by the alteration of the various parameters of osmotic concentration, incubation time and temperature. Alternatives to this method of DNA delivery do exist such as placing the DNA in liposomes and inducing these to fuse with protoplasts (Radford *et al*, 1981) or electroporation of cells as performed in bacteria (Miller *et al*, 1988), but these have not been investigated in most fungal systems. Finally, after incubation, the protoplasts are regenerated within or

on a selective medium which contains an osmotic stabilizer. The variety of selection systems available for use are now large and this will be discussed later in this section.

Of paramount importance is the ability to obtain transformation at frequencies which allow the technique to be exploited for a variety of molecular investigations. For example, it is only possible to clone a gene by complementation of a given mutation following transformation when frequencies are sufficiently high to allow the screening of thousands of colonies in a few experiments. Hence the majority of investigations have focussed upon improving transformation efficiencies.

One method which has been found to result in a marked improvement in the majority of fungi is the linearization of the transforming vector. Unkles *et al* (1989a,b) observed a two to eight times increase in transformation frequency following linearization of the transforming vector in *A. niger* and *A. oryzae*, while Dhawale and Marzluf (1988) noted a three to four times increase in transformation in *N. crassa*. However the site where the circular vector is cut may be important as Dhawale and Marzluf (1988) saw a decrease in efficiency in *N. crassa* if the vector was linearized within the selecting gene and variations in transformation frequency when the vector was cut at different sites.

Another tool which has been widely investigated for increasing transformation efficiency is the development of autonomously replicating vectors via the incorporation of autonomous replication sequences (ARS). Since transformation occurs by integration of the vector into the host chromosome in the vast majority of fungal transformation systems it was hoped that an improvement in efficiency would be obtained if vectors could replicate independently of the host's genome. Hence the ability of DNA sequences from a variety of fungi to confer autonomous replication to vectors within *Saccharomyces cerevisae* has been investigated. ARS sequences have been obtained from a number of filamentous fungi including *A. nidulans* (*ans*-1 sequence; Ballance and Turner, 1985), *Ustilago maydis* (Tsukuda *et al*, 1988), *Mucor circinelloides* (van Heewijck, 1986), *Absidia glauca* (Burmester and Wostemyer, 1987), *N. crassa* (Buxton and Radford, 1984; Paietta and Marzluf, 1985), *Aspergillus amstelodami* (Beri *et al*, 1988) and *C. acremonium* (Skatrud and Queener, 1984; see section 1.1). When used for transformation only the sequences from *A. nidulans* (Ballance and Turner, 1985), *U. maydis* (Tsukuda *et al*, 1988), *M. circinelloides* (van Heewijck, 1986) and *N. crassa* (Buxton and Radford, 1984) resulted in an increase in frequency of transformation. Although there is evidence that the sequences from *A. nidulans* and *N. crassa* allow the maintenance of the vector extrachromosomally for a short period, transformants

still result from the integration of the vector into the genome. However the sequences from *M. circinelloides* and *U. maydis* do result in vectors which are maintained extrachromosomally. One of the only other examples where a filamentous fungus has been transformed by a vector which is non-integrative was when Perrot *et al* (1987) transformed *Podospora anserina* with a linear vector to which the chromosomal telomeres of *Tetrahymena thermophila* had been ligated.

Other relatively simple procedural changes have been made in the transformation protocol to achieve increased transformation frequencies. One of these is the application of heat-shock to the protoplasts of *P. anserina* (Berges and Barreau, 1989) which resulted in a five to 10-fold increase in transformation frequency. Since heat-shock is a vital element of bacterial transformation it seems likely that this technique may be applicable to most filamentous fungi.

DNA mediated transformation systems have been described for many filamentous fungi, the vast majority being Ascomycetes, with a wide range of selection systems. The choice of selection system used varies with the organism investigated and to what use the transformation system will be put. It is probably one of the most important criteria to decide upon when developing a transformation system for a filamentous fungus. Presented below is a brief review of the predominant systems so far used and their possible advantages and disadvantages.

1.3.1 Antibiotic resistance.

By far the most common antibiotic selection system relies on conferring resistance to hygromycin B using the *E. coli* hygromycin phosphotransferase gene, originally isolated by Gritz and Davis (1983). It has been used successfully in *A. nidulans*, *A. niger*, *A. oryzae* (from Gurr *et al*, 1987), *C. acremonium* (Queener *et al*, 1985), *Leptosphaeria maculans* (Farman and Oliver, 1988), *Fusarium oxysporum* (Kistler and Benny, 1988), *Fulvia fulva* (Oliver *et al*, 1987), *Glomerella cingulata* (Rodriguez and Yoder, 1987), *Colletotrichum trifollii* (Dickman, 1988), *U. maydis* (Wang *et al*, 1988) and *Septoria nodorum* (Cooley *et al*, 1988), demonstrating the versatility of this selection system for the transformation of filamentous fungi. Transformation frequencies are low, ranging from one to 10 transformants/µg DNA in the majority of organisms. In *U. maydis* however 50-1000 transformants/µg were achieved when a *U. maydis* heat-shock gene promoter was used to express the hygromycin B resistance gene. This indicates that transformation frequencies are primarily limited by the heterologous *A. nidulans gpd*A used for transformations with this gene.

A number of other systems have been developed using bacterial genes conferring resistance to antibiotics. These include phleomycin selection in *P. chrysogenum* (Kolar *et al*, 1988) and resistance to the antibiotic G418 in *N. crassa* (Bull and Wootton, 1984), *C. acremonium* (Penalva *et al*, 1985), *Absidia*

glauca (Wostemeyer *et al*, 1987) and *Achlya ambisexualis* (Manavathu *et al*, 1988).

Benomyl resistance has been used with some success in a variety of filamentous fungi. For this, a mutant allele of a β-tubulin gene is required which is resistant to the action of benomyl. A mutant allele of the *N. crassa tub*-2 gene has been isolated (Orbach *et al*, 1986) and used to transform *N. crassa* (Orbach *et al*, 1986; Vollmer and Yanofsky, 1986), *C. trifolii* (Dickman, 1988), *P. anserina* (Fernandez-Larrea and Stahl, 1989) and *Gaeumannomyces graminis* (Henson *et al*, 1988), while the *A. nidulans* mutated *ben*A gene has been used for homologous transformations (from Gurr *et al*, 1987). Transformation frequencies were again low in the heterologous experiments (approximately one to 10/μg DNA), although homologous *N. crassa* transformations produced frequencies in excess of 1000 transformants/μg DNA.

A similar system to this is that based on oligomycin C resistance, where a mutant allele of the ATP subunit nine gene is isolated which confers resistance to oligomycin C. Homologous transformations have been achieved in *P. chrysogenum* (Bull *et al*, 1988), *A. nidulans* (Ward *et al*, 1986) and *A. niger* (Ward *et al*, 1988). Despite being homologous, this system results in a difficulty in isolating transformants due to the instability of the resultant transformants.

The above description demonstrates the use of antibiotic resistance as a selectable marker since a

large variety of fungi have been transformed even if very little information is available about the organism and its genetics. Mutants or genes need not be isolated from the organism of investigation for these systems to be used. However, unless promoters more suited to the fungi being transformed are placed in front of the bacterial antibiotic resistance genes, transformation frequencies are low. These systems, except for the last two, also exhibit the problem of using heterologous genes. The problem of using heterologous genes can be overcome when using oligomycin C or benomyl resistance, although it may be problematical to isolate the respective mutant alleles from poorly characterized fungi. It may also be undesirable to use antibiotic resistance genes in industrial organisms, especially those involved in the commercial production of antibiotics, such as *C. acremonium* or *P. chrysogenum*. Finally it may be necessary to use antibiotics within fermentation media to ensure the maintenance of the vector which would be both expensive and environmentally undesirable if the organism was used for industrial scale fermentations.

1.3.2 Uracil auxotrophy

Transformations based on this system result in the transformation of uracil auxotrophs to uracil prototrophy via the orotidine-5-monophosphate decarboxylase gene (OMPase). Hence uracil auxotrophs must be isolated via selection for resistance to the

uracil precurser 5-fluoro-orotic acid. The OMPase of *N. crassa*, *pyr*-4, has been cloned and used for homologous transformation (Buxton and Radford, 1983) and the heterologous transformation of *P. chrysogenum* (Cantoral *et al*, 1987) and *A. nidulans* (Ballance *et al*, 1983). Homologous transformations have also been performed in *A. nidulans* (*pyr*G gene; Ballance and Turner, 1985) and *A. niger* (*pyr*G gene; van Hartingsveldt *et al*, 1987). Additionally, uracil auxotrophs have been isolated from *P. anserina* with a mutated orotidylic acid pyrophosphorylase (OMPppase) gene and transformed with the homologous *ura*5 gene to uracil prototrophy (Begueret *et al*, 1984).

The resultant transformation frequencies when using this system are not very high, however it is especially amenable to use with the *A. nidulans ans*-1 sequence which greatly increases transformation efficiency with this system. The isolation of uracil auxotrophs can be a difficult, time consuming and expensive process. Also, mutants generated by a mutagenic agent may introduce gratuitous mutations into other areas of the genome. This effect is obviously undesirable in an organism which is used for industrial fermentations or for studies on other pathways of the fungus.

1.3.3 Arginine auxotrophy.

The *A. nidulans arg*B gene, encoding ornithine transcarbamylase, has been used to transform the

corresponding arginine auxotrophs of *N. crassa* (Weiss *et al*, 1985), *A. nidulans* (Johnstone *et al*, 1985) and *Trichoderma reesei* (Pentilla *et al*, 1987). This has resulted in high transformation efficiencies but suffers from the problems outlined in Section 1.3.2 of mutant selection.

1.3.4 Tryptophan auxotrophy.

The homologous tryptophan synthase genes have been used to transform tryptophan auxotrophs of *P. chrysogenum* (Picknett *et al*, 1987; Sanchez *et al*, 1987), *A. nidulans* (Yelton *et al*, 1984) and *N. crassa* (Case *et al*, 1979). Although high transformation frequencies have been achieved, the transformants are unstable and difficult to isolate. This system also suffers from the problems outlined in Section 1.3.2 of mutant selection.

1.3.5 Acetamide utilization.

A. nidulans (Tilburn *et al*, 1983; Hynes *et al*, 1983), *A. niger* (Kelly and Hynes, 1985), *A. terreus* (Upshall, 1986), *Cochliobolus heterostrophus* (Turgeon *et al*, 1985), *T. reesei* (Pentilla *et al*, 1987) and *P. chrysogenum* (Beri and Turner, 1987) have all been transformed using the *A. nidulans amd*S gene, coding for acetamidase. Since most organisms grow extremely poorly on acetamide, transformations with the *amd*S gene will result in growth which is greater than sparse background growth of untransformed colonies. This may

be used as a positive selection system and thus has the advantage of not requiring the recipient strain to be mutagenised. Transformation efficiencies useful for research and development have been achieved using this selection system.

1.3.6 Nitrate utilization.

Transformation systems based on the *nia*D gene coding for the nitrate reductase apoprotein have only recently been developed for filamentous fungi. The organisms being *A. nidulans* (Johnstone *et al*, 1990), *A. niger* (Unkles *et al*, 1989b), *A. oryzae* (Unkles *et al*, 1989a) and *F. oxysporum* (Malardier *et al*, 1989). These are homologous systems with the exception of *F. oxysporum* where transformations were performed with the *A. nidulans nia*D gene. Daboussi *et al* (1989) has also transformed *Colletotrichum lindemuthianum*, *Beauveria bassiana*, *Penicillium caseicolum*, *Aphanocladium album*, *Nectria haematococca* and *Pyricularia oryzae* with the *A. nidulans nia*D gene. We have reported the transformation of *P. chrysogenum* with the *A. niger* and *A. nidulans nia*D genes (Whitehead *et al*, 1989), the details of which will be presented in this report.

Whilst heterologous transformation frequencies obtained are quite low, between one to 20/µg DNA, homologous transformation systems can result in frequencies of greater than 1000 transformants/µg DNA. Strains with mutations within the resident *nia*D gene

must be isolated as recipients for this transformation system. However, mutagenesis need not be applied to obtain these mutants, as strains resistant to chlorate (which can be detected at high frequency when grown on chlorate) have a concomitant defect in nitrate assimilation. It is then easy to isolate and characterize *nia*D mutants from the other gene mutations which cause chlorate resistance (see Section 1.4) using phenotypic analysis (Cove, 1979).

Taking into consideration the respective advantages and disadvantages of the above systems it was decided that an attempt would be made to develop a homologous transformation system for *P. chrysogenum* and *C. acremonium* based on the *nia*D gene. This system has several advantages, (i) mutants in nitrate assimilation can be readily and economically isolated by selection for chlorate resistance without recourse to mutagenic treatment, (ii) the *nia*D mutants can be easily distinguished from other chlorate resistant mutants via a phenotypic test, as previously demonstrated in *P. chrysogenum* (Birkett and Rowlands, 1981), (iii) nitrate assimilation is an entirely dispensable pathway under most growth conditions, thus mutations within it should not affect the batch fermentation of industrial strains, (iv) antibiotic resistance genes will not be present and thus the presence of antibiotics will not be required to ensure the maintenance of the genes at any time, which will be environmentally favourable, (v) a wealth of biochemical and genetical data (see Section

1.4) is available on this system in filamentous fungi, (vi) the *niaD* gene from *A. nidulans* has already been cloned and sequenced (Johnstone *et al*, 1990) and is available for use as a heterologous probe to clone the *P. chrysogenum* and *C. acremonium niaD* genes.

1.4 NITRATE ASSIMILATION IN FILAMENTOUS FUNGI.

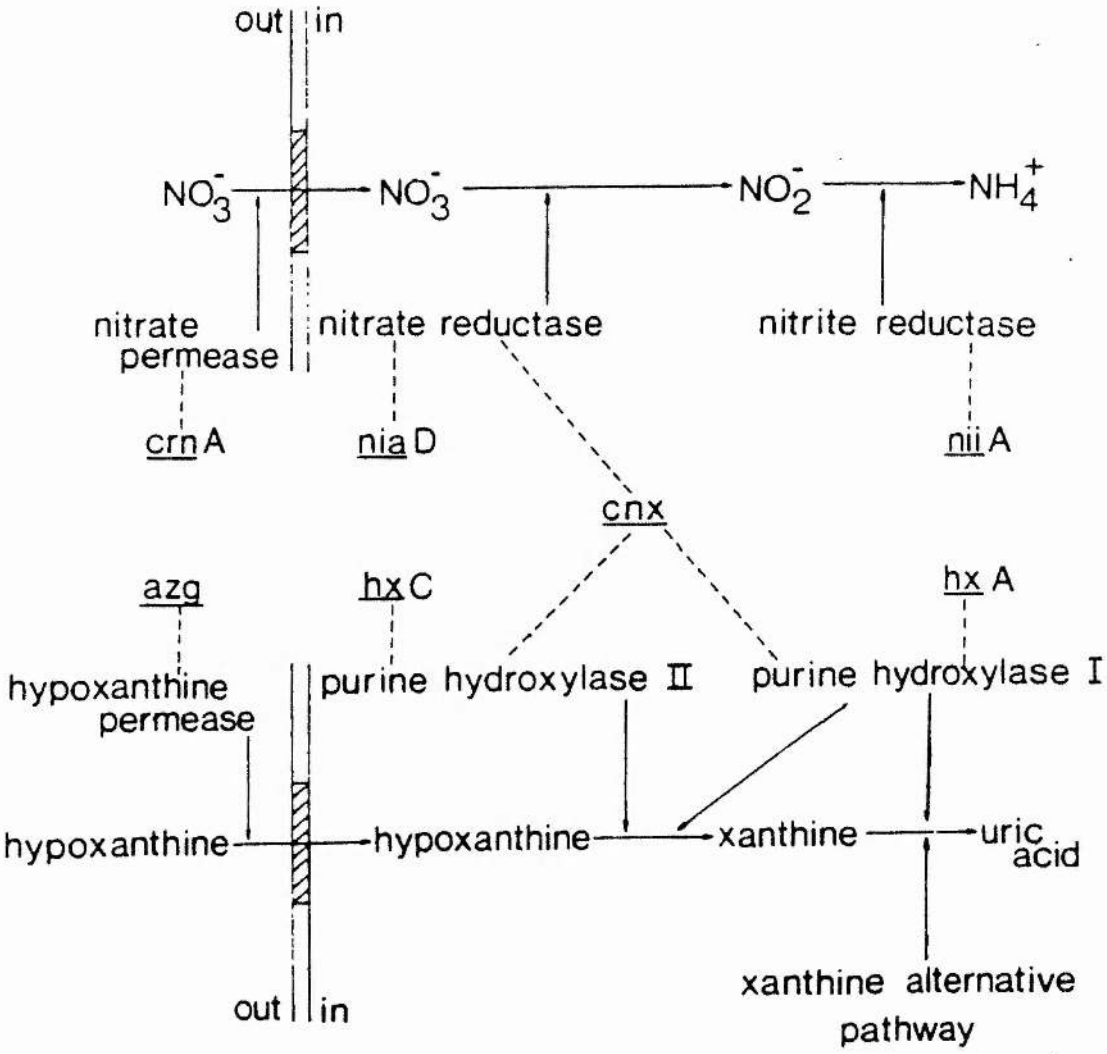
Along with acetamide utilization (Hynes *et al*, 1988), the nitrate assimilation pathway has been the subject of a very detailed study in filamentous fungi, both at a biochemical and genetical level. The two organisms on which most investigations have been performed are *A. nidulans* (Cove, 1979) and *N. crassa* (Dunn-Coleman, 1984).

Figure 1.1 shows the known pathway for nitrate assimilation in *A. nidulans*, with nitrate being internally reduced to nitrite by nitrate reductase and then to ammonium by nitrite reductase.

The nitrate permease responsible for the uptake of nitrate has been identified as being coded for by the *crnA* gene in *A. nidulans* (Brownlee and Arst, 1983), although it is also thought that other systems may play a part in nitrate uptake.

The nitrate reductase enzyme is a complex protein consisting of an apo-protein (most probably existing as a homo-dimer, Cooley and Thomsett, 1985) bound with a molybdenum cofactor along with the cofactors FAD and $cytb_{557}$. The apo-protein is coded for by the *niaD* gene of *A. nidulans*, isolated by

Figure 1.1 The nitrate and hypoxanthine assimilation pathways of *A. nidulans.*



Johnstone *et al* (1989) and the *nit*-3 gene of *N. crassa*, isolated by Fu and Marzluf (1987a).

Mutants defective in the molybdenum cofactor have been found in eight complementation groups in *A. nidulans*, with the putative genes being termed *cnx*A to C, E to H and *cnx*J. The *cnx*A, B and C loci exhibit an overlapping complementation pattern and are thought to be two closely linked genes. This has been found in *A. nidulans* (Cove, 1979), *N. crassa* (Tomsett and Garrett, 1980), *P. chrysogenum* (Birkett and Rowlands, 1981) and *Septoria nodorum* (Newton and Caten, 1988). It is thought that some *cnx* genes code for structural elements of the molybdenum cofactor while others are involved in its assembly and processing. In *A. nidulans* the molybdenum cofactor is also required by the enzymes purine hydroxylase I and purine hydroxylase II. The roles of these enzymes in *A. nidulans* is demonstrated in Figure 1.1. The main activity of purine hydroxylase II is in the conversion of nicotinic acid (the inducer of the enzyme) to 6-hydroxynicotinic acid but has the subsidiary action of converting hypoxanthine to xanthine (Sealey-Lewis *et al*, 1978). Thus the *cnx* genes link the activites of both the nitrate and the hypoxanthine assimilation pathways. Nitrite reductase then converts nitrite to ammonium and is coded for by the *nii*A gene of *A. nidulans*.

It is interesting to note that in *A. nidulans* the *crn*A, *nii*A and *nia*D genes have all been found to be tightly linked at one locus of the genome, although not

under the control of a single operon (Johnstone *et al*, 1990). The *nii*A and *nia*D genes are also linked in *A. niger* (Unkles *et al*, 1989b), *A. oryzae* (Unkles *et al*, 1989a) and *P. chrysogenum* (Birkett and Rowlands, 1981), while in *N. crassa* the *nit*-3 gene is located on a different chromosome to the *nit*-6 gene (Tomsett and Garrett, 1980) coding for nitrite reductase.

The regulation of this pathway has also been the subject of detailed study. A broad spectrum gene coding for nitrogen metabolite repression has been identified which acts as a positive regulator to this system and others involved in nitrogen metabolism (Arst and Cove, 1973). This gene has been cloned from *A. nidulans* (termed the *are*A gene; Caddick *et al*, 1986) and *N. crassa* (termed the *nit*-2 gene; Fu and Marzluf, 1987b). The *N. crassa nit*-2 has also been found to complement *are*A mutants of *A. nidulans* demonstrating the functional and structural similarity between the two regulatory products (Davis and Hynes, 1987). A gene has also been found that is involved in the specific regulation of the *nii*A and *nia*D genes and is called the *nir*A gene in *A. nidulans*. Again an analogous gene has been determined in *N. crassa*, termed the *nit*-4 gene and isolated by Fu *et al* (1989). Recent results (Fu *et al*, 1989) suggest that the *nit*-2 and *nit*-4 genes independently control the expression of the nitrate assimilatory genes, possibly acting at distinct upstream activation sites. It also seems likely that the *nit*-4 gene product has a complex mode of action,

possibly interacting with cellular nitrate, while the gene products of the *nii*A and *nia*D genes may also act in some self-regulatory fashion (Cove, 1979).

One of the most useful tools which has enabled the detailed studying of the nitrate assimilation pathway is that mutations affecting nitrate utilization can easily be found by isolating colonies resistant to chlorate. Since this resistance to chlorate arises spontaneously at high frequency in the presence of chlorate, mutagenesis is not required. It is possible that chlorate acts as an analogue of nitrate and is converted to the toxic chlorite by nitrate reductase and hence organisms without this activity are resistant to chlorate. However it is thought that this is not the only way in which chlorate acts to be toxic to the organism, as it does not sufficiently explain the variety of mutations observed (Cove, 1976), although the exact mechanism of action has not been completely described. Since a number of genes code for elements involved in the control and assimilation of nitrate, chlorate resistant mutants also possess a spectrum of gene mutations. Phenotypic analysis to distinguish some of these mutations were developed by Cove and Pateman (1963), but a comprehensive review of the analysis required is presented by Cove (1979) and this is referred to in other areas of the text. Table 1.1 summarizes the growth tests performed on chlorate resistant mutants to distinguish *nia*D, *nir*A, *are*A, *crn*A, Δ*nia*D*nii*A and *cnx* mutants in *A. nidulans*. This

Table 1.1 The phenotypic analysis of Cove (1979) used to assign genotypes to chlorate resistant mutants of *A. nidulans*.

Gene mutation	Utilization of sole nitrogen source*				
	nitrate	nitrite	ammonium	hypo'thine	glutamate
*crn*A	+	+	+	+	+
*nia*D	-	+	+	+	+
*nir*A	-	-	+	+	+
*are*A	-	-	+	-	-
cnx	-	+	+	-	+
Δ*nia*D*nii*A	-	-	+	+	+

* + denotes wild type levels of growth
- denotes starved growth

analysis has been used with success in a wide variety of filamentous fungi including *N. crassa* (Tomsett and Garrett, 1980) and *P. chrysogenum* (Birkett and Rowlands, 1981) and has proved invaluable in genetic studies on this system.

1.5 ANTIBIOTIC BIOSYNTHESIS IN *C. ACREMONIUM* AND *P. CHRYSOGENUM*.

C. acremonium and *P. chrysogenum* have become famous due to their industrial use as producers of β-lactam antibiotics. They comprise over 50% of the antibiotic market, which is now estimated to be worth $10 billion worldwide. A variety of other organisms have also been found to produce β-lactams, such as the filamentous fungus *A. nidulans*. Although its low level of production is not commercially exploitable, it is useful as a research organism. *Streptomyces* have also been used for the production of β-lactam antibiotics such as cephamycin C (Chen *et al*, 1988) while a wide range of bacteria have been found to produce low levels of antibiotic, such as *Flavobacterium* and *Xanthomonas* (Singh *et al*, 1982). β-lactam antibiotics act by behaving as a substrate analogue for the dipeptide acyl-D-Ala-D-Ala, which is crucial to the final stages of peptidoglycan cell wall synthesis in bacteria.

C. acremonium generally produces cephalosporin C as a by-product, while *P. chrysogenum* produces penicillin G in fermentation media. It is these compounds which, following cleavage of side chains, are

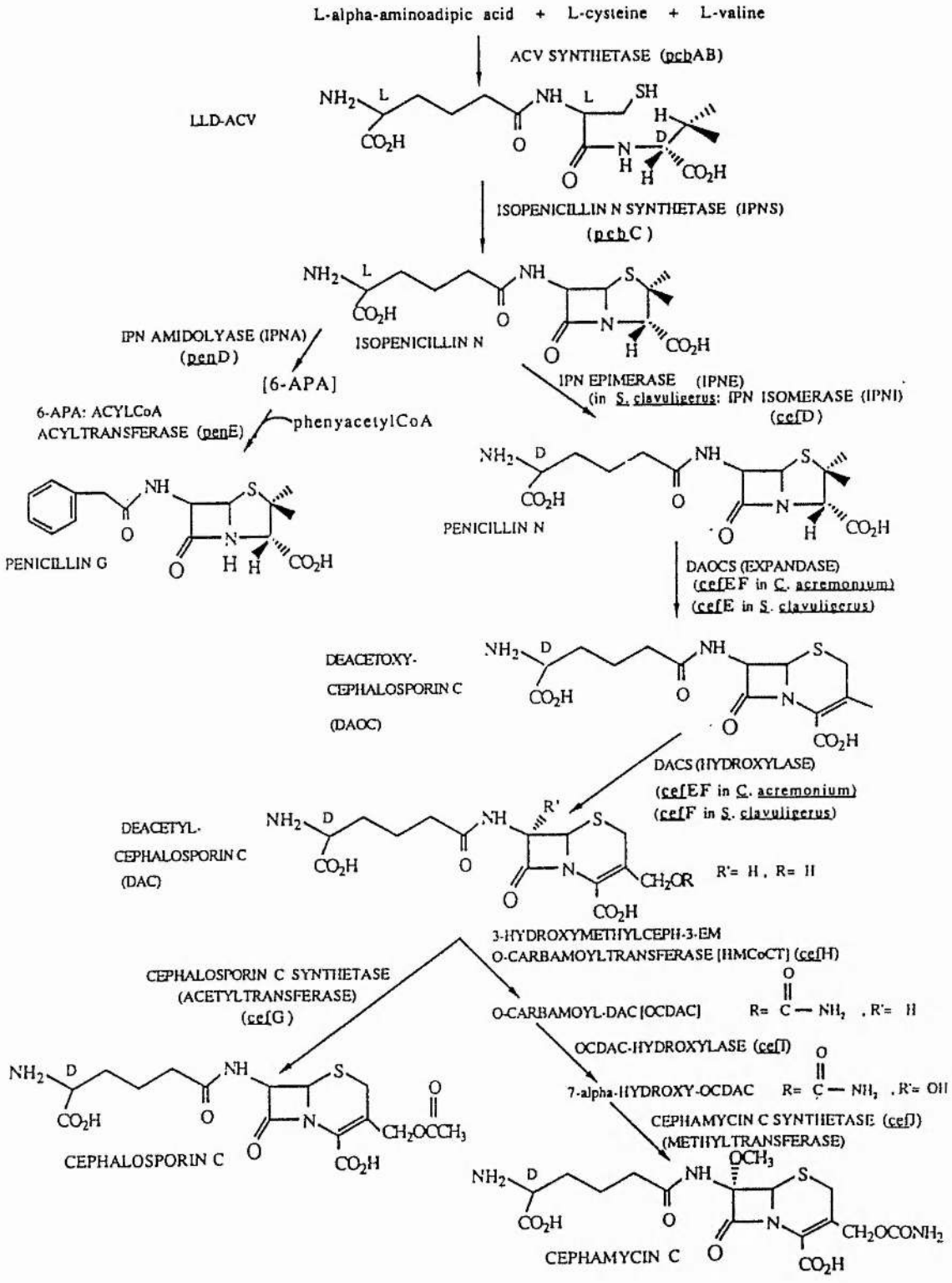
synthetically converted to medically useful antibiotics, such as Cephalothin and Ampicillin.

Extensive research has been carried out on *P. chrysogenum* and *C. acremonium* to determine the biosynthetic pathway for antibiotic production (for review see Ingolia and Queener, 1989). Figure 1.2 shows the antibiotic pathway as known in these two organisms. This figure demonstrates that the pathways are identical in *P. chrysogenum* and *C. acremonium* up until the formation of isopenicillin N where the pathways diverge. In *A. nidulans* and *P. chrysogenum* only penicillin is produced, while in *C. acremonium* only enzymes required for the production of cephalosporin C are found. The notation used for genes are as recommended by Ingolia and Queener (1989). Although genes cloned recently have been termed differently, to avoid confusion they will be referred to as per the Ingolia and Queener (1989) notation throughout this report.

Recently the use of molecular genetic techniques has allowed the cloning and characterization of a number of genes involved in antibiotic biosynthesis, which has greatly increased our knowledge of this pathway in both *C. acremonium* and *P. chrysogenum*.

The first step in β-lactam biosynthesis involves the bioconversion of three amino acids, L-alpha-aminoadipic acid, L-cysteine and L-valine into the tripeptide L-alpha-aminoadipyl-L-cysteinyl-D-valine (ACV). Recently the enzyme responsible for its

Figure 1.2 The antibiotic biosynthetic pathways of *C. acremonium* and *P. chryosgenum*.



formation, ACV synthetase, has been obtained from *A. nidulans* (van Liempt *et al*, 1989). The predicted nucleotide sequence for a region of its gene, *pcb*AB, has been determined from its amino acid sequence. The synthetic oligonucleotides derived from the amino acid sequence have then been used to localise the *pcb*AB gene to be closely linked, 5', to the *pcb*C gene in *A. nidulans* (MacCabe *et al*, 1990). Thus it is highly likely that the *pcb*AB genes from *C. acremonium* and *P. chrysogenum* will be isolated soon by heterologous hybridization.

