A Gene Mediated Transformation System for Nitrate-Utilising Filamentous Fungi of Commercial Importance

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DECLARATION

I, Edward Iain Campbell, hereby certify that this thesis has been composed by myself, that this 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..

CERTIFICATE

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

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I dedicate this work to my parents.

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ABBREVIATIONS

bp	Base pairs
^{o}C	Degrees Celsius
cm	Centimetres
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
et al	*et alia*
EtBr	Ethidium Bromide
g	Gram
hrs	Hours
kb	Kilobase
kg	Kilogram
λ	Lambda
l	Litre
M	Molar
mM	Millimolar
mg	Milligram
mins	Minutes
ml	Millilitre
μCi	Microcurie
μg	Microgram
μl	Microlitre
ng	nanogram
PEG	Polyethylene Glycol
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium Dodecyl sulphate
Tris	Tris (hydroxymethyl) aminomethane
Tween	Polyethylene sorbitan mono-oleate
UV	Ultraviolet
x-gal	5'-Bromo-4'-chloro-3'-indolyl-B-D-galactopyranoside

SUMMARY

Spontaneous chlorate resistant mutants of the industrially important filamentous fungi Aspergillus niger, Aspergillus oryzae, Penicillium chrysogenum were isolated. As the wild-type strain is moderately resistant to chlorate, nitrate reductase deficient mutants of Gibberella fujikuroi were generated using N-methyl-N'-nitrosoguanidine treatment of the conidia followed by the screening for non-growth on nitrate as the sole nitrogen source.

Characterisation of likely genotypic identity by growth tests was applied as described by Cove (1979). Non-reverting (at less than 10^{-8}) niaD mutants were chosen for further experimentation. The A. niger niaD5 non-nitrate utilising mutant strain was transformed to nitrate utilisation at a frequency of up to 1,176 transformants μg DNA^{-1} using the linearised A. niger niaD - containing plasmid (designated pSTA10.) The A. oryzae niaD14 mutant strain using the similarly linearised homologous niaD - containing plasmid, (designated pSTA14) yielded levels of up to 800 transformants μg DNA^{-1}. Heterologous transformation of the P. chrysogenum niaD19 mutant strain and the G. fujikuroi niaD mutant strain niaD11 with the A. niger niaD containing plasmid pSTA10 gave frequencies of up to 9 transformants μg DNA^{-1} and up to 2 transformants μg DNA^{-1} respectively. Southern analyses demonstrated in all cases that vector sequences had integrated into the chromosomal DNA.

1. INTRODUCTION

1.1 Commercial Filamentous Fungi

The filamentous fungi survive and thrive in an exceptionally wide and varied selection of habitats which has allowed them to evolve highly diverse and versatile enzyme systems. Consequently they have been recognised as a rich source of enzymes, antibiotics and hormones with a broad variety of industrial, medical and agricultural applications of great commercial value. Indeed it has been estimated that the present world market for industrial enzymes derived from fungi alone has a value of U.S.$650 million (Berka et al,,1991). With the added financial rewards from antibiotics it can be appreciated that the filamentous fungi are at the heart of a billion U.S. dollar industry. The filamentous fungi employed in this study span the whole spectrum of commercial value, from Aspergillus niger and Aspergillus oryzae which secrete enzymes of industrial and culinary importance to the medical applications of the products of Penicillium chrysogenum to to the agricultural uses of Gibberella fujikuroi products. Until recently, strain improvement to produce higher yields of commercial product from industrial fungi has mainly involved the selection of spontaneous and induced mutant strains or the optimisation of fermentation conditions with varying degrees of success. However as many industrial fungi, such as those in this study, lack a sexual and parasexual cycle it is clear that for greater understanding both genetically and

biochemically, the application of recombinant DNA technology could be of benefit. The primary requirement of genetic analysis and manipulation using molecular genetic methods is a gene-mediated transformation system.

Since the discovery of the process of transformation and transfection which operate in bacteria and phage it has been recognised that the organisation and content of the prokaryotic genome is dynamic in nature. Over the last fifteen years or so significant technological advances have been made in the development of methods (calcium phosphate precipitation, protoplast fusion, liposomes, electroporation *etc*) of introducing DNA from essentially any source into a variety of cultured eucaryotic cells of, amongst others, mammalian, insect, plant, yeast, and fungal origin. These studies have provided compelling evidence that as in the case of its prokaryotic counterpart, the eucaryotic genome, across a broad phylogenetic spectrum, has a remarkable capacity to accommodate additional nucleic acid sequences of endogenous and exogenous origin. Somatic or vegetative cells carrying such an inheritable change are said to have been transformed. At present details of the actual processes of transformation, *i.e.* presentation and uptake across the membrane, transport, stable integration and expression of foreign genes are poorly understood. Despite this, however, the ability to introduce particular changes into the eucaryotic genome by this process of transformation provides a wide range of opportunities from advancing our understanding of many genetically ill-defined organisms to biotechnological strain improvement in which a cloned gene of commercial or medical interest is

expressed in foreign cells. The development of such a system based on nitrate utilisation is the subject of this study as applied to industrially important fungi.

Table1 Examples of Industrial Products of Filamentous Fungi[A]

Category	Product	Source
Primary Metabolites		
	Organic acids-citric,itaconic,gluconic	*Aspergillus niger*
		Aspergillus terreus
	Vitamins β-carotene,riboflavin	*Blakeslea trispora*
		Eremothecium ashbyii
		Ashbya gossypii
	Polysacharides,pullulan,scleroglucan	*Aurobasidium pullulans*
		Sclerotium rolfsii
Secondary Metabolites		
	Antibiotics-penicillins,cephalosporin	*Penicillium chrysogenum*
	griseofulvin, fusidanes,	*Cephalosporium acremonium*
		Penicillium griseofulvin
		Fusidium coccineum
	Plant and animal growth hormones	*Gibberella fujikuroi*
		Gibberella zeae
	Drugs-alkaloids,cyclosporins	*Claviceps purpurea*
		Tolypocladium inflatum
Enzymes		
	Acid proteases	*Aspergillus niger*
		Aspergillus oryzae
		Mucor pusillus
		Mucor miehei
	α-Amylases	*Aspergillus niger*
		Aspergillus oryzae
		Aspergillus awamori
	Catalase	*Aspergillus niger*
	Cellulases	*Trichoderma viride*

	Trichoderma reesei
Fungal rennin	*Mucor miehei*
	Mucor pusillus
β-glucanase	*Aspergillus niger*
Glucoamylases	*Aspergillus niger*
	Aspergillus awamori
	Rhizopus sp.
Glucose oxidase	*Aspergillus niger*
	Penicillium amagasakiense
Invertase	*Aspergillus niger*
	Aspergillus oryzae
Lactase	*Aspergillus niger*
	Aspergillus oryzae
Lipases	*Rhizopus sp.*
	Mucor miehei
	Aspergillus niger
Pectinase	*Aspergillus niger*

Food		
	Edible Mushrooms	*Agaricus bisporus*
		Pleurotus ostreatus
		Lentinus edodes
		Volvariella volvacea
	Mould-ripened cheeses	*Penicillium roquefortii*
		Penicillium camembertii
	Oriental food fermentations	*Aspergillus oryzae*
		Rhizopus sp.
	Single cell protein	*Fusarium,Paecilomyces*

Bio-conversions		
	Steroid hormones	*Rhizopus sp.,*
		Fusarium solani
		Aspergillus ochraceus
		Curvularia lunata
	6-Aminopenicillanic acid	*Penicillium chrysogenum*
		Fusarium sp.

	Cephalosporium sp.
Insecticides	
Colorado beetle control	*Beauvaria bassiana*
Aphid control	*Verticillium lecanii*
Citrus mite control	*Hirsutella thompsonii*
Spittle-bug control	*Metarrhizium ansiloplae*

[A] Compiled from Bigelis (1985), Bennett (1985),Montenecourt and Eveleigh (1985),Brown et. al.,(1987) and Unkles (1989).

1.2 Genetic Transformation of Filamentous Fungi

Although transformation of filamentous fungi was first described by Mishra and Tatum (1973) in N. crassa it was not until transformation protocols which had been developed for the yeast Saccharomyces cerivisciae (Beggs,1978; Hinnen et al.,1978) were adapted by Ballance et al.,(1983), Tilburn et al.,(1983), Case et al.,(1979), John and Peberdy, (1984) and Yelton et al, (1983), for A. nidulans and N. crassa that it became a recognised routine technique for filamentous fungi.

Since then various selection systems have been developed for a variety of filamentous fungi but the basic method of introducing the desired gene into the recipient genome remains essentially the same for all. Fundamental to this process is the removal of the fungal cell wall to generate the protoplast. This is achieved by the use of cell wall digesting enzymes, all of which contain a mixture of hydrolytic enzymes such as chitinase and 1.3-glucanase. Most current protocols rely on a commercial preparation known as Novozyme 234 which is a cocktail of several cell wall digesting enzymes.

Protoplasts may be created from different cell types including germinating microconidia, macroconidia or young mycelia, (for a review, see Fincham,1989). By maintaining the protoplasts in an isotonic solution they retain their physical integrity without the aid of cell walls whose debris is separated from the protoplast by filtration or centrifugation. In general the optimal conditions for uptake of DNA vector involves the concentration of the protoplasts to around $1x10^8$/ml with the plasmid in the presence of

polyethylene glycol and calcium chloride. For different species of fungi optimising the procedure has involved alterations of parameters such as temperature, incubation time, or additional exposure to other cell wall digesting enzymes. Protoplasts are regenerated in the isotonic selection medium. Current selection systems are outlined later in this section in Tables 2,3 and 4.

Considerable effort has focused on the possibility of improving transformation efficiency as this is of importance in enabling the technique to be used for a variety of molecular investigations, e.g. a noticeable increase in transformation frequency in the majority of filamentous fungi has been found when the transforming vector has been linearised. Dhawale and Marzluf (1985) reported a three to four-fold increase in transformation in N. crassa and in this study a two to eight-fold increase in transformation efficiency in A. niger and A. oryzae using linearised vector. The site at which the vector is cut is of prime significance as restriction within the selecting gene leads to a decrease in transforming frequency (Dhawale and Marzluf,1985; Unkles et al.,1989). Cutting in the vector sequences does not seem to increase frequency. Probably as "recombinationally reactive" fungal DNA ends are required. Increase in transforming frequencies have also been noted by other researchers who have reported simple adaptations to the basic procedure. For instance Berges and Barreau(1989) achieved a five- to ten-fold increase in P. anserina transformation by employing heat shock to protoplasts, a technique vital in achieving efficient transformation in the bacterium Escherichia coli.

