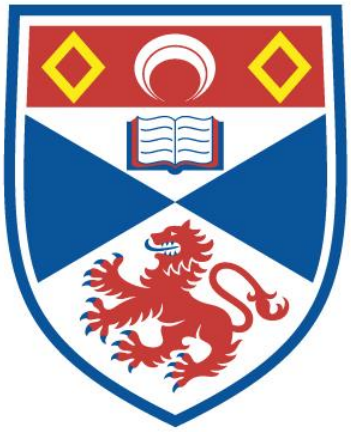
MOLECULAR GENETICAL ANALYSIS OF THE NITRATE GENE CLUSTER OF ASPERGILLUS NIDULANS

PHILIP A. GREAVES

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1989

MOLECULAR GENETICAL ANALYSIS OF THE NITRATE GENE CLUSTER OF *ASPERGILLUS NIDULANS*

PhD THESIS

P.A. GREAVES

1989

Declaration for the Degree of PhD.

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Date. 9/11/89

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ABBREVIATIONS

A	adenine
Ac	acetate
A. nidulans	*Aspergillus nidulans*
ATP	adenosine triphosphate
β-ME	β-mercaptoethanol
bp	base pairs
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
CIP	calf intestinal phosphatase
CM	*Aspergillus* complete medium
dH_2O	sterile distilled water
DMSO	dimethylsulphoxide
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethyleneglycol-bis-D-aminoethylether-N',N',N',N'-tetra-acetic acid
EtBr	ethidium bromide
FAD	flavin adenine dinucleotide
G	guanine
h	hours
K	1000 x
Kb	kilobases
Kdal	kilodaltons
MES	2-(n-morpholino)-ethanesulphonic acid
MM	*Aspergillus* minimal medium
min	minutes
MoCo	molybdenum cofactor
Mw	molecular weight
µCi	microCuries
N	any nucleotide
NADPH	nicotine adenine dinucleotide phosphate
NED	N-1 naphthylethylenediamine dihydrochloride
N. crassa	*Neurospora crassa*
$O.D._{550}$	optical density at 550 nm (wavelength)
o-NP	ortho-nitrophenol
o-NPG	ortho-nitrophenylgalactopyranoside
PEG 4000	polyethyleneglycol of Mw 4000
PMSF	phenylmethylsulfonyl fluoride
R	any purine
R.F.	recombination fraction
rpm	rotations per minute
S	Svedberg unit
SDS	sodium dodecyl sulphate
SSC (20 x)	3M NaCl, 0.3M sodium citrate pH 7.0
T	thymine
TAE	tris.acetate EDTA buffer
TBE	tris.borate EDTA buffer

ABBREVIATIONS cont.

Tris	Tris (hydroxymethyl) aminoethane
Tween-'80	polyethylene sorbitan mono-oleate
v/v	volume per volume
w/v	weight per volume
X-gal	5'-bromo-4'-chloro-3'-indolyl-β-D-galactopyranoside
Y	any pyrimidine

Chemical symbols have their usual meaning and A. nidulans gene symbols are as defined by Clutterbuck (1984). Conventions for gene symbols are taken from Clutterbuck (1973) and Bennett and Lasure (1985). Restriction enzymes are abbreviated in diagrams as follows:

B	BamHI
Bg	BglII
E	EcoRI
H	HindIII
K	KpnI
P	PstI
Pv	PvuII
S	SalI
Sm	SmaI
X	XhoI
Xb	XbaI

SUMMARY

Putative DNA clones harbouring the nitrate gene cluster of Aspergillus nidulans was used in a number of transformation experiments to positively identify the clones. The complementation of several deletions within the nitrate gene cluster ruled out the possibility that a suppressor had been cloned and suggested that extensive regions of the nitrate gene cluster were present on the recombinant plasmid. Construction of several subclones and subsequent transformation of various alleles of the nitrate gene cluster allowed the approximate positions of the three genes (crnA, nitrate permease; niiA, nitrite reductase; niaD, nitrate reductase) to be determined relative to the plasmid. These experiments indicated that a part of the niaD gene was missing, although the entire niiA and crnA genes were present on the plasmid. Subsequently, overlapping clones were isolated and shown to contain the remaining niaD sequences. Genetic analysis of many transformants suggested that the plasmids had mainly integrated into the nitrate gene cluster (corresponding site of homology to the plasmid), although occasionally the plasmids had integrated into other chromosomes and here, therefore, complementation was in trans. Reversion studies on chlorate of two niaD transformants, containing plasmid DNA at the nitrate gene cluster, suggested that these transformants were less stable than the wild-type.

Construction of promoter-β-galactosidase fusions using DNA from the intergenic region between *niiA* and *niaD* suggested that these genes are divergently transcribed. This DNA region had sufficient promoter signals to mediate the nitrate/ammonium regulation of β-galactosidase characteristic to this gene cluster. One transformant, possessing multiple copies of the fusion construct, had elevated levels of β-galactosidase but the maximal nitrate reductase activity was considerably reduced. Evidence obtained, supported the idea that one of the regulatory proteins (*nirA*) was limiting in this multiple copy transformant.

INTRODUCTION

I.1 General *Aspergillus nidulans* Genetics

A. nidulans is a sexually reproducing member of the *Aspergillaceae*. Genetic investigation into this ascomycete started in 1945 and the early development of genetical studies are reviewed by Pontecorvo et al. (1953). One of the main advantages of this organism is that it is a haploid eukaryote capable of forming colonies on simple media and hence can be treated as a micro-organism. Other advantages for studying this organism include a genetic system with both sexual and parasexual cycles (fig. 1), homothallism, fast growth rate, uninucleate conidiospores, haploid and diploid growth phases, metabolic versatility and isogenicity of strains used in different laboratories. This has led to the production of a detailed genetic map, see Clutterbuck (1984).

The metabolic versatility of *A. nidulans* is due to a large number of permease/enzyme systems that are subject to induction and/or repression of synthesis. Such gene regulation is thought, where evidence exists e.g. Winthers et al. (1980), to occur at transcription.