ACV is then converted to isopenicillin N (IPN) by IPN synthetase (IPNS) coded for by the *pcb*C gene. This was the first gene to be isolated from the pathway when Samson *et al* (1985) derived the nucleotide sequence from the isolated IPNS protein and used this to clone the *C. acremonium pcb*C. This gene has then been used to clone the *P. chrysogenum* (Carr *et al*, 1986) and *A. nidulans* (Ramon *et al*, 1987; Weigal *et al*, 1988) *pcb*C genes. The nucleotide and amino acid sequences for these genes have been determined and the similarity between them analysed and compared to the sequence of the *Streptomyces lipmanii pcb*C gene (Weigal *et al*, 1988). The overall similarity between the genes and proteins of the filamentous fungi and the *Streptomyces* is far higher than expected. It has therefore been suggested that the pathway first arose in *Streptomyces* and was then transferred to the fungi long after eukaryotes had diverged from prokaryotes

(Ramon *et al*, 1987; Weigal *et al*, 1988). This scenario is made more likely by the observation of Heinemann and Sprayen (1989) that bacteria can conjugatively transfer plasmids to yeast. Molecular studies on the *pcb*C gene have revealed a variety of information on its structure and function. Samson *et al* (1987a) has demonstrated that following site-directed mutagenesis of the cysteine residues (of which there are two) to serine, the enzyme loses 97% of its specific activity. Investigations into the regulation of expression via promoter elements are currently being performed by Kolar *et al* (1989) and Gomez-Pardo *et al* (1989) who have shown that the *pcb*C gene is regulated at the level of transcription and its expression coincides with the onset of penicillin synthesis.

In penicillin producing organisms, IPN is converted to penicillin G via 6-amino penicillanic acid. It has been thought two enzymes were responsible for this activity, however, recently van Solingen *et al* (1989) cloned a single gene with this activity, the acyltransferase gene of *P. chrysogenum*, *pen*E. This gene has been found to be located 3' to the *pcb*C of *P. chrysogenum*. Thus it seems likely that the 3 genes required for penicillin biosynthesis, *pcb*AB, *pcb*C and *pen*E, are closely linked in *P. chrysogenum* and *A. nidulans*. This has been further supported by Smith *et al* (1989a; 1990) who have expressed this gene cluster from *P. chrysogneum* in *A. niger* and *N. crassa*, two organisms which do not produce antibiotics, with

the resulting transformants exhibiting antibiotic production.

In those organisms which produce cephalosporin C IPN is converted to penicillin N by IPN epimerase (isomerase) which has been recently cloned by Queener (1989), although no details are yet forthcoming. Penicillin N is then converted to deacetoxycephalosporin C (DAOC) by the bifunctional enzyme DAOC synthase/deacetylcephalosporin C (DAC) synthetase (or expandase) coded for by the single gene *cef*EF, cloned from *C. acremonium* by Samson *et al* (1987b). In *Streptomyces* species however, the DAOC synthase and DAC synthetase activites are separable and thought to be coded for by two genes (Ingolia and Queener, 1989).

The terminal step in cephalosporin C synthesis is brought about by cephalosporin C synthetase coded for by *cef*G. It is thought that organisms which produce different end products, such as cephamycin C possess enzymes with different activities at this point.

It can be seen that the biosynthesis of penicillins and cephalosporins is a very complex process and although recent advances have been made in the cloning of genes, much remains unknown about the control and regulation of this pathway. Molecular genetics should reveal much information concerning this. It also offers itself as a useful tool for the manipulation of strains with increased antibiotic production. Skatrud *et al* (1989) have investigated this

by transforming in copies of the *C. acremonium pcb*C and *cef*EF genes into industrial strains of *C. acremonium*. Although the *pcb*C gene had no effect on antibiotic biosynthesis in this strain, the *cef*EF gene resulted in transformants with up to 15% improved capability for cephalosporin C production. It may also be possible to produce novel antibiotics *in vivo* by transforming antibiotic genes from other organisms.

2 MATERIALS AND METHODS

2.1 CHEMICALS AND EQUIPMENT

All chemicals (except where specified) were obtained from either BDH or Sigma and were of analytical grade. Novozym 234 was provided by Novobiolabs, Novo Industries. Restriction enzymes and DNA modifying enzymes were purchased from Pharmacia or NBL.

Centrifugation: For routine DNA isolation and enzyme extraction a Sorvall RC-5C refrigerated superspeed centrifuge was used with either a SS34 or GS3 fixed angle rotor. Ultracentrifugation was performed on a Sorvall Ultracentrifuge (Dupont) using the T865.1 fixed angle rotor for plasmid purification and a Beckman L2-65B ultracentrifuge with the SW65 swing bucket rotor for phage preparations. Protoplasts were isolated using a MSE benchtop centrifuge. Microcentrifuge tubes were centrifuged in a MSE Microcentaur microfuge.

Spectrophotometry: All absorbance readings were performed on a Pye Unicam SP6-550 UV/VIS spectrophotometer.

2.2 MAINTENANCE OF CULTURES

Cultures of *Cephalosporium acremonium* M8650 and *Penicillium chrysogenum* V992, X50 and 26211 (kindly supplied by Dr. S. Harford, Glaxochem U.K. plc) were maintained on complete media (CM, see appendix) at 28^{o}C

and 26°C respectively. The following strains of *Aspergillus nidulans* were also used in this study (gene symbols are as defined by Clutterbuck, 1984): B344 *yA*1; *wA*3 *nia*D10; B5 *biA*1 Δ506 (supplied by Dr. B. Tomsett); *biA*1 as wild type, which were maintained on CM at 37°C.

Escherichia coli DH5α, JM101 or NM259 were maintained on Luria agar (LA, see appendix) at 37°C.

Fungal strains were stored as silica gel stocks at 0-4°C, while *E. coli* strains were stored in glycerol (Maniatis *et al*, 1982) at -70°C.

2.3 ISOLATION AND CHARACTERIZATION OF CHLORATE RESISTANT MUTANTS

2.3.1 Isolation of spontaneous chlorate resistant mutants.

Approximately 10^8 spores were plated onto minimal media (MM, see appendix) supplemented with either 10mM glutamate or arginine and 470mM sodium chlorate (100mM sodium chlorate was used for *P. chrysogenum* X50 and 26211). Chlorate resistant mutants were isolated after 14days incubation for *C. acremonium* and nine days for *P. chrysogenum*.

2.3.2 Isolation of UV irradiated chlorate resistant mutants.

Approximately 10^8 spores were plated on 470mM chlorate MM as above and exposed to UV irradiation to

produce a 99% loss in viability. Chlorate resistant mutants were then isolated as above.

2.3.3 Reversion stability of chlorate resistant mutants.

Suspensions of approximately 10^8 spores of chlorate resistant isolates were plated onto MM (10mM nitrate) and the number of nitrate utilizers recorded after 14 days incubation.

2.3.4 Characterization of chlorate resistant mutants

a) Plate tests.

Single colonies of mutants were patched in a grid pattern onto CM, allowed to grow, then replicated onto MM supplemented with either 10mM nitrate, 10mM nitrite, 10mM glutamate, 10mM ammonium, 2mM inosine, 1mM adenine or 1mM hypoxanthine. Where specified glucose was replaced by either 1% quinic acid, glycerol or sodium acetate.

b) Purine hydroxylase I activity stains.

Cultures were grown in liquid CM (omitting nicotinic acid) for 45hrs at 28°C, 290rpm (New Brunswick orbital shaker). The mycelia were harvested by Buchner filtration through muslin, washed in sterile distilled water and transferred to liquid MM supplemented with 500µM uric acid for 3.5hrs before harvesting. The mycelia was then ground in liquid nitrogen and extracted in two volumes of 100mM phosphate buffer, pH 7.2, 1mM EDTA, 10mM

2-mercaptoethanol and 1% Triton X-100. After centrifugation at 20000rpm for 20mins, the supernatant was assayed for purine hydroxylase I activity as described by Mendel and Muller (1976).

c) NADPH-linked cytochrome c reductase.

Cultures were grown and harvested as above, except the MM was supplemented with 500mM nitrate. The mycelia was ground in liquid nitrogen, extracted in two volumes of 100mM orthophosphate buffer, pH 7.0 and the supernatant assayed for activity using a modification of the published methods (Cove and Coddington, 1965; Wray and Filner, 1970). The reduction of cytochrome c was observed at 550nm in the following reaction mixture: 50µl cytochrome c (2%), 200µl NADPH (1mM), 500µl 100mM Tris buffer (pH 8.5, 4mM EDTA), 50µl FAD (100µM), 200µl enzyme extract.

The reaction was started by the addition of cytochrome c, with NADPH being omitted from negative controls.

d) *In vivo* complementation of chlorate resistant mutants.

i) Protoplast fusion.

This was performed as described in Anné and Peberdy (1976). Protoplasts were prepared as in Section 2.5.2. After fusion protoplasts were plated on either CM or MM (10mM nitrate), osmotically buffered with 10.8% sucrose.

ii) Anastomosis.

Mutants were inoculated onto MM (10mM nitrate) in a pairwise manner, approximately 1cm apart and within 2cm of the petri dish edge. Growth was assessed after 14days incubation.

e) *In vitro* complementation of chlorate resistant mutants.

Cell free extracts from a *Neurospora crassa nit*-1 mutant were prepared by the method of Mendel *et al* (1981). Cell free extracts of *C. acremonium* were prepared as in Section 2.3.4c), except the mycelia was extracted with 1.95ml 50mM phosphate buffer, pH 7.5, 5mM EDTA, 50µl 1M sodium molybdate and 3.1mg reduced glutathione. The extract was heated to 80°C for 90secs under nitrogen and cooled on ice before 50µl was added to 100µl *N. crassa nit*-1 extract in the presence of 5µl 20mM NADPH. This mixture was incubated for 40mins at 25°C under nitrogen. 50µl 100mM nitrate and 100µl 100µM FAD were then added and the mixture incubated for a further 30mins at 25°C under nitrogen. Nitrite determinations were performed as in Wray and Filner (1970).

2.4 INVESTIGATION OF ALTERNATIVE TRANSFORMATION SYSTEMS FOR *C. ACREMONIUM*.

2.4.1 Determination of antibiotic resistance.

Approximately 10^6 spores of *C. acremonium* were plated out on CM containing either hygromycin B

(Calbiochem), phleomycin (CAYLA) or benomyl at various concentrations. The presence or absence of regenerating colonies was noted after 15 days incubation at 28°C.

2.4.2 Determination of carbon and nitrogen substrates of acetamidase.

Spores of *C. acremonium* were inoculated onto MM supplemented with various carbon and nitrogen sources and the amount of growth determined and recorded after ten days incubation at 28°C. Colonies showing good growth (equivalent to growth shown on 1% glucose as carbon source and 10mM nitrate as nitrogen source) were scored a five, while colonies showing no growth, or starved growth were scored a zero with intermediate scores for intermediate growth.

2.5 TRANSFORMATION OF CHLORATE RESISTANT MUTANTS

2.5.1 Transformation of *P. chrysogenum*.

Mycelia was grown in liquid CM for 20hrs, 290rpm at 26°C before being harvested, washed in sterile distilled water and placed in 50mls 1M $MgSO_4$, 10mM phosphate buffer, pH 5.8 and 5mg/ml Novozym 234 for 3hrs with gentle shaking at room temperature. The contents were then transferred to 25ml universal tubes, overlayed with 800mM $MgSO_4$ and centrifuged at 4000rpm for 20mins. Floating protoplasts were removed and washed three times in 700mM KCl, with centrifugation at 2500rpm for 10mins. Washed protoplasts were transformed

using a modification of the method of Cantoral *et al* (1987). Approximately $5x10^7$ protoplasts were suspended in 500µl KCM (0.7M KCl, 50mM $CaCl_2$, 10mM MOPS, pH 5.8) with 1-10µg transforming DNA and 50µl PCM (50% PEG 8000, 50mM $CaCl_2$, 10mM MOPS, pH 5.8). After 30mins incubation at 4°C, 2mls of PCM were added and the protoplasts given a further 20mins incubation at 20°C, before being plated on MM supplemented with nitrate (10mM) and 700mM KCl.

2.5.2 Transformation of *C. acremonium*.

Mycelia was grown on a minimal base media (Queener *et al*, 1985; see appendix) for 36hrs at 28°C, 290rpm. Approximately 2g (wet weight) of mycelia was harvested, washed in sterile distilled water and placed in 50ml citrate/phosphate buffer (McIlvaine, 1921), pH 7.1, 10mM DTT and gently shaken at 25°C for 30mins. After harvesting, the mycelia was washed in 700mM KCl and placed in 50ml citrate/phosphate buffer, pH 5.8, 700mM KCl and 5mg/ml Novozym 234. After 70mins gentle shaking at 25°C, the protoplasts were separated from undigested mycelia by filtration though sintered glass (pore size No. 1). Protoplasts were collected by centrifugation at 2500rpm for 10mins and washed three times in 700mM KCl. Approximately 10^8 protoplasts were resuspended in 100µl 700mM KCl, to which $CaCl_2$ was added to 50mM. Transforming DNA (1-40µg) and 10µl PEG solution (50% PEG 4000, 50mM $CaCl_2$, 50mM Tris, pH 7.5) were added and the protoplasts incubated for 20mins at

room temperature before 900µl of PEG solution was added. After a further 20mins incubation the protoplasts were spread plated on MM supplemented with 10mM nitrate, 20% sucrose and 2% agar. After overnight incubation at 15°C, plates were transferred to 28°C. When selecting for hygromycin B resistant transformants protoplasts were regenerated overnight at 15°C on CM osmotically buffered with 20% sucrose and 2% agar before being overlayed with 5mls CM containing 0.5% agar and 75µg/ml hygromycin B. Alterations in this transformation procedure are explained in the results section.

2.5.3 Transformation of *Aspergillus nidulans*.

Transformation was performed using an adaptation of the method of Johnstone *et al* (1990). After growth in liquid MM supplemented with 10mM ammonium for 16hrs at 30°C, 200rpm, the mycelia was harvested, washed in sterile distilled water and placed in 50mls 800mM $MgSO_4$, 10mM phosphate buffer, pH 5.8, 5mg/ml Novozym 234 and 0.01% 2-mercaptoethanol. Protoplasts were released by gentle shaking at 25°C for 2hrs before being harvested as *P. chrysogenum* protoplasts (see 2.5.1). Floating protoplasts were removed and washed three times in transformation buffer (1.2M sorbitol, 10mM $CaCl_2$, 10mM Tris, pH 7.5) and suspended to a final concentration of $3x10^8$ protoplasts/ml. Transforming DNA and 100µl of PEG solution (50% PEG 4000, 10mM $CaCl_2$, 10mM Tris, pH 7.5) was added to 1ml of protoplasts and

incubated at room temperature for 20mins. Then a 10 times volume of PEG solution was added and the protoplasts given a further 20min incubation. Transformed protoplasts were plated directly into molten MM supplemented with nitrate (10mM) and 1.2M sorbitol at 45°C

2.6 NITRATE REDUCTASE DETERMINATIONS

Nitrate reductase assays were performed on *P. chrysogenum* and *A. nidulans* wild type mycelia grown at 290rpm, 26°C or 200rpm, 30°C respectively in liquid MM containing one of the following nitrogen sources: 20mM sodium nitrate, 20mM sodium glutamate, 10mM ammonium tartrate or 20mM sodium nitrate with 10mM ammonium tartrate. Samples of mycelia were taken at various time intervals, harvested, washed and stored in liquid nitrogen. *P. chrysogenum* transformants were grown as above and harvested aftr 26hrs growth.

C. acremonium wild type and transformants were initially grown in CM for 38hrs at 28°C, 290rpm before being harvested, thoroughly washed and transferred to MM containing the same nitrogen sources as above and incubated for 5hrs before harvesting. Wild type *C. acremonium* was also grown in MM containing the above nitrogen sources and incubated at 28°C, 290rpm with samples of mycelia being harvested at various time intervals and stored in liquid nitrogen.

The harvested mycelia was ground in liquid nitrogen and extracted in 10 volumes of 100mM phosphate

buffer, pH 7.5 and the supernatant assayed in triplicate as Cove (1966).

2.7 PROTEIN DETERMINATIONS

All protein determinations were carried out using the method devised by Bradford (1976). Fresh standard curves against bovine serum albumin were prepared for each batch of determination solution.

2.8 β-GALACTOSIDASE ACTIVITY INVESTIGATIONS IN *C. ACREMONIUM*.

C. acremonium wild type was grown on a minimal bacterial medium, M9 (see appendix) or MM containing either 1% lactose, 1% glucose or 0.2% glucose and 10mM nitrate, 10mM glutamate or 10mM ammonium. X-gal dissolved in methanol was added (40mg/litre of media) as a colour indicator for β-galactosidase activity. The plates were inspected after 7 days incubation at 28°C.

2.9 BIO-ASSAY ASSESSMENT OF *C. ACREMONIUM*

Colonies of *C. acremonium* required for investigation were inoculated onto 5mm diameter plugs of fermentation media (see appendix). Such plugs were incubated at 25°C in a humid atmosphere before being tested for antibacterial capability. Bio-assay plates were set up by adding 2mls of *Bacillus subtilis* (five day culture) and 1ml of 0.5% tetrazolium chloride to 200mls of bio-assay media (see appendix) at 45°C. After mixing, this media was poured into large (20cm x 20cm)

plates. Once set, the plugs of fermentation medium were placed (at 3cm intervals) on the surface of the bio-assay medium and the plates incubated overnight at 37°C. Antibacterial activity was assessed by the presence or absence of germinated *B. subtilis*, as exhibited by clear zones.

2.10 GENERAL MOLECULAR GENETICS TECHNIQUES

2.10.1 Transformation of *E. coli*.

Transformation of *E. coli* was carried out using an adaptation of the method described in Maniatis *et al* (1982). Cells competent to take up DNA were obtained by inoculating 100ml of Luria broth (LB, see appendix) with 1ml of an overnight culture of *E. coli* grown in LB at 37°C. This was incubated at 37°C, 200rpm, for approximately 2hrs until the $O.D._{600nm}$ was between 0.175 and 0.225. The cells were harvested by centrifugation at 5000rpm, 4°C for 10mins and resuspended in 25mls of ice cold 100mM $MgCl_2$ and then pelleted as before and resuspended in 25mls of ice cold 100mM $CaCl_2$. After 1hr on ice, the cells were again pelleted and taken up in 2.5mls ice cold 100mM $CaCl_2$, 14% glycerol and decanted into 100µl aliquots into pre-cooled eppendorfs. The competent *E. coli* was stored at -70°C and used as required.

DNA (0.1-1µg) was added to the 100µl aliquot of competent *E. coli* and left on ice for 40mins. After heat-shocking the cells for 2mins at 42°C, 100µl of LB

was added and the mixture incubated for 1hr at 37°C. Suitable dilutions were plated onto LA containing 50µg/ml ampicillin and incubated overnight at 37°C to select for transformants.

2.10.2 Plasmid preparation from *E. coli*.

Large scale plasmid isolations were adapted from Maniatis *et al* (1982). An overnight culture of *E. coli*, containing the plasmid of interest, was grown up and 10mls inoculated into 1l of LB (supplemented with 50µg/ml ampicillin). Cells were harvested by centrifugation at 8000rpm at 4°C for 5mins after overnight growth at 37°C, 200rpm. The supernatant was discarded and the cell pellet resuspended in 50mls of 50mM glucose, 25mM Tris, pH 8.0, 10mM EDTA, before 80mls of 200mM NaOH, 1.5% SDS was added. After gentle mixing the solution was incubated on ice for 5mins and 40mls of 3M KAc (pH 4.8, adjusted with glacial acetic acid) added, the solution was gently mixed and centrifugation repeated. The supernatant was decanted through muslin and 0.6 volumes of ice cold propan-2-ol added. The precipitated DNA was pelleted at 8000rpm, 4°C for 10mins before being resuspended in 10mls of 10mM Tris, pH 7.5, 1mM EDTA (TE). To this, 50µg/ml RNase (prepared as Maniatis *et al*, 1982) was added and the DNA solution incubated at 37°C for 2hrs before being treated with phenol and chloroform as Maniatis *et al* (1982). The DNA was precipitated with the addition of 0.1 volumes 3M NaAc (pH 5.8, adjusted with glacial

acetic acid) and three volumes of ice cold 96% ethanol, followed by incubation at -70°C for 2hrs. The precipitate was pelleted at 15000rpm, 15 mins, washed with ice cold 70% ethanol and repelleted.

The plasmid DNA was then isolated and purified by ultracentrifugation. After dissolving in 4mls of distilled H_2O the DNA was added to 31.4mls of distilled H_2O containing 37g of $CsCl_2$ and 1.6ml of EtBr (10mg/ml) added. The contents were mixed and split equally between four ultracentrifuge tubes (T865.1 Sorvall). These were spun at 50000rpm for 24hrs, resulting in a separated plasmid DNA band which was extracted from the side of the tube by a hypodermic syringe. EtBr was removed by six extractions with butan-1-ol. The DNA was dialysed against a large volume of TE at 4°C for 24hrs before being precipitated with NaAc as above and dissolved in an appropriate volume of TE for use.

2.10.3 Isolation of fungal chromosomal DNA.

Chromosomal DNA was isolated using an adaptation of the method of Tilburn *et al* (1983). Approximately 2g of mycelia was harvested after being grown in liquid CM. This was ground in liquid nitrogen to a fine powder and 50mls of solution I (500mM sucrose, 25mM Tris pH 7.5, 20mM EDTA, 2% sarkosyl NL30) added. After incubation at 65°C for 1hr, cell debris was pelleted at 10000rpm for 10mins and the supernatant removed and incubated overnight at 30°C with 60µg/ml proteinase K.

50mls solution II (30% PEG 6000, 1.5M NaCl) was then added, mixed and the solution kept on ice for 90mins before the DNA was pelleted by centrifugation at 15000rpm, 15mins. The DNA was then gently resuspended in 10mls TE, treated with RNase, phenol and chloroform extracted as in section 2.6.2 before being precipitated with 3M NaAc (see section 2.6.2) and dissolved in TE to the required concentration.

2.10.4 Restriction enzyme digestion of DNA.

Restriction enzyme digests were performed with the recommended assay buffers at 37°C for 2-4 hrs for plasmid or phage DNA. For restriction mapping purposes a combination of single, double and triple digests were performed. Where possible the digest was carried out in a common buffer. If this was not possible the enzyme requiring the lowest salt concentration was used for digestion first and then the required salt added with the second enzyme. Large scale digests of plasmids or fungal chromosomal DNA were carried out for 18hrs with the addition of fresh enzyme after 10hrs. The digestion was terminated with the addition of 1/6 volume of loading buffer (0.025% bromophenol blue, 0.025% xylene cyanol, 15% Ficoll, type 400) or by the precipitation of the DNA (see section 2.6.2) if required for ligations.

2.10.5 Electrophoresis of DNA.

DNA was electrophoresed through a horizontal 0.8% agarose (ultrapure, BRL) gel in 1xTBE (89mM Tris, 89mM boric acid, 2mM EDTA) containing 500ng/ml EtBr, with the electrophoresis buffer also being 1xTBE. Electrophoresis was performed at between 20-70V until the bromophenol blue dye was approximately two-thirds through the length of the gel.

2.10.6 Recovery of DNA fragments.

After the DNA had been digested to completion it was electrophoresed, as in section 2.10.5 through 1% low melting point agarose (ultrapure, BRL). After separation the gel was stained in an EtBr solution (200ng/ml) for 10mins, before being visualized. The fragment band of interest was excised from the gel and transferred to an eppendorf. The agarose was melted by heating to 65°C for 30mins before cooling to 37°C. Phenol and chloroform extraction was performed at 37°C to removed the agarose. The resulting DNA was then recovered by precipitation with 3M NaAc (see section 2.10.2).

2.10.7 Ligation and subcloning of DNA fragments.

Using the equation:

$$Mw = \left(\frac{51.1}{j/i[DNA]} \right)^2$$

where M_w=molecular weight of DNA sample (660kdal=1kb)

j=the concentration of one end of a molecule relative to the other molecules ends

i=the total concentration of ends

[DNA]=the total DNA concentration g/l,

(as determined by Dugaiczyk *et al*, 1975), it is possible to determine the theoretical levels of DNA required to optimize ligations. In practice a number of ligations were carried out with different molar ratios around the theoretical optimum, with an excess of insert to vector.

To prevent religation the plasmid was initially treated with calf intestinal phosphatase (CIP). The vector (0.1-1mg) in 50µl TE was added to 10µl 10xCIP buffer (500mM Tris pH 7.4, 100mM $MgCl_2$, 200mM DTT. 10mM ATP, 50µg/ml BSA), 5µl CIP (0.1 units/µl) and 35µl sterile water and incubated at 37°C for 30mins. A further 5µl of CIP was then added and incubation carried out for a further 30mins before 20µl 100mM EGTA was added and the mixture heated to 68°C for 10mins to stop the reaction. The DNA was then isolated by extraction with phenol and ether and precipitation with 3M NaAc (see section2.10.2).

1µl of insert DNA (10-500ng) was ligated with 1µl of vector (10-500ng) by 1µl T4 DNA ligase (1 unit/µl), 1µl ATP (10mM), 1µl CIP buffer amd 5µl of sterile water, by overnight incubation at 14°C. The ligated mixtures were transformed into *E. coli* (see section2.10.1) which was plated onto LA containing

50µg/ml ampicillin, 0.036% IPTG and 320µg/ml X-gal. If the vector was M13 based, then after transformation (note: the 1hr incubation in LB was not required) competent *E. coli* JM101 was added to 3mls LA (0.7% agar) at 45°C with 200µl of freshly grown *E. coli* and IPTG and X-gal as above and then poured onto LA plates.

2.10.8 Hexa-prime ^{32}P labelling of DNA.

DNA was labelled using an Amersham Multiprime kit (RPN 1601) from the method of Feinberg and Vogelstein (1983, 1984). After heating to 100°C for 10mins and cooling on ice, 10µl of DNA was added to 18µl sterile water, 10µl 5x buffer (10mM ATP, 10mM GTP, 10mM TTP in 100mM Tris pH 7.8, 100mM $MgCl_2$ and 2-mercaptoethanol), 5µl primer (random hexanucleotides), 5µl ^{32}P-dCTP (50µCi) and 2µl Klenow enzyme (1 unit/µl in phosphate buffer, pH 6.5, 10mM 2-mercaptoethanol and 50% glycerol) and incubated at room temperature for at least 7hrs. Unincorporated nucleotides were removed using a commercial nick^{TM} column (Pharmacia).

2.11 SOUTHERN TRANSFER AND PROBING OF GENOMIC DNA

After digestion and electrophoresis (see sections 2.6.4 and 2.6.5) the DNA in the agarose gel was depurinated in 250mM HCl for 15mins, washed, denatured in 500mM NaOH, 1.5M NaCl for 1hr, washed and finally neutralized in 500mM Tris pH 7.5, 3M NaCl for 1hr. The DNA was then Southern blotted onto a nitrocellulose (Hybond C-Amersham) or a nylon (Hybond

N-Amersham) filter as described in Maniatis *et al* (1982). Transfer was allowed to proceed overnight before the DNA was fixed to the filter, by baking *in vacuo* for 2hrs for nitrocellulose or exposure to a UV transilluminator for 4mins for nylon filters. Hybridization and prehybridization were carried out in either of the following solutions:

A) 2.5ml 20xSSC (3M NaCl, 300mM Na citrate, pH 7.0)
   1ml 50xDenhardts
   400µl boiled, sonnicated herring sperm DNA (10mg/ml)
   1ml phosphate buffer (500mM, pH 6.5)
   5.1ml H_2O

or

B) 2.5ml 20xSSPE (3.6M NaCl, 200mM $Na_2H_2PO_4$, pH 7.4, 20mM EDTA, pH 7.4)
   2ml 30% PEG 6000
   500µl 10% skimmed milk (Cadbury's Marvel)
   200µl 5% NaP_4O_7
   1ml 10% SDS
   250µl boiled, sonicated herring sperm DNA (10mg/ml)
   3.3ml H_2O

The filters were pre-wet in 2xSSC prior to prehybridization for 8hrs in a sealed polythene bag with either of the above solutions. Hybridization was carried out overnight after the addition of the denatured ^{32}P-labelled probe. This treatment was performed at between 55-68^{o}C depending on the hybridization conditions required. Following hybridization, the filters were washed sequentially in

25ml 5xSSC, 0.1% SDS; 3xSSC, 0.1% SDS; 1xSSC, 0.1% SDS and if required 0.1xSSC, 0.1%SDS at the same temperature at which hybridization was performed.

Filters were placed in a polythene bag and placed over Fuji (RX film) Autoradiograph film in a Kodak film cassette between two intensifying screens. The cassette was stored at -70°C before the films were developed using a Fuji X-ray film processor.

2.12 ISOLATION OF *C. ACREMONIUM NIAD* GENE

2.12.1 Screening of EMBL3 gene bank.

A gene bank of *C. acremonium* constructed in the vector EMBL3, was kindly supplied by Glaxochem UK. *E. coli* NM259 was infected and the gene library screened using an adaptation of the method of Davis *et al* (1986). Bacteria for transfection were prepared by adding 1ml of an overnight culture to 100mls LB and growing at 37°C, 200rpm until an OD_{600nm} of 0.175-0.225 was obtained. *E. coli* was harvested by centrifugation at 4000rpm for 10mins and resuspended in 10mls 10mM $MgSO_4$. To 500µl aliquots of *E. coli*, 500µl aliquots of phage (containing 2000 viable phage) were added, the solution mixed and incubated at 37°C for 15mins. After absorption, the bacteria were added to 2.5mls LA (0.5% agar) at 45°C and poured onto LA plates and incubated overnight at 37°C.