Generally, however the procedure for transformation is similar for all filamentous fungi with a number of selection systems now available for filamentous fungi (Tables 2, 3, 4)

Table2 Auxotrophic Complementation Selection Systems

System	Filamentous fungus	Reference
Adenine	*Neurospora crassa*	Alic et al., 1990
	Phanerochaete chrysoporium	
Arginine	*Aspergillus nidulans*	John & Peberdy , 1984 Johnstone 1985, Upshall et al., 1986
	Aspergillus niger	Buxton et al., 1985
	Aspergillus oryzae	Hahm & Batt , 1988
	Neurospora crassa	Weiss et al , 1985
Glutamate	*Neurospora crassa*	Kinsey & Rambosek , 1984, Grant et al. , 1984
Inositol	*Neurospora crassa*	Feher et al., 1986
Methionine	*Aspergillus oryzae*	Iimura et al., 1987
Nitrate	*Aspergillus niger*	Unkles et al., 1989 (a), Campbell et al., 1989
	Aphonocladium album	Daboussi et al., 1989
	Beauveria bassiana	Daboussi et al., 1989
	Colletotrichum lindemuthianum	Daboussi et al., 1989
	Fusarium oxysporum	Malardier et al., 1989, Daboussi et al., 1989, Langin et al., 1990
	Nectria haematcocca	Daboussi et al., 1989
	Penicillium caseicolum	Daboussi et al., 1989
	Penicillium chrysogenum	Whitehead et al., 1989

	Pyricularia oryzae	Daboussi et al., 1989
Proline	*Aspergillus nidulans*	Durrens et al., 1986
Quinate	*Neurospora crassa*	Case et al., 1989,Case , 1982
	Aspergillus nidulans	Da Silva et al., 1986
Tryptophan	*Coprinus cinereus*	Binninger et al., 1987
	Neurospora crassa	Case et al., 1979, Kim & Marzluf, 1988
	Penicillium chrysogenum	Sanchez et al., 1987 Picknett et al., 1987
	Aspergillus nidulans	Yelton & Timberlake, 1983
Uracil	*Aspergillus nidulans*	Ballance et al., 1983, Ballance & Turner , 1985
	Aspergillus niger	Goosen et al., 1987, van Hartingsvelt et al., 1987
	Aspergillus oryzae	de Ruiter-Jacobs , 1989
	Neurospora crassa	Buxton & Radford , 1983
	Penicillium chrysogenum	Beri & Turner , 1987, Cantoral et al., 1987, Diez et al., 1987
	Podospora anserina	Tudzynski et al., 1980, Perrot et al., 1987
	Trychoderma reesei	Smith et al., 1991 Gruber et al., 1990

Table3 Antibiotic Resistance Selection Systems

System	Filamentous fungus	Reference
Benomyl	*Aspergillus nidulans*	Dunne & Oakley , 1988
	Neurospora crassa	Orbach et al., 1986,
		Vollmer & Yanofsky , 1986
	Gaeumannomyces graminis	Henson et al., 1988
G418	*Neurospora crassa*	Bull & Wooton , 1984
	Cephalosporium acremonium	Penalva et al., 1985
Hygromycin	*Aspergillus nidulans*	Cullen et al., 1987
	Aspergillus niger	Punt et al , 1987
	Cephalosporium acremonium	Queener et al., 1985
	Fusarium oxysporum	Kistler & Benny , 1988
	Fulvia fulva	Oliver et al., 1987
	Colletrotrichum lindemuthianum	Rodriguez & Yoder , 1987
	Ustilago maydis	Leong et al., 1987
		Wang et al., 1988
Kanamycin	*Ustilago maydis*	Suarez & Eslava , 1988
Oligomycin	*Aspergillus nidulans*	Ward et al., 1986
	Aspergillus niger	Ward et al., 1988
	Penicillium chrysogenum	Bull et al., 1988

Table4 Substrate Utilisation Selection Systems

System	Filamentous fungus	Reference
Acetate	*Aspergillus nidulans*	Turner et al., 1985, Ballance & Turner, 1986
Acetamide	*Aspergillus nidulans*	Kelly & Hynes , 1985, Tilburn et al., 1983
	Aspergillus niger	Kelly & Hynes , 1985
	Aspergillus terreus	Upshall , 1986
	Cochliobolus heterostrophus	Turgeon et al., 1985

1.3 Molecular Aspects of Aspergillus niger

Aspergillus niger is widely used commercially for the production of extracellular enzymes (Table 1) such as pectinase, β-glucanase, and glucoamylase (Bennett,1985). It's main use however, is in the production of organic acids, particularly citric acid for the food industry. As a highly efficient extracellular protein secretor it is also of great interest to the pharmaceutical sector.

Successful heterologous transformation systems have been developed for A.niger based on the rescue of arginine auxotrophy using the Aspergillus nidulans argB gene (Buxton et al., 1985), utilisation of acetamide with the introduction of the A.nidulans amdS gene (Kelly & Hynes, 1985), the reversal of uracil auxotrophy with the Neurospora crassa pyr4 gene (Goosen et al., 1987) and resistance based on the bacterial gene apt (Punt et al., 1987). In addition homologous systems using pyrG for uracil auxotrophic repair (Goosen et al.,1987, van Hartingsveldt et al., 1987) and the oliC gene for oligomycin resistance have been developed. However the frequency of homologous transformation is rather low, ranging from 0.3-2 transformants μg DNA^{-1} with oliC and between 40 and 50 transformants with pyrG.

1.4 Molecular Aspects of Aspergillus oryzae

Aspergillus oryzae is of broad interest to the commercial world due to the diversity of its extracellular secretion products (see Table 1) and its use in the food industry especially in the Far East where it is used in making soy sauce, miso and sake (Heseltine, 1983).

Fermentation procedures for this organism have been long established, the first patented process describing the production of A. oryzae enzymes being in1894. The high levels of protein secretion raise the possibility of exploiting A. oryzae as a vehicle for secreting foreign proteins by genetic manipulation.

Owing to their long association with the food industry A. oryzae and A. niger are two of the few filamentous fungi to have been given status *Category 1 "Generally Regarded As Safe"* (GRAS) from a health standpoint, for commercial purposes (World Health Organisation,1987).

Heterologous transformation systems have been developed for A. oryzae based on the A. niger pyrG gene (Mattern et al.,1987),the A. nidulans argB gene (Hahn & Batt,1988) as well as a homologous system using the A. oryzae met gene for the repair of methionine auxotrophy (Iimura et al.,1987). The frequencies of transformation for all these systems were never observed to be any higher than 5-20 transformants $\mu g\ DNA^{-1}$.

1.5 Molecular Aspects of Penicillium chrysogenum

Since the 1940,s there has been extensive research into Penicillium chrysogenum and the production of penicillin whose antibacterial activity had such a profound beneficial effect in the fight against disease, infection and on the general health of the human and animal population as a whole. As in the case of A. niger and A. oryzae progress in genetic studies of P. chrysogenum have been limited by the lack of a sexual stage. Parasexual analysis using protoplast fusion technology has identified six genetic loci thought to be involved in antibiotic production (Saunders & Holt,1987). These genetic studies of loss-of- function mutants have up until the present been of no assistance in the isolation of improved strains for industrial use. On the other hand the selection of mutants with increased penicillin titres has been successful although the basis of such mutants was unknown (Saunders & Holt 1987).

As a number of gene transformation systems for P. chrysogenum have recently been reported progress in strain improvement and investigation of this organism using molecular biology techniques is now a real possibility. In this regard four heterologous systems have been described based on transformation using the N. crassa pyr4 gene (Cantonel et al.,1987), the bacterial Tn5 phleomycin resistance gene (Kolar et al.,1988), the N. crassa mutated tubulin gene, tub2 conferring resistance to benomyl (Chiang,1988),and the amdS gene from A. nidulans (Ben & Turner,1987). Two other homologous systems have

also been reported based on the trpC gene (Sanchez et al.,1987; Picknett et al.,1987), and oliC (Bull et al.,1989).

1.6 Molecular Aspects of Gibberella fujikuroi

The filamentous fungus Gibberella fujikuroi (= Fusarium moniliforme) is a commercially important source of gibberellins which have plant growth-promoting properties (Jefferys,1973; Hedden et al.,1978). It is also of further agricultural interest in that it is pathogenic to rice and related grasses (Marasas et al.,1984).

For similar reasons as in the other fungi in this study i.e. lack of sexual cycle, limiting the use of classical genetic techniques, the understanding of the gibberellin biosynthetic pathway and further industrial strain improvement would best be facilitated using molecular biological methods. Up until this study (Sanchez-Fernando et al.,1991) no transformation system for G. fujikuroi has been published.

1.7 Nitrate Assimilation in Filamentous Fungi.

The nitrate assimilation pathway has been studied both genetically and biochemically in filamentous fungi, particularly in Aspergillus nidulans (Cove, 1979). Fig 1 shows the known pathway for nitrate assimilation in A. nidulans with the nitrate permease gene crnA being responsible for uptake of external nitrate which is thereafter internally converted to nitrite by the nitrate reductase gene niaD before being further converted to ammonium by the nitrite reductase gene niiA. A powerful tool in the study of this pathway is provided by the fact that mutations affecting nitrate utilisation can be so easily found by selection of colonies resistant to chlorate. It is thought that chlorate may act as an analogue of nitrate whereby it is converted by nitrate reductase to toxic chlorite thus the organisms without this enzyme activity are resistant to chlorate. This however does not completely explain the variety of mutations observed (Cove, 1979) and thus far the exact mechanism is not totally understood. But it is important to note that as resistance to chlorate arises spontaneously at high frequency mutagenisis is not normally required for the selection of mutants in this pathway.

As a number of genes code for elements involved in the assimilation and control of nitrate, chlorate resistant mutants have a spectrum of gene mutations which may easily be differentiated from each other by simple growth tests (Cove, 1979) and shown in Table 5.

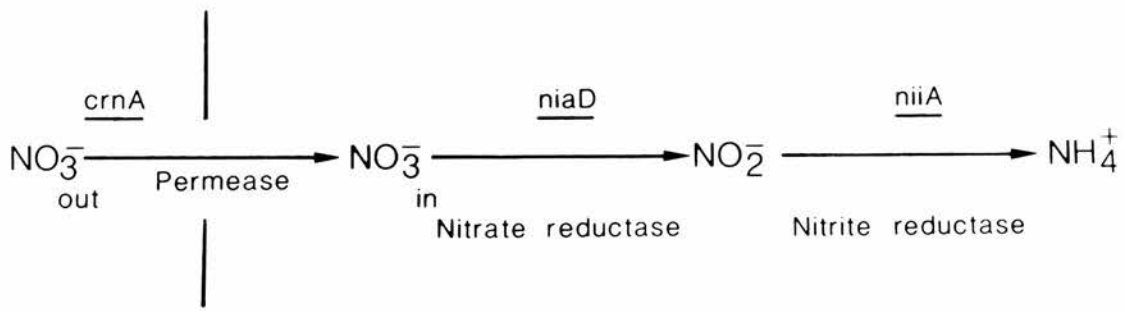

Figure 1 The nitrate assimilation pathway in Aspergillus nidulans.

External nitrate enters the cell via the permease gene crnA before being internally reduced to nitrate by the nitrate reductase gene niaD and then finally converted to ammonium by the nitrite reductase gene niiA.

Table5 Assignation of Genotypes of Chlorate Resistant Mutants of Aspergillus nidulans using the Phenotype Analysis of Cove (1979)

	Utilisation of sole nitrogen source				
Gene mutation	nitrate	nitrite	ammonium	hypoxanthine	glutamate
crnA *	+	+	+	+	+
niaD	-	+	+	+	+
nirA	-	-	+	+	+
areA	-	-	+	-	-
cnx	-	+	+	-	+
ΔniaD/ niiA	-	-	+	+	+

+ denotes wild type levels of growth

- denotes starved growth

* The *crnA* mutant probably has a wild type phenotype because it has a further nitrate transporter.

1.8 Advantages of the Nitrate System for Fungal Transformation

There are certain disadvantages with some selection systems outlined in Tables 2,3, and 4 particularly in the case of industrial fungi. For auxotrophic complementation, mutants first must be generated. This can be very difficult and tedious especially in poorly characterised organisms. In addition, mutagenesis may cause disturbances within general metabolic systems thereby reducing yields of desirable products within the industrial strain. Screening of such mutants can also be both time consuming and costly. Finally, industrial fungi are often diploid or even aneuploid and therefore recessive auxotrophic mutations would not be revealed. Although selection for drug resistance appears at first to overcome the problems associated with auxotrophic complementation, i.e. the wild-type strain can be used, it too has distinct disadvantages. As resistance genes are usually of bacterial origin commercial products in the food and pharmaceutical industry derived from this process would require lengthy and expensive clinical trials before being passed as safe by the authorities. In this regard, transformation of fungi using the nitrate system has some distinct advantages over other systems in that-

A. Mutants can be easily and economically isolated by selection for chlorate resistance without the necessity for mutagenisis.