As a simple eukaryote, *A. nidulans* has a genome size estimated at about 2.6×10^4 Kb with a low repetitive DNA content (Timberlake 1978). With the recent advances in molecular biology, this means that *A. nidulans* is quite tenable as a system to investigate

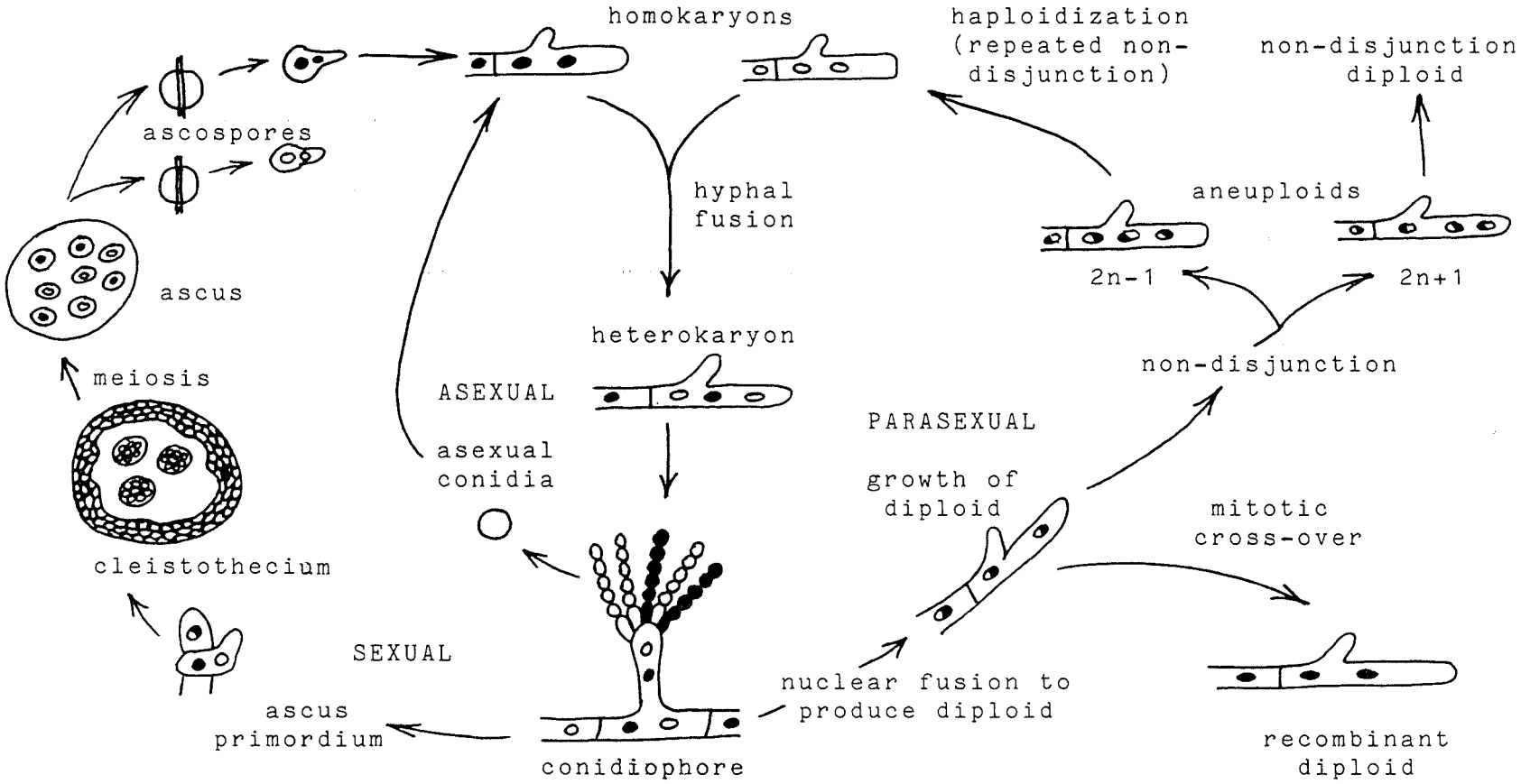

homokaryons
haploidization (repeated non-disjunction)
non-disjunction diploid
ascospores
hyphal fusion
aneuploids
2n-1
2n+1
ascus
heterokaryon
non-disjunction
meiosis
ASEXUAL
PARASEXUAL
asexual conidia
growth of diploid
mitotic cross-over
cleistothecium
SEXUAL
nuclear fusion to produce diploid
ascus primordium
conidiophore
recombinant diploid

Fig. 1. Life cycle of the filamentous fungus A. nidulans. In the asexual cycle, the asexual spores (conidia) are uninucleate. The conidia germinate to produce a branched multinucleate mycelium where all the nuclei are of the same genotype (= homokaryon). Fusion of hyphae (anastomosis) from different homokaryons produces a heterokaryon (different nuclear genotypes present). The conidia are produced from the conidial head (conidiophore) which may be heterokaryotic or homokaryotic (haploid or more rarely diploid). The sexual cycle results in fruiting body (cleistothecium) formation. The unordered asci produced in a single cleistothecium all result from a single nuclear fusion event in the ascus primordium. As A. nidulans is homothallic this fusion may be between nuclei of the same or differing genotypes. The ascospores produced are binucleate. The parasexual cycle arises from the rare fusion of nuclei and mitotic division of the diploid nucleus resulting in a diploid sector/colony. Failure of the chromosomes to segregate normally during mitosis (non-disjunction) produces slow growing aneuploids. Selection of haploid sectors from these aneuploids can give rise to recombinant haploid homokaryons. Mitotic crossing-over in the diploid can give rise to recombinant diploid sectors/colonies.

gene regulation at the molecular level compared with the complexity of higher eukaryotes.

I.2 Biochemistry of Nitrate Assimilation

One system, particularly amenable to study, is that of the enzymes in nitrate assimilation, see fig. 2.

The enzymes of this pathway, nitrate reductase (E.C.1.6.6.3) and nitrite reductase (E.C.1.6.6.4) have been studied in both filamentous fungi (e.g. Aspergillus nidulans and Neurospora crassa) and plants; and are reviewed in Cove (1979) and Dunn-Coleman et al. (1984). A brief summary of these enzymes and the nitrate permease will be presented here.