To screen the library by Southern hybridization the plates were incubated for 2-4hr at 4°C after

overnight incubation before numbered nitrocellulose filters were placed on them and left for 2mins. The filters were then air dried for 30mins (colony side uppermost) and then sequentially denatured for 20sec the following solutions:

1) 200mM NaOH, 1,5M NaCl

2) 2xSSC, 400mM Tris pH 7.4

3) 2xSSC

The DNA was fixed to the nitrocellulose filters as in Section 2.7. The filters were then probed by hybridization as described in Section 2.7.

2.12.2 Preparation of phage DNA.

E. coli NM259 was inoculated into 200ml LB with approximately 10^{10} viable phage and grown overnight at 37°C, 350rpm to produce good lysis. Chloroform (2ml) was added and the culture shaken for a further 5mins before the bacterial debris was pelleted by centrifugation at 7000rpm for 20mins. To the supernatant 1µg/ml of DNaseI and RNase was added and the solution gently shaken at 37°C for 60mins. The phage were removed by centrifugation at 16000rpm for 90mins at 6°C and each pellet resuspended in 400µl SM buffer (100mM NaCl, 15mM $MgSO_4$, 50mM Tris pH7.5, 0.01% gelatin), and the suspension spun at 2500rpm for 5mins to remove any debris. The supernatant was then gently layered onto the following CsCl step gradient:

1.25ml, 4.5g CsCl in 10ml SM

750µl, 8.2g CsCl in 10ml SM

750µl, 6.4g CsCl in 5ml SM

and spun at 35000rpm, 25°C for 2hrs in a swing out rotor. The resultant phage band was isolated and dialysed against TE overnight. The dialysate was adjusted to 100mM NaCl, extracted with phenol and chloroform and again dialysed overnight against 10mM Tris pH 7.5. The DNA was then precipitated as in section 2.6.2.

2.13 SEQUENCE ANALYSIS OF THE *C. ACREMONIUM NIAD* GENE

2.13.1 Preparation of single stranded DNA.

After preparing subclones of the *C. acremonium niaD* gene in M13 vectors mp18 and mp19 as described in section 2.6.7, stocks of the phage recombinants were stored at 4°C until required. To 10mls of LB, 100µl of an overnight culture of JM101 and 50µl of phage stock solution was added and shaken at 37°C, 300rpm for 5hrs, before the suspension was centrifuged at 10000rpm for 10mins. The supernatant was carefully removed and 2mls of 2.5M NaCl, 20% PEG 6000 added, mixed and left to stand for 15mins at room temperature. This was again centrifuged at 10000rpm for 10mins before the supernatant was discarded and the pellet re-centrifuged for a further 2mins. Any residual supernatant was very carefully removed with a Pasteur pipette and the pellet resuspended in 500µl of TE. The DNA was then extracted with phenol and chloroform before precipitation with 3M NaAc (see section 2.6.2). The DNA was visualized on an

agarose gel to ensure it was single stranded and adjusted to 500ng per 7µl.

2.13.2 Di-deoxy sequencing of single stranded DNA.

Sequencing was performed using the Sequenase™ kit (Cambridge bio-labs). To 7µl (500ng-1µg) of single stranded DNA, prepared in section 2.13.1, 2µl Sequenase buffer and 1µl universal M13 primer were added and the mixture incubated at 65°C for 2mins before being cooled to 35°C in the same water bath. To this annealed primer 2µl of labelling mix (containing dCTP, dTTP, dGTP), 500nl of ^{35}S dATP (high specific activity) and 2µl of Sequenase was added and the mixture incubated at room temperature for 5mins. 3.5µl of this solution was added to 2.5µl of either dideoxy ATP, dideoxy CTP, dideoxy TTP or dideoxy GTP. These mixtures were incubated at 37°C for 5 mins before the reaction was stopped by the addition of 4µl loading solution. Immediately before loading on a polyacrylamide gel for sequence analysis the solutions were heated to 75-80°C for 2mins.

The polyacrylamide gel consisted of 24g urea, 3.8g acrylamide, 200mg bis-acrylamide, 300µl 10% ammonium persulphate and 5ml 10xTBE per 50mls. The sequence reactions were electrophoresed through the gel for between 4-12hrs at 1500V before the polyacrylamide gel was gently washed in 10% methanol, 10% acetic acid for 20mins. The gel was then dried against 3MM Whatman paper under vacuum before being placed next to Fuji (RX

film) Autoradiograph film for 24hrs before the film was developed using a Fuji X-ray film processor.

The predicted amino acid sequence of the determined nucleic acid sequence and its similarities with other predicted amino acid sequences of nitrate reductase enzymes was determined using Staden analseq and analsep computer programs (Staden, 1982; 1984).

2.14 DETAILS OF VECTORS USED DURING THIS STUDY SUPPLIED BY OTHER RESEARCHERS

2.14.1 *nia*D gene vectors

a) pSTA10: This contains the entire *A. niger* *nia*D gene on a 7kb fragment sub-cloned into pUC8 (Unkles *et al*, 1989b).

b) pSTA12: This contains the *A. nidulans ans*-1 sequence which can increase transformation efficency in *A. nidulans* (Ballance and Turner, 1985) inserted into the *Pst*1 site of the pUC8 polylinker of pSTA10.

c) pSTA14: This contains the entire *A. oryzae* *nia*D gene sub-cloned into pUC18 (Unkles, *et al*, 1989a).

d) λAN8a: This contains the entire *A. nidulans* *nia*D gene within a 19.6kb fragment within the phage vector EMBL3 (Johnstone *et al*, 1990).

2.14.2 Plasmids conferring antibiotic resistance.

a) pIH1: This contains the *E. coli* hygromycin phosphotransferase gene (Gritz and Davies, 1983), linked to a 600 bp fragment of the *C. acremonium* *pcb*C

gene promoter within the vector pUC18 (supplied by Dr. Ramsden.).

b) pBT6: This contains the entire *N. crassa* mutated β-tubulin gene which confers resistance to benomyl (Orbach *et al*, 1986) kindly supplied by Prof. J. Peberdy.

c) pGA3: This contains the *N. crassa* mutated β-tubulin gene which confers resistance to benomyl, linked to the *A. niger* glucoamylase promoter (Unkles, per.comm.).

d) pAN8-1: This contains the phleomycin resistance gene from *Streptoalloteichus hindustanus* linked to the *gpd*A promoter and *trp*C terminator of *A. nidulans*.

e) pUT-701: This contains the phleomycin resistance gene from *S. hindustanus* linked to the **CYC1** terminator of *S. cerevisae*.

f) pUT-332: This contains the phleomycin resistance gene from *S. hindustanus* linked to the **TEF1** promoter and **CYC1** terminator of *S. cerevisae*.

2.14.3 *E. coli lacZ* gene fusion plasmid.

pAN5-41B: This contains the *E. coli lacZ* gene fused to the *A. nidulans gpd*A gene promoter (van Gorcom *et al*, 1986).

2.14.4 Acetamidase containing vector.

p3SR2: This contains the entire *A. nidulans amd*S gene subcloned into pBR322 (Hynes *et al*, 1983).

2.14.5 Plasmids possessing antibiotic biosynthetic genes.

a) pSTA200: This contains the entire *A. nidulans* *pcb*C and *pen*E genes together with part of the *pcb*AB gene (MacCabe *et al*, 1990).

b) pSTA201: This contains the entire *pcb*AB and *pcb*C genes of *A. nidulans* (MacCabe *et al*, 1990).

c) pSTA204: This contains the entire *pcb*C gene of *A. nidulans* (MacCabe *et al*, 1990).

d) pEXPAND: This contains the entire *cef*EF gene of *C. acremonium* (supplied by Dr. Illing).

e) pIPNS: This contains the entire *pcb*C gene of *C. acremonium* (supplied by Dr. Illing).

f) pNNII1: This contains both the *pcb*C gene and the *nia*D gene of *C. acremonium* (supplied by Dr. Illing).

g) pNNII2: This contains both the *pcb*C gene and the *nia*D gene of *C. acremonium*, in the opposite orientation to each other as pNNII1 (supplied by Dr. Illing).

h) pNNII3: This contains two copies of the *C. acremonium pcb*C gene and one copy of the *C. acremonium nia*D gene (supplied by Dr. Illing).

i) pNNXX6: This contains both the *cef*EF gene and the *nia*D gene of *C. acremonium* (supplied by Dr. Illing).

3 RESULTS AND DISCUSSION

3.1 ISOLATION AND CHARACTERIZATION OF CHLORATE RESISTANT MUTANTS OF *P. CHRYSOGENUM*.

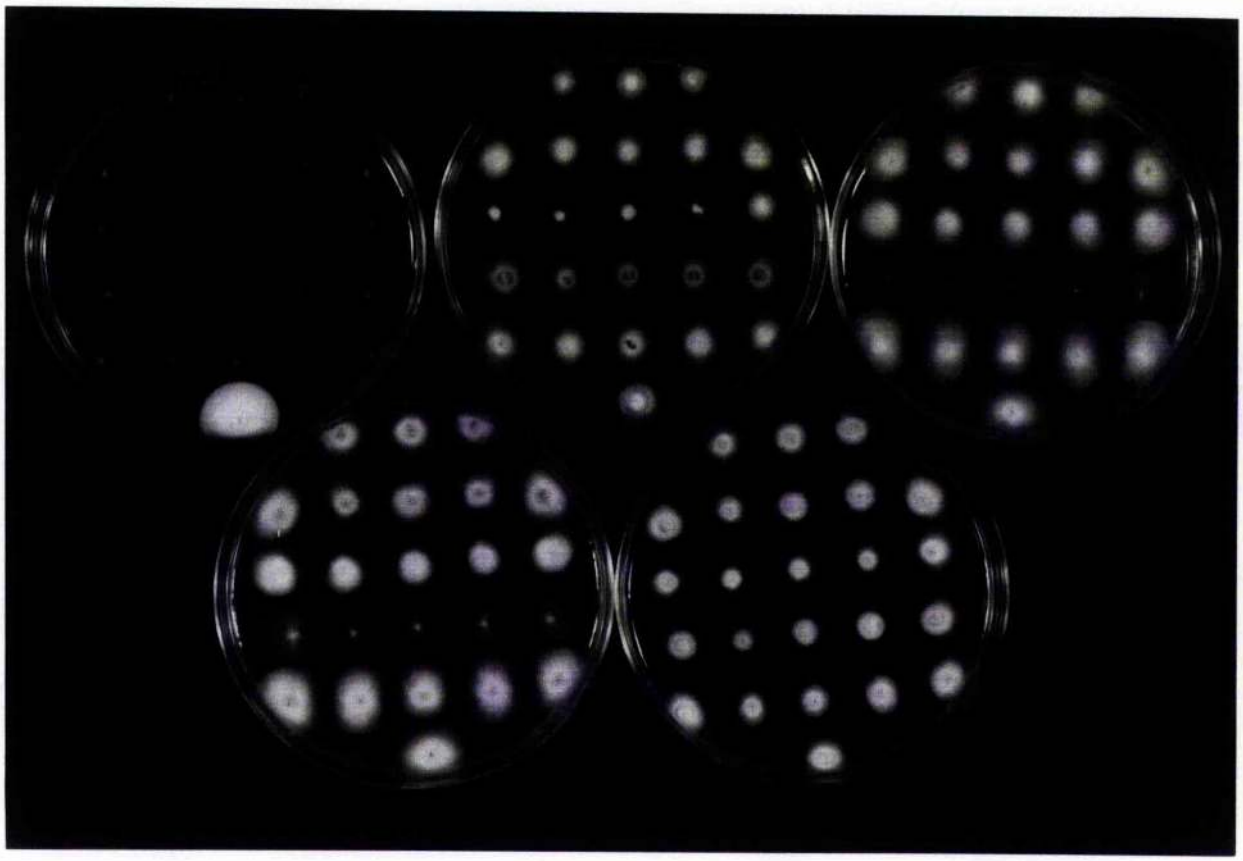
A total of 56 spontaneous chlorate resistant mutants of *P. chrysogenum* were isolated, 34 from strain V992, 13 from 26211 and nine from strain X50. Phenotypic analysis described by Cove (1979) was applied to these mutant strains, and the genotypic classifications assigned as shown in Table 3.1. Figure 3.1 shows the growth of several representative mutants on the test media. The majority of mutants obtained are either of the *nia*D or *cnx* type as Birkett and Rowlands (1981) found when investigating *P. chrysogenum*. Since glutamate was used as the nitrogen source in this investigation, these results would tend to support Birkett and Rowlands (1981) theory that contrasting nitrogen sources (L-arginine and uric acid in their study) have no significant effect on the distribution of mutant types in *P. chrysogenum*. This is in contrast to the effect observed in *A. nidulans* where the available nitrogen source has a profound effect on mutant type (Cove, 1976a). As with Birkett and Rowlands (1981) *nir*A mutants were observed, however approximately 5% of mutants were found to be of the *crn* genotype, while they did not observe any.

A number of *nia*D mutants were then tested for stability and those which showed reversion to nitrate utilization at less than 1 in 10^8 spores were retained for later transformation studies.

Table 3.1 Designated genotypes of chlorate resistant mutants of *P. chrysogenum*.

	Strain			
Mutant type	V992	X50	26211	Total
niaD	16	1	5	22
cnx	16	8	4	28
crnA	1	0	2	3
Δ*niiA,niaD*	0	0	0	0
nirA	1	0	0	1
areA	0	0	0	0

Figure 3.1 Growth of *P. chrysogenum* wild type and chlorate resistant mutants on MM containing, from left to right, top to bottom, 10mM nitrate, 10mM nitrite, 1mM hypoxanthine, 1mM adenine, 10mM ammonium.



row 1 = *nia*D mutant
row 2 = *nia*D mutant
row 3 = *nir*A mutant
row 4 = *cnx* mutant
row 5 = *nia*D mutant
row 6 = wild type

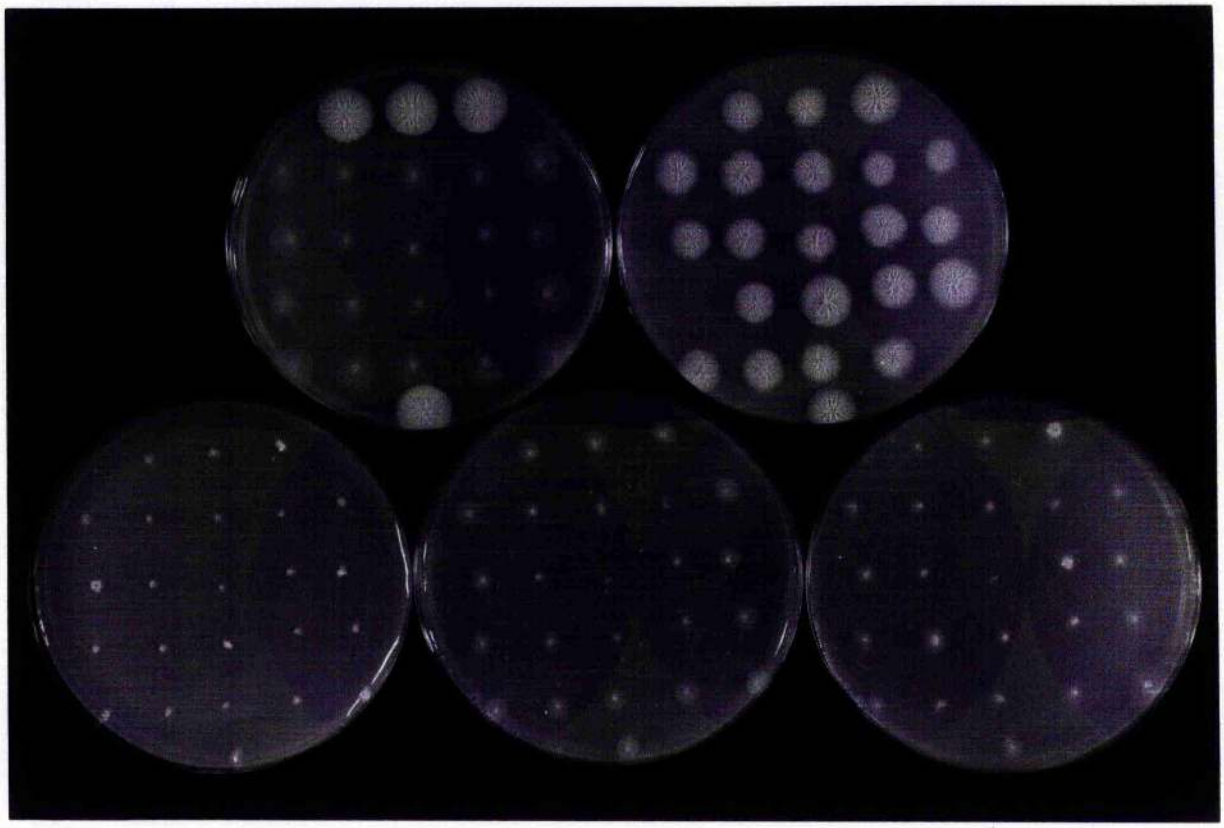
3.2 ISOLATION AND CHARACTERIZATION OF CHLORATE RESISTANT MUTANTS OF *C. ACREMONIUM*

3.2.1 Characterization using phenotypic analysis as described by Cove (1979).

From $1.2x10^8$ non-mutagenised spores, 394 chlorate resistant colonies were isolated with glutamate as the sole nitrogen source (designated CSG) while 491 mutants were generated from $7.7x10^7$ spores grown on arginine (designated CSA) of which only 200 were analysed further. When UV mutagenesis was applied to the spores, 230 chlorate resistant strains were generated from $1.9x10^7$ viable spores on glutamate containing media (designated CMG), while 161 mutants were obtained from $2.5x10^7$ spores with arginine as sole nitrogen source (designated CMA).

All of the above mutants, except where mentioned, were analysed using the phenotypic test of Cove (1979). However it was found that *C. acremonium* wild type does not grow on MM containing hypoxanthine or adenine (100μM-5mM) sufficiently to allow differentiation betwen *cnx* and *nia*D mutations, (Figure 3.2). The results of this analysis, shown in Table 3.2, therefore have chlorate resistant mutants which utilize ammonium and nitrite and yet do not utilize nitrate classified as *cnx/nia*D mutants. These results show that the overwhelming majority of mutants fall into the *cnx/nia*D mutant class. A similar distribution of mutations was observed by Cove

Figure 3.2 Growth of *C. acremonium* wild type and chlorate resistant mutants on MM containing, from left to right, top to bottom, 10mM nitrate, 10mM nitrite, 1mM hypoxanthine, 1mM adenine, 10mM ammonium.



row 1 = wild type
row 2 = *cnx/niaD* mutant
row 3 = *cnx/niaD* mutant
row 4 = *cnx/niaD* mutant
row 5 = *cnx/niaD* mutant
row 6 = wild type

Table 3.2 Designated genotypes of chlorate resistant mutants of *C. acremonium*.

	Mutant origin				
Mutant type	CSG	CSA	CMG	CMA	Total
niaD/cnx	326	141	154	130	751
crnA	68	59	66	21	214
nirA	0	0	5	9	14
Δ*niiA,niaD*	0	0	0	0	0
areA	0	0	0	0	0

(1976b) when he selected for chlorate resistant mutants of *A. nidulans* with arginine and glutamate as sole nitrogen sources. Under these conditions he also observed a lack of *nir*A$^-$ mutants, yet found a low percentage of Δ*nii*A*nia*D deletion mutants. Cove's (1976b) results formed the basis for our strategy of using arginine and glutamate as nitrogen sources in the isolation of *C. acremonium* chlorate resistant mutants. The majority of mutants produced would be *nia*D mutants, and furthermore some Δ*nii*A*nia*D mutants would be produced if the genes were contiguously or tightly linked as found in *A. nidulans* (Cove, 1979), while *nir*A$^-$ mutants (which have a similar phenotype to Δ*nii*A*nia*D mutants) would be kept to a minimum. Interestingly, despite the analysis of a large number of mutants (some of which had received UV mutagenesis) none could be found with the Δ*nii*A*nia*D phenotype, indicating these two genes are unlinked in *C. acremonium* as is the case with *N. crassa* (Tomsett and Garrett, 1980). These genes have been found to be linked in fungi other than *A. nidulans*, such as *P. chrysogenum* (Birkett and Rowlands, 1981), *A. niger* (Unkles *et al*, 1989b) and *A. oryzae* (Unkles *et al*, 1989a).

Although Cove (1979) defined *A. nidulans nir*A$^-$ mutants as those showing no growth on nitrate or nitrite (equivalent phenotype to Δ*nii*A*nia*D mutants) Birkett and Rowlands (1981) observed that *nir*A$^-$ mutants of *P. chrysogenum* showed limited but significant growth on nitrite. Hence *C. acremonium* mutants were classified as being *nir*A$^-$ when they exhibited this type of growth. It is possible that

these are infact *niaD/cnx* mutants with an additional mutation which reduces the utilization of nitrite, this is however unlikely since the utilization of other nitrogen sources such as glutamate and ammonium was not affected.

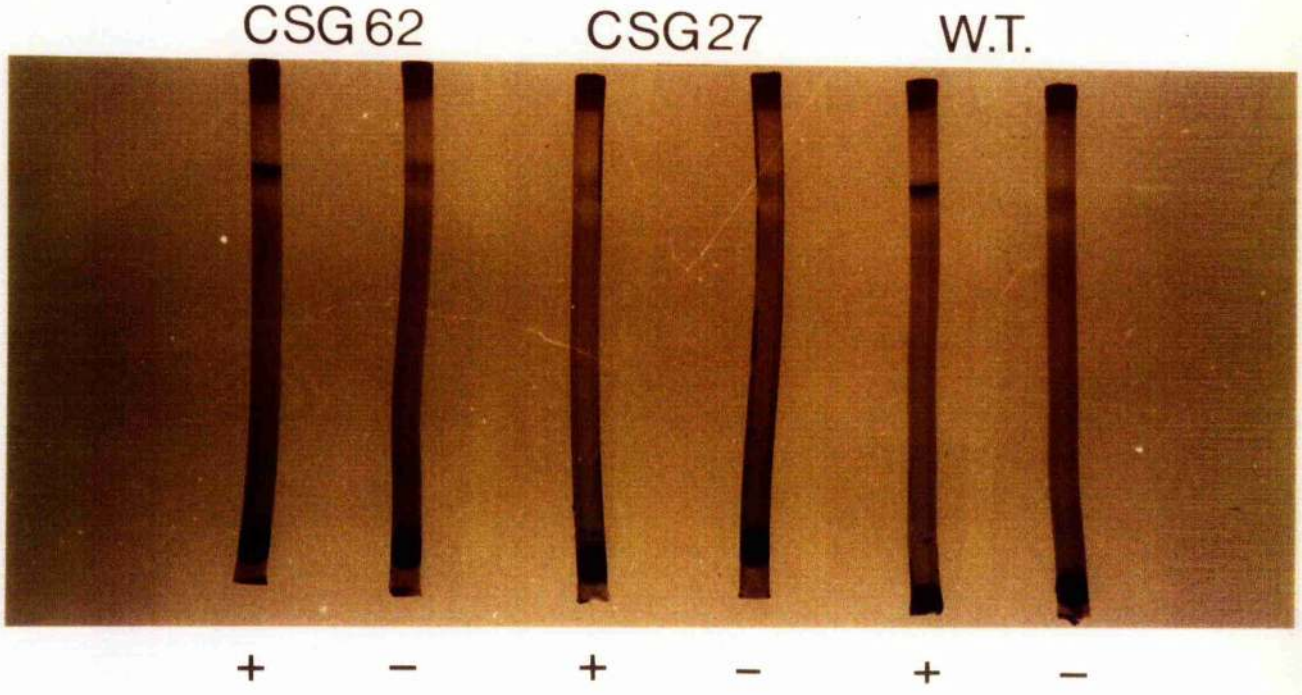
3.2.2 Assignment of *cnx* and *niaD* mutant genotypes.

An investigation was undertaken to find a test which would distinguish between *cnx* and *niaD* mutants of *C. acremonium*. Although *C. acremonium* wild type does not grow on MM with hypoxanthine the activity of the enzyme purine hydroxylase I (which utilizes hypoxanthine) was looked for. Crude extracts of *C. acremonium* wild type were prepared from mycelia induced with uric acid (the inducer of purine hydroxylase I in *A. nidulans*, Lewis *et al*, 1978) since it was found that *C. acremonium* utilizes uric acid as the sole nitrogen source in MM. It should be noted that nicotinic acid was omitted from the media since this is known to induce purine hydroxylase II (which is mainly involved in the conversion of nicotinic acid to 6-hydroxynicotinic acid but has a subsidiary activity of converting hypoxanthine to xanthine, Sealy-Lewis *et al*, 1978). When this activity was assayed for by activity staining in polyacrylamide gels in the presence of the substrate hypoxanthine, the extract was found to possess activity. In the absence of hypoxanthine no activity was found. Since this result allowed us to conclude that *C. acremonium* wild type possessed purine hydroxylase I, its activity was assayed in 20 *cnx/niaD*

C. acremonium mutants and in *P. chrysogenum* wild type, *nia*D19 and *cnx*51 mutants which acted as known comparisons. All except two *C. acremonium* mutants were found to possess activity and were thus regarded as putative *nia*D mutants while the two which lacked activity were termed putative *cnx* mutants. Figure 3.3 shows the representative results from the wild type organism, a putative *cnx* mutant (CSG27) and a putative *nia*D mutant (CSG64), while Figure 3.4 shows the results of *P. chrysogenum* wild type, *nia*D19 and *cnx*51.

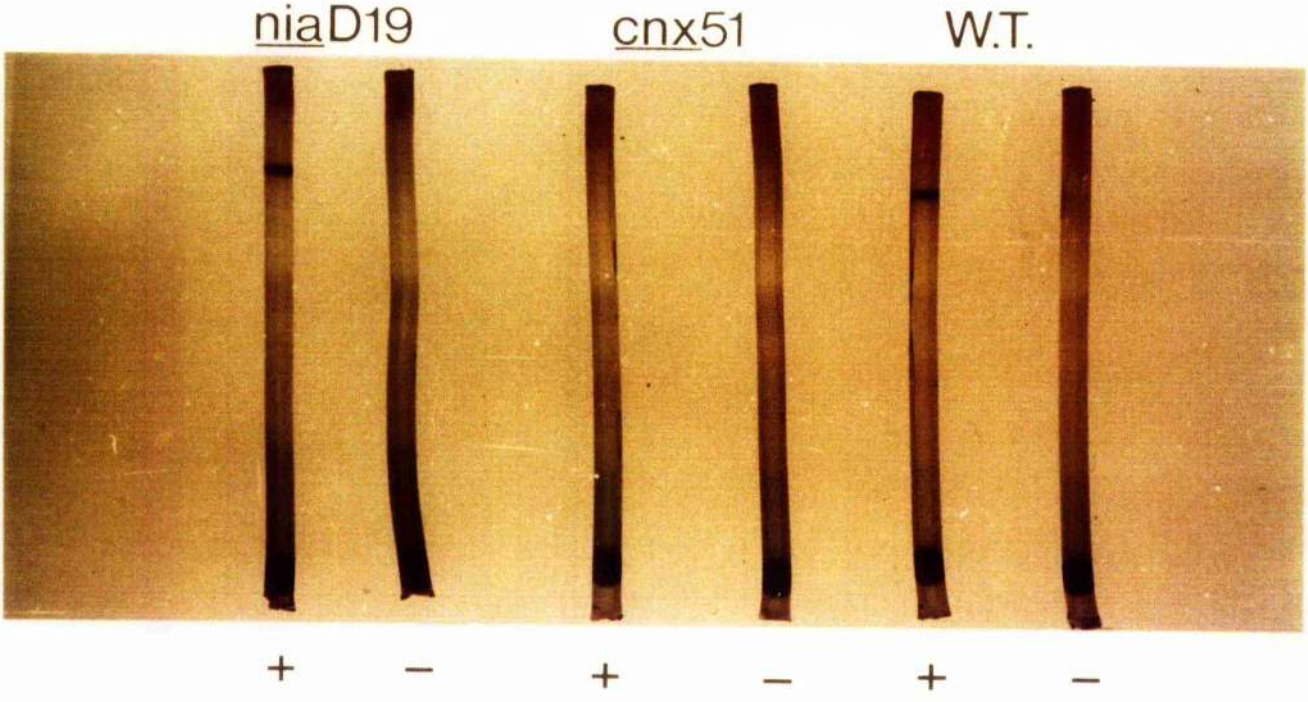
A further biochemical test was performed to confirm this analysis. It has been found that the *nia*D apoprotein possesses an NADPH associated cytochrome C reductase activity (NADPH cytochrome C oxidoreductase, E.C.I.6.2.3) in both *A. nidulans* (Cove and Coddington, 1965) and *N. crassa* (Kinsky and McElroy, 1958). This activity was investigated in the *C. acremonium* mutants CSG62 and CSG27 and *P. chrysogenum nia*D19 and *cnx*51. The results presented in Table 3.3 show that both CSG62 and *nia*D19 exhibit reduced cytochrome C reductase activity compared to the wild type organisms, confirming that CSG62 possesses a mutation within the *nia*D gene. In comparison CSG27 and *cnx*51 express activity at an equivalent level to the wild type organisms thus showing CSG27 to be a *cnx* mutant. That the cytochrome C reductase levels within the *cnx* mutants is in fact greater then that of the wild type organisms is consistent with previous work performed with *A. nidulans* (MacDonald *et al*, 1974).

Figure 3.3 Purine hydroxylase I activities of *C. acremonium* wild type and mutants CSG62 and CSG27.



+ indicates staining in the presence of hypoxanthine
- indicates staining in the absence of hypoxanthine

Figure 3.4 Purine hydroxylase I activities of *P. chrysogenum* wild type and mutants *nia*D19 and *cnx*51.



+ indicates staining in the presence of hypoxanthine
- indicates staining in the absence of hypoxanthine

Table 3.3 NADPH-linked cytochrome C reductase activities of *C. acremonium* and *P. chrysogenum* chlorate resistant mutants.