B. niaD mutants are readily distinguishable from other chlorate resistant mutants by means of simple phenotypic tests.

C. The fact that the nitrate assimilation pathway is entirely dispensable under most growth conditions reduces the possibility of any metabolic disturbance during batch fermentation of industrial strains.

D. Foreign genes of bacterial origin need not be introduced.

E. The nitrate assimilation pathway has been extensively investigated genetically and biochemically. The niaD gene of A. nidulans has been cloned and sequenced (Johnstone et al., 1990) and was kindly made available to us as by Dr. I.Johnston, University of Glasgow as a heterologous probe to clone the A. niger, A. oryzae, and P. chrysogenum, genes.

2. Materials and Methods

2.1 Chemicals and Equipment

All chemicals (except where specified) were obtained from either British Drug Houses or Sigma and were of analytical grade. Novozyme 234 was provided by Novo Biolabs, Novo Industries. Restriction enzymes were purchased from Pharmacia or NBL.

Centrifugation: For routine genomic and plasmid DNA isolation Sorvall RC-5C/B (Dupont) refrigerated superspeed centrifuges were used with either a SS34, SA600, or GS3 fixed angle rotor. Ultracentrifugation was performed on a Sorvall Ultracentrifuge using the T865.1 fixed angle rotor. Phage DNA was isolated using a Beckman L2-65B ultracentrifuge with the SW65 swingout bucket rotor. Protoplasts were separated using a MSE benchtop centrifuge Microcentrifuge tubes were centrifuged in an Eppendorf microfuge.

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

2.2 Strains Media and Plasmids

A. niger wild-type strain IMI 60286 (ATCC 10864), A. oryzae wild-type strain IMI144242 (= ATC91002, α-amylase overproducing strain), P. chrysogenum strain V992 (= Q176),

and G. fujikuroi strain IMI58289, were used for selection of recipient mutants.

Escherichia coli strain DH5 (F^-, endA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1, r_k^-, m_k^-) was used for construction, propagation, and amplification of hybrid plasmids (Frischauf et al. 1983; Maniatis et al. 1982). E. coli strains NM 538 and NM 539 were used for the construction of the A. oryzae gene library.

Plasmids used in this research including pUC8, pUC13, and pUC18 were as described in Maniatis et al. (1982) as were the λ vectors such as EMBL3 used for the growth of genomic banks.

Standard media for cultivation of bacteria was as described in Maniatis et al.,(1982), media for A. niger, A. oryzae, P. chrysogenum were as described in Cove (1979). ICI medium for G. fujikuroi was described by Geissman et al., (1966) with the modification that 276g/l glucose, 1g/l yeast extract, and 1g/l peptone was added. CMP buffer was reported by Harris (1982). Minimal medium was described by Avalos et al., (1985).

2.3 Isolation and Characterisation of niaD Mutant Strains

With the exception of G. fujikuroi, nitrate assimilation defective mutants were obtained by positive selection to chlorate (Cove 1979). Approximately 10^9 spores were harvested in 10ml saline containing 0.08% Tween 80. Aliquots (0.2ml) were spread out on minimal medium

containing 470mM sodium chlorate with 5mM sodium glutamate as the sole nitrogen source. Chlorate resistant mutants were isolated between seven and fourteen days, depending on the species.

Single colonies of putative mutants were patched in a grid pattern onto complete medium, allowed to grow, replicated onto minimal medium supplemented with either 10 mM nitrate, 10mM nitrite, 10mM ammonium, 1mM adenine or1mM hypoxanthine.

Those mutants which failed to grow on nitrate but grew as wild-type on other sole nitrogen sources were designated niaD and checked for stability.

2.4 Reversion Stability of Chlorate Resistant Mutants

Suspensions of approximately 10^8 spores of chlorate resistant isolates were plated onto minimal medium containing 10mM nitrate and the number of nitrate utilisers recorded after fourteen days. Those with a reversion frequency of $<10^{-8}$ were chosen as recipients for transformation experiments.

2.5 Protoplast Preparation and Transformation procedures

As there are differences in the general culturing and protoplasting procedure for each organism a full description for each is given below.

2.5.1 A.niger and A. oryzae

The A. niger and A. oryzae protoplasting procedure was developed from the A. nidulans method (Ballance & Turner 1983; Tilburn et al., 1983; Yelton et al.,1984; Marion & Peberdy 1984) and modified from a previous A. niger procedure (Unkles et al., 1989) by Campbell et al.,(1989) Conidia from a mature culture grown on complete medium (one 9 cm diameter Petri dish) were harvested and used to inoculate 250ml minimal medium containing 20mM ammonium tartrate as sole nitrogen source. This culture was grown at 30^{0}C for 12h in a New Brunswick orbital shaker at 250 r.p.m. The resulting cells were collected by harvesting through sterile gauze and washed with soln. A (0.8M $MgSO_4$ in 10mM PO_4 buffer pH 5.8). To the cell suspension, in a sterile universal container, was added 20ml of soln. A and 0.1g Novozyme 234. This was placed in the orbital incubator at 200 r.p.m. for 45min at 30^{0}C. The suspension was centrifuged at 3,000 r.p.m. for 10min at room temperature. The suspension was divided into 4 equal aliquots in universal containers and 20ml soln. B (1.2M sorbitol, 50mM $CaCl_2$, 10mM Tris pH 7.5) added and centrifuged at 2,000 r.p.m. at room temperature for 5min. The supernatant was discarded and the small pellets of protoplasts were gently resuspended and combined in soln. B to a total volume of 20ml and centrifuged at 2,000 r.p.m. for 5min. This procedure was repeated twice more to remove residual Novozyme. The cells were resuspended in 100µl soln. B and transferred to a 1.5ml sterile microfuge

tube. 10μg DNA (volume no greater than 10μl) was added and gently mixed by tapping the tube wall. 12.5μl soln. C (50% PEG 4,000, 50mM $CaCl_2$, 10mM Tris pH 7.5) was added, The solution was gently mixed and incubated on ice for 20min. The mixture was transferred to a sterile test tube containing 1ml soln. C, after a gentle shake 2ml soln. B was added (to dilute PEG), gently mixed and added to precooled (55^0C) selection medium (minimal medium containing 1.2M sorbitol and 10mM sodium nitrate as sole nitrogen source). An aliquot of the 10^{-1} dilution of untransformed protoplasts was added to selection medium as a control. This dilution as determined by haemocytometer counting was usually around 1.5 X 10^7 protoplasts. An aliquot of the 10^{-4} dilution of untransformed protoplasts was added to 20ml of non-selective medium (minimal medium containing 1.2M sorbitol and 10mM ammonium tartrate as sole source of nitrogen). All solutions were freshly made before use.

2.5.2 P. chrysogenum

Mycelia was grown in liquid complete medium for 20h at 26^0C in an orbital shaker at 290 r.p.m. before being harvested in sterile gauze washed with sterile distilled water and placed in 50ml 1M $MgSO_4$, 10mM PO_4 buffer, pH 5.8 containing 5mg/ml Novozyme 234 for 3h with gentle shaking at room temperature. The contents were transferred to sterile universals, overlayed with 800mM $MgSO_4$ and centrifuged at 4,000 r.p.m. 20min at room temperature. Floating protoplasts were removed and washed three times

in 700mM KCl, with centrifugation at 2,500 r.p.m. for 10 mins. Washed protoplasts were transformed using a modification of the method of Cantoral et al.,(1987).

Approximately 5 X 10^7 protoplasts were resuspended in 500µl KCM (0.7M KCl, 50mM $CaCl_2$, 10mM MOPS, pH 5.8) with 1-10µg transforming DNA and 50µl PCM (50% PEG 8,000, 50mM $CaCl_2$, 10mM MOPS, pH 5.8). After 30 min incubation on ice, 2ml of PCM was added and the protoplasts given a further incubation at room temperature before being spread on minimal medium supplemented with 10mM sodium nitrate as sole nitrogen source and osmotically stabilised with 0.7M KCl. Control dilutions were prepared as in the case of A. niger and A. oryzae.

2.5.3 G. fujikuroi

ICI medium (50ml) with 10mM ammonium tartrate as sole source of nitrogen was inoculated with 2 X 10^6 spores in a 500ml flask and incubated in an orbital shaker (250 r.p.m) for 40h at 30^0C. The mycelium was harvested by retention in sterile gauze, washed with minimal medium and finally resuspended in 10ml ICI medium. This suspension was sonicated for 20s and centrifuged in 2ml CMP buffer (Harris, 1982) containing Novozyme 234 at final concentration 5mg/ml and Lyticase (1,000 units; Sigma). This was incubated with gentle shaking 4h at 30^0C. Protoplasts were separated from mycelial debris through a sterile nylon net, resuspended in 20ml soln B.

The method of transformation was the same as in A. niger.

Transformed cells were plated out on selective medium (minimal medium with 3g/l sodium nitrate as sole nitrogen source and 1.2M KCl as the osmotic stabilising agent). Control dilutions were plated out on selective and non-selective medium as in A. niger. Cultures were incubated at 30^{0}C for 10-15 days.

2.6 General Molecular Genetics Techniques

2.6.1 Transformation of E. coli.

Transformation of E. coli was performed using an adaptation of the method described in Maniatis et al., (1982). Competent cells were obtained by inoculating 100ml Luria Broth (LB) with 1ml of an overnight culture of E. coli grown in LB at 37^{0}C. This was incubated in an orbital shaker at 300 r.p.m., 37^{0}C to O.D.$_{600nm}$ 0.175 to 0.225.
The cells were centrifuged at 4000 r.p.m., 4^{0}C for 10min and resuspended in 25ml ice cold 100mM $MgCl_2$ then centrifuged as above and resuspended in 25ml ice cold 100mM $CaCl_2$. Following incubation on ice for 1h the cells were again centrifuged and resuspended in 2.5ml ice cold 100mM $CaCl_2$, 14% glycerol and aliquoted in 100µl amounts into pre-cooled microfuge tubes for storage at -70^{0}C DNA (0.1-1 µg) was added to a 100µl aliquot of competent cells and incubated on ice for 30min before heat-shocking for 2min at 42^{0}C. 1ml of LB was added to the cells and incubated for 45min at

37°C. Appropriate dilutions were spread onto Luria medium containing 50μg/ml ampicillin and incubated overnight at 37°C.

2.6.2 Plasmid DNA Isolation from E. coli

Isolation of plasmid DNA was adapted from the method described by Maniatis et al., (1982). 200ml of LB containing 50μg/ml ampicillin was inoculated with 10ml of an overnight culture OD_{600} 1-1.5 of E. coli. After 12 to 14h shaking at 300 r.p.m., 37°C the cells were harvested by centrifugation at 4000 r.p.m. at 4°C for 10min. The cell pellet was resuspended in 4ml of soln I (50mM glucose, 10mM EDTA, 25mM Tris pH 8.0) and incubated at room temperature for 5min. The cells were lysed by the addition of 8ml soln II (0.2M NaOH, 1% SDS) and incubated on ice for 5min. Cell debris, protein and chromosomal DNA were removed by centrifugation at 12,000 r.p.m., 40min at 4°C following precipitation on ice for 10min with the addition of 6ml ice cold 3M potassium acetate pH 4.8. The supernatant was decanted through muslin and an equal volume of isopropanol added. After 15min the precipitated DNA was pelleted by centrifugation at 12,000 r.p.m., 18°C for 20min. The pellet was dried, resuspended in 4ml TE (10mM Tris pH 7.5, 1mM EDTA) containing RNase to a final concentration of 100μg/ml for 1h at 37°C. The plasmid DNA was further purified by the use of a commercial anion exchange resin, Qiagen-tip 500 (Hybaid cat. no. 10063) following manufacturers instructions and resuspended in 200μl TE.