Nitrate reductase. This is a complex protein which is probably a homodimer of subunit Mwt. 91 Kdal (Cooley and Tomsett 1985). Studies with this and the N. crassa enzyme (subunit Mwt. 115 Kdal - Garrett and Nason 1969) have established that assimilatory nitrate reductase is a soluble and an electron transferring protein. The two electron transfer is thought to be mediated by enzyme-bound heme iron, an easily dissociable FAD and a molybdenum cofactor (Garrett and Nason 1969, McDonald and Coddington 1974). These components function as electron carriers between the physically separated pyridine nucleotide (NADPH specific) oxidation site and the nitrate reduction site.

In addition to this physiological activity (sedimentation coefficient of 7.6 S), apparent non-

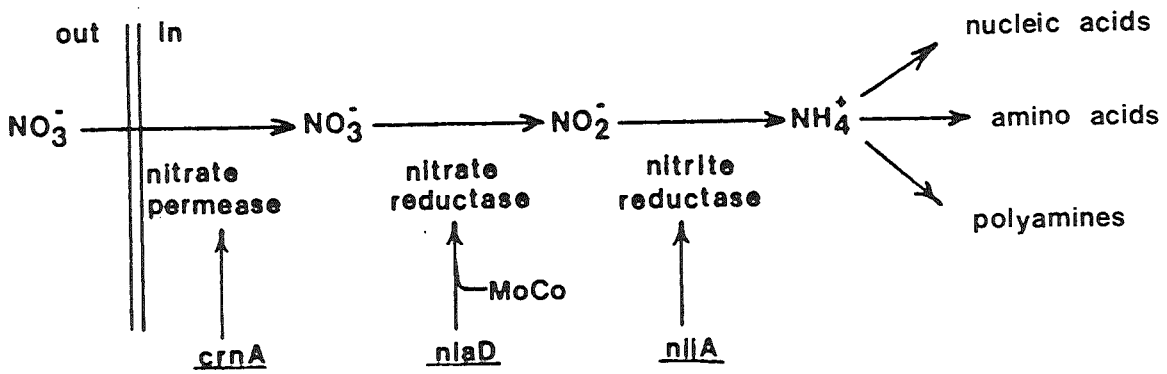


Fig. 2. The genetics and biochemistry of the nitrate assimilation pathway in A. nidulans. Nitrate may be imported into the conidium by the nitrate permease (encoded by the crnA gene product). Internal nitrate is reduced by nitrate reductase (formed by the niaD gene product and the molybdenum cofactor whose synthesis requires the cnx genes) to nitrite. This nitrite is reduced further by nitrite reductase (encoded by the niiA gene product) to ammonium. Regulation of this pathway is by two positively-acting regulatory genes nirA and areA (see text).

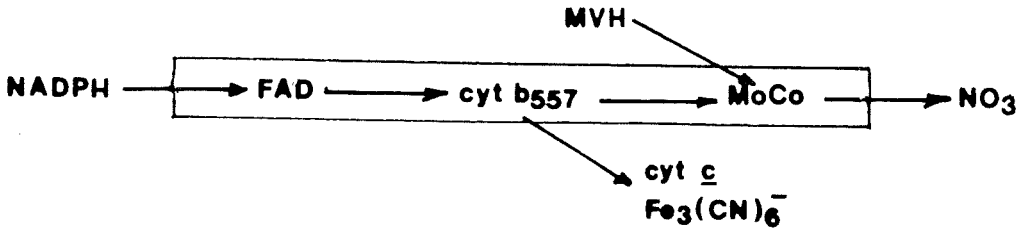


Fig. 3. The general features of a typical assimilatory nitrate reductase indicating the flow of electrons. Also shown are the in vitro electron acceptors cytochrome c, ferricyanide and the electron donor methyl viologen dye.

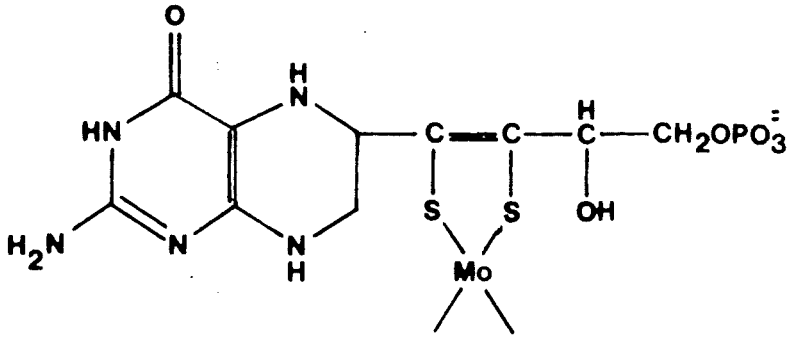


Fig. 4. Proposed structure for the molybdenum cofactor, showing a reduced pterin ring system with a position 6-alkyl substituent. The two sulphur atoms are thought to interact with the molybdenum atom. (see Johnson and Rajagopalan 1982, 1987).

physiological activities can be shown in vitro. The first is a dehydrogenase (diaphorase) activity in which nitrate reductase can mediate the pyridine nucleotide-linked reduction of one- or two-electron acceptors such as ferricyanide, cytochrome c or dichlorophenolindophenol. The molybdenum moiety is not involved in these reactions (Cove and Coddington 1965). The second type of activity involves the reduction of nitrate with reducing power generated either reduced flavins or viologen dyes. The pyridine nucleotide reduction site is not involved in this reductase activity and electrons are probably added to either heme or molybdenum cofactor (Pateman et al. 1967). These partial activities have been shown to be differentially inhibited by various reagents, Garrett and Nason (1969), revealing the following scheme in fig. 3. The terminal electron acceptor of the nitrate reductase protein is thought to be the molybdenum cofactor. Recent studies suggest that the structure is most likely a low molecular weight, molybdenum binding urothione like molecule, termed molybdopterin (Johnson and Rajagopalan 1982). Although many of the biochemical features of this and other assimilatory nitrate reductases appear well established, the actual role of the components involved in electron flow is only poorly understood. Elucidation of these aspects, however, involves the use of large quantities of protein, a formidable task given the low cellular concentration and relative instability of all nitrate reductases.

dependence and is partially defective in strains carrying the crnA (permease) mutation in conidia or young mycelia. The broad pH dependency is consistent with this notion, and the kinetics of nitrate uptake suggest systems with different affinities for nitrate.