Organism	NADPH-linked cytochrome C reductase activity*	Genotype
C. acremonium wild type	245	W.T.
mutant CSG27	301	*cnx*$^-$
mutant CSG62	57	*niaD*$^-$
P. chrysogenum wild type	257	W.T.
mutant *cnx*51	291	*cnx*$^-$
mutant *niaD*19	84	*niaD*$^-$

*nmol/min/µg

In parallel, an *in vitro* complementation analysis was carried out on the *C. acremonium cnx/nia*D mutants. For this extracts from a molybdenum cofactor mutant of *N. crassa* (*nit*-1, Dunn-Colemann *et al*, 1984), which should just contain the nitrate reductase apo-protein, were added to extracts from wild type and *cnx/nia*D mutants of *C. acremonium*. This has been shown to result in the molybdenum co-factor from the wild type and the *nia*D mutants complementing with the N. *crassa* extract producing nitrate reductase activity while the *cnx* mutants would be unable to do so. Unfortunately, reproducible results could not be obtained. This may have been caused by the molybdenum co-factor of *C. acremonium* being sufficiently different so as not to allow complementation with the *N. crassa* apo-protein (although this is unlikely since extracts from the plant *Nicotiana tabaccum* have been shown to complement this mutation, Mendel *et al*, 1981). The most likely explanation is that an oxygen free atmosphere was not maintained within the experiments described, thus activity was not observed (as the molybdenum co-factor is extremely oxygen sensitive).

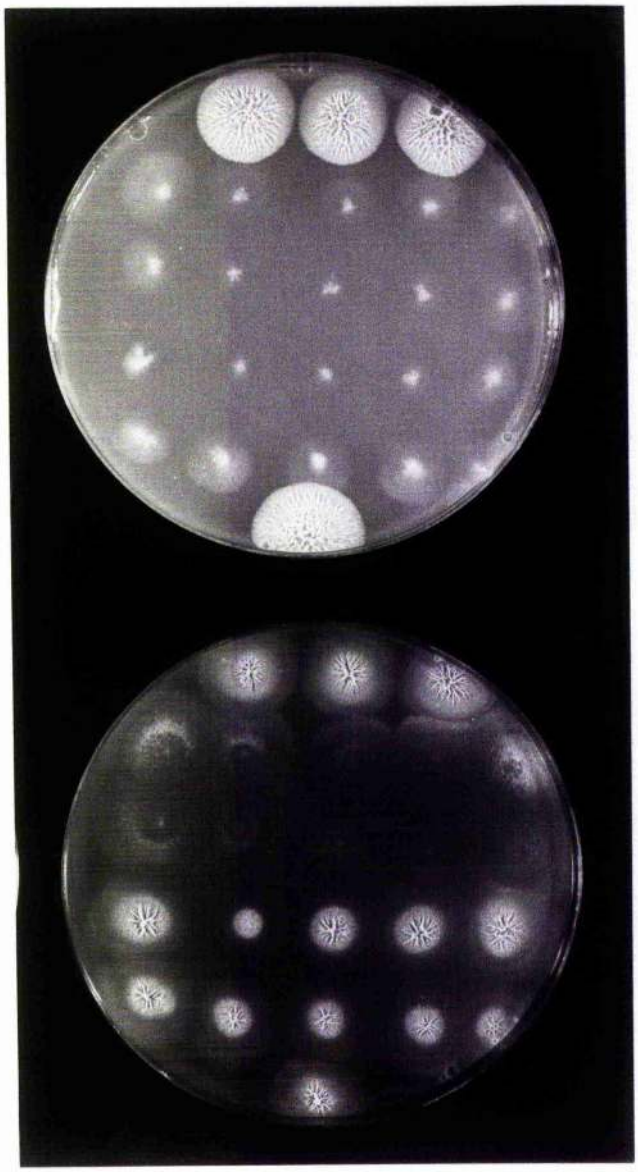
These series of results therefore showed that whilst *C. acremonium* possesses a purine hydroxylase I which can utilize hypoxanthine, paradoxically it cannot grow on MM with hypoxanthine as the sole nitrogen source. It was therefore postulated that the reason for this lack of growth was that glucose within the MM was acting by carbon catabolite repression to prevent the utilization of hypoxanthine. Thus glucose was replaced within MM with

either 1% glycerol, 1% acetate or 1% quinic acid and with 10mM nitrate as the nitrogen source and *C. acremonium* wild type tested for growth on these media. Figure 3.5 shows that *C. acremonium* can grow on such carbon sources, but quinic acid provides the optimum growth. Therefore *C. acremonium* wild type was grown on quinic acid MM (QAMM) containing 1mM hypoxanthine and after about five weeks incubation, growth could be detected. Figure 3.6 shows the growth of *C. acremonium* wild type, CSG62 and CSG27 on QAMM, 1mM hypoxanthine and demonstrates how this medium can be used as a test to distinguish between *cnx* and *niaD* mutants. However such distinguishing growth could only be seen after six weeks incubation. Since this period of incubation resulted in infection and was inconvenient, an alternative to hypoxanthine was investigated. Inosine is a precurser to hypoxanthine (containing a ribose added to a hypoxanthine molecule) and is known to be far more soluble than hypoxanthine. Hence this was used as a sole nitrogen source with MM and QAMM for *C. acremonium* wild type. Again no growth on MM with inosine (2mM) as a nitrogen source was observed but the growth on QAMM was better than that achieved with hypoxanthine. Good growth could be seen in less then three weeks as shown in Figure 3.7 which demonstrates how QAMM 2mM inosine is utilized by *C. acremonium* wild type and CSG62 yet not used by CSG27. The biochemical analysis performed on CSG62 and CSG27 demonstrated these to be *niaD* and *cnx* mutants respectively, thus growth on QAMM 2mM inosine provides a useful phenotypic analysis for

Figure 3.5 Growth of *C. acremonium* on 10mM nitrate MM in which glucose has been replaced by, from left to right, 1% quinic acid, 1% acetate and 1% glycerol.

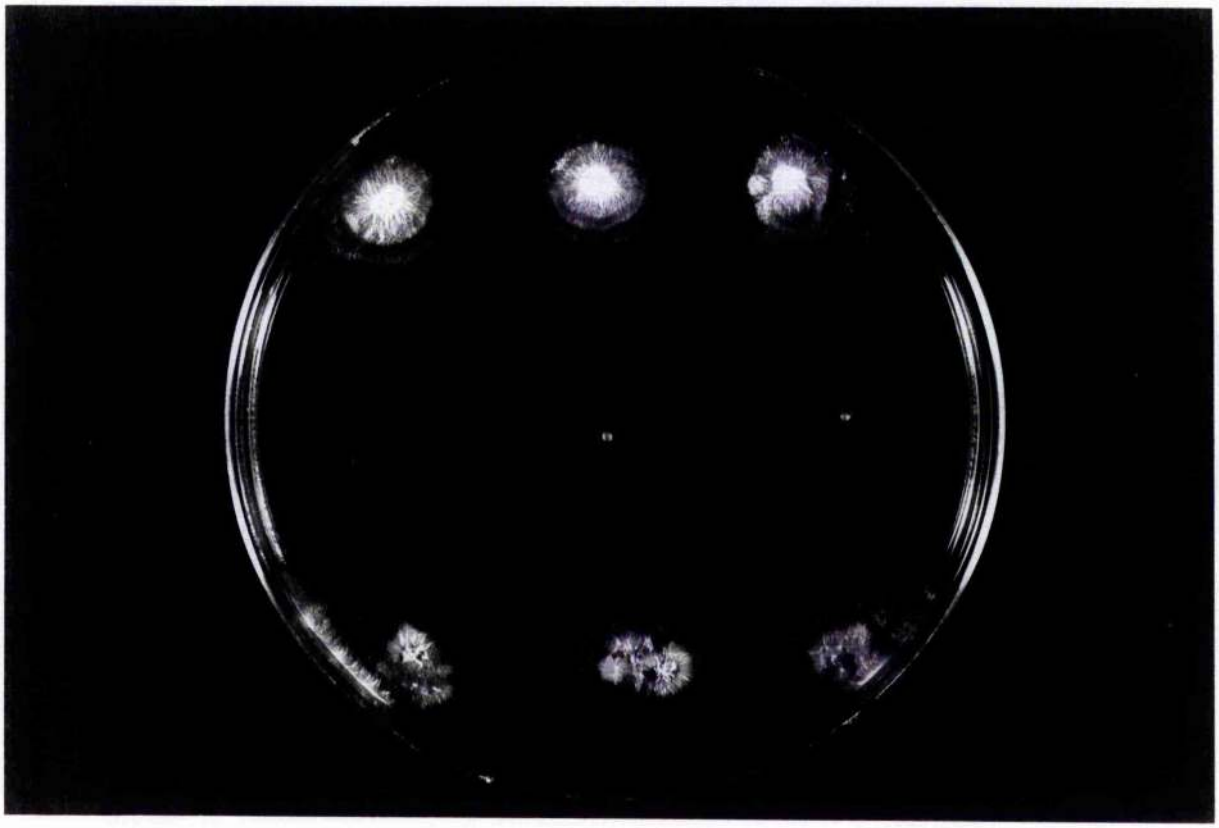
Plates shown after 14 days incubation

Figure 3.6 Growth of *C. acremonium* wild type and mutants CSG62 and CSG27 on QAMM containing 10mM nitrate (top) and 1mM hypoxanthine (bottom).



row 1 = wild type
row 2 = CSG27
row 3 = CSG27
row 4 = CSG62
row 5 = CSG62
row 6 = wild type

Figure 3.7 Growth of *C. acremonium* wild type and mutants CSG62 and CSG27 on QAMM containing 2mM inosine.



row 1 = wild type
row 2 = CSG27
row 3 = CSG62

distinguishing *cnx* and *nia*D mutants of *C. acremonium*.

The results of these studies indicate that glucose is probably acting by carbon catabolite repression to prevent the utilization of hypoxanthine (and inosine). The exact mechanism of action has not been determined but it is possible that since *C. acremonium* wild type possessed purine hydroxylase I activity when induced with uric acid in medium containing glucose, yet would not grow on glucose and hypoxanthine plates, the transport of hypoxanthine is being inhibited by glucose (since uric acid is taken up by a different uptake system in *A. nidulans*, Darlington and Scazzocchio 1967).

This phenotypic analysis was then used to distinguish 100 chlorate resistant nitrate non-utilizing *cnx*/*nia*D mutants and the genotype assigned. From this analysis only six chlorate resistant mutants were determined to be *cnx* mutants, while the remaining 94 were found to be of the *nia*D type. The ratio of a predominance of *nia*D mutants to very few *cnx* mutants agrees with the results of Cove (1976b) in *A. nidulans* when arginine or glutamate act as the sole nitrogen source when selecting for chlorate resistance.

A number of *nia*D mutants were then tested for stability and those mutants which showed reversion to nitrate utilization at less then 1 in 10^8 spores were retained for later transformation studies.

3.2.3 Further investigations of chlorate resistant mutants.

Since *C. acremonium* lacks a sexual cycle experimentation to determine if *cnx* and *nia*D mutations occur in genes which are unlinked or linked and to assess the number of genes or orientation of allelic mutations cannot be carried out by formal genetic analysis (see section 1.1). Hence protoplast fusion was attempted between a *cnx* mutant CSG27 and a number of non-reverting *nia*D mutants, namely CSG98, CSG116, CSG117 and CSG128. Pairwise crosses were made between the *cnx* mutant and each *nia*D mutant, between each *nia*D mutant and each mutant crossed to itself. The results presented in Table 3.4 show the percentage of nitrate utilizing colonies obtained from each cross.These results show that complementation between *cnx* and *nia*D mutants is relatively high and is probably due to non-allelic complementation.

Table 3.4 also demonstrates that the *nia*D mutants only complement each other at very low levels. This is indicative of allelic complementation caused by the intra-chromosomal recombination effected by crossing-over during mitosis. Hence it is likely that these mutations are located within a single gene. It is important to note that no nitrate utilizing colonies were obtained when the mutants were self crossed, showing that reversion can be ruled out.

The percentage frequency of complementation between *cnx* and *nia*D mutants is of a very similar value when using different auxotrophic markers, as

Table 3.4 Complementation of *C. acremonium* chlorate resistant mutants via protoplast fusion

Strains fused	% of viable protoplasts showing growth on nitrate MM
CSG27(*cnx*) x CSG128(*niaD*)	0.44
CSG117(*niaD*) x CSG27	0.19
CSG116(*niaD*) x CSG27	0.45
CSG98(*niaD*) x CSG27	0.76
CSG98 x CSG128	0.0008
CSG98 x CSG117	0.0023
CSG117 x CSG128	0.0068
CSG116 x CSG117	0.0005
CSG27 x CSG27	0
CSG128 x CSG128	0
CSG117 x CSG117	0
CSG116 x CSG116	0
CSG98 x CSG98	0

found by Anné and Peberdy (1976) who originally described this method of protoplast fusion in *C. acremonium*. They presumed the complementation was due to heterokaryon formation, however it is now thought that this is unlikely. The only research studies to report heterokaryosis in *C. acremonium*, following an induced parasexual cycle are those by Nüesch *et al* (1973) who found less than 10% of hyphal segments to exist as heterokaryons. They also reported the presence of diploids. However when other groups analysed recombinants (Hamlyn and Ball, 1979) only haploid recombinants and heterozygotes were observed . It is now presumed the products of protoplast fusion tend to be stable recombinant haploids and unstable heterozygotes of undefinable ploidy (Peberdy *et al*, 1986). It was most likely a mixture of these which were observed in this experiment.

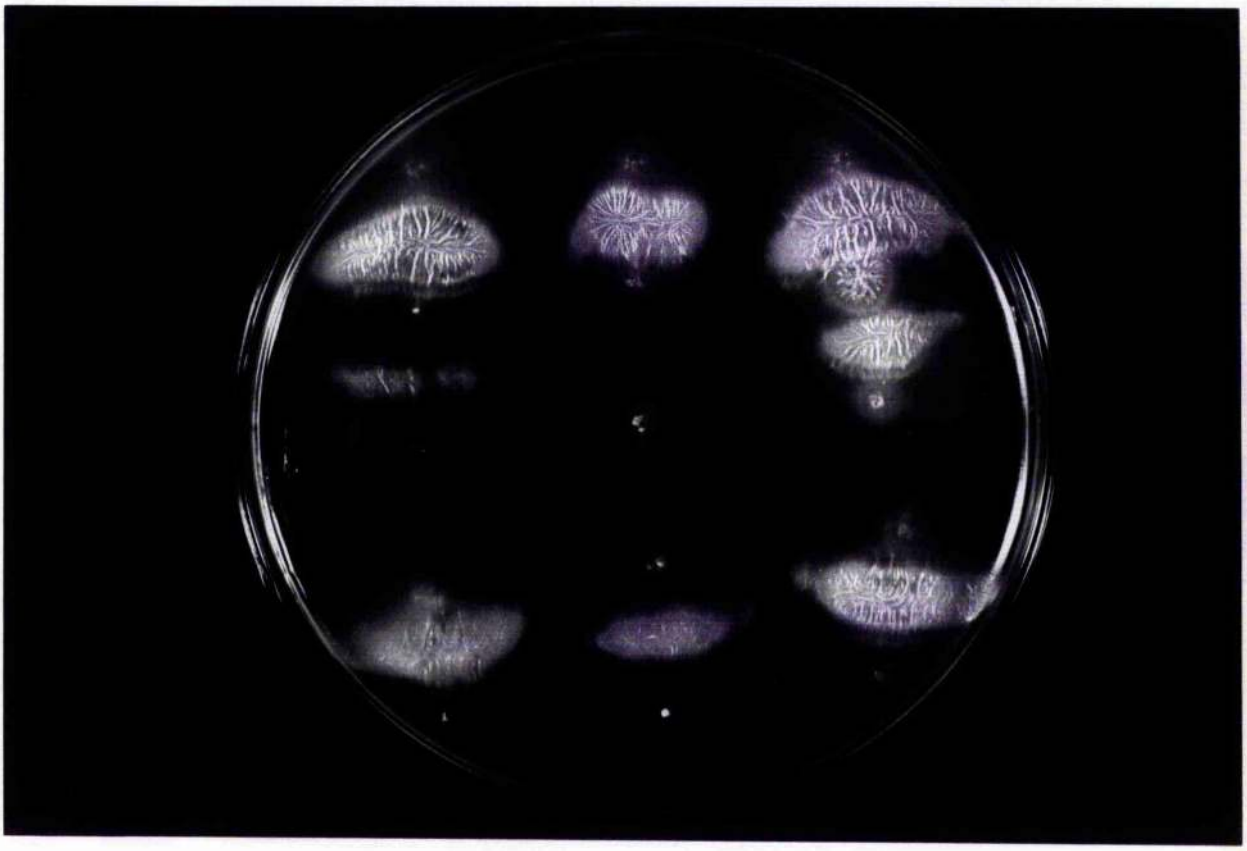
As reported in section 3.2.2, six *cnx* mutants were isolated from 100 chlorate resistant mutants analysed. These six (designated CSG2, CSG24, CSG27, CSG54, CSG57, CSG70) were tested for their ability to complement a *niaD* mutant (CSG128) and each other via hyphal anastomosis which Peberdy (1987) reported as being readily inducible in *C. acremonium*. Table 3.5 shows that all the *cnx* mutants are able to complement CSG128 yet only CSG24 and CSG70 are able to complement each other and these cannot complement the other *cnx* mutants CSG2, CSG27, CSG54, and CSG57. Figure 3.8 demonstrates complementation on MM (10mM nitrate) following hyphal anastomosis. Thus *cnx*

Table 3.5 Pairwise crosses of *C. acremonium cnx* mutants via hyphal anastomosis.

Mutant	CSG2	CSG24	CSG27	CSG54	CSG57	CSG70	CSG128
CSG2	-	-	-	-	-	-	+
CSG24	-	-	-	-	-	+	+
CSG27	-	-	-	-	-	-	+
CSG54	-	-	-	-	-	-	+
CSG57	-	-	-	-	-	-	+
CSG70	-	+	-	-	-	-	+
CSG128	+	+	+	+	+	+	+

CSG128 = *niaD* mutant
+ indicates growth
- indicates no growth

Figure 3.8 Growth of *C. acremonium* mutants on 10mM nitrate MM following hyphal anastomosis.



row 1 = CSG24
row 2 = CSG128
row 3 = CSG27
row 4 = CSG2
row 5 = CSG128

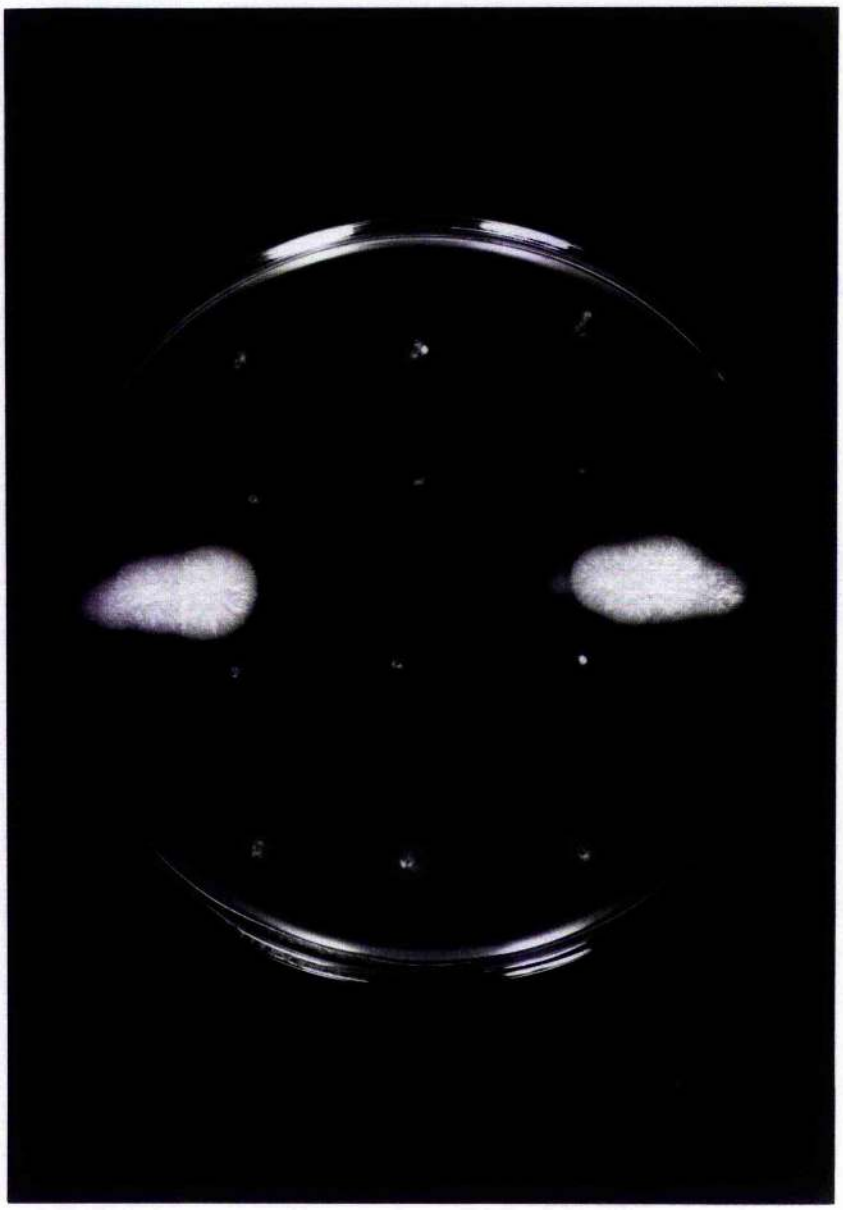
mutants can be said to fall into the following complementation classes: Class I (CSG24) which complements only Class III mutants; Class II (CSG2, CSG27, CSG54, CSG57) which fails to complement either Class I or Class III mutants; and Class III (CSG70) which complements only Class I mutants. This complex overlapping complementation pattern has also been observed in *A. nidulans* (Cove, 1979), *N. crassa* (Tomsett and Garrett, 1980), *P. chrysogenum* (Birkett and Rowlands, 1981) and *Septoria nodorum* (Newton and Caten, 1988). Thus the Class I, II and III *cnx* mutants of *C. acremonium* can be termed to be the equivalent of the *A. nidulans cnx*ABC gene class. Such a complex pattern of complementation is thought to occur because two *cnx* genes are closely linked, thus mutations in Class I or III genes are in each separate gene and thus can complement each other. Mutations in Class II are most probably deletion or overlapping mutations which affect both genes and thus complementation cannot occur (Cove, 1979). It is interesting to note that Cove (1976b) found a similar pattern of mutant distribution within the *cnx* mutants he isolated using either arginine or glutamate as sole nitrogen source, that is the majority of *cnx* mutants being of the *cnx*ABC class.

This genetic classification was further confirmed after analysis of the six *cnx* mutants for repair of the mutation by molybdate. Cove (1979) describes that *A. nidulans cnx*E mutations can be repaired by supplementation of the growth medium with 33mM molybdate

(as do Birkett and Rowlands, 1981 for *P. chrysogenum*). However none of the *C. acremonium cnx* mutants were able to utilize nitrate on MM in the presence of 33mM molybdate and thus cannot be classified as *cnx*E mutants.

Further analysis was performed using hyphal anastomosis whereby the ability of the putative *nir*A mutants (CMG10, CMG19 and CMG21) to complement *cnx* (CSG27) and *nia*D (CSG62) mutants was determined. Figure 3.9 shows that CMG10 is able to complement the mutation of CSG27 but not that of CSG62 (as was found to be the case with CMG19 and CMG21). However this complementation is much less than that observed between a *nia*D and a *cnx* mutant as a zone of growth takes up to five days longer to appear and the growth formed is more diffuse and less distinct as shown by the growths in Figure 3.8 and Figure 3.9. This pattern of complementation would be expected following hyphal anastomosis, since a *nir*A mutant could complement a *cnx* mutant due to the molybdenum cofactor being communicated between compartments. Yet to allow a *nia*D mutant to complement a *nir*A mutation communication must occur between the nuclei of the two hyphal compartments. A similar result has been observed by Cove (1979) when heterokaryons of *A. nidulans cnx* and *nir*A mutants were found to complement while *nir*A and *nia*D mutant heterokaryons complemented extremely poorly. That the complementation between a *cnx* mutation and the putative *nir*A mutant is less than that found between a *cnx* and a *nia*D mutant of *C. acremonium* is expected since only one of the hyphal compartments is being

Figure 3.9 Growth of *C. acremonium* mutants on 10mM nitrate MM following hyphal anastomosis.



row 1 = CMG10
row 2 = CSG27
row 3 = CSG62
row 4 = CMG10

complemented. Thus these results help to confirm the idea that the putative *nir*A mutants are not in fact Δ*nia*D*nii*A mutants (as was postulated in section 3.2.1) but are indeed *nir*A type mutations.

3.3 INVESTIGATION OF ALTERNATIVE TRANSFORMATION SYSTEMS FOR *C. ACREMONIUM*.

3.3.1 Determination of toxicity of hygromycin B to *C. acremonium*.

Toxicity analysis was performed using hygromycin B at a concentration of 949 units/mg (as determined by microbiological assay). Subsequent experiments using different batches of hygromycin B were adjusted to maintain this concentration. The resistance of *C. acremonium* wild type to hygromycin B was determined in two ways. First, between 10^6-10^7 spores of *C. acremonium* were plated onto CM and incubated overnight at room temperature before 5mls CM (0.5% agar) containing hygromycin B was overlayed onto the plates. Table 3.6 shows that hygromycin B does not affect the growth of *C. acremonium* until at a concentration of 15µg/ml and does not completely inhibit germination until at 50µg/ml. The second analysis involved plating the 10^6-10^7 spores on CM containing hygromycin B. The results in Table 3.7 show that hygromycin B is more toxic when used in this manner as growth is reduced at 5µg/ml and germination inhibited at 25µg/ml. Hence hygromycin B was used at a concentration of 75µg/ml in all subsequent overlay

Table 3.6 Resistance of *C. acremonium* to hygromycin B present in overlays.

Hygromycin B concentration (µg/ml)	Growth characteristics
0	5
5	5
10	5
15	3
25	2
35	1
50	0
75	0
100	0

5 indicates good growth
0 indicates no growth

3.7 Resistance of *C. acremonium* to hygromycin B present in plates.

Hygromycin B concentration (µg/ml)	Growth characteristics
0	5
1	5
2	5
5	3
7.5	3
10	2
15	1
20	1
25	0
50	0

5 indicates good growth
0 indicates no growth

transformation experiments and at 50µg/ml in all plate growth experiments to confirm hygromycin B resistance. These levels should ensure any colonies growing on these media are hygromycin B resistant.

3.3.2 Determination of toxicity of phleomycin to *C. acremonium*.

As in section 3.3.1, the resistance of *C. acremonium* to phleomycin was determined by the two methods of introducing phleomycin in an overlay or directly into the plates. Tables 3.8 and 3.9 demonstrate that phleomycin acts to prevent germination at concentrations of 150µg/ml and 75µg/ml in agar overlays and in direct plating experiments respectively. Hence phleomycin was used at a concentration of 200µg/ml in all transformations involving overlaying, and at 100µg/ml in all plate growth experiments to confirm phleomycin resistance.

3.3.3 Determination of toxicity of benomyl to *C. acremonium*.

The toxicity of benomyl was determined by plating 10^6-10^7 spores of *C. acremonium* onto CM containing benomyl. Table 3.10 shows that the growth of *C. acremonium* is hardly affected until the benomyl concentration is 5µg/ml. Once this level is present, growth and germination are completely inhibited. Therefore a concentration of 10µg/ml in osmotically buffered media was used in transformation experiments.

Table 3.8 Resistance of *C. acremonium* to phleomycin present in overlays.

Phleomycin concentration (µg/ml)	Growth characteristics
0	5
25	5
50	4
75	2
100	1
150	0
200	0
250	0
300	0

5 indicates good growth
0 indicates no growth

Table 3.9 Resistance of *C. acremonium* to phleomycin present in plates

Phleomycin concentration (µg/ml)	Growth characteristics
0	5
25	4
50	1
75	0
100	0

5 indicates good growth
0 indicates no growth

Table 3.10 Resistance of *C. acremonium* to benomyl present in plates.

Benomyl concentration (µg/ml)	Growth characteristics
0	5
0.5	5
1	5
2	3
5	0
10	0
25	0
50	0

5 indicates good growth
0 indicates no growth

3.3.4 Investigation of carbon and nitrogen sources utilized by acetamidase in filamentous fungi.

The utilization of a variety of carbon and nitrogen sources by the acetamidase of *C. acremonium* was investigated to determine a suitable selection medium involving the *A. nidulans amdS* gene (Tilburn *et al*, 1983) as the selectable marker. This gene has been used as a transformation vector in the filamentous fungi *A. nidulans* (Tilburn *et al*, 1983), *A. niger* (Kelly and Hynes, 1985) and *P. chrysogenum* (Beri and Turner, 1987; see section 1.3.5). The growth of some of these organisms on the same media as *C. acremonium* was also investigated to provide an indication of the best selection media.

These results presented in Table 3.11 show that *C. acremonium* generally exhibits a similar level of growth on the acetamide and acrylamide MM as *A. oryzae* and *A. nidulans*. Since acetamide (10mM) is used successfully as the selection media in transformations with the *A. nidulans amdS* in these fungi it becomes the obvious choice for *C. acremonium* transformation experiments. Encouragingly this is the nitrogen source used in the *P. chrysogenum* transformation procedure with the *amdS* gene, and as shown in Table 3.11, *P. chrysogenum* exhibits no growth on this media. Thus if the *A. nidulans amdS* gene allows *P. chrysogenum* (which cannot naturally use acetamide) to grow on acetamide then it should show activity within one that can, such as *C. acremonium*. It was also decided to use 10mM acrylamide as a selection system for *C. acremonium* transformation as detecting

Table 3.11 Growth of *C. acremonium, P. chrysogenum, A. oryzae* and *A. nidulans* on various minimal media.