2.6.3 Isolation of Fungal Chromosomal DNA

Isolation of chromosomal DNA from wild-type and transformed strains was adapted from the method of Tilburn et al., (1983). Approximately 5g mycelium was harvested from a 250ml overnight culture grown in A. nidulans complete medium. This was ground to a fine powder in liquid nitrogen and 20ml of soln I added (0.5M sucrose, 50mM EDTA, 25mM Tris pH 7.5). Sarkosyl NL30 was added to a final concentration of 4% and SDS to 0.5%. After incubation at 60°C for 1h the cell debris was pelleted by centrifugation at 10,000 r.p.m. for 10min, the supernatant removed and incubated overnight at 35°C with 400μg/ml proteinase K. An equal volume of soln II (30% PEG 6,000, 1.5M NaCl) was added, mixed well and incubated on ice for 1h before pelleting the DNA by centrifugation at 15,000 r.p.m. for 20min, 4°C. The DNA was then gently resuspended at 37°C with the addition of 10ml TE containing RNase to a final concentration of 100μg/ml overnight followed by four to five phenol/chloroform extractions before precipitation with 3M sodium acetate and EtOH at -20°C. The precipitated DNA was pelleted by centrifugation at 10,000 r.p.m., 10min at 4°C, washed with 70% EtOH, dried and finally resuspended in 1ml TE.

2.6.4 Isolation of Phage DNA

E. coli LE392 was inoculated into 250ml NZYDT medium (Gibco) with approximately 10^{10} viable phage and grown 12h at 37°C,

350 r.p.m. to produce efficient lysis. Chloroform (4ml) was added and the culture shaken for a further 2min and the bacterial debris pelleted by centrifugation at 8,000 r.p.m., 4^{0}C for 10min. To the supernatant, PEG 6,000 was added to 10% w/v and NaCl to 1M and dissolved. This mixture was incubated on ice for 30 to 40min before centrifugation at 7,000 r.p.m. for 15min and resuspension in 5ml TE containing 10mM $MgCl_2$. An equal volume of chloroform/isoamylalcohol (24:1) was added, mixed well and centrifuged 4 to 6,000 r.p.m.,16^{0}C for 5 to 10min.The aqueous phase was removed and centrifuged at 35,000 r.p.m. for 20min in a SW55 rotor. The pellet was resuspended in 600ml TE, 10mM $MgCl_2$ and microfuged at 14,000 r.p.m. for 2min. To the supernatant was added 3μl of 20% SDS, 8μl of 0.5M EDTA and 10 to 20μl RNase (20 mg/ml) and the mixture incubated at 70^{0}C for 10 to 15 min. After 1 phenol and 2 chloroform/isoamylalcohol extractions the purified DNA was precipitated with 0.1 vol 5M sodium acetate and 2 vol EtOH at -20^{0}C.

2.6.5 Restriction Enzyme Digestion of DNA

Restriction enzyme digests were performed with the recommended assay buffers at 37^{0}C for 2-4h for plasmid or phage DNA. For restriction site mapping purposes a combination of single, double or triple digests were performed Where appropriate, the digest reaction was carried out in a common buffer. If this was not possible then the enzyme with the lowest salt requirement was digested first with the salt

concentration increased for the second enzyme. Digests of fungal chromosomal DNA were carried out overnight with addition of fresh enzyme after 10h. Depending on circumstances digestions were terminated by :-

1. Precipitation of the DNA with sodium acetate/EtOH.
2. Addition of EDTA.
3. Heat inactivation at 75^0 C.
4. Phenol extraction.
5. The addition of 1/10 volume of loading buffer (0.025% bromophenol blue, 0.025% xylene cyanol, 15% Ficoll)

2.6.6 Electrophoresis of DNA

DNA was electrophoresed through a horizontal 0.8% agarose (NBL) gel in 1 X TEA (0.04M Tris-acetate, 0.0001M EDTA) containing 500ng/ml ethidium bromide, the running buffer also being 1 X TEA. Electrophoresis was performed at convenience, usually between 20-100V until the bromophenol blue dye had migrated approximately two-thirds through the length of the gel.

2.6.7 Recovery of DNA Fragments

After the DNA had been digested to completion it was electrophoresed, as in section 2.6.5 through 1% low melting point agarose containing 500 ng/ml ethidium bromide. The band of interest was excised from the gel and transferred to

a microfuge tube. It was then eluted and bound to a commercial matrix (Prepagene from NBL) before purification following manufacturers instructions. The resulting DNA was then recovered by EtOH precipitation with 0.3M sodium acetate.

2.6.8 Ligation and Subcloning of DNA Fragments

Ligation and subcloning of DNA fragments were performed essentially as described by Maniatis et al., (1982).

2.6.9 Southern Analysis of Genomic DNA

Following conventional Southern blotting (Maniatis et al., 1982) on to nylon membranes (Hybond-N, Amersham U.K.), hybridisations were performed in plastic sandwich boxes containing 2-3ml prehybridisation/hybridisation solution per square centimetre. This solution contained 5 X SSPE, 6% PEG 6,000 (BDH Chemicals, Poole, U.K.), 0.5% skimmed milk (Marvel, Cadbury, U.K.), 0.1% $Na_4P_2O_7$, 1% SDS, and 250μg/ml sheared, boiled, herring sperm DNA. After prehybridisation for 4-5h, the probe was added and incubated overnight. Hybridisation temperature was usually 65^{0}C for homologous probing and 54^{0}C for heterologous probing. The DNA was labelled using an Amersham Multiprime kit (RPN 1601) modified from the method of Feinberg & Vogelstein (1983, 1984). After heating to 100^{0}C for 2min and cooling on ice, 50ng (10μl) of DNA was added to 18μl sterile water, 10μl 5 X buffer (10mM ATP, 10mM GTP, 10mM TTP, in 100mM Tris pH 7.8, 100mM $MgCl_2$ and 2-β-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-β-mercaptoethanol and 50% glycerol) and incubated at room temperature for 4-5h or 37^{0}C for 30min. Unincorporated nucleotides were removed using a commercial Nick™ column (Pharmacia). For low or high stringency, hybridisation and washing temperatures were 54^{0}C and 65^{0}C respectively. Membranes were washed in 5 X SSC, 0.1% SDS, 0.1% $Na_4P_2O_7$ for low stringency or 0.2 X SSC, 0.1% SDS, 0.1% $Na_4P_2O_7$ for high stringency.

2.7 Construction of Gene Libraries

2.7.1 A. niger

Chromosomal DNA for library construction was isolated from nuclei essentially by the method of Garber & Yoder, (1983). Following partial *Sau*3A digestion, fragments 6-12 kb were isolated from 1% agarose by centrifugation through nitrocellulose and ligated to *Bam*HI digested pUC8 to yield a library containing 19,500 clones in total.

2.7.2 A. oryzae

A gene bank of A. oryzae wild-type strain (IMI44242) was kindly supplied by Dr. Van Hartingsveldt , TNO, Netherlands and is decribed in Van Hartingsveldt et al., (1987). A primary library of 98,000 plaques, representing 40-50 copies of the

genome, was obtained.

2.8A Lambda Libraries

E. coli strain LE 392 was prepared for transfection by adding 1ml of an overnight culture to 100ml NZYDT broth and growing at 37^{0}C, 300 r.p.m. until an OD_{600} of 0.45 was obtained. The cells were harvested by centrifugation at 4,000 r.p.m. for 10 min and resuspended in 0.15 vol SM buffer. Initial screening was performed in 250ml bioassay plates. 2.5ml fresh plating bacteria was infected with 10^4 pfu in universals and shaken gently for 20 min at 37^{0}C, added to 50ml Top NZYDT (0.7% agarose) at 50^{0}C, and quickly poured onto pre-warmed bioassay plates before incubation at 37^{0}C for 7-9 hrs until the resulting plaques were a reasonable size.

The plates were stored at 4^{0}C for 2 hrs to allow the top agarose to harden before being overlayed with dry sterile nylon membrane. The membranes were quickly removed and placed upside down on 3MM paper dampened with denaturing soln for 20 sec and then on 3MM paper dampened with neutralising soln for 20 sec before washing in 3 X SSC, drying and binding under UV for 5 min. Heterologous hybridisation was as described in 2.6.9.

2.8B Plasmid Libraries

Transfected colonies containing plasmid were plated on LB medium with antibiotic and grown 16 hrs at 37^{0}C. Circular

nylon hybridisation membrane was placed evenly over the colonies and orientation holes in an asymetric pattern made with a syringe needle.

After 1-2 mins filters were lifted and placed colony side up onto 3MM paper saturated with denaturing solution for 5 mins. The filters were then blotted briefly of excess liquid on paper towel before being placed colony side up on 3MM paper soaked in neutralising solution for 5 mins. Excess liquid was removed as before and the filter finally placed on 3MM paper soaked in 1.5M NaCl and 2 X SSC before being air-dried on the bench for 1hr.

Hybridisation of membranes was as described in 2.6.9. Positive hybridising colonies on the autoradiogram were aligned to the original colonies on the plates using the orientation marks.

2.9 Di-deoxy Sequencing of Double Stranded DNA

Sequencing of denatured double stranded DNA was performed following the Sequenase™ Version 2.0 T7 DNA polymerase sequencing protocol (USB, U.K. supplier Cambridge Bio-labs) supplied by the manufacturer.

Prior to electrophoresis through a polyacrylamide sequencing gel 3μl of each sample was heated to 75-80^{0}C for 2mins. Electrophoresis was performed at 2000V for 4-8h before briefly washing the gel in 10% methanol,10% acetic acid and drying onto 3MM Whatman paper. After 24h exposure against the dried gel the autoradiograph film was developed and the nucleotide sequence determined.The nucleotide sequence and predicted amino acid sequence was

analysed using the programs supplied as part of the University of Wisconsin Genetics Computer Group sequence package and accessed through the Science and Engineering Council SEQNET system.

3. Results and Conclusion

3.1A Isolation of niaD Mutants.

Spontaneous chlorate resistant mutants were seen to arise at a frequency of 1 in 10^6 spores. Approximately 100 mutants of each organism under investigation were tested for phenotypic identity as described in Materials and Methods 2.3 by their ability to grow on nitrate, nitrite, ammonia, hypoxanthine, proline, or glutamate as sole nitrogen source. In general around 50% of chlorate resistant mutants were found to be *nia*D as judged by these plate tests. Several *nia*D mutants from each organism were tested for reversion to nitrate prototrophy with those having a reversion frequency of less than 1 in 10^7 viable spores being selected as suitable recipients for transformation experiments.

3.1B Isolation and Characterisation of the Aspergillus niger niaD Gene.

The A. niger genomic library was divided into nine pools, four of which contained 3000 clones, the remainder containing 1500 clones each. DNA isolated from each pool was digested with *Eco*RI or*Bam*HI, subjected to 0.8% agarose gel electrophoresis followed by Southern blotting and hybridisation to a 2.7kb *Xba*I fragment of pSTA8. This fragment contains the 5'-coding region of the A. nidulans niaD gene subcloned from the pniiA *Eco*RI fragment in p*ILJ*16. (Johnstone et al, 1990).

Only one pool showed strong hybridisation to the probe which was selected for standard colony hybridisation using the same probe. DNA from putative niaD clones was isolated and preliminary restriction enzyme analysis showed these to be identical. A detailed restriction map of the A. niger clone pSTA10 is shown below in Fig. 2.

The approximate 5' position of the initiation codon of the A. niger niaD gene as judged by hybridisation to the 0.9kb *Xba*I -*Kpn*I probe derived from the extreme 5' end of the A. nidulans nitrate reductase gene, is indicated. The direction of transcription shown by the arrow was inferred by hybridisation of a 3.5kb *Sal*I fragment of λAN8a representing the 3' end of the A. nidulans gene. In addition, probing pSTA10 with a 0.8kb *Eco*RI fragment containing an internal stretch of the A. nidulans nitrite reductase structural gene (niiA) hybridised strongly to the region of pSTA10 suggesting that, as in A. nidulans, the A. niger nitrate reductase and nitrite reductase are contiguous and transcribed in opposite directions.

pSTA10 digested with *Hin*dIII was back-hybridised to wild-type A. niger genomic DNA cleaved with six restriction endonucleases (Fig. 3). By comparison with the wild-type there were no indications of rearrangements of the cloned DNA in pSTA10.