I.3 Genetical Studies of Nitrate Assimilation

niaD. Although mutation in many genes can prevent or reduce utilization of nitrate as a nitrogen source, only mutations in niaD abolish nitrate reductase activity alone (Cove 1979). Nitrate reductase mutants (including niaD) may be isolated on the basis of chlorate resistance (an example of a positive selection system). The mechanism of chlorate resistance may be the absence of reduction to chlorite (toxic) but other factors may also be important (Cove 1976a). Temperature sensitive niaD mutants have been isolated which show a thermolabile enzyme activity in vitro, providing strong supporting evidence that the niaD gene is indeed the structural gene (McDonald and Cove 1974).

cnx genes. Like niaD, mutations may also be selected on the basis of chlorate resistance. It is thought that the cnx genes encode the enzymes (as yet unknown) involved in the synthesis and processing of the molybdenum cofactor (Cove 1979 and Scazzocchio 1980). The cnx mutants are also unable to utilize hypoxanthine, as both purine hydroxylases require the molybdenum cofactor MoCo (Pateman et al. 1964). The mutants fall

into eight complementation groups cnxA-C, E-H and J. The cnxA, B and C genes show an overlapping complementation pattern and have been shown to be tightly linked, the other cnx genes being scattered about the genome (Cove 1979). The cnxE mutants are repairable by high concentrations of molybdenum and this gene has been implicated in producing a gene product which mediates the insertion of molybdenum into the cofactor (Arst et al. 1970). Two cnxJ mutations have been characterized by Arst et al. (1982b) who suggest that this may be a regulatory gene or a MoCo carrier protein. Temperature sensitive alleles of the cnx genes have been isolated (Cove 1979 and Arst et al. 1982b) but only cnxH temperature sensitive alleles show a thermolabile nitrate reductase activity. However given the structure of the MoCo proposed by Johnson and Rajagopalan (1982 and 1987) in fig. 4 it is difficult to see where such a gene product may be incorporated into the cofactor.

niiA. Mutants are unable to utilize either nitrate or nitrite as a nitrogen source, but grow like wild-type on ammonium. The niiA mutants can be distinguished from nirA$^-$ mutants by staining for nitrite excretion (Cove 1976b). The nitrite reductase from a temperature sensitive niiA strain (niiA72) has been shown to be less stable in vitro than the wild-type by Rand and Arst (1977), again suggesting that this is the structural gene.

nirA. Mutations in nirA can lead to the inability

to utilize nitrate or nitrite as a nitrogen source, or more rarely constitutive expression of nitrate and nitrite reductases. Allelism is difficult to prove, though the mutations are consistent in mapping to one locus (Cove 1970). The existence of two contrasting types of mutant alleles might suggest that the nirA gene product is regulatory. The fact that null alleles are recovered more frequently and these mutants are unable to grow on nitrate suggests that the mode of action is a positive one. Dominance relationships of $nirA^{c}$, $nirA^{+}$ and $nirA^{-}$ in diploids and heterokaryons suggests only partial dominance. This is most easily explained by a gene dosage model where the nirA gene product is present in near limiting concentrations (Cove 1969). This conclusion is consistent with the finding that $nirA^{-}$ strains complement only weakly with $niaD^{-}$ and $niiA^{-}$ strains in heterokaryons.

Recently mutations in nirA, designated $nirA^{d}$ in Tollervey and Arst (1981), have been characterized that relieve nitrogen metabolite repression and bypass the requirement for the areA product (see below). The nirA alleles, designated $nirA^{c/d}$, resulting in both constitutivity and derepression have been constructed by mutation and intragenic complementation, suggesting that the nirA gene product contains two separate domains; a co-inducer region being defined by constitutivity mutations, and a region somehow interacting with the areA gene product or its site of action. An alternative

explaination is that the nirA gene is regulated by areA and the derepression mutations occur in the promoter region of nirA. Recently work by Lockington et al. (1987) suggest that the regulatory gene alcR is subject to carbon catabolite repression (and alcR may also induce its own synthesis).

Constitutivity of nitrite reductase and cytochrome c reductase (nitrate reductase apoprotein) can also be seen in some niaD and some cnx mutations. With the exception of cnxE, cnx mutants with constitutive expression seem to show a monomeric form of cytochrome c reductase (4.5 S), (McDonald et al. 1974). This had led to the idea that the holoenzyme of nitrate reductase itself is implicated in its own regulation (autoregulation) possibly in the absence of nitrate by interacting with the nirA gene product and prevention of activation of genes in the nitrate gene cluster. The suggestion that protein-protein interactions may inactivate a regulatory gene product is not unique. Other examples include N. crassa, where the qa-1F protein may be the target for the qa-1S protein in the absence of the inducer, but in the presence of inducer the qa-1S protein is no longer able to exert its repression of the qa-1F activator (Giles et al. 1985). In yeast, the GAL4 activator appears to be the target for the GAL80 protein. In the presence of galactose GAL80 is no longer able to interact with the GAL4 regulator.

areA. Different mutant alleles of the areA gene are associated with a considerable variety of phenotypes. Two general classes can be distinguished: one, areA^{r} showing a repressed phenotype, the strains only able to utilize ammonium; and two, the rarer areA^{d} allele leading to derepression of one or more, normally ammonium repressable activities. Because the areA gene could mutate to either phenotype Arst and Cove (1973) proposed that the areA gene product played a direct role in the mediation of ammonium repression. This is also supported by the fact that some alleles e.g. areA102, alter the extent of ammonium repression for different structural genes. Reversion of this allele produced new areA mutations that reversed the extent of ammonium repression for the structural genes (Hynes 1975). Thus regulation by areA shows no hierarchy and hence the areA gene product must be directly involved in nitrogen metabolite repression. It seems probable that its mode of action is a positive one (on the basis of frequency of mutant phenotypes) which is prevented by ammonium, or more likely the metabolite glutamine (Grove and Marzluf 1981). The active areA gene product is probably a protein on the basis that polypeptide chain termination alleles are available (Al Taho et al. 1984). The corresponding N. crassa nit-2 gene product has been isolated as a nuclear, DNA-binding protein specifically eluted by L-gutamine (Grove and Marzluf 1981). Evidence would suggest that the areA protein is not limiting

because complementation involving *areA*^{r} alleles occurs readily in heterokaryons (Arst and Cove 1973), and multiple copies of the *amdS* promoter do not titrate out the *areA* protein (Kelly and Hynes 1987).