Media	Organism			
	C. acremonium	*P. chrysogenum*	*A. oryzae*	*A. nidulans*
MM+2mM formamide	2	5	4	5
MM+2mM acetamide	2	0	2	2
MM+2mM acrylamide	0	0	1	1
MM+2mM ammonium	3	3	5	5
MM+2mM nitrate	5	3	4	4
MM+10mM acrylamide	0	0	2	1
MM+10mM acetamide	2	0	4	3
MM+50mM acrylamide	0	0	3	1
MM+50mM acetate, 2mM nitrate	1	3	3	2
MM	0	0	0	0

5 indicates good growth
0 indicates no growth

increased transformant growth against a background growth may be problematical. Acrylamide allows no background growth in *C. acremonium* and proves a good indicator of transformation by the *amdS* gene in the other fungi.

It is interesting to note that the utilization of formamide as a nitrogen source and acetate as a carbon source is quite poor in *C. acremonium*. This contrasts with the results found in the other three fungi.

3.3.5 Development of a medium for use in β-galactosidase investigations.

Since it is likely that *C. acremonium* possesses a native β-galactosidase which would be capable of hydrolysing X-gal and hence producing the characteristic blue colour of its breakdown product, it was important to develop a medium on which *C. acremonium* did not produce a β-galactosidase. It is likely that glucose would be sufficient to repress a β-galactosidase, therefore standard MM, and MM in which 1% glucose has been replaced by 0.2% glucose were analysed along with M9 media (traditionally used for bacterial β-galactosidase investigations, Maniatis *et al*, 1982) with the same nitrogen sources. To act as a positive control the above media also had glucose replaced by the β-galactosidase substrate and inducer 1% lactose. Either nitrate (10mM) or ammonium (10mM) were used as nitrogen sources.

As expected, an intense blue colour was produced in both media when lactose was present, indicating that *C. acremonium* does infact possess a native

β-galactosidase. With 0.2% glucose as the carbon source an intense blue colour was seen in M9 medium, whereas only a faint blue colour was seen in MM. Finally, when 1% glucose acted as the sole carbon source a faint blue colour was seen with M9 medium and no blue colour could be observed with MM. Hence MM (10mM nitrate) was to be used in any investigations involving expression vectors linked to *E. coli* β-galactosidase activity.

3.4 TRANSFORMATION OF *C. ACREMONIUM* WITH ALTERNATIVE SELECTION SYSTEMS TO NITRATE UTILIZATION.

3.4.1 Transformation and selection for antibiotic resistance.

Section 1.1 describes the development of a transformation system for *C. acremonium* based on the *E. coli* hygromycin B resistance gene (Skatrud *et al*, 1987). We used this system to demonstrate that transformation was possible using the *niaD* strain CSG116 within our laboratory. Using the transformation procedure outlined in Section 2.5.2, experiments were performed using the vector pIH1. A regeneration frequency of 0.5-10% was achieved in all experiments, with the transformants appearing after six to 12 days incubation. The efficiency of transformation varied between one to 10 transformants/μg DNA. This frequency of transformation is almost a hundred times greater than that achieved by Queener *et al* (1985), however, their system used a plasmid construct linking the *E. coli* hygromycin

resistance gene to the *S. cerevisiae* phosphoglycerol kinase gene promoter. When Skatrud *et al* (1987) replaced this promoter with the *C. acremonium pcb*C promoter to form a plasmid very much like pIH1 they achieved transformation frequencies of around 20/µg DNA. Hence we have achieved transformation at a very similar level to Skatrud *et al* (1987). When transformants exhibiting hygromycin B resistance were isolated from the regeneration medium, only 75% exhibited growth on CM containing 50µg/ml hygromycin B, thus only these were counted as true transformants. The remaining 25% had most probably grown through an area of thin selective overlay and thus were not transformants. Figure 3.10 demonstrates the growth of a number of transformants on hygromycin B containing media.

A level of 20 transformants/µg DNA is similar to that that found in other fungi, such as *Colletotrichum trifolii* (Dickman, 1988) and *Fusarium oxysporum* (Kistler and Benny, 1988), when using hygromycin B selection. However in these systems heterologous promoters were used. When Wang *et al* (1988) transformed *Ustilago maydis* to hygromycin B resistance using a homologous heat shock promoter they achieved frequencies of up to 1000/µg. These results would tend to indicate that *C. acremonium* may be more difficult to transform efficiently than the fungi mentioned above, since a homologous promoter is being used for the *C. acremonium* transformation. It should be noted however, that the low transformation frequencies achieved by Queener *et al* (1985) with a

Figure 3.10 Growth of *C. acremonium* transformants on CM (right) and CM containing hygromycin B (left) .

row 1 = *C. acremonium* pIH1 transformants
row 2 = *C. acremonium* pIH1 transformants
row 3 = CSG116 (left) and wild type (right)

heterologous promoter is similar to that achieved by Rodriguez and Yoder (1987) using a heterologous promoter linked to the hygromycin B resistance gene when transforming *Glomerella cingulate*. *C. acremonium* therefore may not be unique in the problem of low transformation efficiency.

A second positive selection system investigated was that of phleomycin resistance conferred by a number of vectors, pUT322, pAN8-1 and pUT701. The constructs contain the *S. hindustanus* phleomycin resistance gene linked to a *S. cerevisae*, an *A. nidulans* and its natural promoter respectively. Transformation was achieved with all three vectors, however the frequencies in each case were lower than 0.1/μg DNA. Hence further investigations were not performed with this system .

These results are in contrast to those found by Kolar *et al* (1988) who used phleomycin resistance for transformation of *P. chrysogenum* with resultant frequencies of up to 20 transformants/μg.

The ability of the *N. crassa* mutated β-tubulin gene to transform *C. acremomium* to benomyl resistance was also studied. When using the vector pGA3 the protoplasts were initially regenerated on MM (10mM nitrate) in which glucose had been replaced by starch. This was intended to induce the *A. niger* glucoamylase promoter to which the *N. crassa tub*-2 gene was connected. However no transformants were observed so the protoplasts were instead plated onto osmotically stabilized CM containing benomyl. This was also the procedure when the vector pBT6

was used. Again no transformants were observed with either vector. The benomyl concentration in all these experiments was 10µg/ml. Since this may be applying too high a selective pressure, the concentration was reduced to 5µg/ml for a number of transformations with the vector pBT6. However, the benomyl concentration was not sufficient to cause inhibition of germination in the transformations and therefore no transformants were observed. This result is surprising since this vector construct has been used to transform the Ascomycete fungi *Gaeumannomyces graminis* (Henson *et al*, 1988) and *C. trifolli* (Dickman, 1988) to benomyl resistance at frequencies of 1-4/µg DNA.

3.4.2 Transformation of *C. acremonium* with the *A. nidulans amdS* gene.

Transformation was performed as described in Section 3.4.1. The protoplasts were spread onto osmotically stabilised MM supplemented with either 10mM acetamide or 10mM acrylamide. Despite numerous experiments no vigorously growing colonies were observed on either medium. This result is unusual since this system has been used successfully for the transformation of *Trichoderma reesei* (Pentilla *et al*, 1987), *Cochliobolus heterostrophus* (Turgeon *et al*, 1985), *G. cingulata* (Rodriguez and Yoder, 1987), *P. chrysogenum* (Beri and Turner, 1987), *A. niger* (Kelly and Hynes, 1985) aswell as *A. nidulans* (Tilburn *et al*, 1983). This result, taken in conjunction with those from section 3.4.3

demonstrates that *C. acremonium* is extremely difficult to transform when a heterologous gene or promoter is used. The only heterologous promoters to successfully transform *C. acremonium* were those linked to the phleomycin resistance gene or the hygromycin B resistance gene (Queener *et al*, 1985). Neither the *N. crassa tub*-2 or the *A. nidulans amdS* genes were expressed in *C. acremonium*. Thus these results would indicate that *C. acremonium* will only transcribe a gene at very low frequency when a foreign promoter is present. It also seems that *C. acremonium* has difficulty expressing the products of more complex genes than bacterial genes. This may be due to an inability to recognise or process foreign introns.

3.5 ISOLATION AND CHARACTERIZATION OF THE *C. ACREMONIUM NIAD* GENE.

3.5.1 Isolation of the *C. acremonium niaD* gene.

A gene library of *C. acremonium* wild type constructed in the phage vector EMBL3 was kindly supplied by Glaxochem plc. This was plated out to produce 30000 individual clones which were transferred to nitrocellulose. By screening this number of clones there should be a greater than 99.99% probability that the *C. acremonium niaD* is present. This library was screened by heterologous hybridization to a 2.4kb *Xba*I fragment of the *A. nidulans niaD* gene (performed by S. Gurr, unpublished) which contains purely protein coding sequences (and introns) of the 5′ region of the gene

(Johnstone *et al*, 1989). It was known that there was extremely low homology between this fragment and *C. acremonium* genomic DNA (Gurr and Spence, per. comm.), hence hybridization conditions between the *A. nidulans* *Xba*I fragment and the gene library were such that the stringency was low. From this first screen around 60 positively hybridizing clones were observed. All of these were isolated and subjected to a secondary screen under the same conditions (performed by S. Gurr, unpublished). This screen revealed 11 strongly hybridizing clones (which also appeared on a duplicate screen). Four of these clones were taken (designated λSTA1, λSTA4, λSTA6, and λSTA7) and the DNA isolated. Each clone was then digested with each of the restriction enzymes *Hin*dIII, *Bam*HI, *Eco*RI and *Sal*I and these digests again hybridized to the *A. nidulans* 2.4kb *Xba*I fragment (performed by S. Gurr, unpublished). In each of the four clones a strongly hybridizing 8.6kb *Eco*RI fragment could be identified. This fragment was therefore isolated from λSTA6 and sub-cloned in to the *Eco*RI site of pUC18 and the new construct termed pSTA700 (performed by S. Gurr, unpublished). It was this vector that we presumed to contain the *C. acremonium nia*D gene. However this had to be shown by the further analysis outlined below.

3.5.2 Restriction endonuclease mapping of the *C. acremonium* subclone.

pSTA700 was taken and restriction enzyme digested with various enzymes (including double and triple

digestions) until the position of the site of cutting of each enzyme could be determined. Figure 3.11 shows the restriction endonuclease map of the insert of pSTA700. It is present within the *Eco*RI site of pUC18, with the rest of the multi-cloning site being on the 3′ side of the insert as it is shown. Additionally it was found that this insert contained no internal *Hin*dIII site.

3.5.3 Hybridization of pSTA700 to *C. acremonium* genomic DNA.

To confirm that the insert of pSTA700 was *C. acremonium* DNA it was hybridized under stringent conditions to *C. acremonium* DNA which had been digested with either *Kpn*I, *Bgl*II or *Eco*RI. Figure 3.12 demonstrates that the pSTA700 fragment hybridizes to a 8.6kb *Eco*RI genomic fragment, 1.2kb, 2.4kb and 6.4kb *Bgl*II fragments and 2.8kb, 3.8kb and 8.6kb *Kpn*I fragments. Since the 8.6kb *Eco*RI fragments, the 1.2kb *Bgl*II fragments and the 3.8kb *Kpn*I fragments are seen as internal sites within the restriction map of pSTA700 (Figure 3.11) the clone of pSTA700 contains *C. acremonium* DNA in which no gross rearrangements have occurred during its cloning. It is noteworthy that the insert of pSTA700 did not hybridize to *A. nidulans* or *P. chrysogenum* genomic DNA under the same hybridization conditions (Gurr, per. comm.). It was also found that this fragment hybridized to a 20kb *Hin*dIII *C. acremonium* genomic fragment. This will be demonstrated in later analysis.

Figure 3.11 Restriction endonuclease map of the insert of pSTA700.

B = *Bam*HI	K = *Kpn*I
Bg = *Bgl*II	S = *Sal*I
Bs = *Bsc*II	Sa = *Sac*II
E = *Eco*RI	X = *Xba*I

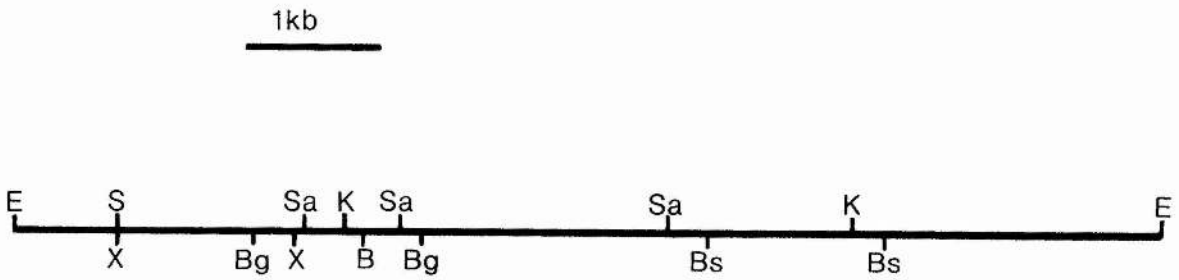
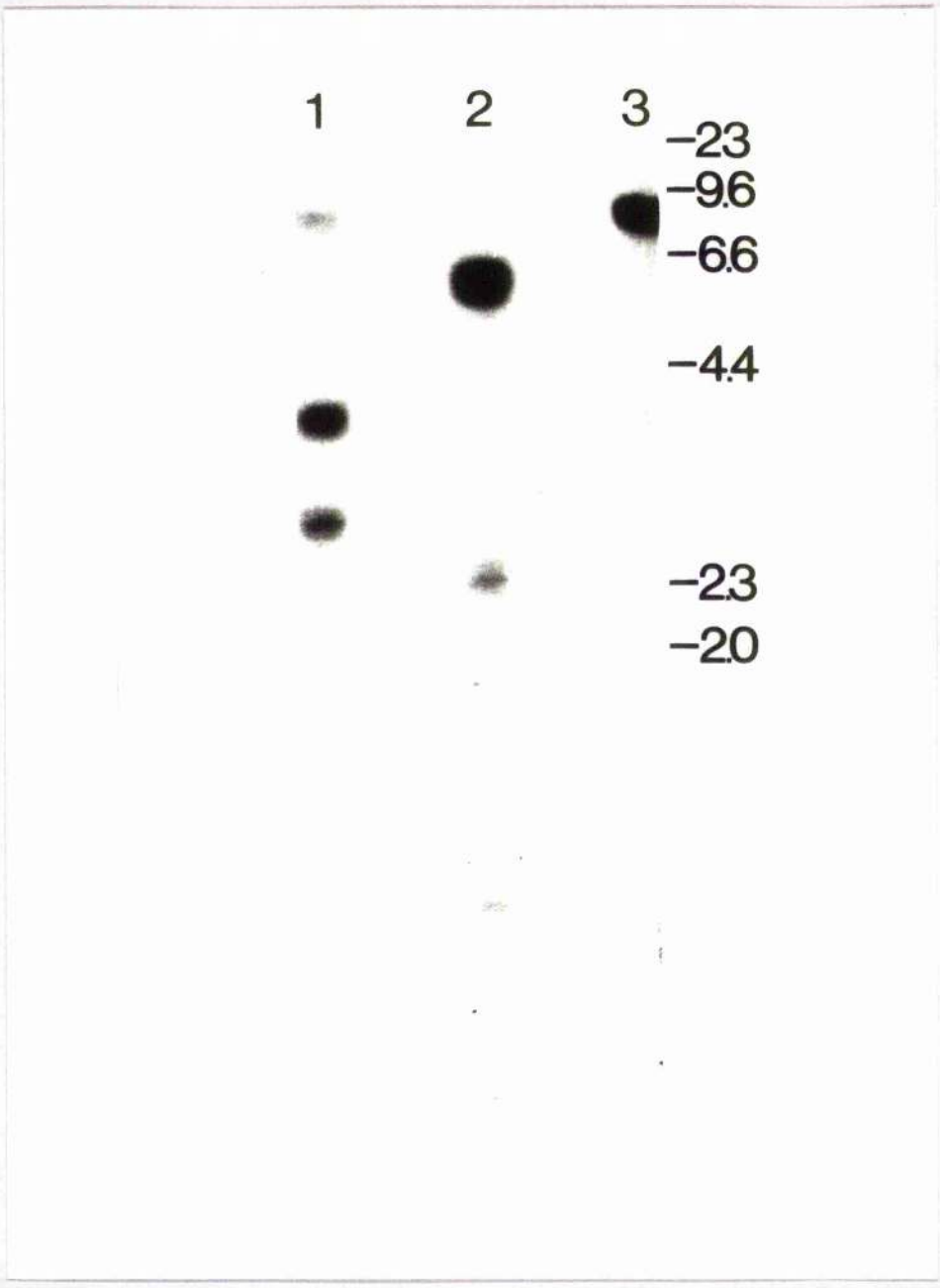


Figure 3.12 Hybridization of pSTA700 insert to *C. acremonium* wild type genomic DNA digested with lane 1, *Kpn*I; lane 2, *Bgl*II and lane 3, *Eco*RI.



3.5.4 Sequence analysis of the insert of pSTA700.

The nucleotide sequences for the plant nitrate reductase genes from *Arabidopsis thaliana* (Crawford *et al*, 1988) and *Nicotiana tabacum* (Calza *et al*, 1987) and the *A. nidulans niaD* gene (Johnstone *et al*, 1989) are now available. Kinghorn and Campbell (1989) have recently converted these into the predicted amino acid sequence for each protein and then compared by dot matrix similarity the homology between the three proteins. This revealed that there were quite large regions of similarity between the three genes. It was found that over a region at the start of the enzymes (between intron two and intron three of the *A. nidulans niaD* gene) an area of high homology could be seen contained within a 124bp *PstI-AccI* fragment of the *A. nidulans niaD* gene which coded for this region. The predicted amino acid sequence of this region is shown in Figure 3.13 along with the homology it shows to the plant amino acid sequences.

The codon usage in translation is known for two genes of *C. acremonium*, namely *pcbC* and *cefEF* (from Gurr *et al*, 1987). Using the bias found in these two genes the predicted *C. acremonium* nucleotide sequence was determined for the amino acid sequence within the *PstI-AccI* fragment. Comparing this to the *A. nidulans* nucleotide seqence within the *PstI-AccI* fragment it was found that there would be a 25% mismatch betweem the two genes over this region. Hence this *PstI-AccI* fragment was isolated and hybridized to pSTA700 which had been

Figure 3.13 Comparison of the predicted amino acid sequence of the *A. nidulans* nitrate reductase protein between the *Pst*I and *Acc*I restriction enzyme site of the *A. nidulans niaD* gene to the *N. tabacum* and *A. thaliana* amino acid sequences of this region (Kinghorn and Campbell, 1989).

```
                          ***************    ** ** *  **
A. thaliana     VSEFAYREFAATLVCAGNRRKEQNMVKKSKGFNWGSAGVST
N. tabacum      VNEFPCRELPVTLVCAGNRRKEQNMVKQTIGFNWGAAAVST
A. nidulans     LQNYDQITAPITLVCAGNRRKEQNTVRKSKGFSWGSAALST
```

*Indicates homology between the 3 sequences

digested with various restriction enzymes under low stringency hybridization conditions (performed by S. Gurr, unpublished). Hybridization was found to occur to the 1.2kb *Bgl*II fragment of pSTA700. Since it was presumed that this fragment therefore contained a region of the *C. acremonium nia*D gene, it was subcloned into M13 and partial sequence analysis was carried out via di-deoxy sequencing. A continuous 246bp stretch of nucleotide sequence was confirmed on one strand only and then compared along to the *A. nidulans nia*D gene sequence, along with the predicted amino acid sequence. This is shown in Figure 3.14. Of note is the presence of a stop codon at amino acid position 29, which can be explained by one of two reasons. Firstly it may be present within an intron at this point. This is an interesting possibility since the amino acids around this region do not show high homology with the *A. nidulans* amino acids and seem to be inserted as an extra region into the gene compared to the *A nidulans* sequence. To be an intron it requires a 5′ start site of GT and a 3′ end site of AG, with an internal lariat formation sequence of CTAAC (Gurr *et al*, 1987). There is one possible intron termination site of TAG between amino acid 30 and 31. Close to this is a possible lariat sequence of CTGTAACC, which would conform to the consensus lariat sequence of PyGCTAACN determined by Gurr *et al* (1987). The distance of this sequence from the 3′ splice site is 4 bp, which agrees with the consensus distance of less than 22 bp in filamentous fungi, as determined by Gurr *et al* (1987).A

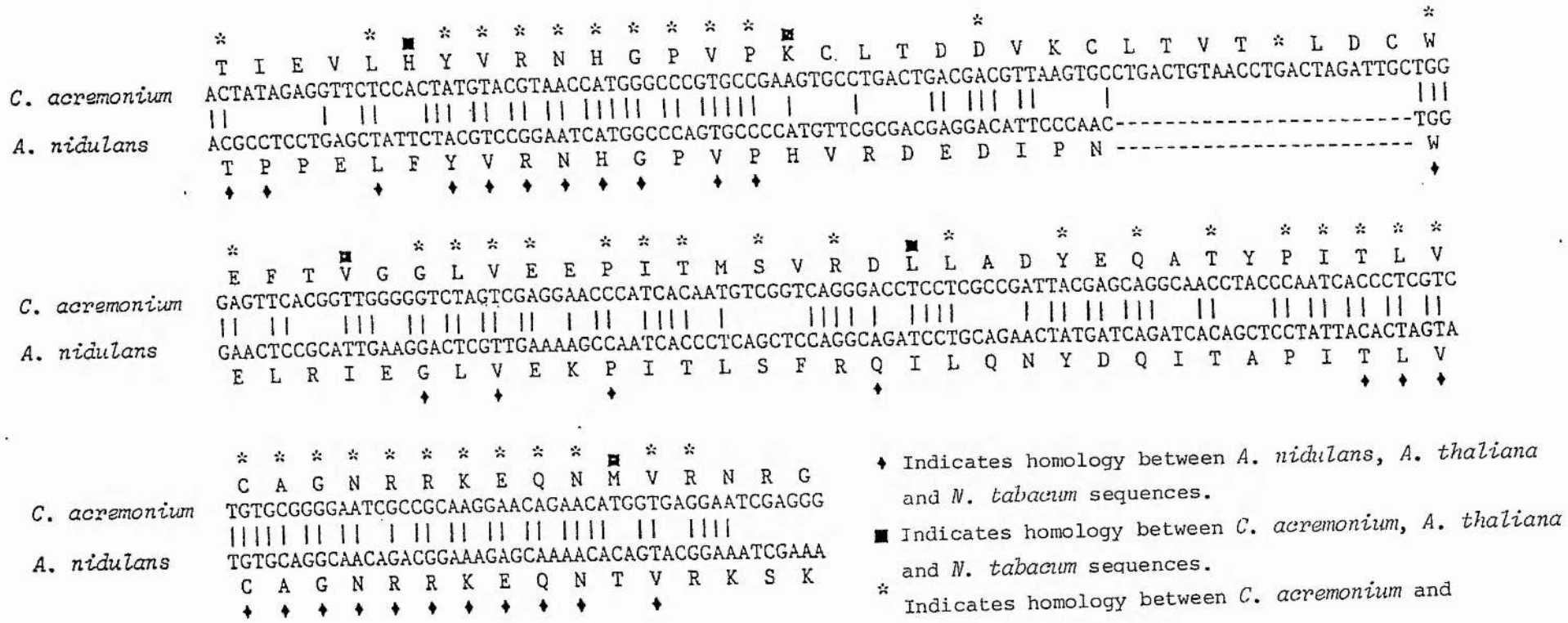


Figure 3.14 Comparison of the nucleic acid sequence and predicted amino acid sequence of part of pSTA700 to the nucleic acid sequence and predicted amino acid sequence of nitrate reductase from *A. nidulans*.

possible start site for the intron could be the GTT at amino acid 22. An alternative start site could be the GTG between amino acids 16 and 17, however this would then exclude some amino acids which show homology to the other nitrate reductase amino acid sequences and thus this site may be unlikely. If the intron start site was at amino acid 22 then the intron size would only be 28 bp long. This would make it the smallest intron so far discovered in filamentous fungi. The next smallest introns being the first intron of the *alc*A gene (Gwynne *et al*, 1987) and the sixth intron of the *ben*A gene of *A. nidulans* which are 48 b.p. long (from Gurr *et al*, 1987).

The alternative to the scenario of the TGA termination site being within an intron is that a mistake has been made within the sequencing of this region, however this does not seem likely as this region was determined twice and clearly gave the TGA sequence. Ideally the opposite strand to the one sequenced should be determined as this would enable us to confirm the sequence to be correct. Unfortunately time pressures did not allow this undertaking.

When the determined amino acid sequence of the *C. acremonium* clone is compared with the *A. nidulans* nitrate reductase sequence, areas of similarity can be observed. It is important to note that where these areas of homology occur, similarity is also seen with the plant nitrate reductase proteins. This therefore indicates that this similarity does not occur by chance and that the clone pSTA700 does in fact contain sequences coding for

nitrate reductase. The overall amino acid homology between *A. nidulans* and *C. acremonium* is 59% over this region. This compares to 39% homology between *A. nidulans* and the plant enzymes and 43% between *C. acremonium* and the plant enzymes. These results show that as expected, there is greater homology between the two fungal proteins than between the fungal and plant proteins. Interestingly there is also greater homology between *C. acremonium* and the plant proteins than between *A. nidulans* and the plant enzymes. However due to this only being a small amount of sequence it would be impossible to conclude that the whole protein shows greater similarity.

Despite an amino acid homology of 59% between *C. acremonium* and *A. nidulans* the nucleotide homology is only 52%. This lack of nucleotide homology is more clearly demonstrated in the region between amino acids 62 and 79 which exhibit 94% amino acid homology while the nucleotide homology is only 66%. This indicates that the two organisms are not phylogenetically closely related. This confirms the analysis of Chen *et al* (1984) who, after studying 5S rRNA sequences placed *C. acremonium* as a Pyrenomycete while *A. nidulans* was classified in the dissimilar class of Plectomycetes. It also agrees with the analysis of Weigal *et al* (1988) who detemined *C. acremonium* to be phylogenetically dissimilar to both *A. nidulans* and *P. chrysogenum* after analysis of the *pcb*C gene structure.

Another characteristic of the *C. acremonium* sequence which agrees with results previously observed in

C. acremonium is the percentage GC content. The GC value for the *C. acremonium* genome has been found to be 55% (Salinas and Penalva, unpublished; from Ramon *et al*, 1987) and the percentage GC from this region of pSTA700 is 56%.

Although this data strongly indicates that the gene cloned is the *C. acremonium nia*D gene it does not prove it. Evidence for this will come from the use of pSTA700 to transform *C. acremonium nia*D mutants to nitrate utilization.

3.6 TRANSFORMATION OF CHLORATE RESISTANT MUTANTS.

3.6.1 Transformation of *A. nidulans*.

A number of transformations were performed on the *A. nidulans nia*D mutant B344 to determine the performance of vectors containing the available *nia*D genes. This mutant was successfully transformed with the vectors λAN8a, pSTA10 and pSTA12, a summary of which is shown in Table 3.12. These levels of transformation are quite low considering that λAN8a contains the homologous *nia*D gene and the plasmid vectors contain the *nia*D gene from the phylogenetically close *A. niger*. Transformation in *A. nidulans* using other selectable markers, for example the *arg*B gene, Johnstone *et al* (1985), tend to result in higher efficiences. However, the frequencies obtained with the *nia*D vectors are similar to that observed by Greaves (per. comm.) using λAN8a in this and other mutant *nia*D strains.

Table 3.12 Summary of *niaD* transformations in *A. nidulans*.

Vector	Maximum transformation efficiency*
λAN8a	2.5
pSTA10	2.7
pSTA12	1.1
linear pSTA10	8.1

* transformants/μg DNA

It is interesting to note that the frequency of transformation obtained with pSTA12 was not increased despite the presence of the *ans*-1 sequence. This is in contrast to other *A. nidulans* transformation systems where *ans*-1 has been found to increase efficiencies by up to 100 fold (Ballance and Turner, 1985; Cullen *et al*, 1987).

When the vector pSTA10 was linearized via restriction at a unique *Nru*1 site, transformation frequencies were increased by up to four fold. This was expected, since linearization of pSTA10 resulted in increased transformation in *A. niger* (Unkles *et al*, 1989b; see section 1.3).

3.6.2 Transformation of *P. chrysogenum*.

The non-reverting *P. chrysogenum niaD* mutant designated *niaD*19 was used a a recipient strain in all transformations. Initially protoplasts were regenerated in osmotically stabilized agar overlays. Under these conditions regeneration of protoplasts on non-selective media occurred at less than 0.1%. Hence protoplasts were subsequently spread onto osmotically stabilized media. In these experiments regeneration varied between 5-40% with the average being approximately 15%. Successful tranformation was achieved using the *niaD* vectors pSTA10, pSTA12, pSTA14, pSTA700 and λAN8a. A summary of the transformation results are shown in Table 3.13.

Transformations with vectors pSTA10, pSTA12, pSTA14 and λAN8a resulted in approximately 40% of

Table 3.13 Summary of *nia*D transformations in *P. chrysogenum*.