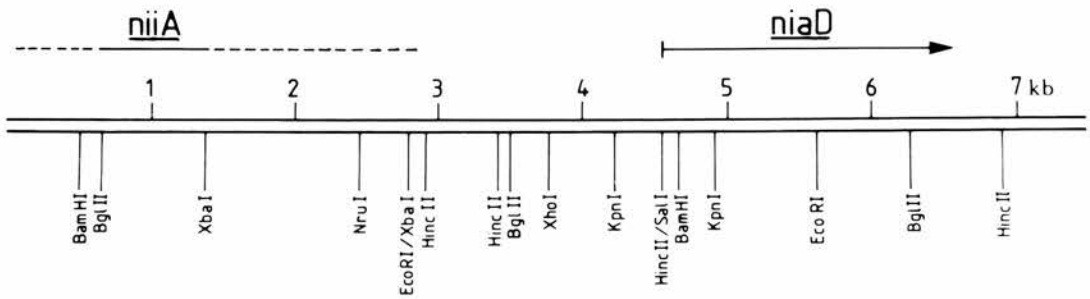

Fig 2. Restriction Map of A.niger Nitrate Reductase Clone,pSTA10.

The map was determined by single and double digests of the enzymes indicated. Vector sequences are not included. The *Bam*HI site on the polylinker to the right of the clone was disrupted during the cloning procedure. No sites were found for enzymes *Acc*I, *Pst*I, *Sma*I, or *Xma*I. *Eco*RI/*Xba*I and *Hin*dIII/*Sal*I restriction sites are very close and could not be distinguished by restriction analysis. The approximate position of the 5' end of the A. niger niaD gene was determined by hybridisation of the digested, Southern-blotted pSTA10 to an extreme 5' A. nidulans niaD probe. The arrow indicates the direction of transcription as judged by hybridisation to a 3' A. nidulans niaD probe. The position of niiA gene is inferred by hybridisation to an A. nidulans niiA probe. Hybridisation was performed at 54^{0}C followed by washing to 3 x SSC.

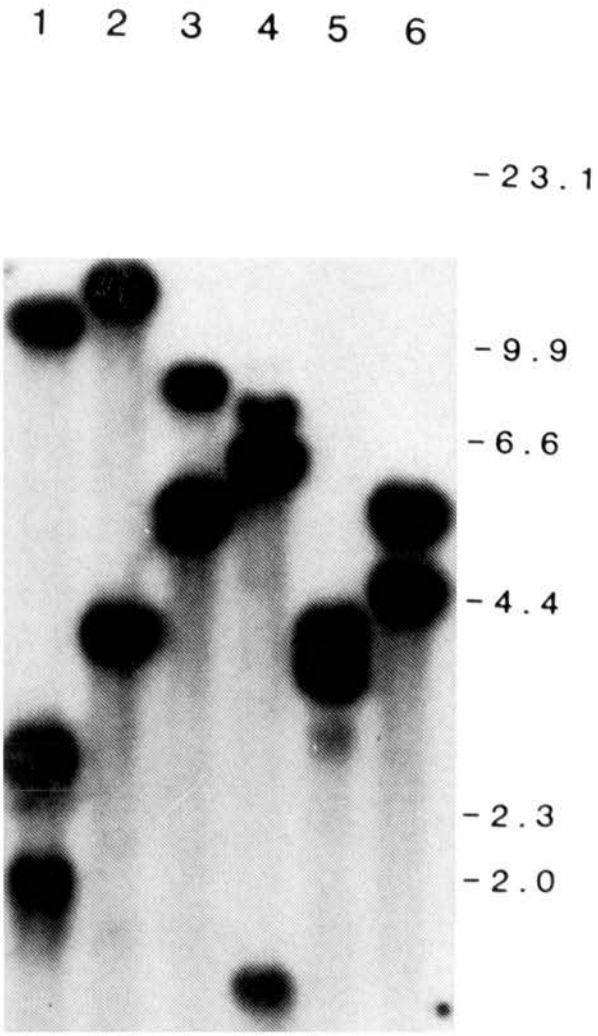

Fig 3. Hybridisation of pSTA10 to A.niger Genomic DNA

A. niger genomic DNA was digested with *Eco*RI (lane1), *Xho*I (lane 2), *Nru*I(lane 3), *Xba*I (lane4), *Bam*HI (lane5) and *Sal*I (lane6), and the resulting fragments separated by electrophoresis in 0.8% agarose. After blotting onto nitrocellulose and baking for 2h at 80^{0}C under vacuum, the filter was probed with pSTA10 linearised with *Hin*dIII. Hybridisation was carried out at 65^{0}C followed by washing to 0.2 x SSC.

3.1B Frequency of Transformation and Fate of Transforming DNA in Aspergillus niger

Transformation of A. niger with circular pSTA10 resulted in up to 46 transformants per μg DNA^{-1} with STAN5 as recipient strain (Table 6). Fewer transformants were routinely obtained when the mutant strain STAN6 was used as recipient. Fig. 4 shows the background growth of the mutant on nitrate to be extremely poor, supporting the notion that the nitrate system is amenable for effective transformation selection. Furthermore, unlike some other systems such as the amdS (Kelly and Hynes, 1985) or pyrG genes (Van Hartingsveldt et al., 1987), no background of abortive transformants was visible. Some variation (up to tenfold) in transformation frequencies was obtained between experiments. However this is not an uncommon observation. For example, there is a sixfold variation in transformation frequencies of trpC into Penicillium chrysogenum (Picknet et al., 1987) and almost twenty-fold for hygromycin B into Fulvia fulva (Oliver et al., 1987). Plasmid pSTA10 was restricted with *Nru*I which cleaves the molecule at a single site 5' to the probable position of the nitrate reductase gene (Fig 2). Transformation frequencies initially, although again variable, were always between two and eight-fold higher in mutant strain STAN5 and two to seven-fold higher in strain STAN6 than frequencies obtained using circular plasmid. Subsequent improvements in the transformation procedure described in MATERIALS AND METHODS resulted in further increases of up to eight-fold over the previous procedure using linearised plasmid.

Dhawale and Marzluf, (1985) likewise observed a three to four-fold increase in transformation frequency with the introduction of linearised plasmid into Neurospora crassa, as did Skatrud et al, (1987) in Cephalosporiun acremonium. Increase in transformation frequency probably reflects the position of the cut, giving rise to homologous ends which are more "recombinogenic" rather than marker-specific effects or increased efficiency of DNA uptake.

Fig. 5. shows in a representative number of transformants (designated T1 to T6), obtained after transformation of strain STAN5 with circular pSTA10, the presence of bacterial sequences revealed by pUC8 probing of *Bam*HI-digested genomic DNA.

Integration occurred at a similar site in T1, T2, T4, T5 and T6, giving a diagnostic 6kb DNA fragment. Transformant T6 has an additional signal at 5.8kb, suggesting a non-homologous integration event within the fungal sequences of pSTA10. Transformant T3 gave two different fragments (10kb, 7.6kb) indicating either recombination within pUC8 sequences or two separate non-homologous integration events within the fungal sequences. Six further transformants examined (T7 to T12; data not shown) showed similar patterns to T1, T2, T4 and T5, while wild-type DNA did not hybridise to pUC8. Therefore, in all twelve transformants, examined by hydridisation, bacterial sequences are present, showing that recombination by gene conversion has not taken place, at least, in any of these cases.

Table 6 Transformation Frequencies of Circular and Linear Plasmids.

Strain designation	Mutant allele	Plasmid	Conformation[a]	Number[b]
STAN5	*niaD*5	pSTA10	circular	46
		pSTA10	linear	106
STAN6	*niaD*6	pSTA10	circular	7
		pSTA10	linear	50
STAN5	*niaD*5	pSTA12	circular	9
		pSTA12	linear	50

[a] Circular refers to plasmid preparations untreated with a restriction enzyme; linear refers to plasmid cut with *Nru*I.

[b] Maximum number of transformants per μg of plasmid DNA obtained.

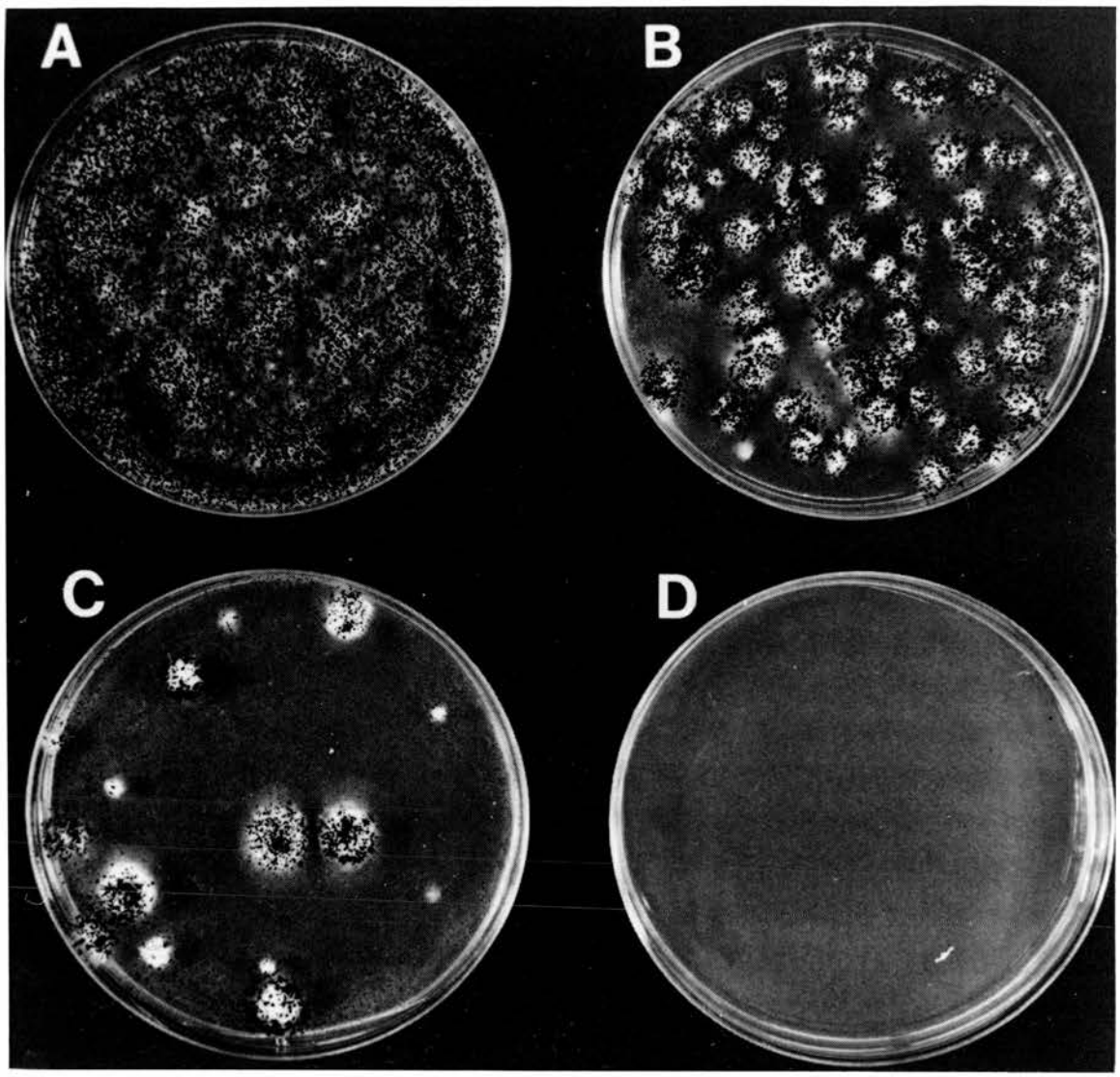

Fig 4. Appearance of Control and Transformed niaD5 Strains Growing on Nitrate and Ammonium.