The phenotypes of *A. nidulans* mutants lacking NADP-linked glutamate dehydrogenase (*gdhA*) or glutamine synthetase (*glnA*) are consistent with glutamine being the co-effector molecule with the *areA* gene product in nitrogen metabolite repression (McDonald 1982 and Arst et al. 1982a).

The nitrate gene cluster. The structural genes which encode nitrate reductase, nitrite reductase and nitrate permease are very closely linked (1%). Tomsett and Cove (1979) generated a detailed fine structure genetic map of the *niaD-niiA* region by deletion mapping, part of which is shown in fig. 5. They found that approximately 1% of spontaneous chlorate-resistant mutants behaved as *niaD-niiA* double mutants, which were subsequently shown to be deletions and non-revertable, thus confirming the extremely tight linkage of these genes. Such a cluster of related genes is unusual for eukaryotes although it is unlikely the genes function as an operon. In *N. crassa* the genes are present on separate chromosomes. In *A. nidulans* it would appear that the structural genes are separately regulated as *nirA*^{c} mutants studied to date lead to much higher constitutive expression of nitrate reductase than nitrite reductase. Also, observations with *nis-5* (an

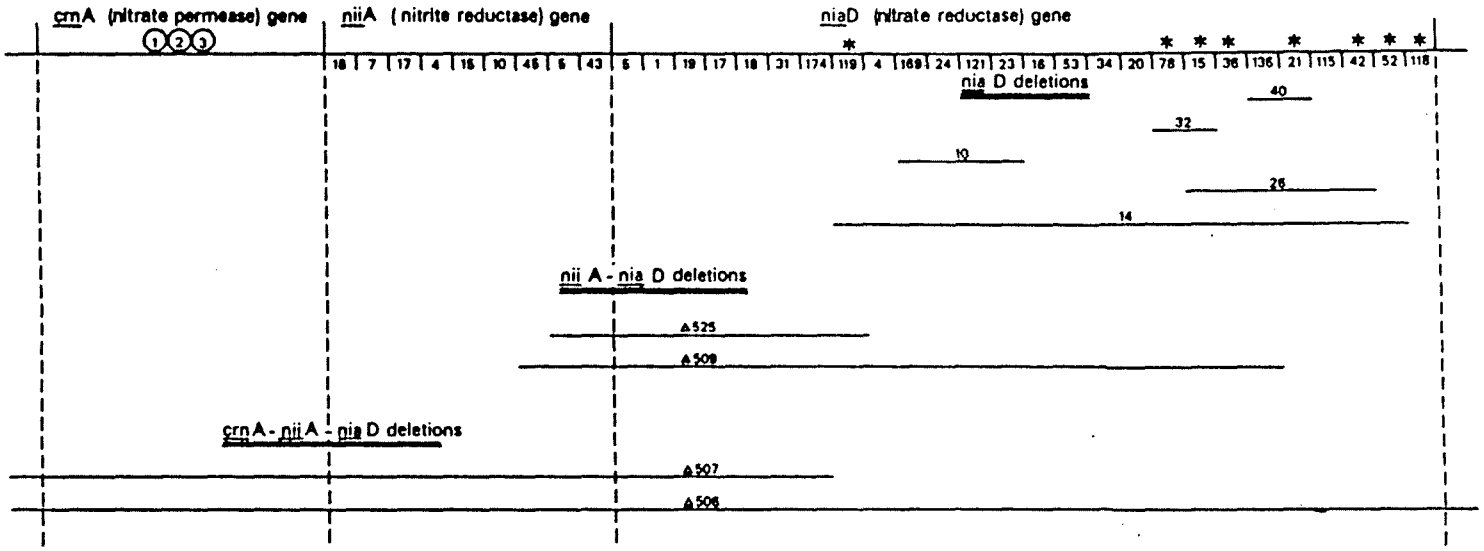


Fig. 5. Deletion map of the nitrate gene cluster of A. nidulans modified from Tomsett and Cove (1979). Deletion intervals are represented by one point mutation allele. Deletion intervals with temperature sensitive alleles are indicated by an asterisk. Deletions are represented by bars below the deletion intervals indicating the missing deletion intervals. Unlocated point mutations are circled.

insertional translocation between niiA and niaD) suggest that the two genes are regulated separately but in a parallel manner (Arst et al. 1979). The crnA gene seems to be regulated differently in that it does not appear subject to nirA control and is under developmental control, expressed only in conidia or young mycelia (Brownlee and Arst 1983). Other gene clusters (encoding related enzymes) are known e.g. the proline cluster in A. nidulans (Jones et al. 1981), the quinic acid gene cluster in N. crassa (Giles et al. 1985), and several more. In other cases gene fusions have occurred to form multiple enzyme aggregates, e.g. the arom gene in A. nidulans (Hawkins 1987). Such gene clusters may reflect the recent acquisition of multi-step pathways for synthesis or degradation of novel metabolites.

I.4 Gene Regulation at the Genetic Level

Britten and Davidson (1969) presented a model in which parallel positive-acting regulatory genes, termed integrator genes, control overlapping sets of structural genes, each mediating induction of its set under certain conditions. This type of regulatory circuit requires only a single copy of each structural gene rather than as many copies as there are conditions when expression of the structural gene is required. In A. nidulans there are many such sets identified which appear to fit this model, not only the regulation of nitrate assimilation genes but circuits involved in acetate utilization

(facB), w-amino acid utilization (amdR (intA)), proline utilization (prnA) etc. (Arst 1984 and references therein). These, and nirA, control a subset of structural genes and have been termed pathway specific or minor regulatory genes. Regulatory genes such as areA that control a wide range of structural genes are referred to as wide domain or major regulatory genes (see also Marzluf 1981 and Wiame et al. 1985).