	Vector					
	pSTA10	pSTA12	pSTA14	pSTA700	λAN8a	linear pSTA10
Maximum* transformation efficiency	9.0	5.8	3.0	2.5	19.4	15.6
Average*+ transformation efficiency	6.2	5.2	2.1	1.7	14.5	13.3

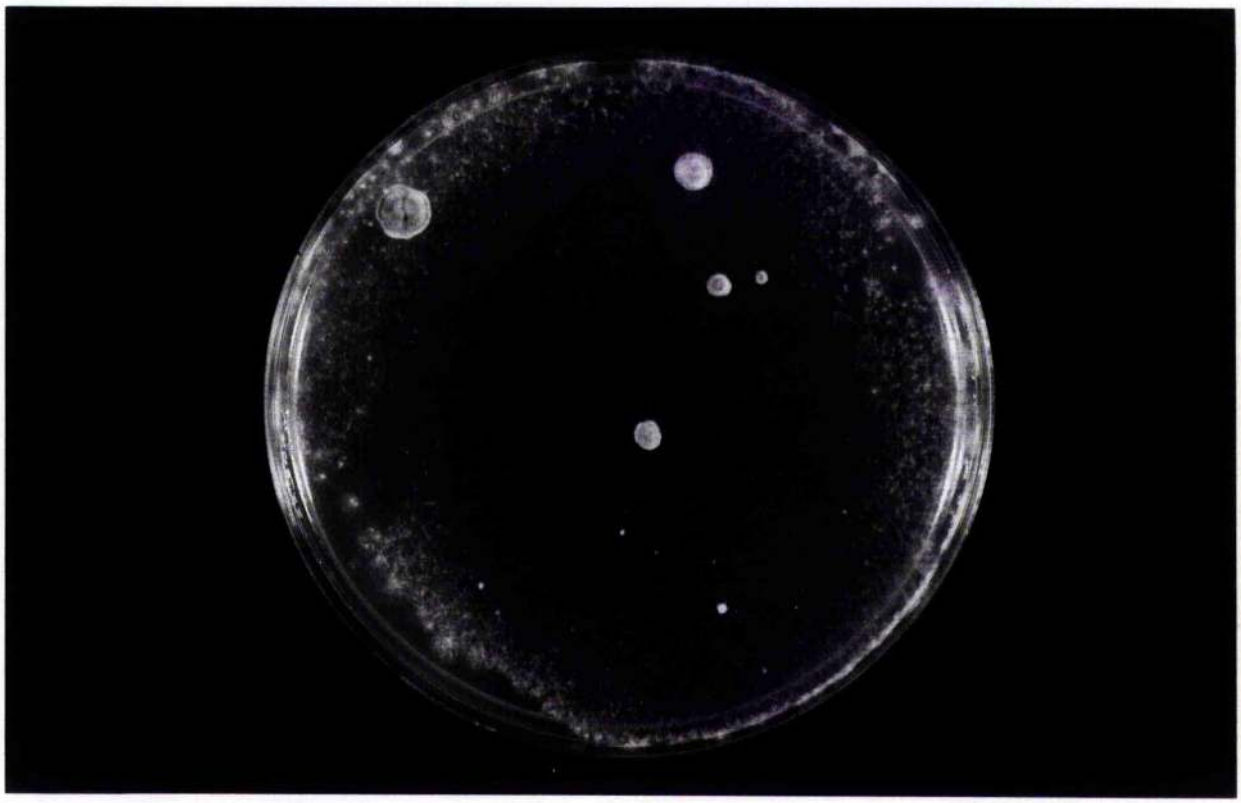
* transformants/μg DNA
\+ average of three independent experiments

transformants appearing after seven days incubation, while the remaining slower growing colonies appeared over 14 days. Transformants obtained using pSTA700, however did not regenerate until after 12 days growth, and continued to appear for up to 25 days. Both classes developed to mature colonies on further incubation, and continued to grow on nitrate MM on subsequent inoculation via growth on non-selective CM. Less than 5% of transformants exhibited mitotic instability by failure to grow following sub-culture. The background growth was extremely low since the growth of the recipient *niaD* strain on nitrate as sole nitrogen source is very poor. Transformant growth was therefore obvious to detect, as shown by Figure 3.15, with the transformants being easily isolated from background growth.

This system produced some differences to those previously reported for *P. chrysogenum*, resulting in some advantages and disadvantages. In all other *P. chrysogenum* transformations, regeneration is performed in agar overlays, except the *trpC* based systems of Picknett *et al* (1987) and Sanchez *et al* (1987) which plate out protoplasts onto solidified media. However Picknett *et al* (1987) found that only transformation was affected by use of agar overlays and not regeneration. Thus this inability to regenerate in agar overlays is particular to this strain of *P. chrysogenum*.

The period after which transformed colonies appears varies with the selection system. Most *P. chrysogenum* transformation systems result in

Figure 3.15 Regeneration of *P. chrysogenum niaD* transformants on osmotically stabilized nitrate MM.



transformants appearing between three to eight days incubation. The *niaD* system tends to require longer periods of incubation before all the transformants are revealed. This reflects the severity of the selection system and that the protoplasts require a longer period of incubation to allow gene expression. The transformants exhibit good growth and isolation from the background is easy as shown by Figure 3.15. This is in contrast to the findings of Picknett *et al* (1987) and Bull *et al* (1987) who experienced problems in isolating from background growth. Another problem incurred in some transformation systems is the appearance of "false" transformants (such as may occur by natural mutation or by the inherent inefffectiveness of the selection system) or abortive colonies. No abortive type colonies were observed in any *niaD* transformations and greater than 95% of isolated colonies were stable on selective media after growth on non-selective media. It is noteworthy that no nitrate utilizing colonies were observed when the vectors were absent from the transformation procedure.

One of the most important comparisons to make with other *P. chrysogenum* transformation systems is that of efficiency. The levels of transformation achieved with all vectors is of a similar level to the systems of Carramolino *et al* (1989), Bull *et al* (1988) and Beri and Turner (1987) and only slightly less than the *trpC* system of Sanchez *et al* (1987) and Picknett *et al* (1987; as only 14% of the 300-1800 transformants/µg DNA could be isolated). The *pyr4* based system of Cantoral *et al* (1987)

produced frequencies of greater than 1000 transformants/µg DNA. However, this frequency was obtained using a vector containing the *A. nidulans ans*-1 sequence. When the *ans*-1 sequence was incorporated into the vector pSTA12, a slight decrease in transformation efficiency was observed. This may be due to the *ans*-1 sequence having no effect in this strain of *P. chrysogenum* under our transformation conditions or may be due to the position and orientation of the *ans*-1 sequence within the vector.

It is possible to relate the transformation frequencies obtained with the vectors pSTA10, pSTA14, pSTA700 and λAN8a to one another. Vectors pSTA10 and λAN8a produce greater transformation frequencies when transformations were performed in parallel with more than one vector. Therefore it may be postulated that the *A. oryzae* and *C. acremonium niaD* genes are expressed less efficiently than the *A. nidulans* and *A niger niaD* genes in *P. chrysogenum*.

A comparison of these transformation frequencies can also be made with those achieved in other organisms using the *niaD* selection system. Similar levels of efficiency, between 1-10/µg DNA, have been achieved by Malardier *et al* (1989) in *Fusarium oxysporum* and by Daboussi *et al* (1989) in *Colletotrichum lindemuthianum, F. oxysporum, Beauveria bassiana, Penicillium caseicolum, Aphanocladium album, Nectria haematococca* and *Pyricularia oryzae* using the *A. nidulans niaD* gene. This indicates that these levels are standard for heterologous

transformation using this system. Higher frequencies of homologous transformation have been achieved recently in *A. oryzae* (Unkles *et al*, 1989a) and *A. niger* (Unkles *et al*, 1989b; Campbell *et al*, 1989) with frequencies in excess of 1000 transformants/μg DNA. Frequencies such as these were only obtained when the vector (pSTA10) was linearized. Table 3.13 demonstrates that an increase in transformation efficiency is also observed in *P. chrysogenum* when pSTA10 is linearized (approximately two fold). This phenomenon has been previously observed in other filamentous fungi (see section 1.3). It may also explain why transformation with λAN8a results in higher levels of transformation than pSTA10, since this is a linear vector. Unkles *et al* (1989b) performed transformations of *A. niger* with pSTA12, with very similar results to those found in *P. chrysogenum*. This confirms that the *ans*-1 sequence is not in the correct position for its activity to be seen or that it does not work in conjunction with *nia*D selection system. This last supposition is likely due to the observation of Cantoral *et al* (1987) that the *ans*-1 helps to stabilize integration in *P. chrysogenum* with the result of fewer abortive transformants. Since abortive colonies have not been observed in any *nia*D transformation systems the *ans*-1 sequence cannot exert any effect.

A total of 12 *P. chrysogenum* transformants, six derived from *A. niger* gene transformations (designated PTG) and six from *A. nidulans nia*D gene transformations (designated PTD) were analysed for insertion of the

vectors into the transformants genome, since this is by far the most common form of vector stabilization in filamentous fungi (see section 1.3). Transformant and wild type genomic DNA was isolated and restricted with *Hin*dIII and probed with linearized pUC18 for *A. niger* gene transformants (Figure 3.16) or with *Hin*dIII digested λ for *A. nidulans* gene transformants (Figure 3.17). As expected all transformants showed integration of the vectors into the host genome. This also indicates that integration of the *nia*D gene has not occurred solely via a gene conversion (type III integration; from Fincham, 1989) as bacterial or bacteriophage sequence are seen in all transformants. It is impossible to say whether integration has occurred by homologous (type I) integration or via integration at an ectopic site (type II integration). The only indication of what may have occurred is in the case of transformant PTG2. As shown by Figure 3.16, PTG2 contains only a single band of homology to pUC18 showing that the break point for integration has occurred within the *A. niger* insert of pSTA10, since only one *Hin*dIII site is present in pSTA10 at the pUC polylinker region. This does not automatically indicate that homologous integration has taken place since the *A. niger* insert contributes 75% of the vector, thus even ectopic integrations are more likely to occur via a break point within this region. Figure 3.17 shows that all *A. nidulans nia*D gene transformants exhibit common 23kb and 6.5kb fragments, except PTD3 which lacks a 23kb band. These fragments correspond to the left (23kb) and right

Figure 3.16 Southern hybridization analysis of DNA from *A. niger niaD* gene, *P. chrysogenum* transformants using pUC18 as the probe to *Hin*dIII digested DNA from PTG1 (lane 1), PTG2 (lane 2), PTG3 (lane 3), PTG4 (lane 4), PTG5 (lane 5), PTG6 (lane 6) and wild type (lane 7).

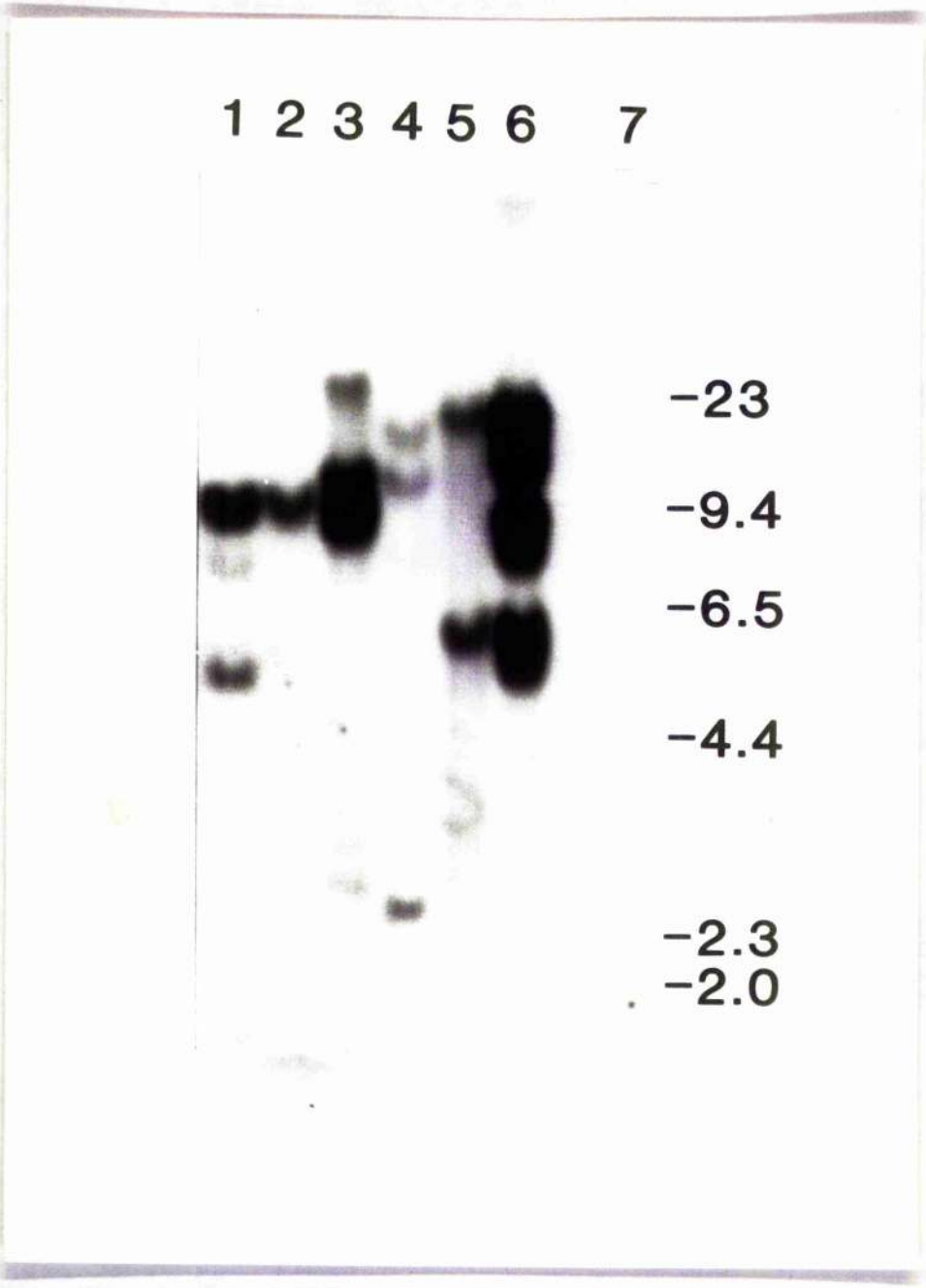
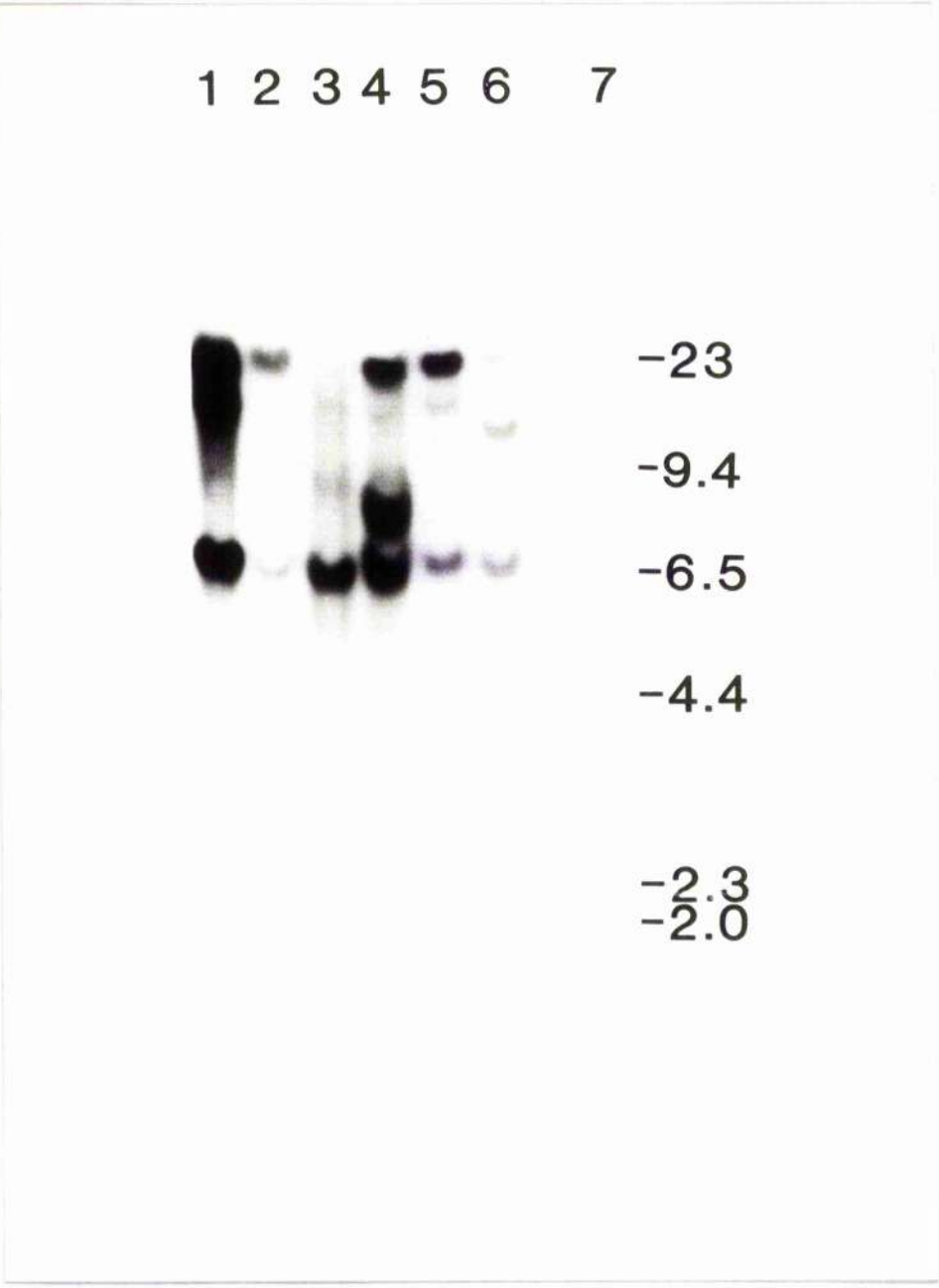


Figure 3.17 Southern hybridization analysis of DNA from *A. nidulans niaD* gene, *P. chrysogenum* transformants using *Hin*dIII digested λ as the probe to *Hin*dIII digested DNA from transformants PTD1 (lane 1), PTD2 (lane 2), PTD3 (lane 3), PTD4 (lane 4), PTD5 (lane 5), PTD6 (lane 6) and wild type (lane 7).



(6.5kb) arms of EMBL3 to the first and from the last *Hin*dIII sites of the *A. nidulans* recombinant molecule λAN8a, respectively (Johnstone *et al*, 1990). That the 23kb band is missing from PTD3 indicates that DNA rearrangements have taken place during the integration of the vector into the transformants genome.

It is obvious from the analysis of Figures 3.16 and 3.17 that various levels of integration have occurred. For example transformants PTG3, PTG6 and PTD1 are likely to be the result of multiple integration events due to the number and intensity of the positively hybridizing signals. In contrast, transformants PTG2, PTG4, PTG5, PTD2 and PTD6 are likely to be single or low copy number transformants. This phenomenon of multiple and tandem integration and rearrangements have been observed by Sanchez *et al* (1987), Beri and Turner (1987), Bull *et al* (1988) and Picknett *et al* (1987) in previous *P. chrysogenum* transformations.

3.6.3 Transformation of *C. acremonium*.

A non-reverting *nia*D mutant, CSG116, was chosen as the recipient in all *C. acremonium* transformations to nitate utilization, except where mentioned.

Vectors pSTA10, pSTA14 and λAN8a were all used to transform either CSG116 or CSG98 (also a non-reverting *nia*D mutant), however, despite numerous attempts, no transformants were observed. The fact that the *A. nidulans*, *A. niger* and *A. oryzae nia*D genes failed to complement *C. acremonium nia*D mutants may be due to

C. acremonium being unable to recognise their promoter and regulation sequences or being unable to correctly process the genes. Alternatively this may be due to the heterologous nitrate reductase apo-protein not operating correctly with the necessary co-factors (such as the molybdenum cofacter) of *C. acremonium*. The latter explanation does not appear likely since fungal *nia*D gene products can complement *in vitro* with plant molybdenum cofactors (Mendel *et al*, 1981).

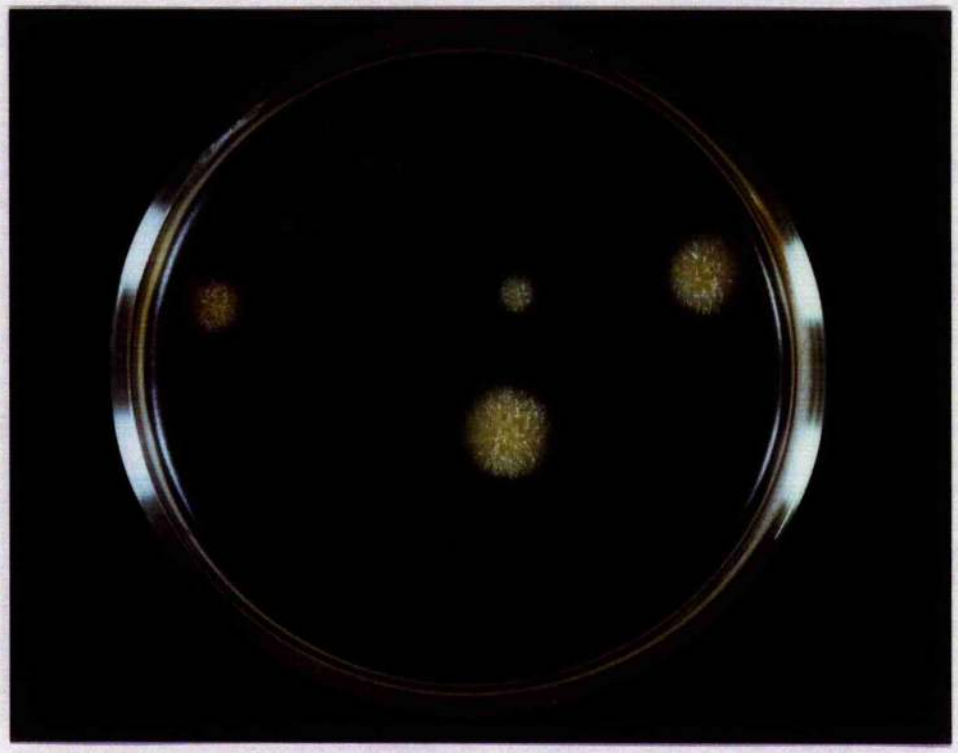
The only vectors able to complement a *C. acremonium nia*D mutant were λSTA6 and pSTA700, which were presumed to contain the *C. acremonium nia*D (see section 3.5). As complementation does occur it seems likely that pSTA700 does contain the *C. acremonium nia*D in an active form, especially when this result is taken in conjuction with those from Section 3.5. Transformation using the method outlined in section 2.5.2 resulted in nitrate utilizing colonies being detected after 10 days incubation, approximately 75% of the total number of transformants observed, while the remainder appeared over a further eight days. The regeneration of protoplasts varied between 1-10% on non-selective media in all experiments. Table 3.14 summarizes the transformation frequencies obtained using this system. Transformants were easily isolated since the background growth was extremely poor and no abortive colonies were observed, as demonstrated by Figure 3.18. It should be noted that no nitrate utilizing colonies were found in non-transformed protoplasts. Both the slower growing transformants and

Table 3.14 Summary of *niaD* transformations in *C. acremonium.*

	pSTA700	Vector λSTA6	linear pSTA700
Maximum* transformation efficiency	47	6.2	19.1
Average*+ transformation efficiency	36.3	-	9.6

* transformants/μg DNA
\+ mean of 3 independent experiments

Figure 3.18 Regeneration of *C. acremonium niaD* transformants on osmotically stabilized nitrate MM.



those which appeared after 10 days incubation, developed to mature colonies on further incubation and continued to grow on nitrate MM on subsequent inoculation. This was found to be the case with *P. chrysogenum niaD* tranformants which exhibited this growth pattern (section 3.6.2; Whitehead *et al*, 1989). Approximately 95% of transformants exhibited mitotic stability due to continued growth on selective medium after growth on non-selective media. This indicates that only a few transformants were unstable.

The transformation efficiencies achieved (Table 3.14) were up to 47 transformants/µg DNA, with an average of approximately 36/µg DNA with pSTA700. These levels are similar to that of Skatrud *et al* (1987) and the results presented in Section 3.4.1 when using the *E. coli* hygromycin B resistance gene linked to the *C. acremonium pcbC* promoter. They are also very similar to the frequencies achieved by Unkles *et al* (1989a,b) when transforming *A. oryzae* and *A. niger* with their respective homologous *niaD* genes (using circular vector). However, when Unkles *et al* (1989a,b) and Skatrud *et al* (1987) linearized the transforming vectors, a large increase in transformation efficiency was observed, two to three fold increases in *C. acremonium* and up to eight fold increases in *A. oryzae* and *A. niger*. It was hoped that a similar effect would be observed in transformations using pSTA700. Table 3.14 demonstrates that when pSTA700 was linearized with *Hin*dIII no effect on transformation frequency was found. *Hin*dIII restricts pSTA700 once only in the pUC18

polylinker region and not within the *C. acremonium* DNA region. Therefore rearrangements within the *nia*D gene would not occur. Other attempts were therefore made to try to improve transformation efficiency in this system.

The first series of experiments to improve transformation frequencies was performed via a novel method of DNA introduction to the protoplasts. This involved creating protoplasts as previously described and then subjecting them to electroporation in the presence of DNA. Electroporation is the application of a high intensity electric field for a short duration to the protoplasts, with a resultant reverse permeabilization of the protoplast membrane, which should hopefully result in DNA uptake. This technique is commonly used to transfer DNA into mammalian (Potter *et al*, 1984) and bacterial cells (Miller *et al*, 1988). A commercial "Electroporater"^{TM} (kindly supplied by BRL) was used to perform the transformations. Approximately $1x10^8$ protoplasts were subjected to a large variety of elctroporation conditions in the presence of 10µg of pSTA700. The protoplasts were then spread onto selective media. No transformants were observed under any conditions. When the protoplasts were instead given a 20 min incubation in PEG solution prior to incubation on selective media, transformation was observed at a very low frequency, less than 1/µg DNA. However it could not be concluded whether this transformation was due to electroporation or incubation in the presence of PEG. Since the frequency obtained was far lower than that

achieved using standard procedures it was decided not to investigate this system any further.

An investigation into the effect of temperature shock was performed. It has been found that applying heat shock (Berges and Barreau, 1989) or cold shock (Cantoral *et al*, 1987) to protoplasts can improve transformation in filamentous fungi. The conditions used, along with the results obtained are outlined in Table 3.15. These results demonstrate that both heat shock and cold shock applied to the protoplasts during the PEG incubations have a minimal effect on transformations. However, applying a heat shock to the protoplasts prior to the addition of DNA and PEG resulted in a marked increase in transformation efficiencies (approximately a three fold increase) to 130 transformants/µg DNA. Such a pattern whereby heat shock produces an effect only when applied before the addition of DNA was also observed by Berges and Barreau (1989) when applied to *Podospora anserina*. It was found that this system was not reproducible as on two occasions (out of the six experiments performed) no increase in transformation was observed. This inconsistency is probably due to slight variations in temperature and duration of incubations caused by experimental pressure. Once this method of transformation is used with regularity to achieve frequencies of greater than 100 transformants/µg DNA it would become an extremely useful system. These frequencies are far higher than the heterologous system of Skatrud *et al* (1987) and

Table 3.15 Summary of attempts to improve *niaD* transformations in *C. acremonium*.

Treatment of protoplasts	Maximum transformation efficiency*
Standard transformation	47
1st PEG incubation at 0°C	13.4
2 mins at 35°C after 1st PEG incubation	25.7
Above two treatments together	13
2 mins at 45°C after 1st PEG incubation	14.2
2 mins at 45°C 10 mins before 1st PEG incubation	136.5
5 mins at 45°C 10 mins before 1st PEG incubation	35
10 mins at 45°C 10 mins before 1st PEG incubation	19.9
10 mins at 37°C 10 mins before 1st PEG incubation	43.6

* transformants/µg DNA

are equal to some homologous systems found in other filamentous fungi.

It has been mentioned previously that 95% of all transformants isolated from selective media continued to grow on nitrate MM, demonstrating that the transformants are mitotically stable when sub-cultured. However it would be useful to know if the majority of spores were stable since single spore isolation from a *C. acremonium* colony is extremely difficult, if not impossible. To this end, spores were taken from transformants of CSG116, formed into a suspension in 0.01% Tween 80 solution and plated out on MM (10mM nitrate) or MM (10mM glutamate, 470mM chlorate). It was found that almost 100% of spores from CSG116 transformants (namely CT1, CT5, CT7 and CT10) could germinate on nitrate MM, however this was also observed for regeneration on chlorate containing MM. Over 100 transformants were then analysed for this phenomenon of growth on both nitrate MM and MM containing chlorate from strains CSG116 and CSG98 (to ensure it was not strain dependent). All transformants exhibited growth on chlorate glutamate MM and nitrate MM. This situation may have been caused by the chlorate resistant mutants *niaD* mutation causing a concomittant effect on the uptake of chlorate. To investigate this, the transformants were inoculated onto MM containing 10mM nitrate, 470mM chlorate. No growth of the transformants, *C. acremonium* wild type or CSG116 was observed on this medium, indicating that chlorate was being taken up by these transformants. It is important to note that CSG116 and

the transformants exhibited starvation growth (sparse mycelia, growing to an approximate one cm radius) while the growth of the wild type strain was inhibited. When mycelia from the starved colonies was then cut out of the agar and placed on MM containing nitrate alone, the transformants grew while CSG116 did not. These results suggest that a heterogeneous mixture of nuclei exist within the transformants, with some containing the transformed *niaD* gene and others lacking it. When a transformant grows on nitrate, it predominantly proliferates the *niaD*$^{+}$ nuclei, with a few *niaD*$^{-}$ nuclei being maintained by communication of solutes through the hyphal compartments. The opposite of this would then be true when the organism was grown on chlorate with the low level of *niaD*$^{+}$ nuclei not being sufficient to inhibit the growth of the colony. This situation is likely to arise during the transformation protocol because protoplasts tend to fuse on the addition of PEG. A transformant colony would therefore contain nuclei from a number or protoplasts of which maybe only one had been transformed to the *niaD*$^{+}$ genotype. Unfortunately this does not explain why such high levels of chlorate resistant colonies were found when a spore suspension of transformants grown on nitrate produced almost 100% growth on chlorate MM. This result is probably due to it being almost impossible to obtain single spores of *C. acremonium*. Despite uninucleate spores being produced, they form within heads covered in slime and thus cannot be separated (Peberdy, 1987). It is possible that these

spores showed instability of incorporation of the *nia*D gene and were excising the insert from the genome when placed under selective chlorate pressure. Only when both these systems were operating would it explain the results obtained.