A. untransformed on 10mM ammonium tartrate; B. transformed with linear pSTA10 on 10mM $NaNO_3$; C. transformed with circular pSTA10 on 10mM $NaNO_3$; D. untransformed cells on 10mM $NaNO_3$.

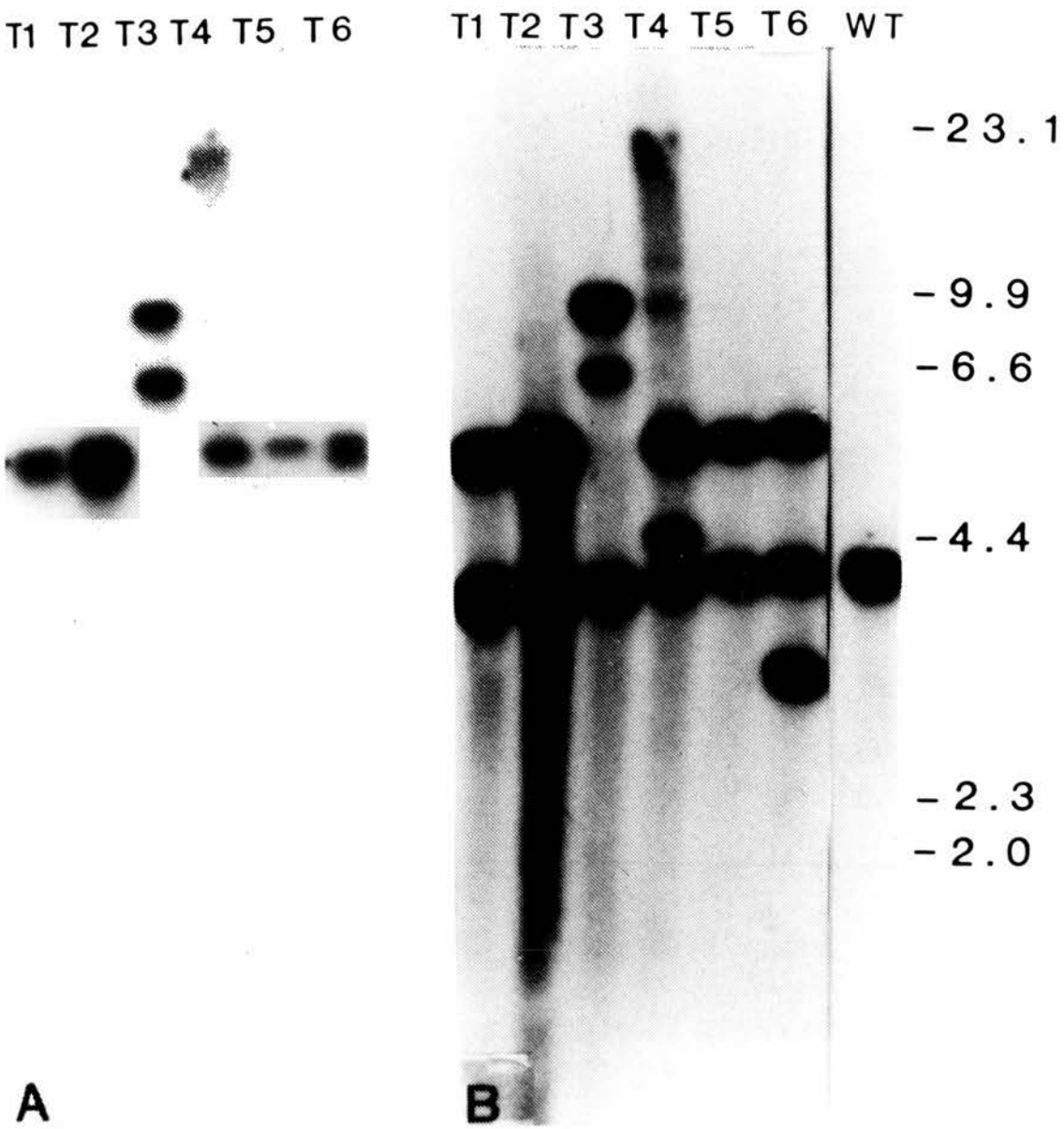

Fig 5. Molecular Analysis of A. niger niaD Transformants.

Genomic DNA from six transformants designated T1-T6 and wt A. niger were digested with *Bam*HI and separated on 0.8% agarose and subjected to hybridisation.
Panel A: *Eco*RI-digested pUC8 probe. Panel B: Re-hybridisation of the filter to a 1.9kb *Eco*RI niaD internal fragment of pSTA10 (Fig. 2). DNA size markers are shown.

The filter was reprobed with the A. niger niaD internal 1.9kb *Eco*RI fragment of pSTA10. This fragment extends from the *Eco*RI site of the niaD gene (Fig. 2.) to the 3' *Eco*RI site on the polylinker of pUC8 (not shown). Fragments of a similar size to those with the pUC8 probe were seen as expected, representing the newly introduced niaD copy. Additional niaD sequences are seen in transformants T4 (4.7kb), T6 (3kb), T10 (5kb, not shown) and T12 (5.4kb,not shown).

3.1D Mitotic Stability of Aspergillus niger Transformants

Previous studies of transformant stability in filamentous fungi have examined the frequency of loss of a gene after several rounds of replication on non-selective medium. Such studies suffer the disadvantage that often only small numbers, usually a few hundred cells, are phenotypically screened for the presence or absence of the exogenous gene. Furthermore, normally only a few transformants are studied since screening of cells derived from transformants can be laborious, especially with auxotrophic markers. As nitrate non-utilising strains can be selected directly by growth on chlorate, a much larger sample can be analysed and this therefore allows a more precise investigation of the stability of the exogenous gene. This is a usefull feature of the nitrate system, in that it can reveal, quickly and easily assay for the instability of a niaD gene contained in just a few nuclei within a large population. Of course, chlorate resistance would result from mutation in the resident niaD locus, and also other nitrate utilisation genes (such as cnx and nir), but account can be taken of this by simultaneous determination of the frequency of chlorate resistance in the wild-type A. niger. The difference in numbers of chlorate-resistant colonies in wild-type and transformants, therefore, should be due only to niaD instability, e.g. deletions, re-arrangements, point mutation in the transforming niaD gene. Table 6. shows the frequency of reversion of 12 niaD transformants to chlorate resistance compared with the mutation frequency of wild-type A. niger (i.e., below 2×10^{-6}).

Only two transformants, T6 and T9, showed wild-type frequencies. Transformants T2 and T4 were less stable, with reversion frequencies of around 3×10^{-5}. The other transformants were notably unstable at 1×10^{-4} and above. These figures represent the lowest frequency of instability that could be expected, since the transformants were maintained under selective conditions. As expected, significantly lower stability frequencies are found in cells grown under non-selective conditions (data not shown).

These results demonstrate that even under selective pressure i.e., growth on nitrate, the integrated niaD gene would appear to be less stable than the resident wild-type copy. No direct correlation could be made between stability and location of the integrated niaD. gene.

Table 6 Mitotic Stability of A. niger Transformants

Strain[A]	Reversion rate (x 10^{-6})[B]	
	Exp. 1	Exp. 2
wild-type	< 2	< 2
T1	875	125
T2	27	27
T3	780	1300
T4	84	33
T5	400	980
T6	< 3	< 2
T7	1250	360
T8	630	470
T9	< 2	< 7
T10	580	200
T11	126	1500
T12	1800	430

[A] Strains T1 to T12 are randomly selected A. niger transformants, transformed with circular plasmid DNA.

[B] Conidia were taken from five day cultures grown on minimal medium containing 10mM $NaNO_3$ as sole nitrogen source. Ten-fold dilutions were made in saline-Tween. Viable counts were obtained by plating on complete medium. All counts were as a result of three individual dilution and platings. Experiments 1 and 2 are independent.

3.2A Isolation and Characterisation of the Aspergillus oryzae niaD Gene.

A 2.3kb *Xba*I fragment containing the A. nidulans niaD nitrate reductase structural gene was hybridised against various genomic DNAs digested with *Eco*RI. Unique bands of 6kb, 2.8kb, and 2.5kb were observed with A. niger , A. oryzae and P. chrysogenum respectively (Fig. 6). From an A. oryzae gene library of 40,000 plaques hybridised with the A. nidulans 2.3kb *Xba*I fragment 17 positive clones were identified. Two of these designated (λSTA51 and λSTA62) were purified for further studies. Both contained *Sal* I fragments (5.5kb and 8.2kb respectively) which hybridised strongly to the A. nidulans niaD probe and these overlapping *Sal*I fragments were subcloned into the *Sal*I site of pUC18 to give plasmids pSTA13 and pSTA14 respectively. Only pSTA14 (Fig. 7) was capable of phenotypic rescue of the A. oryzae niaD14 mutant to nitrate utilisation and so it was assumed that pSTA14, but not pSTA13, contained the entire structural gene for nitrate reductase. Radiolabelled *Xba*I-digested pSTA14 was hybridised to a Southern blot of wild-type A. oryzae DNA. Identical bands were observed using either pSTA14 or the *Xba*I fragment of A. nidulans niaD (data not shown). This showed positive identification of the clone. No evidence for rearrangements of the clone sequences in pSTA14 (by comparison with wild type genomic sequences) was detected In this regard the expected internal fragments, 2.5kb and 1.8kb *Bam*HI, 2.5kb *Eco*RI and 4.2kb *Bgl*II, were observed.

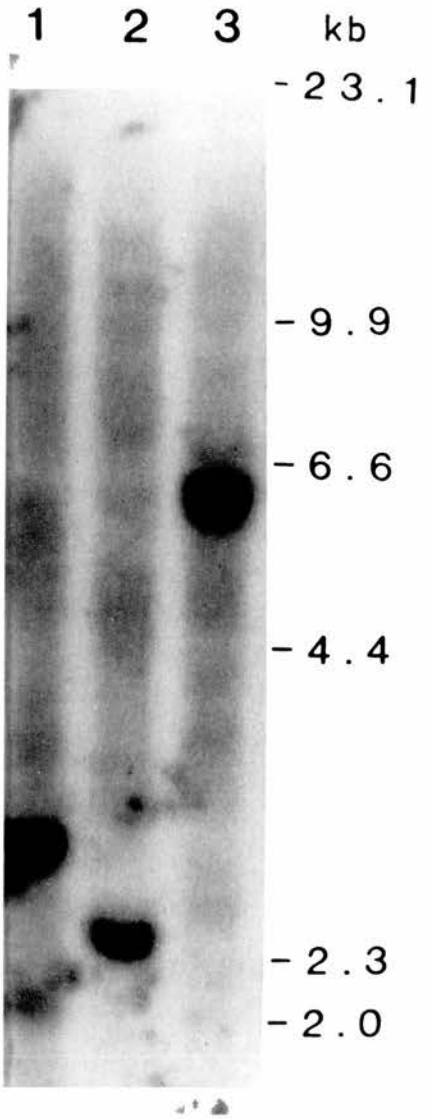

Fig 6. Heterologous Hybridisation of the Aspergillus nidulans niaD Gene

Genomic DNA from (1) A. niger, (2) A. oryzae, and (3) P. chrysogenum was digested with *Eco*RI and fragments separated on 0.8% agarose. Following transfer to nylon, the filter was probed with a 2.3kb ^{32}P-labelled *Xba*I fragment containing the A. nidulans niaD gene. Hybridisation was at low stringency (54^{0}C, washed once with 5 X SSC) The positions of λ*Hind*III molecular size markers are indicated.