A prediction of such a model system is that adjacent to such structural genes should be receptor sites for the regulatory gene products (proteins where evidence exists). Such cis regulatory mutations have been isolated for a number of systems. e.g. amdS (Hynes and Davis 1986), uapA (Arst and Scazzocchio 1975) and gabA (Bailey et al. 1979). The model would also suggest that the regulatory gene product should have affinity for the co-effector molecule. As seen, the nit-2 protein in N. crassa, which mediates nitrogen metabolite repression, is specifically eluted from DNA by glutamine. Another regulatory protein, the uaY gene product, has been isolated as a DNA-binding protein:uric acid complex (Philippides and Scazzocchio 1981).

Thus for the expression of the nitrate assimilatory genes in the presence of nitrate, the nirA protein is able to bind to the regulatory sequences and concomitantly the areA protein, in the absence of ammonium, binds to adjacent sequences (and/or to nirA sequences?) and both are able to effect transcription of

the nitrate gene cluster genes. The nitrate reductase enzyme is also involved in the regulation of this pathway, possibly by interacting with the nirA protein. It is unlikely that nitrate accumulation in niaD and cnx mutants is the cause for constitutive expression of nitrite reductase and cytochrome c reductase, as induction with nitrate of mycelia of the mutant strains which had previously been growing on ammonium shows no effect on enzyme levels. Expression of crnA does not appear to be under nirA control but is still regulated by the areA protein and is dependent on some, as yet unknown developmental control.

I.5 Gene Cloning and Molecular Biology

The enormous wealth of genetics has led to some idea of the gene products required for expression of the nitrate assimilation pathway in A. nidulans (and N. crassa). However, the detailed molecular mechanism of regulation at the transcription level remains elusive. The recent technology of recombinant DNA has allowed a number of approaches to be made as regards cloning of genomic DNA (or cDNA) from filamentous fungi.

Until recently the number of cloned genes in A. nidulans was limited to those genes which could complement corresponding auxotrophs of E. coli (qut - Hawkins et al. 1985; trpC - Yelton et al. 1983; arom - Kinghorn and Hawkins 1982) or those of yeast (argB - Berse et al. 1983). Other genes have been isolated by

differential hybridization (*amdS* - Hynes et al. 1983; *areA* - Caddick et al. 1986; *oliC* - Ward et al. 1986). However, the development of a homologous transformation system for *A. nidulans* has opened up the possibility of exploitation of the enormous wealth of *A. nidulans* genetics. Use of mainly three systems, namely: *trpC* (Yelton et al. 1984), *argB* (Johnstone et al. 1985), and *pyr-4* (Ballance and Turner 1985), has enabled initial selection of transformants to take place. The *pyr-4* gene is from *N. crassa* (isolated by heterologous complementation of the corresponding auxotroph of *E. coli*) and is able to complement *pyrG* mutations in *A. nidulans*, despite little sequence homology (Ballance and Turner 1985).

Transformation of *A. nidulans* involves generation of protoplasts from mycelia using a cell wall degrading enzyme (e.g. novozyme 234) in the presence of an osmotic stabilizer (KCl or $MgSO_4$). The protoplasts are purified from the mycelial cell wall debris by either filtration or bouyant density centrifugation. The protoplasts are then treated with $CaCl_2$, DNA and polyethylene glycol. The mechanism by which transformation takes place is unknown, but the polyethylene glycol also induces protoplast fusion and calcium precipitated DNA may be internalized along with the membrane during this process. The methods of production and regeneration of protoplasts were initially developed for protoplast fusion experiments. Protoplasts thus produced, in the