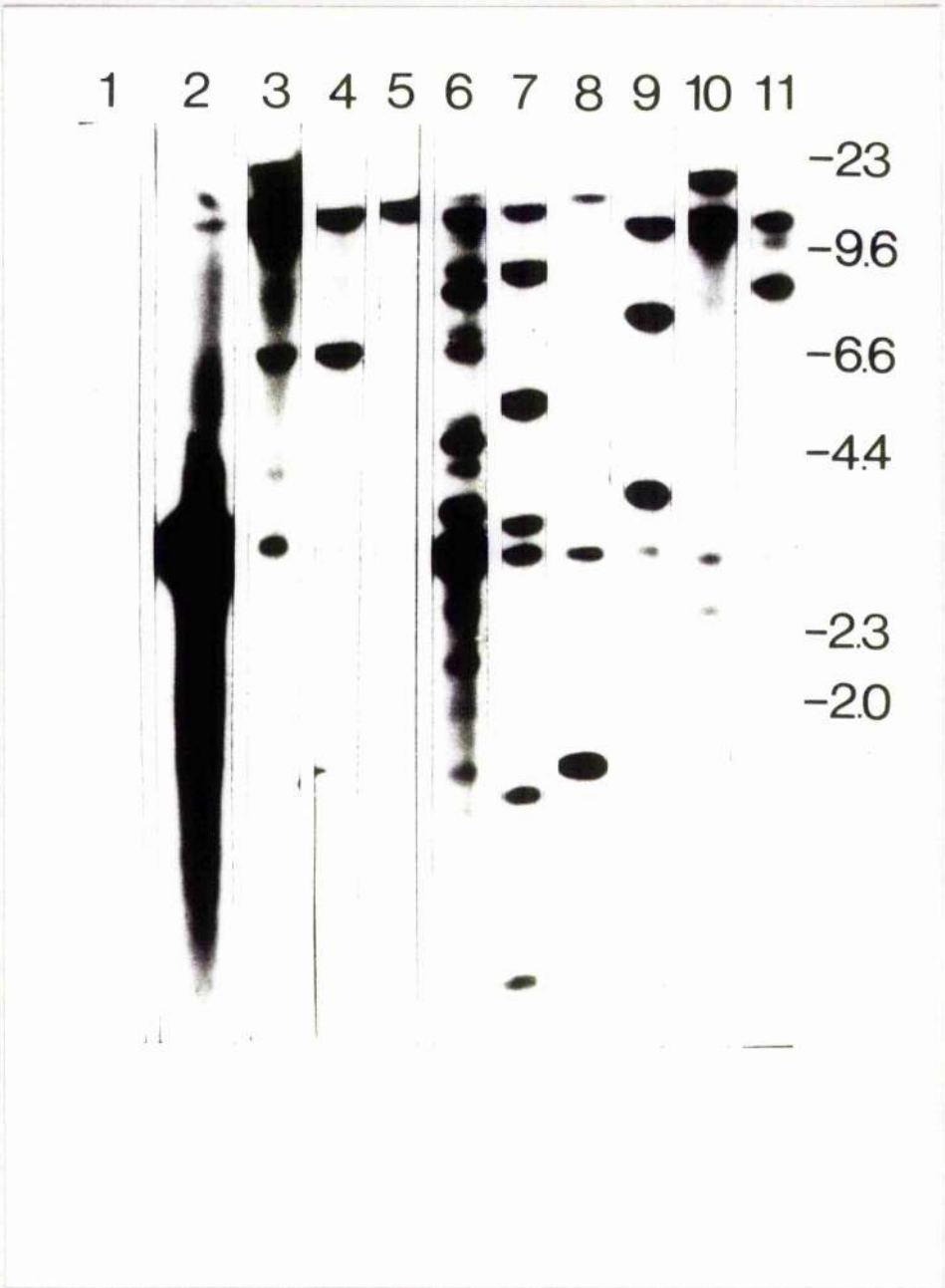
These results may imply that *C. acremonium nia*D transformants are mitotically unstable. It was found, however, that when transformants were maintained on non-selective CM and spore suspensions from these cultures again inoculated onto CM to form individual colonies, all colonies tested grew on nitrate MM (over 100 colonies were analysed), indicating that *C.acremonium nia*D transformants are mitotically stable unless the organism is put under selective pressure to lose the *nia*D^{+} genotype, such as by growth on chlorate.

Previous investigations into the stability of *nia*D transformants have been carried out by Unkles *et al*, (1989b) and by Malardier *et al* (1989) on the organisms *A. niger* and *F. oxysporum* respectively. They observed similar results with their *nia*D transformants producing chlorate resistant colonies at high frequencies. Levels of one in 1000 for *A. niger* (where transformants were maintained on selective media prior to testing) and one in 100 to one in 10 for *F. oxysporum* (where transformants were maintained on non-selective media prior to testing) were found for the majority of transformants analysed. A low level of transformants, approximately 10-20%, were found to exhibit stability on nitrate media and only produced chlorate resistant colonies at the same rate as

the wild type organism. These results are therefore different to those found in *C. acremonium*, although it would be interesting to determine if either *A. niger* or *F. oxysporum* were as stable on nitrate media as *C. acremonium*.

DNA isolated from 10 randomly selected transformants (designated CT1 to CT10), restricted with *Hin*dIII, Southern blotted and probed with pUC18. All 10 transformants were found to contain bacterial sequences as demonstrated in Figure 3.19. This shows that all transformations had not occurred solely by gene conversions (type III integrations, from Fincham, 1989). The variety of restriction patterns produced from this hybridization indicate that integrations have occurred at multiple ectopic regions to the resident *nia*D locus. The intensity and number of hybridization signals is indicative of tandem integration of several copies of pSTA700, seen in transformants CT1 and CT5, since the DNA in each lane is approximately constant. One feature of this hybridization pattern which is hard to explain is the presence of the 3.1kb hybridization signal in all transformants. This band of hybridization is most intense in CT1 where a 3.1kb band can be seen in the DNA digest banding pattern, which is not observed in any other transformants, CSG116, or the wild type. Why this appears cannot be explained by referring to the restriction enzyme map of pSTA700 since this contains only one HindIII site which is within the pUC18 polylinker. Thus one explanation for its presence is that pSTA700 has

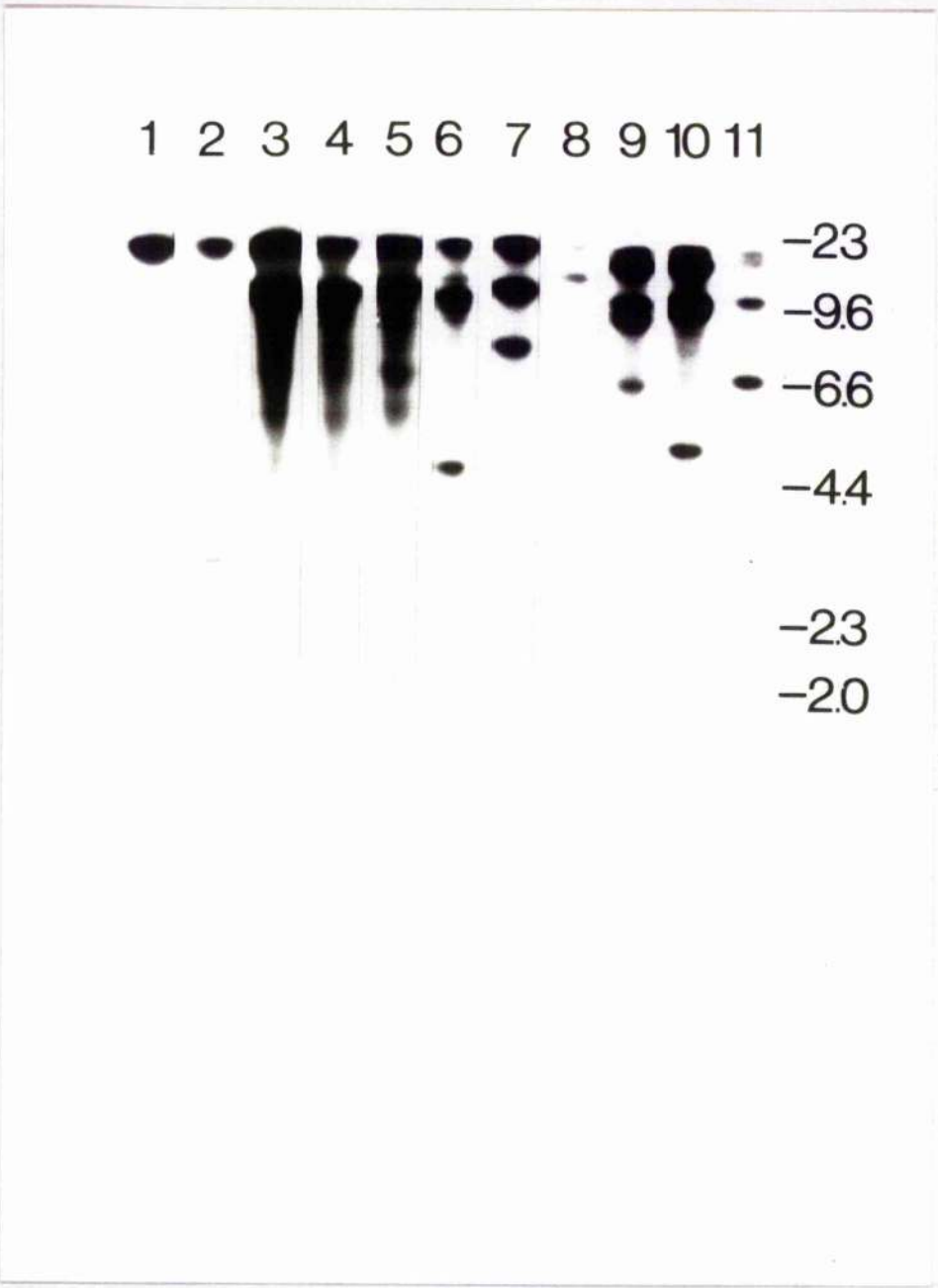
Figure 3.19 Southern hybridization of *Hin*dIII digested genomic DNA of *C. acremonium* wild type and transformants probed with pUC18: lanes 1, wild type; 2, CT1; 3, CT2; 4, CT3; 5, CT4; 6, CT5; 7, CT6; 8, CT7; 9, CT8; 10, CT9 and 11, CT10.



inserted into at least one constant part of the genome in all the transformants. This area could be the homologous *nia*D locus or a "recombination hot-spot". The latter explanation is more credible due to the intensity of this band in a number of the transformations and in particular in CT1. After insertion of pSTA700 into this area of the genome, proliferation of the region occurred due to its "hot-spot" nature and large quantities of repetitive DNA have been produced.

An investigation into whether homologous type I integration had occurred in any of the transformants CT1 to CT10 was also undertaken. It was found that the resident *nia*D locus of *C. acremonium* wild type was within an approximately 20kb *Hin*dIII fragment. Thus if integration has occurred at this area an unnatural *Hin*dIII restriction site would have been introduced causing a shift in the restriction pattern of the wild type organism when hybridized to the *C. acremonium nia*D gene. Figure 3.20 shows the restriction pattern of the wild type organism and transformants when probed with the 8.6kb *Eco*RI fragment of pSTA700. It should be noted that 5µg of non-labelled pUC18 was placed in the hybridization solution to prevent any hybridization of labelled pUC18 which may have been present due to carry over from the isolation of the internal 8.6kb *Eco*RI fragment from pSTA700. The large 20kb *Hin*dIII fragment of the wild type *C. acremonium* (lane 1) does not appear to be shifted in the transformants indicating that integration most probably has not occurred at the homologous site. Figure

Figure 3.20 Southern hybridization of *Hin*dIII digested genomic DNA of *C. acremonium* wild type and transformants probed with the 8.6kb *Eco*RI insert of pSTA700: lanes 1, wild type; 2, CT1; 3, CT2; 4, CT3; 5, CT4; 6, CT5; 7, CT6; 8, CT7; 9, CT8; 10, CT9 and 11, CT10.



3.20 also demonstrates that multiple copies of the *C. acremonium niaD* gene have integrated into the transformants genome, with some of the hybridization patterns matching those seen in Figure 3.19 when the transformants were probed with pUC18, as would be expected. The common 3.1kb band is not highlighted (except in CT1 where the quantitiy of this band is so large that hybridization to residual carry over of labelled pUC18 could not be prevented by non-labelled pUC18) in this hybridization indicating that it does not contain any of the 8.6kb *Eco*RI region of pSTA700. For this to be the case, the break point for recombination must have occurred within the pUC18 region of pSTA700, again indicating that this pattern of integration has not been caused by homologous integration.

The overall pattern of integration found in the transformations is one of random ectopic and possible tandem integration events at multiple integration sites. This result is in contrast with the results observed in homologous *niaD* transformations in *A. niger* (Unkles *et al*, 1989b), *A. oryzae* (Unkles *et al*, 1989a) and *A. nidulans* (Johnstone *et al*, 1989) where single or low copy integrations tend to occur at the homologous site. However this pattern of multiple integration is observed in other *C. acremonium* transformations (Queener *et al*, 1985, Skatrud *et al*, 1987) and transformations involving this strain of *C. acremonium* (Ramsden, per. comm.). It has also been seen in heterologous *niaD* transformations

for *F. oxysporum* (Malardier *et al*, 1989) and *P. chrysogenum* (Whitehead *et al*, 1989).

Several gene mediated transformation systems do not allow direct selection of the transformants. To cirumvent the problem of complex plasmid construction by incorporating the non-selectable gene on the same vector as the selectable marker, co-transformation is often performed, (Werners *et al*, 1987). The frequency of co-transformation was investigated for *C. acremonium*. Table 3.16 demonstrates that irrespective of which plasmid is selected for during transformation (either pIH1 hygromycin B resistance or pSTA700 nitrate utilization) the frequency of co-transforamtion is approximately 25% when an equimolar ratio of plasmids is used. As one would expect the co-transformation frequency also increases and decreases when the molar ratio of plasmids is altered in favour of the non-selected plasmid and the selected plasmid, repectively. The percentage co-transformation obtained is considerably higher than that found in *A. niger* (16%; Campbell *et al*, 1989) or *A. oryzae* (9%; Unkles *et al*, 1989a) when using their respective homologous *nia*D genes at a 1:1 molar ratio to the non-selected plasmid. Transformation frequencies were not adversely affected at a 1:1 molar ratio, thus making this an extremely useful system for the isolation of transformants with non-selectable markers. When the ratio of non-selected plasmid was increased above that of the selected marker, transformation frequencies did decrease

Table 3.16 Co-transformation frequency of pSTA700 and pIH1 in *C. acremonium*.

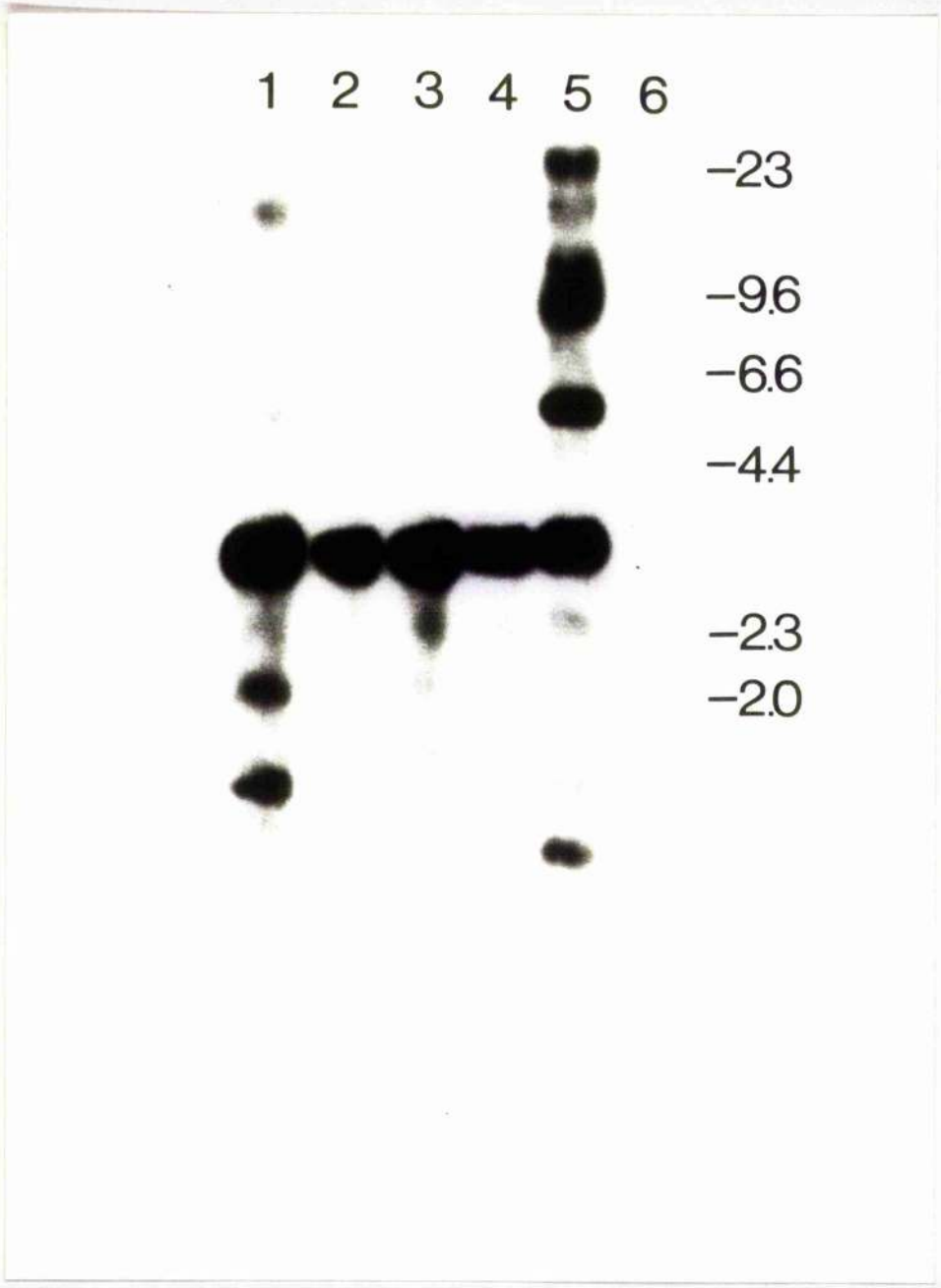
Molar ratio of vectors			Co-transformation %*	no. analysed
Selected vector	:	non-selected vector		
1 pSTA700	:	2 pIH1	40	100
1 pSTA700	:	1 pIH1	23	100
2 pSTA700	:	1 pIH1	10	100
4 pSTA700	:	1 pIH1	2	75
1 pIH1	:	2 pSTA700	50	50
1 pIH1	:	1 pSTA700	27	75

* Percentage of transformants exhibiting properties of the non-selected vector.

to 1-5/μg DNA when selecting for transformation with pSTA700.

A further investigation was performed via co-transformation. This was to determine if a vector which had previously been found not to be expressed in *C. acremonium*, under direct selection, could be selected for. For this, co-transformations were performed at a 1:1 molar ratio with pSTA700 and pBT6, conferring resistance to benomyl, which had previously been found not to allow direct selection for benomyl resistant colonies (see section 3.4.1). 25% of co-transformants were found to be resistant to benomyl at 10μg/ml, a level at which CSG116 and *C. acremonium* wild type could not grow. To check these were transformants and not spontaneous mutations to benomyl resistance, the DNA was isolated from 5 transformants (CTB1-CTB5), restricted with *Hin*dIII, Southern blotted, and then probed with a 3.1kb *Hin*dIII fragment of pBT6 which contains purely *N. crassa* DNA sequences. Figure 3.21 demonstrates that all the transformants contain the 3.1kb *Hin*dIII fragment while the wild type organism shows no homology to the probe at the high stringency of hybridization employed. That other size bands of homology are seen in some transformants is again indicative of random integration. One would expect that the major band detected would be the 3.1kb *Hin*dIII fragment since when integration occurs it would have to be via a breakpoint in the pUC sequences of pBT6 outwith the 3.1kb *Hin*dIII *N. crassa* DNA region so that the benomyl resistance gene will be intact and therefore

Figure 3.21 Southern hybridization of *Hin*dIII digested genomic DNA of *C. acremonium* wild type and pBT6 co-transformants probed with the 3.1kb *Hin*dIII fragment of pBT6: lanes 1, CTB1; 2, CTB2; 3, CTB3; 4, CTB4; 5, CTB5 and 6, wild type.



expressed correctly. This result is, in itself, interesting since benomyl resistant colonies could not be detected in transformations where benomyl resistance was directly selected for, with benomyl at 10μg/ml. It indicates that the *N. crassa* gene can be expressed efficiently in *C. acremonium* but by applying direct selection to protoplasts, their growth was inhibited before the gene could be expressed. This may be the case for other vectors previously investigated in this study (see section 3.4).

The vector pAN5-41B was then analysed for co-transformation ability. This vector contains the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene promoter linked to the *E. coli* lacZ gene, and would therefore give an indication if *A. nidulans* promoters are expressed within *C. acremonium*. From a transformation using a 1:1 molar ratio of pSTA700 to pAN5-41B, 50 nitrate utilizing colonies were isolated and analysed for β-galactosidase activity as outlined in section 3.3.5. No transformants produced the characteristic blue colour of β-galactosidase activity, indicating that this promoter was not efficient in its expression within *C. acremonium*. This result provides further evidence that *A. nidulans* gene systems do not operate within *C. acremonium* confirming results found in previous investigations (see sections 3.4 and 3.6.3). It should be noted that pAN5-41B has been used in co-transformation experiments in *P. chrysogenum* with 90% of transformants being co-transformed with pAN5-41B, when used at a 1:1 molar ratio

with the selecting plasmid, *amdS* in this case (Kolar *et al*, 1988).

3.7 NITRATE REDUCTASE ACTIVITIES IN WILD TYPE AND TRANSFORMANT STRAINS OF *A. NIDULANS*, *P. CHRYSOGENUM* AND *C. ACREMONIUM*.

3.7.1 *A. nidulans* activities.

A wealth of data is available on nitrate reductase activities in *A. nidulans* (Cove, 1979 and references therein). The nitrate reductase levels of *A. nidulans* were determined to use as a comparison with the analysis in *P. chrysogenum* and *C. acremonium*. Table 3.17 shows the nitrate reductase activities of *A. nidulans* wild type when grown in MM containing either nitrate, glutamate, ammonium and nitrate and ammonium, with samples of mycelia being taken at the time intervals indicated. The results show the expected pattern of nitrate reductase being induced in the presence of nitrate and repressed in the presence of ammonium and compare closely with the mRNA levels of the *nia*D gene in this strain of *A. nidulans* (Johnstone *et al*, 1990).

3.7.2 *P. chrysogenum* activities.

P. chrysogenum wild type exhibits a very similar pattern of nitrate reductase induction and repression to that of *A. nidulans* as demonstrated by the results presented in Table 3.18. To determine if the *A. nidulans* and *A. niger nia*D genes are also subject to this control

Table 3.17 Nitrate reductase activities* of *A. nidulans* wild type grown at 30°C, 200 rpm.

	Grown in MM containing			
Time (hrs) mycelia harvested	10mM nitrate	10mM glutamate	10mM ammonium	10mM nitrate + 10mM ammonium
7.75	195	8.8	0	2
9.5	278	7.8	0	1.8
11.5	373	8.1	0	12
13.5	235	7.1	0	19
15.5	181	5.8	0	29
17.5	182	8.8	0	33
19.5	205	6.3	0	30
27	0	0	0	0

* nmol nitrite formed/min/mg protein

Table 3.18 Nitrate reductase activities* of *P. chrysogenum* wild type grown at 26°C, 290rpm.

	Grown in MM containing			
Time (hrs) Mycelia harvested	10mM nitrate	10mM glutamate	10mM ammonium	10mM nitrate+ 10mM ammonium
15	73	6.8	0	7.6
17	118	7.4	0	6.1
19	97	5.1	3.5	21
21	144	4	5.8	12
23	176	4	0	16
24	131	2.8	0	33

* nmol nitrite formed/min/mg protein

once transformed into the *P. chrysogenum niaD* mutant, nitrate reductase activities were assayed for in a number of transformants. These being PTG2, PTG3, PTG4 and PTG6 from *A. niger* gene transformations and PTD1, PTD2, PTD4 and PTD6 from *A. nidulans* gene transformations. Table 3.19 shows the results obtained for these transformants and the wild type *P. chrysogenum*. All transformants exhibit the same control of activity, that is induction in the presence of nitrate and repression in the presence of ammonium except for PTG3, PTG4 and PTG6. These show partially derepressed levels of nitrate reductase when grown in medium containing ammonium and nitrate. PTG3 and PTG6 also exhibit partial derepression when grown on ammonium alone. These results can be compared to the level of integration seen in the transformants shown in Figure 3.16. PTG3 and PTG4, which are two possible high copy number transformants, may exhibit derepression due to copies of the genes being inserted such that the controlling elements have been rearranged, thus preventing correct repression for those copies.

It may be expected that similar control mechanisms regulating nitrate reductase would be found in the three related filamentous fungi, *A. nidulans*, *A. niger* and *P. chrysogenum*, and that the heterologous genes would be regulated correctly. That the product of the *N. crassa nit-2* gene (the wide domain regulatory gene for ammonium or nitrogen metabolite repression) complements mutations in the analagous *areA* mutants of *A. nidulans* (Davis and Hynes, 1987) supports this theory. When induced with

Table 3.19 Nitrate reductase activites* of *P. chrysogenum* wild type and transformant strains.

Vector	Strain	Grown in MM containing			
		10mM nitrate	10mM glutamate	10mM ammonium	10mM nitrate+ 10mM ammonium
None	wild type	110	4	0	2
None	*nia*D19	0	0	0	0
pSTA10	PTG2	71	1	1	6
pSTA10	PTG3	91	12	21	24
pSTA10	PTG4	68	1	0	14
pSTA10	PTG6	78	14	26	22
λAN8a	PTD1	165	23	0	0
λAN8a	PTD2	69	4	0	0
λAN8a	PTD4	62	8	1	1
λAN8a	PTD6	83	7	0	0

* nmol nitrite formed/min/mg protein

nitrate, all transformants, except PTD1, show a moderately reduced level of nitrate reductase activity (15%-45% reduced) compared with the wild type organism. This distribution of activities in transformants has been observed previously in *A. niger* (Unkles *et al*, 1989b). The majority of transformants showed moderately reduced activities even in homolgous *A. niger* transformations. The cause of this reduction in both *A. niger* and *P. chrysogenum* is probably the position of the inserted gene not being conducive to allow full expression or induction of the *nia*D gene. Thus the reduction in *P. chrysogenum* transformant activities is not likely to be caused by failure of signal recognition in the promoter region by controlling elements of *P. chrysogenum*. Transformant PTD1, a putative high copy number transformant (Figure 3.17) showed significantly increased nitrate reductase levels (50% greater than wild-type), greater than that seen in even homologous *A. niger nia*D transformations (Unkles *et al*, 1989b). PTD1 remained subject to nitrogen metabolite repression. This indicates that it is possible to increase enzymic activities through the transformation of *P. chrysogenum* and yet ensure the transformed gene stays under the required genetic controls.

3.7.3 *C. acremonium* activities.

The nitrate reductase activities of *C. acremonium* wild type following growth in the same medium as *A. nidulans* and *P chrysogenum* was determined. Table 3.20

Table 3.20 Nitrate reductase activities* of *C. acremonium* wild type grown at 28°C, 290 rpm.

	Grown in MM containing			
Time (hrs) mycelia harvested	10mM nitrate	10mM glutamate	10mM ammonium	10mM nitrate + 10mM ammonium
20	239	45	0	0
25	231	100	0	0
30	684	96	6	3
35	500	29	0	0
40	159	33	0	0

* nmol nitrite produced/min/mg protein

demonstrates that *C. acremonium* follows a similar pattern of induction by nitrate and repression by ammonium, and that the induction to maximum activity is preceeded by a lag period, and followed by a decrease to low activity levels. The activities however, are two to three times greater than that observed in *A. nidulans*. One striking feature found in Table 3.20 is the high level of activity when grown in the presence of glutamate. This phenomenon is most manifest after 25hrs growth when the nitrate reductase activity of mycelia grown in glutamate is almost 50% that of the nitrate grown mycelia. Stark contrast can be seen between this result and that observed in *A nidulans* (see Section 3.7.1; Cove, 1979) and *N. crassa* (Tomsett and Garrett, 1981) where the nitrate reductase activity is very low, if detectable at all, when grown in glutamate as the sole nitrogen source.

A number of *C. acremonium* CSG116 transformants complemented with pSTA700 were investigated for nitrate reductase activity, these being CT1, CT7, CT8, CT9 and CT10 (see section 3.6.3, Figure 3.19 and 3.20). The mutants were grown in MM containing either nitrate, glutamate, ammonium or nitrate and ammonium as in a previous manner to the wild type organism. It was discovered that all the transformants (CT1 in particular) grew slower than the wild type organism under these conditions, making comparison between the wild type and transformant activities difficult. The transformants grew equally well on solid MM (10mM nitrate) as the wild type organism.

To enable a meaningful comparison of nitrate reductase activities, the transformants and wild type were grown in CM before being harvested, washed in sterile water and placed in MM containing the nitrogen sources used before. The mycelia was isolated and tested for activity after five hrs growth in MM. The results from this analysis are presented in Table 3.21. These results demonstrate that mutant CSG116 exhibits no nitrate reductase activity while the wild type shows a similar pattern of activity to that found when grown in MM, Table 3.20. One difference is the level of activity when placed in glutamate, with this being a much higher percentage of the nitrate grown mycelial activity in the switch experiment (Table 3.21). These results were observed in numerous experiments (data not shown). An unusual feature of the results presented in Table 3.21 is the higher nitrate reductase activities found in transformants CT7, CT8, CT9 and CT10 with up to 10 times (in the case of CT8) the wild type activity. Transformant CT1 shows greatly reduced activity with only 20% of wild type activity. This transformant contains the highly elevated presence of the 3.1kb hybridizing band in Southern analysis (see section 3.6.3, Figure 3.19). It is likely that the genomic integrity or general metabolic balance has been adversely affected and thus low levels of nitrate reductase activity were observed. Alternatively, only one complete copy of pSTA700 may have integrated into CT1 and that copy may not carry out efficient gene expression. Another alternative may be

Table 3.21 Nitrate reductase activities* of *C. acremonium* wild type and transformant strains, experiment 1.