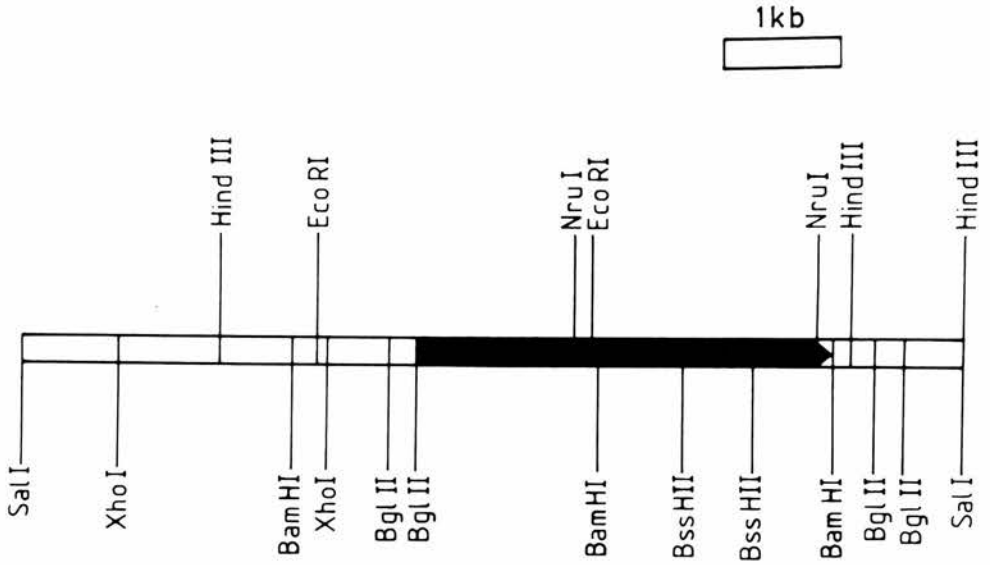

Fig 7. Restriction Map of pSTA14 Containing the Aspergillus oryzae niaD gene

This map was determined by single and double digestions using the enzymes indicated. Vector sequences are not included. No sites were found for enzymes *Bsc*I, *Pst*I, *Sma*I, *Xba*I, or *Xma*I. The approximate position of the niaD gene within pSTA14 is indicated by the *solid bar.* The start of the niaD gene was determined by hybridisation to an extreme 5' A. nidulans niaD fragment. Hybridisation experiments were carried out at 54^{0}C followed by washing in 5 x SSC.

3.2B Frequency of Transformation and Fate of Transforming DNA in Aspergillus oryzae

Transformation of A. oryzae mutant niaD14 with circular plasmid molecules of pSTA14 yielded an average of 64 transformants per μg DNA^{-1} (Table 7).

The background growth of this strain with nitrate as sole nitrogen source, was extremely poor. Additionally no abortive transformants were observed. Certain transformants initially grew slower than the others but eventually developed into mature colonies. Transformation efficiency increased giving an average of 455 transformants per μg DNA^{-1} when pSTA14 was linearised with *Hin*dIII (Table. 7). Other research groups have likewise observed increases in fungal transformation frequencies using linearised plasmid molecules- the frequency depending on the position of the site of cleavage, ie within bacterial or fungal sequences (Dhawale and Marzluf1985; Skatrud et al. 1987; Wang et al 1988).

To determine the types of integration events which occurred using uncut pSTA14 to transform the niaD14 strain, chromosomal DNA of 22 nitrate-utilising transformants were subjected to Southern analysis using bacterial or A. oryzae niaD probes. Chromosomal DNA was cleaved with *Pst*I which does not cut the recombinant clone, but does have a site in the polylinker of pSTA14 (Fig. 7). The expected genomic *Pst*I fragment is 9.7kb Briefly three types of hybridisation profiles were observed, representative examples are shown in Fig. 8. First, nine transformants, such as TO27, lacked pUC sequences and contained a niaD-hybridising *Pst*I band of approximately 9.7kb

which was indistinguishable from the recipient band (or the wild-type, not shown). Such transformants are more likely to be as a result of a gene conversion event. A second group exemplified by TO24 (five transformants) showed a single band of 12kb hybridising to pUC while the resident niaD band was replaced by two other bands of 12kb and 8.9kb. It would seem that a single pSTA14 copy had integrated into the resident niaD site. Third, eight transformants, similar to TO26, were seen where the resident niaD band was replaced by three bands, border fragments of 12kb and 8.9kb as well as the band representing the unit length of pSTA14 (11kb), indicating that two copies of the plasmid had integrated in tandem at the niaD locus. No examples were seen of integration into a non-homologous site.

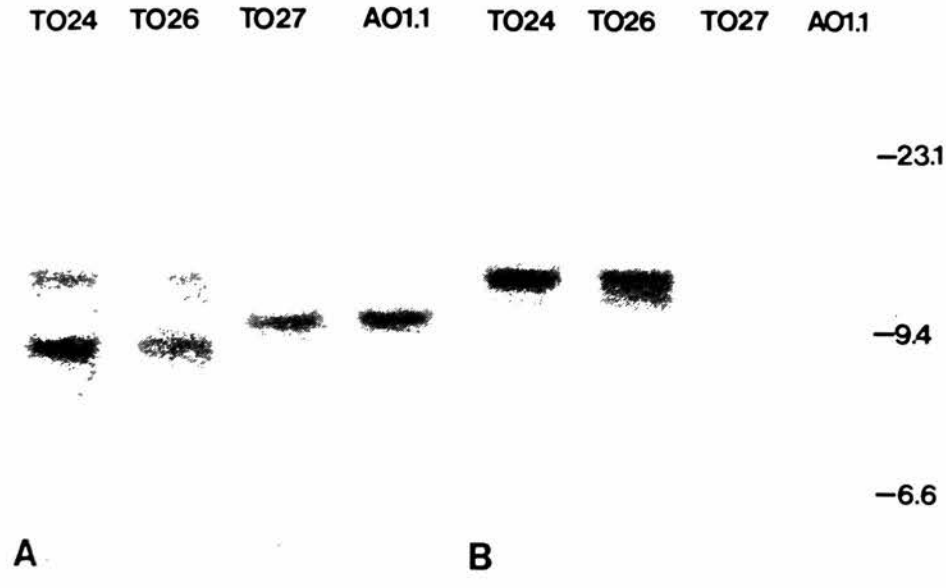

Fig 8. Analysis of Aspergillus oryzae Transformants.

A. Genomic DNA from three representative transformants and the recipient strain A01.1 was digested with *Pst*I and fragments separated on 0.8% agarose. After Southern transfer to nylon, the blot was probed first with a ^{32}P-labelled, 5.5kb *Hin*dIII fragment of pSTA14.
B. Following boiling twice for 10min in distilled water containing 1% SDS, the blot was probed again with pUC18. Hybridisation was carried out at 65^{0}C with washes down to 0.2 X SSC. The positions of λ*Hin*dIII molecular size markers are indicated.

Table 7. Transformation of Aspergillus oryzae niaD14[A] Mutant Strain with the Homologous niaD Gene

Plasmid	Form	Frequencies[B]	Mean
pSTA14	Circular[C]	64, 77, 51, 67	64
pSTA14	Linear[D]	236, 406, 386, 789	454

[A] A. oryzae strain A01.1 (niaD14)

[B] Number of transformants per μg DNA. These represent results from four independent experiments.

[C] Uncut plasmid

[D] Plasmid digested with *Hin*dIII

3.3 Frequency of Transformation and Fate of Transforming DNA in Penicillium chrysogenum.

Using clone λAN8a, containing the A. nidulans niaD gene in the EMBL3 vector, nitrate-utilising transformants of the P. chrysogenum niaD strain designated STP19 were obtained at frequencies of up to 20 per μg DNA^{-1} (Table 8). With pSTA10 the plasmid carrying the A. niger niaD gene the maximum efficiency was 9 transformants per μg DNA^{-1}. Such transformation frequencies are similar to those achieved with the heterologous system of Beri and Turner (1987), although less than reported by Cantoral et al., (1987). The latter system, however, did include the A. nidulans ans-1 sequence which has been shown to increase transformation efficiency in A. nidulans (Ballance and Turner 1985). A plasmid construct (pSTA12) containing the ans-1 sequence inserted upstream from the A. niger niaD gene did not result in improved transformation frequencies in this system.

Table 8 Transformation Frequencies of P. chrysogenum Using Heterologous niaD Fungal Genes[A]

Vector	Circular pSTA10 (A. niger)	Linear pSTA10 (A. niger)	Circular pSTA12 (A. niger)	λAN8a (A. nidulans)
Maximum transformation efficiency	9.0	15.6	5.8	19.4
Average transformation efficiency [B]	6.2	13.3	5.2	14.5

[A] Transformants per μg plasmid DNA

[B] Mean of 3 independent experiments

A total of 13 P. chrysogenum transformants, 6 derived from A. niger niaD gene transformations (designated PTG) and 6 from A. nidulans niaD gene transformations (designated PTD), were analysed by Southern hybridisation. Transformant and wild-type genomic DNA was restricted with *Hin*dIII and probed with linearised pUC18 for the detection of A. niger gene transformants (Fig.9), or with *Hin*dIII-digested λ for A. nidulans gene transformants. (Fig.10). From the results of these experiments, several conclusions can be made. First, it is clear that none of the transformants examined is the result of gene conversion as bacterial or bacteriophage sequences were detected in all A. niger niaD or A. nidulans niaD transformed strains. Second, from the number and intensity of positive hybridisation signals, it is likely that multiple integration has occurred in a number of isolates (such as PTG3, PTG6, and PTD1), while in others single integration events seem to have taken place. Third, PTG transformants (pSTA10) could be a result of integration at the resident or ectopic sites. This is in contrast to the pattern of integration shown previously in A. niger and A. oryzae. However, in the latter cases, transformation was based on homologous niaD genes. Since we failed to clone the P. chrysogenum niaD gene a meaningful comparison could not be made. Integration outside the pUC portion of pSTA10 has most probably taken place in transformant PTG2 as a single band only was observed. Fourth and with regard to PTD transformants (λAN8a), common 23kb and 6.5kb fragments represent left and right arms to the first and from the last *Hin*dIII sites of the A. nidulans recombinant molecules respectively (Johnstone et al., 1989).

That the 23kb band is not present in PTD3 suggests that DNA rearrangements may have taken place.

The results show that the A. nidulans and A. niger niaD genes are expressed in P. chrysogenum in a similar manner to the endogenous niaD gene in these organisms.