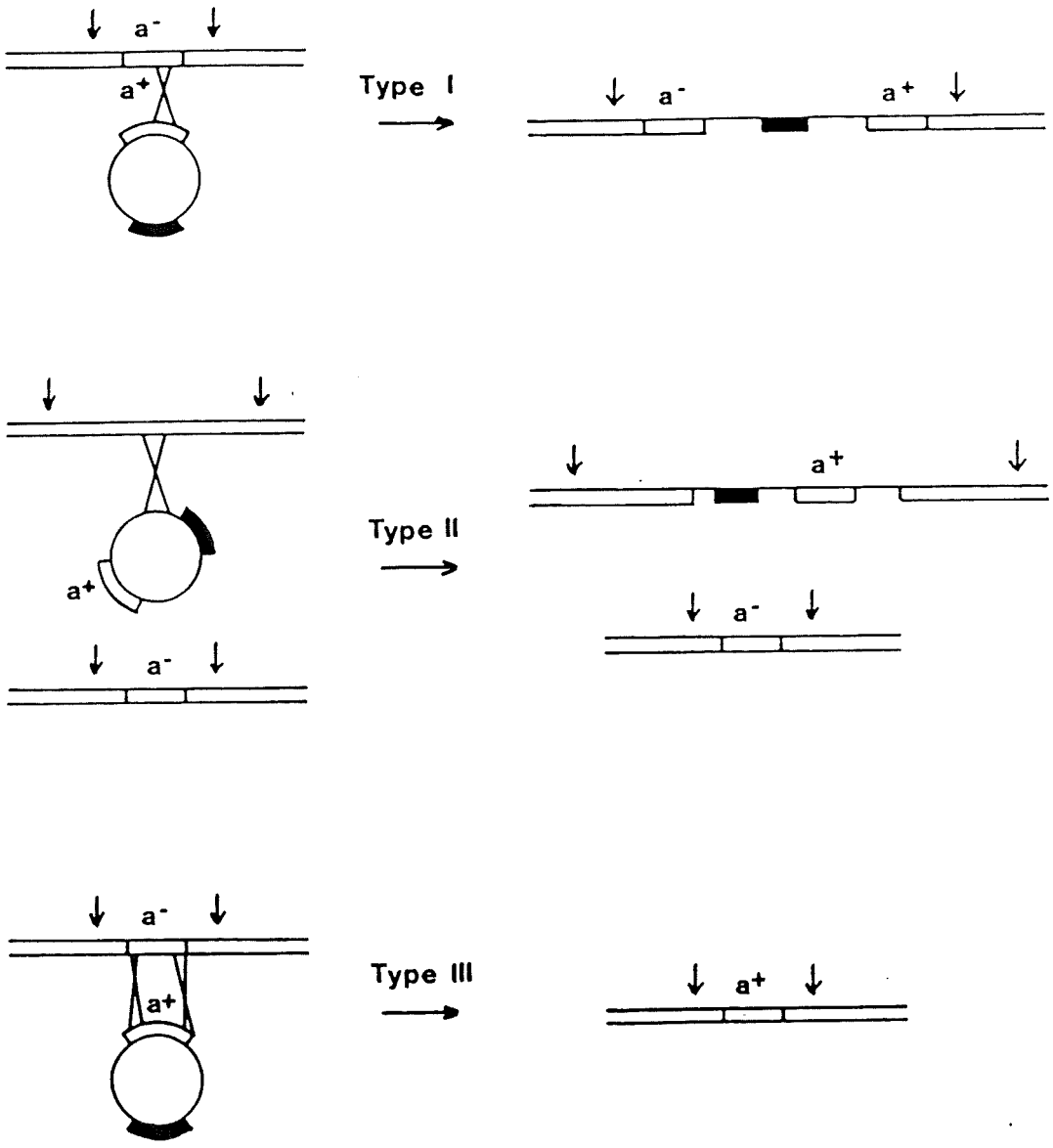


Fig. 6. Modes of integration of plasmid DNA into the chromomsome (modified from Hicks et al. 1979). $\underline{a}^-$ represents the null allele while $\underline{a}^+$ is the wild-type allele. The dark shaded region represents the marker gene on the plasmid. Restriction sites (that cut outside the cloned region) are represented by arrows.

In type I integration a direct repeat of the $\underline{a}$ alleles is produced and the restriction fragment size is increased.

In type II integration a new restriction fragment is produced, in addition to $\underline{a}^-$, so on Southern blotting (and probing with $\underline{a}^+$) two fragments are seen.

In type III integration the $\underline{a}^-$ allele is replaced by the $\underline{a}^+$ allele and no change in restriction fragment size is seen (unless $\underline{a}^-$ is a large deletion). This event is indistinguishable from reversion.

presence of osmotic stabilizer, will regenerate on appropriate solid medium to form healthy colonies. For transformation and complementation of the mutant to occur the expression and stable maintenance of transforming genetic material is required. Abortive transformants, small colonies which do not maintain the selected marker on subculture, are frequently observed and may arise as a result of transient expression. In A. nidulans (and other filamentous fungi) maintenance is achieved largely by integration of plasmid sequences into the genome. Like the yeast system (integrating vectors), a number of integration events can arise (Hicks et al. 1979) - see fig. 6: Type I integration where a single crossover at site of homology produces a direct repeat; type II integration where a single crossover occurs at another site (non-homologous); and type III integration where a double crossover produces a gene conversion event. The relative frequencies of the three classes of transformant vary with the organism, selection system used, the nature of the resident mutation, and possibly the genetic background of the recipient strain. In classes I and II, either single or multiple plasmid integrations can occur and disruption of plasmid sequences has been reported (Wernars et al. 1985, and Ballance and Turner 1985). Deletions of genomic sequences may also occur (Durrens et al. 1986) and there is some evidence for free plasmid being expressed in N. crassa transformants (Grant et al. 1984).

The transformation of E. coli with total genomic DNA from the transformant is usually sufficient to produce a few antibiotic resistant colonies and this, again, suggests the presence of plasmid molecules in the fungus. Alternatively, manipulated excision and recircularization of integrated plasmids may be used for recovery of recombinant molecules. Thus all the requirements for a shuttle vector are met. Indeed, it is possible to rescue genomic sequences adjacent to the site of integration and recover the resident mutant allele (Johnstone et al. 1985).

As discussed above, most of the original methods of selection rely on complementation of mutant alleles. It would be advantageous for complementation of wild-type strains to be possible, avoiding the necessity of strain construction. Use of resistance markers like ben^r by Vollmer and Yanofsky (1986) in N. crassa has been successfully used in transformation of wild-type strains. Other improvements to the transformation system involve the use of cosmids (e.g. Yelton et al. 1985) which have the advantage that the screening numbers are reduced (particularly useful for a time-consuming screen like that of antibiotic production). In conjunction with the use of cosmids, an ordered library (where recombinant plasmids are subcultured separately and may be pooled together for transformation experiments) is advantageous as isolation of the recombinant clone by reducing the pool size used in transformation allows the

clone to be obtained directly from the library (i.e. not passaged through the fungus), so rearrangements are less likely (e.g. Vollmer and Yanofsky 1986). If an effective screening system is available then increased efficiency of transformation would be beneficial. Ballance and Turner (1985) selected the ans-1 sequence on the basis of conferring autonomous replicating ability to the vector pBF6 in yeast. Despite this, no plasmid replication (as in the yeast system) occurs in A. nidulans, although a higher frequency of transformation is observed with this vector (two orders of magnitude). Concomitant with this is the reduction in the number of abortive colonies. The means by which ans-1 affects transformation frequency in A. nidulans is unclear but similar results have been obtained in Penicillium chrysogenum (Cantoral et al. 1987). In some instances bacterial sequences may be deleterious to transformation and Johnstone et al. (1985) found that replacement of pBR322 with pUC8 sequences increased the transformation efficiency fifty fold. Addition of telomeres from Tetrahymena thermophila to a Podospora integrative vector and subsequent transformation of protoplasts yielded (about 50%) linear, extrachromosomal plasmids in some transformants. The transformants generated in this way are mitotically unstable and only contain one copy of the plasmid for several nuclei under selective conditions (Perrot et al. 1987). It remains to be seen whether the addition of further sequences to increase

the plasmid size (at present only about 10 Kb) will have any effect on stability. Also, the use of centromeric sequences would be interesting in this regard.