Strain	Grown in MM containing			
	10mM nitrate	10mM glutamate	10mM ammonium	10mM nitrate + 10mM ammonium
wild type	111	85	14	30
CSG116	0	0	0	0
CT1	20	4	0	0
CT7	666	207	17	62
CT8	1210	857	18	25
CT9	980	750	391	512
CT10	717	681	208	455

* nmol nitrite produced/min/mg protein

that CT1 produces large quantities of an inactive form of the *nia*D gene product which combines with the molybdenum cofactor excluding active apoprotein from so combining.

Despite the elevated levels of nitrate reductase in CT7 and CT8, they remain subject to ammonium repression. Transformants CT9 and CT10, however, do not and exhibit substantial derepression in the presence of ammonium, the activity under these conditions being approximately 30% of the activity of nitrate grown mycelia. It is difficult to compare these results with levels of integration of pSTA700 within these transformants (Figure 3.20) since they all appear to be the result of low copy number integrations. The transformants also exhibit high nitrate reductase activities when grown in glutamate, indicating that this is an inherent characteristic of *nia*D gene expression in *C. acremonium*.

Due to the unusual nature of these results it is important to comment on their reproducibility. The results presented for *A. nidulans*, *P. chrysogenum* and *C. acremonium* are produced by the average of triplicate assays. All assays showed very little variation amongst any one mycelial extract. The variation found with *A. nidulans* was almost zero, while that for *C. acremonium* was between 1-10% (mostly 1-5%). This decrease in reproducibility in *C. acremonium* assays was expected since the assay conditions were developed for *A. nidulans* (Cove, 1966). When experiments were repeated for *A. nidulans* and *P. chrysogenum* the results obtained were

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Table 3.22 Nitrate reductase activities* of *C. acremonium* wild type and transformant strains, experiment 2.

	Grown in MM containing	
Strain	10mM nitrate	10mM glutamate
wild type	282	256
CSG116	0	0
CT1	11	4
CT7	1291	307
CT8	1566	904
CT9	817	700
CT10	1920	1130

* nmol nitrite produced/min/mg protein

very similar and thus the findings presumed to be correct and accurate. However when the switch experiments were repeated for *C. acremonium* (Table 3.22), the nitrate reductase activities recorded were found to vary considerably to the first experiment. The absolute numbers for the nitrate reductase activities can be seen to be different yet the percentage differences between transformants and the wild type organsism are constant. For example, in the second experiment, *C. acremonium* wild type possessed a nitrate reductase activity of approximately 280 nmol/min/mg when induced in MM containing nitrate alone. This level of activity is different to that seen in Table 3.21 for the first experiment. The differences between CT7 and the wild type however are approximately the same, that is CT7 showing six times the activity of the wild type organism when grown under the same conditions. Therefore when assessing the results presented in Table 3.21 it should be kept in mind that the nitrate reductase activity levels vary. Conclusions should therefore be drawn from the differences seen between transformants and the wild type organism.

3.8 THE UTILIZATION OF THE NITRATE SELECTION SYSTEM TO INTRODUCE GENES REQUIRED FOR ANTIBIOTIC BIOSYNTHESIS INTO *C. ACREMONIUM*.

The suitability of the nitrate selection system for the introduction of antibiotic biosynthetic genes was investigated. This will be a major requirement of the

system for research and development in *C. acremonium*. Transformations were performed in two ways, via co-transformation (vectors were used at a 1:1 molar ratio or a 2:1 ratio in favour of the non-selectable antibiotic biosynthetic gene) or via vectors constructed to contain both the *C. acremonium niaD* gene and an antibiotic biosynthetic gene (such plasmid constructs were supplied by Glaxochem Ltd.).

Transformation was performed using the standard procedure outlined in section 2.5.2. Nitrate utilizing colonies were isolated and analysed for the ability to inhibit bacterial growth (see section 2.9). Two classes of antibiotic biosynthetic genes were investigated, those isolated from *A. nidulans* and those obtained from *C. acremonium*. The *A. nidulans* genes analysed were the *pcb*C gene within pSTA204, the *pcb*C and the *pen*E gene within pSTA200 and the *pcb*AB and *pcb*C gene within pSTA201 (MacCabe *et al*, 1990). These vectors were all introduced via co-transformation. The *C. acremonium* genes used were *pcb*C within pIPNS used with co-transformations and pNNII1, pNNII2 and pNNII3 (containing copies of *pcb*C) all introduced by direct selection. The *cef*EF gene was contained upon pEXPAND and pNNXX6 which were introduced by co-transformation and direct selection, respectively.

All transformants were analysed via bioassay experiments and those which demonstrated a 20% increase (as determined by the diameter of the zone of inhibition of bacterial growth) over and above the capability of the untransformed strain CSG116 were classed as possessing

increased antibacterial action. Table 3.23 summarises the number of transformants analysed from each transformation and details the numbers found to show an increase in antibacterial capability. Figure 3.22 demonstrates how this increase in activity was observed when screening a large number of transformants via bioassay.

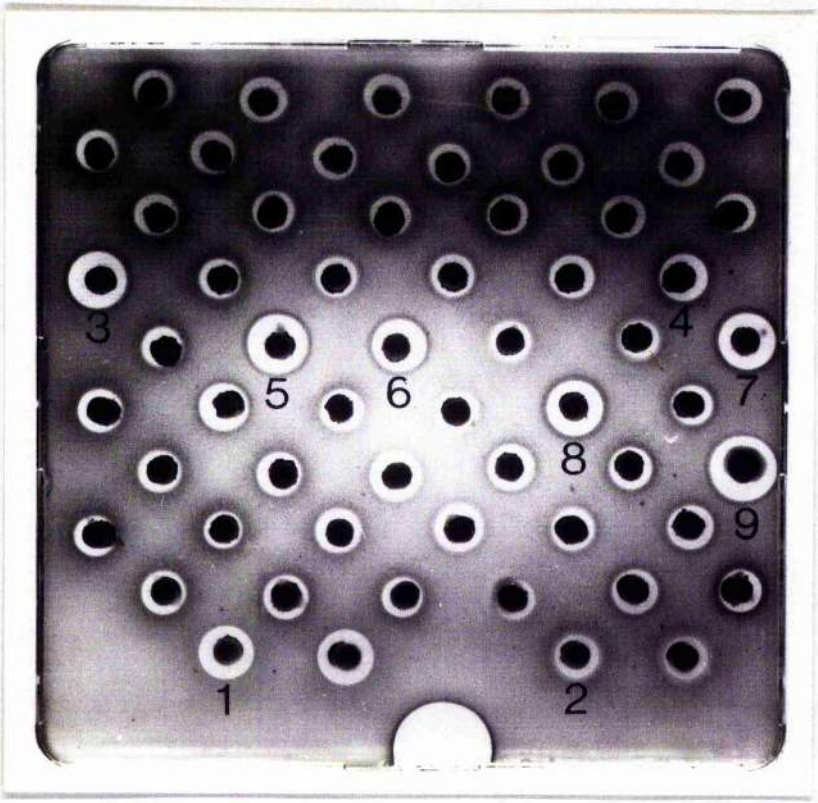
Smith *et al* (1989a; 1990) was able to transform *P. chrysogenum* antibiotic biosynthetic genes into both *N. crassa* and *A. niger* (which do not possess antibiotic production capability) with some resultant transformants being able to produce penicillin V. Additionally Beckman *et al* (1989) has transformed and expressed the *S. clavuligerus* deactoxycephalosporin C synthetase gene in *P. chrysogenum*. It was hoped, therefore,that the *A. nidulans* antibiotic biosynthetic genes would be able to be expressed and active enzymes produced even if not all the specific co-factors were present within *C. acremonium*. Table 3.23 shows that when pSTA200 is co-transformed into *C. acremonium* a large percentage of transformants demonstrate increased antibacterial activity. Due to pSTA200 containing both the *pcb*C and the *pen*E gene of *A. nidulans*, either could be responsible for the increase in activity. However when the *pcb*C gene was introduced via pSTA210 and pSTA204 only one transformant out of 124 showed increased activity. This transformant may include a natural mutation which has caused this increase or it may have been the result of the introduction of *pcb*AB since this is also on pSTA201. It is probable that the increase in

Table 3.23 Summary of transformation of *C. acremonium* with antibiotic biosynthetic genes.

Vector used	Number of transformants analysed	Number exhibiting increased bioassay capability *	%
pSTA700 + pSTA200	124	22	18
pSTA700 + pSTA201	101	1	1
pSTA700 + pSTA204	23	0	-
pSTA700 + pIPNS	62	2	3
pSTA700 + pEXPAND	70	2	3
pNNXX6	256	15	6
pNNII1	183	4	2
pNNII2	175	17	10
pNNII3	122	6	5

* Increased by greater than 20% diameter of inhibition compared to untransformed CSG116.

Figure 3.22 Bioassay of *C. acremonium* transformants. Plugs numbered are inoculated with the following strains: 1, wild type; 2, CSG116; 3, CTC25; 4, CTC26; 5, CTC27; 6, CTC28; 7, CTC29; 8, CTC32 and 9, CTC37.



Mycelia were incubated on the plugs for 5 days prior to bioassay assessment.

antibacterial activity produced by the introduction of pSTA200 is not due to the action of *pcb*C. A more detailed analysis of this will be shown later in this section.

Interpretation of the results achieved from transformation using *C. acremonium* biosynthetic genes is not straightforward. Skatrud *et al* (1986; 1989) did not observe an increase in cephalosporin C yields after transformation of a *C. acremonium* wild type strain and an industrial strain with the *C. acremonium pcb*C gene. The results presented in Table 3.23 are at variance with these results since an increase in antibacterial activity was found in a small percentage of transformants (5% on average). The difference in the results be explained by isopenicillin N synthetase not being a rate limiting step in cephalosporin C biosynthesis in the stains used by Skatrud *et al* (1986; 1989) while it could have been, at least to some extent, in CSG116. Smith *et al* (1989b) has shown that a mutant strain of *P. chrysogenum*, which exhibits increased antibiotic biosynthesis, possesses between eight and 16 copies of the *pcb*C gene, demonstrating that increased copies of *pcb*C can result in increased antibiotic biosynthesis.

The results from transformations with vectors pEXPAND and pNNXX6 are also in contrast with those found by Skatrud *et al* (1989) when transforming an industrial *C. acremonium* strain with the *cef*EF gene. They observed that an increase in cephalosporin C titre was commonly seen , for example, one in a batch of eight transformants tested showed a 40% increase in titre. That

a low number of transformants showed an increase in antibacterial activity following transformation with pEXPAND could be due to these transformants being co-transformants. Thus only a percentage of transformants analysed would contain any integrations of pEXPAND. A further reason for the low number of transformants isolated possessing increased antibacterial activity is the nature of the bioassay analysis. The transformants isolated by Skatrud *et al* (1989) were analysed for increased cephalosporin C titre via HPLC analysis, this also revealed a concommitant decrease in penicillin N concentration. Both these compounds possess antibacterial activity, therefore analysis via bio-assay may not reveal a total increase in antibacterial activity. It can be surmised that the increase in bio-assay capability of the *cef*EF transformants was most probably caused by the presence of the *C. acremonium* antibiotic biosynthetic gene since the percentage that exhibit this is greater than that found by natural or artificial mutation of strains of *C. acremonium* (Skatrud *et al*, 1989). It can be concluded that since the percentage of transformants recovered with increased antibacterial activity was low in comparison with the results of Skatrud *et al* (1989) the bioassay method of assessment probably has severe drawbacks.

The most striking feature of Table 3.23 is that co-transformants of pSTA200 show the greatest percentage of transformants with increased antibacterial activity. These transformants also exhibited the greatest

percentage increase in antibacterial activity when compared with that of CSG116. The diameter of inhibition was 20-55% (55% in the case of CTC37 shown in Figure 3.22) increased in some pSTA200 transformants while transformants from other vectors showed an increase of 20-40%. Since pSTA200 contains only *A. nidulans* antibiotic biosynthetic genes and previously it had been shown that *A. nidulans* genes are poorly expressed, if at all, in *C. acremonium* (see section 3.4.2 and 3.6.3) this is a somewhat surprising result. It has been presumed that the *pcb*C gene contained within pSTA200 has little, if any, effect on bio-assay capability (earlier in this section). It can therefore be concluded that the *pen*E gene was responsible for this activity.

The product of the *pen*E gene is the enzyme acyltransferase which produces penicillin G (or derivative dependent on the sidechain supplied) in *A. nidulans* from isopenicillin N (see section 1.5). Transformants of pSTA200 which showed this increase in activity (namely CTC25, CTC26, CTC27, CTC28, CTC29, CTC32 and CTC37) were investigated for the presence of penicillins.These transformants were grown in fermentation media and broth samples analysed for natural penicillins (Bycroft and Shute, 1987) and penicillin G by HPLC and TLC (performed at Glaxochem Ltd.). No penicillins could be found by this analysis. The levels of production of penicillin N and cephalosporin C were also investigated in these transformants by HPLC (performed at Glaxochem Ltd) and compared to the levels

found in CSG116. No difference could be detected between the transformants and CSG116, however since this is a wild type strain, detecting any antibiotic levels was problematical since the HPLC was set up for analysis of industrial strains. There is therefore some doubt as to whether transformation with pSTA200 results in the production of penicillins in *C. acremonium* via the *pen*E gene. An alternative to this gene being responsible for the marked increase in antibacterial activity is for pSTA200 to contain another gene influencing antibiotic activity. Considering pSTA200 contains at least 8kb of DNA 3′ to the *pcb*C gebe (MacCabe *et al*, 1990) and the *pen*E gene is most probably situated similarly in relation to *pcb*C as it is in *P. chrysogenum* (*pen*E is 1.5kb long and the start is within 1.5kb of the 3′ end of *pcb*C; van Solingen *et al*, 1989) ample room is available for a further gene. However no such discoveries have yet been reported.

Despite the uncertainty concerning the analysis of antibiotic biosynthesis within transformants it is possible to conclude that the nitrate selection system is an efficient and useful method for the introduction of homologous and heterologous antibiotic biosynthetic genes. Some transformants exhibit an increase in antibacterial activity compared to the untransformed strain, thus making this system advantageous in comparison to selection by classical means.

4 CONCLUSION.

Chlorate resistant mutants have been isolated from both *C. acremonium* and *P. chrysogenum*. The frequency with which spontaneous resistant mutants appear varies between one in 10^5 to one in 10^7 spores as observed by Unkles *et al* (1989a; b) in *A. oryzae* and *A. niger* and by Tomsett and Cove (1979) in *A. nidulans*, although this is less than the mutation rate of one in 1000 found in *F. oxysporum* (Malardier *et al*, 1989). This high level of spontaneous mutation to chlorate resistance in this and other studies could be caused by either of two factors. Chlorate itself may act as a mutagen within the cell resulting in the high number of mutations. This could be further investigated by analysing chlorate resistant mutants for mutations in other pathways and comparing this to a control group which had not been exposed to chlorate. Alternatively the spores exposed to chlorate may have mechanisms for directing mutations to result in chlorate resistance. That organisms have this ability to direct mutations has been reported by Cairns *et al* (1988). It would appear that the nitrate assimilation pathway would provide an excellent vehicle for research into this possibility.

It has been demonstrated that the *nia*D mutants of both *P. chrysogenum* and *C. acremonium* can be characterized from a background of a variety of chlorate resistant mutants. That *P. chrysogenum* mutants

can be characterized using the approach devised by Cove (1979) was expected due to the investigations of Birkett and Rowlands (1981). However it was not forseen that *C. acremonium* would be unable to utilize hypoxanthine in the presence of glucose since this has not been observed in any other fungi previously investigated. Evidence has been presented which indicates that such inability to utilize hypoxanthine is due to carbon catabolite repression affecting hypoxanthine uptake. This could be further investigated by determining the effect of cAMP in this environment, since cAMP has been found to mimic the effect of glucose in the repression of glucoamylase in *A. awomori* (Bhella and Altosaar, 1988). It should also be noted that carbon catabolite repression has been found in other investigations of *C. acremonium* (S. Harford, pers. comm.). It is important that a simple phenotypic test has been developed to distinguish *C. acremonium* *nia*D mutants to allow easy isolation and characterization of *nia*D mutants from a variety of industrial strains of *C. acremonium*. The authenticity of this new test has been proved by the series of biochemical analyses performed.

Further analysis of *C. acremonium* chlorate resistant mutants showed that the genetics of nitrate assimilation in this organism resemble that found in other filamentous fungi. This was shown by the presence of three mutant *cnx* alleles which show the overlapping complementation pattern observed in other filamentous

fungi. Results also indicated that the *nii*A and *nia*D genes were unlinked as found in *N. crassa* (Tomsett and Garrett, 1980). The presence of other genes involved in the control of nitrate assimilation was not demonstrated. No *are*A mutants were found and the mutants classified as putative *nir*A were not demonstrated to be unequivocably of this genotype. It is therefore possible that the mechanisms of control in *C. acremonium* may be different to other filamentous fungi. This possibility being highlighted by the unusually high nitrate reductase activities when grown in the presence of glutamate.

The next stage in developing homologous transformation systems for these organisms was the isolation of the corresponding *nia*D genes. Unfortunately time pressures did not allow the isolation of the *P. chrysogenum nia*D gene. However this should prove relatively easy and could be accomplished either by direct complementation of a *P. chrysogenum nia*D mutant or by heterologus hybridization to the *A. nidulans nia*D gene.

Evidence has been presented that the gene isolated by heterologous hybridization to the *A. nidulans nia*D gene is indeed the *C. acremonium nia*D gene. First the clone contains *C. acremonium* DNA and no gross rearrangements in structure have occurred during the cloning process. Second the gene shows sequence homology to the *A. nidulans nia*D gene and exhibits very high amino acid homology at conserved areas of the

nitrate reductase protein when this sequence is converted into its predicted amino acids. This data though cannot be taken as proof of the identity of the gene as only one strand of the clone has been sequenced accurately. Further sequence analysis would therefore have confirmed the gene's authenticity. However the main use of such investigations would be in revealing the nature of gene structure in *C. acremonium*. The preliminary investigations presented in this study indicate that some difference may be found. For example the presence of the putative intron within the region sequenced was only 28 bp long, the smallest so far observed in filamentous fungi. It would also be interesting to see if any similarities in 5' regulation and activation sequences could be observed between the *C. acremonium nia*D gene and other fungal *nia*D genes. The final proof obtained which confirmed the identity of the *C. acremonium nia*D gene was the ability of this clone to transform both *C. acremonium* and *P. chrysogenum nia*D mutants to nitrate utilization.

It would also be possible to investigate the location of the *nia*D gene within the genome of *C. acremonium*. Skatrud and Queener (1989) have developed an electrophoretic molecular karyotype for *C. acremonium* and thus it would add to the sparse genetic information available for *C. acremonium* to know which chromosome the *nia*D resided on and if it was linked to any other genes of interest.

Despite not being able to obtain the *P. chrysogenum niaD* gene it was still important to demonstrate the ability of heterologous genes to transform *P. chrysogenum*. This showed that the *niaD* gene transformation system is possible within *P. chrysogenum* and exhibits various advantages over other *P. chrysogenum* transformation systems. Most importantly the efficiency of transformation is similar to that of a number of other systems, namely those developed by Carramalino *et al* 1989), Bull *et al* (1988) and Beri and Turner (1987). Transformation frequencies are sufficiently high enough to enable the majority of molecular investigations to be performed. If the homologous *niaD* gene was isolated and used, this would hopefully raise transformation frequencies. It would then be possible to construct a *P. chrysogenum* gene library which contained this gene as the selectable marker for the library.

The *P. chrysogenum* transformants investigated contained a variety of levels of integration. This would enable both single or high copy number transformants to be isolated, allowing the study of gene dosage effects in this organism. Some of the transformants exhibited wild type expression of the transformed genes. Thus it may be useful to link a *niaD* promoter to a heterologous gene and thereby control the expression of a protein to a high degree. This could be especially beneficial if the protein to be expressed was toxic to *P. chrysogenum*.

The investigations into transformation of *C. acremonium* demonstrate the difficulties of developing transformation systems for organisms which have not been well characterized. The only genes which were found to transform *C. acremonium* in direct selection experiments were the *E. coli* hygromycin B resistance gene linked to the *C. acremonium pcb*C promoter (pIH1), the *S. hindustanus* phleomycin resistance gene linked to its native promoter, the *A. nidulans gpd*A promoter and the *S. cerevisae* **TEFI** promoter and the *C. acremonium nia*D gene. The phleomycin system could immediately be discounted as a viable selection system due to the low level of transformation frequency. That vectors containing the *A. nidulans nia*D or *amd*S genes, *A. oryzae nia*D gene, *A. niger nia*D gene or *N. crassa* mutated *tub*-2 gene linked to the *A. nidulans* glucoamylase promoter could not be expressed within *C. acremonium* indicates that *C. acremonium* may have difficulty in expressing heterologous genes. This could be due to a failure to recognise promoter and activation sequences or be caused by problems in processing mRNA such as caused by an inability to excise introns due to failure in recognising the required sequences. It is most probably a combination of these features that result in the absence of transformation. It was found that the *N. crassa tub*-2 gene (pBT6) could transform *C. acremonium* to benomyl resistance when not directly selected for in co-transformation experiments, but not

when directly selected for. This could indicate that there is a lag in expression time for heterologous promoters and that this lag results in protoplasts being unable to regenerate before their growth is inhibited by the antibiotic. This would explain why some of the vectors investigated could not transform *C. acremonium*. However when the vector pAN5-41B, containing *the A. nidulans gpd*A promoter linked to the *E. coli* β-galactosidase gene, was co-transformed into *C. acremonium*, no expression of the gene was observed.This indicates that for some vectors the lack of expression was caused by a failure to recognise promoter sequences. This is likely to be the case with pAN5-41B since it is probable that the *E. coli* β-galactosidase gene would be translated and transcribed correctly as it lacks introns.

An investigation into whether the *C. acremonium nia*D gene can be expressed in *A. nidulans* or the *P. chrysogenum nia*D gene expressed in *C. acremonium* should be undertaken. This may indicate if it is an inherent property of *C. acremonium* to have difficulty expressing heterologous genes or if this phenomenon is found in *A. nidulans* when transformed with *C.acremonium* genes. It is likely that the problem lies within *C. acremonium* as a great many heterologous transformations have been performed with success in other filamentous fungi (Fincham, 1989 and references therein).

Once transformation in *C. acremonium* had been found using pSTA700 (containing the *C. acremonium niaD* gene) efficiency of transformation was improved to over 100 transformants/µg DNA. This level is sufficient to allow the *niaD* system to be used for a whole spectrum of transformation experiments. For example a *C. acremonium* gene library could be made within a vector containing the *C. acremonium niaD*, this would then provide a useful tool for cloning genes of *C. acremonium*.

It is likely that increases in transformation frequencies could be achieved in *C. acremonium* by optimising the heat shock conditions or by developing better procedures for producing and handling protoplasts as observed by Skatrud *et al* (1987). That linearization of pSTA700 with *Hin*dIII did not produce an increase in transformation was disappointing. However it may be that an increase in transformation could be obtained by the linearization of pSTA700 at other restriction sites as Dhawale and Marzluf (1985) found that altering the restriction site at which a vector was cut varied the transformation frequencies obtained in *N. crassa* using the *qa*-2 gene. Dhawale and Marzluf (1985) also observed a difference in the percentage of transformants showing homologous and heterologous integration events following linearization of the transforming vector. Thus this may be a method of inducing homologous or low copy number integrations into this strain of *C. acremonium*. It is desirable to

further investigate the type of integration events found in *C. acremonium* as some manipulations may require single or low copy number integrations or the ability to direct the integration to the homologous site. Kim and Marzluf (1988) observed a difference in the type of integration events when different strains of *N. crassa* were transformed with the *trp*-1 gene. The problem of getting multiple integration events may be negated by using a different strain of *C. acremonium* as would be the case in transformations of industrial strains.

One extreme example of a transformant being the result of a multiple integration event was transformant CT1, although this phenomenon may have been caused by a DNA amplification event following integration of the vector. Such a transformant would not be of use for studies of nitrate reductase activity in *C. acremonium* due to the unknown nature of the integration. However it could be useful for genetically marking this organism since the 3.1kb hybridization band can be observed in the DNA digest alone. Thus the presence of this organism could be detected in a mixed background of other *C. acremonium* colonies without resorting to detailed analysis. This strain could therefore be used for investigations into how mixed strains of *C. acremonium* interact with each other.

The results from the nitrate reductase activity analysis of transformants demonstrate that it is possible to achieve greatly increased (up to 10 times)

enzyme activities in *C. acremonium*, following transformation. The knowledge that this result is possible offers hope for further experiments with *C. acremonium*. For example it means that enzymes with low activity levels, which may be the rate limiting step of a biosynthetic pathway, can have their activities increased following transformation. It may be possible to fuse the *nia*D gene promoter to a gene of interest and achieve high expression of that gene via induction with nitrate.

One of the more unusual observations in this study was the finding that *C. acremonium nia*D transformants retained chlorate resistance. Why this should occur was not experimentally proved, however the most likely explanation was due to the transformants existing in a heterokaryotic state with the majority of nuclei being either *nia*D^+ in the presence of nitrate or *nia*D^- in its absence. In the experiments conducted, stability of the *nia*D^+ genotype did not appear to be adversely affected when the transformant was maintained on non-selective media. Thus the transformants were assumed to be stable. However it would be desirable to carry out longer term investigations and also experiments on mycelia grown in non-selective liquid culture before the transformants could be said to be unequivocably stable.

Co-transformation was achieved in *C. acremonium* at a frequency which enabled easy isolation of transformants containing the non-selected vector. This

ability allows various transformation experiments to be performed without the need to resort to costly and time consuming vector constructions.

The final experiments described involved the transformation of antibiotic biosynthetic genes into *C. acremonium* via the nitrate transformation system. A small percentage of the resultant transformants exhibited increased antibacterial capability following transformation with the *C. acremonium pcbC* and *cef*EF genes. These results demonstrate the potential for increasing cephalosporin C production in industrial strains of *C. acremonium*. It was also found that genes from the *A. nidulans* antibiotic biosynthetic gene cluster could also result in increased antibacterial activity in *C. acremonium*. Although it was not determined which enzymic activity was responsible for the increase in antibacterial capability, the results demonstrate the potential for developing industrial strains of *C. acremonium* with increased antibiotic biosynthetic capabilities. They also offer the possibility of developing novel antibiotics in *C. acremonium* as has been previously achieved in *Streptomyces* (Floss, 1987) by the expression of antibiotic biosynthetic genes from a heterologous organism

In conclusion a successful heterologous transformation system has been developed for *P. chrysogenum* and the first homologous transformation has been developed for *C. acremonium* based on the *nia*D

gene of the nitrate assimilation pathway. Both systems allow transformation at frequencies enabling a variety of genetic manipulations to be performed. Finally this system allows the introduction of antibiotic biosynthetic genes with resultant transformants demonstrating increased antibacterial activity.

APPENDIX

Luria Broth (per litre): 10g Tryptone
5g Yeast extract
10g NaCl
adjusted to pH 7.5 with NaOH.

Agar was added to a final concentration of 1.2% w/v for Luria Agar.

Complete Medium (per litre): 10g D-glucose
2g Mycological peptone
1g Yeast extract
1g Casein hydrolysate
20mls Salt solution
1ml Trace element solution
1ml Vitamin solution
adjusted to pH 6.5 with NaOH.

Agar was added to a final concentration of 1.5% w/v.

Minimal Medium (per litre): 10g D-glucose
20mls Salt solution
1ml Trace element solution
adjusted to pH 6.5 with NaOH

Agar was added to a final concentration of 1.5% w/v. Minimal media for use with *P. chrysogenum* also contained 1ml vitamin solution /litre.

Salt Solution for CM and MM (per litre) :

26g KCl

26g $MgSO_4.7H_2O$

76g KH_2PO_4

Trace Element Solution for CM and MM (per litre):

1.1g $Na_2Mo_7O_{24}.2H_2O$

11.2g H_3BO_4

1.6g $CoCl.6H_2O$

1.6g $CuSO_4.5H_2O$

50g EDTA

5g $MnCl_3.4H_2O$

22g $ZnCl_2.7H_2O$

heated to boiling, cooled to 60°C and adjusted to pH 6.5 with 1M KOH.

Vitamin Solution for CM and MM (per litre):

1.5g Aneurine

2.5g Biotin

2.5g Nicotinic acid

20g Choline.HCL

0.8g PABA

1.0g Pyrodoxine. HCL

2.5g Riboflavin

2.0g Pantothenate (Ca salt)

Minimal Base Medium: 100mls Solution A

2mls Solution B

4mls Solution C

Solution A (per litre): 36g Sucrose

7.5g L-asparagine

15g KH_2PO_4

21g K_2HPO_4

0.75g Na_2SO_4

0.18g $MgSO_4.7H_2O$

0.06g $CaCl_2$

adjusted to pH 7.5 with NaOH

Solution B (per litre): 108g D-glucose

Solution C (per litre): 25g Sucrose

5.5g NH_4 Acetate

5g $CaCO_3$

M9 Medium (per litre): 6g Na_2HPO_4

3g KH_2PO_4

0.5g NaCl

adjusted to pH 7.4 with NaOH

Agar was added to a final concentration of 1.2% w/v. After autoclaving the following were added:

2mls 1M $MgSO_4$

100µl 1M $CaCl_2$

Carbon source as in Section 2.8

Fermentation Medium (per litre): 35g Lactose

10g $CaCO_3$

4.5g $MgSO_4.7H_2O$

2.1g Corn steep liquor

adjusted to pH 6.8 with NaOH.

Agar was added to a final concentration of 4% w/v.

Bio-assay Medium (per litre): 5g Bacteriological peptone
3g Lablemco (Oxoid)
1g Na Citrate

adjusted to pH 7.5 with NaOH

Agar was added to a final concentration of 1.2% w/v.

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