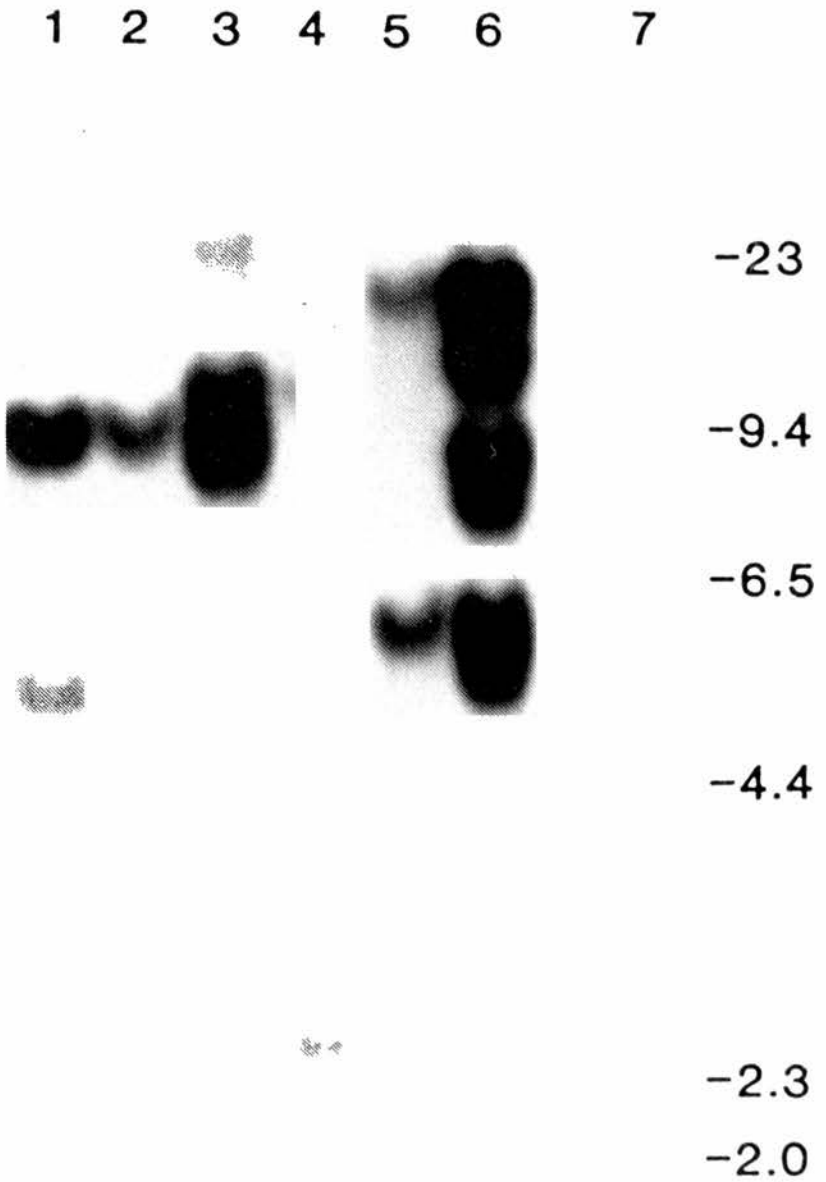

Fig 9. Molecular Analysis of A. niger niaD Transformants using pUC18 as a Probe.

20μg *Hin*dIII-digested DNA from transformants PTG1 (lane1),PTG2 (lane2), PTG3 (lane3), PTG4 (lane4), PTG5 (lane5), PTG6 (lane6), and V992 wild type (lane7). λ*Hin*dIII molecular size markers are indicated.

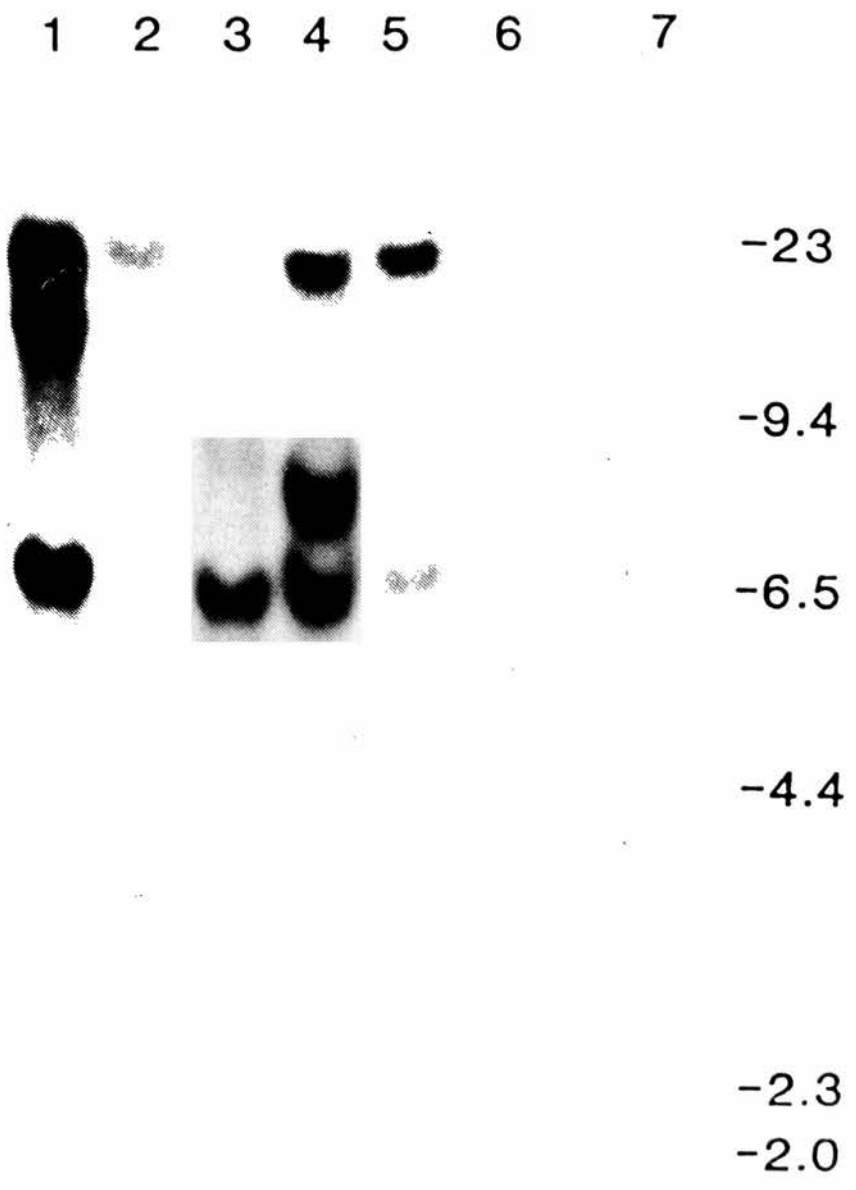

Fig 10. Molecular Analysis of A. nidulans niaD Transformants using *Hin*dIII-digested λ as a Probe.

20μg *Hin*dIII-digested DNA from transformants PTD1 (lane1), PTD2 (lane2), PTD3 (lane3), PTD4 (lane4), PTD5 (lane5),PTD6 (lane6), and V992 wild type (lane7). λ*Hin*dIII molecular weight markers are indicated.

3.4 Frequency of Transformation and Integration of Transforming DNA in Gibberella fujikuroi.

Heterologous transformation of the Gibberella fujikuroi strain niaD11 with the plasmid vector pSTA10 containing the entire A.niger niaD gene yielded one to two transformants per μg DNA^{-1} when nitrate utilising colonies were selected on minimal medium with nitrate as the sole nitrogen source. Southern blot analysis of DNA from five putative $niaD^+$ transformants showed that vector DNA had integrated into the chromosomal DNA. Figure 11 shows a Southern blot of *Xba*I - digested genomic DNA isolated from these transformants, designated GT1-5, and the recipient strain. *Xba*I cleaves pSTA10 twice within the niiA structural gene, part of which is also present on the pSTA10 clone. The probe used was *Eco*RI-digested pUC13. Using the 2kb *Eco*RI fragment of pSTA10 containing the 3'end of the niaD gene as a probe the same band patterns were obtained, except for transformant GT2 in which only the larger band was evident (data not shown). No hybridisation to the endogenous Gibberella niaD gene was observed at the stringency used. This result shows that the $niaD^+$ colonies are genuine transformants and that the A..niger gene is expressed in G. fujikuroi. The band patterns of transformants GT3 and 5 are consistent with single copy integrations, the pUC and niaD probes both hybridising to the same 8.8kb fragment generated most likely by cross-over of pSTA10 between the *Xba*I sites. Transformant GT4, with an 8.5kb fragment hybridising to both probes is probably also a single integration but the cross-over point is neither

between the *Xba*I sites or within the 2kb *Eco*RI fragment used as niaD probe. Transformant GT1 showing two bands of 8.5kb and 7.8kb hybridising to pUC and niaD, suggests integration of two copies of pSTA10 into different sites. Transformant GT2 probably arose by integration via pUC sequences to give two pUC- hybridising bands only one of which (the larger) hybridised to the niaD probe. Alternatively two copies could have integrated at different sites, one of which had deleted part of the pSTA10 insert containing the 2kb *Eco*RI fragment.

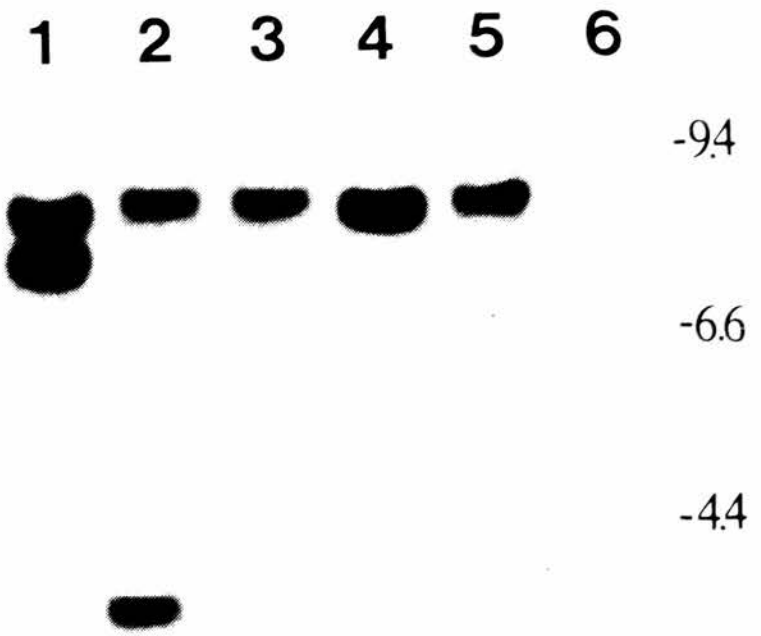

Fig 11. Southern Analysis of Gibberella fujikuroi transformants.

Genomic DNA (5µg) from transformants GT1-5 (lanes 1- 5, respectively) and recipient strain niaD11 (lane 6) was isolated, digested with *Xba*I and fragments electrophoresed through 0.8% agarose. Following transfer and UV binding to nylon membrane, the filter was probed with *Eco*RI digested pUC13.
Hybridisation was carried out at 65°C with washing to 0.2 x SSC.
λ *Hin*dIII molecular size markers (kb) are indicated.

3.5 Conclusion

(i) Chlorate resistant mutants have been isolated from the industrially important filamentous fungi Aspergillus niger, Aspergillus oryzae, Penicillium chrysogenum, Spontaneous mutation to chlorate resistance in these studies may be caused by chlorate acting as a mutagenic agent within the cell, or the spores, having been exposed to chlorate may activate mechanisms for directing mutations to result in chlorate resistance. Selection for mutants by this method is not universal for all filamentous fungi however, as demonstrated in this study by the necessity to resort to an external mutagenic agent such as N-methyl-N'- nitrosoguanidine in this case for Gibberella fujikuroi

(ii) The approach devised by Cove (1979) for characterisation of niaD mutants has been shown to be not only effective for the closely related Aspergillus species but also for the more distantly related species of Gibberella.

(iii) Heterologous hybridisation using the A. nidulans niaD gene as probe has resulted in the cloning of the corresponding gene from A. niger, and A. oryzae, gene banks.

(iv) Homologous transformation of niaD mutants of A. niger, and A. oryzae, has been developed with complementation frequencies ranging from to 800 per μg DNA^{-1} for A. oryzae and 1000 per μg DNA^{-1} for A. niger.

(v) The A. oryzae niaD gene is functionally expressed in A. nidulans, A. niger, and P. chrysogenum, while the A. niger niaD gene is similarly expressed in A. nidulans, A. oryzae, P. chrysogenum and G. fujikuroi. Accordingly it can be said that the nitrate system offers a broad based transformation selection system for nitrate assimilating fungi of industrial importance.

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