Analysis of transformants may proceed by sexual crosses to strains with linked markers, or by parasexual analysis of diploids (constructed with a master strain - genetic marker on each chromosome) to reveal the site of integration of the plasmid (Clutterbuck 1974). Molecular analysis by Southern blotting and hybridization may reveal novel bacterial sequences not normally present; and disruption of genomic fragments may reveal the site of integration (Tilburn et al. 1983).

Genes isolated on the basis of homologous DNA trnasformation in A. nidulans include brlA (Johnstone et al. 1985), yA (Yelton et al. 1985), acuD and fwA (Ballance et al. 1986), and riboB (Oakley et al. 1987) with many more no doubt to follow. The isolation of genes in this way provides the means for analysis of gene structure and function at the molecular level.

At present in A. nidulans a number of genes have been cloned, of which a few have been sequenced, reviewed in Gurr et al. (1988). Yet the understanding of the molecular mechanisms of gene regulation in this organism have some way to go before approaching that of yeast systems (e.g. Struhl 1987), or that of 'phage lambda (Ptashne 1986b).

From sequence studies, general consensus sequences of the filamentous fungal gene have emerged. Some, but

not all, genes from filamentous fungi have sequences related to the TATA sequence (30-70 bp upstream from the start point) and a CAAT region (70-100 bp upstream). The ATG start codon is normally preceeded by an A at -3 and sometimes by a C at -4. Some highly expressed genes also have a pyrimidine rich region (CT box) in the 5' region (Punt et al. 1988). The role of these sequences in transcription, however, has yet to be determined by in vitro mutagenesis experiments. Fungal genes, unlike yeast, typically contain a few introns, but these are usually short (less than 100 bp) containing a consensus splicing signal sequence of 5'-GTRNGT---NRCTRAC--YAG-3'. Codon bias is not particularly evident in A. nidulans, although there is a preference for a G or C in the third position. The classical polyadenylation signal AATAAA has not been found in all fungal genes to date.

While sequences are available for such comparisons, much less information is available at the functional level. However, a few systems will be given here to show some idea of the progress and approaches being made in this field so far.

The nit-2 regulatory gene (N. crassa) has been cloned via homologous transformation and complementation (Stewart and Vollmer 1986). Subcloning of the nit-2 gene and Northern analysis by Fu and Marzluf (1987a) indicated that nit-2 encodes a mRNA of 3.5 Kb (is this equal to the 22 Kdal glutamine eluted DNA-binding protein of Grove and Marzluf (1981)?) which shows a

three fold increase in mRNA levels in nitrogen derepressed conditions compared to those of nitrogen repression. A nit-2 mutant similarly showed elevation of mRNA levels in nitrogen derepressed conditions; hence suggesting that nit-2 is not autoregulated (Fu and Marzluf 1987a). That nit-2 is at all differentially expressed seems surprising since one might expect such genes to be constitutive under the Britten and Davidson model (1969). It is quite possible that in A. nidulans the situation may be different (i.e. constitutive) as there are a number of elements different in nitrogen metabolite regulation (e.g. the nmr-1 allele which results in derepression of enzymes under nit-2 control, and hence is negative-acting (Debusk and Ogilvie 1984)). Recently Marzluf and coworkers have isolated the nit-3 gene (Fu and Marzluf 1987b) and the nmr^+ gene (Fu et al. 1988), both by complementation. Northern analysis of the nit-3 gene (encoding nitrate reductase) of N. crassa wild-type shows transcriptional regulation of nit-3 mRNA, and analysis of nit-2 and nit-4 mutants indicates that this transcription is dependent on functional gene products from these two genes (Fu and Marzluf 1987a and 1988). Although the nmr-1 allele does not effect nit-2 expression, it does appear to derepress nit-3 mRNA levels (Fu and Marzluf 1988). Northern analysis of the nmr^+ gene has indicated that the gene expresses a 1.8 Kb mRNA species constitutively (Fu et al. 1988). Experiments by Davis and Hynes (1987) where the nit-2

gene of N. crassa was used to complement areA$^-$ regulatory gene mutations in A. nidulans, resulted in transformants that were less sensitive to nitrogen metabolite repression. This would suggest that the nit-2 gene is indeed analogous to areA, but might suggest that the regulation by these genes might be subtly different. Further work on isolation of the nit-4 gene and purification of the regulatory gene products will help in establishing the molecular mechanism of this system.

The amdS system takes advantage of a number of in vivo mutations from the fine structure map (Hynes 1979) at the 5' end, which show altered regulation of the amdS gene (reviewed in Hynes and Davis 1986). The amdI93 mutation eliminates amdR control of amdS (mediating w-amino acid induction), amdI9 increaes acetate inducibility of amdS expression (eliminated by facB mutations), while amdI66 acts in cis to cause high levels of amdS expression only in strains carrying amdA mutations. This latter mutation does not affect amdR or facB mediated controls, again, indicating the independent controls regulating amdS expression. For control the areA gene appears to be essential but not sufficient for high levels of expression, while amdR, facB and amdA genes activate amdS expression in decreasing order of strength. With sequencing the amdS gene (Corrick et al. 1987) and the sequence analysis of the cis acting mutations has revealed the nature and the position of the mutation (Hynes et al. 1988). The amdI93

mutation is a deletion of base pairs -181 to -151, the amdI9 mutation results from a single base change at base pair -210, and the amdI66 mutation is due to a duplication of base pairs -107 to -90. Transformation with a plasmid deleted for sequences upstream from -111 bp was found to have lost amdR and facB mediated controls but was still regulated by amdA and the areA genes (Hynes et al. 1988). This is in agreement with above and additionally places the site of action of the areA gene to within -111 and -1 bp of the promoter region. On selecting for multiple copies of amdS, titration of the amdR gene product is observed suggesting that this regulator is limiting. No such phenomenon is seen with the areA gene product and facB titration is only observed with multiple copies of amdI9 (increased affinity for the facB gene product) are present but not with amdS^{+}. Recent cloning of the amdR gene by Andrianopolous and Hynes (1988) has established that the amdR gene is constitutively expressed and not subject to autoregulation (amdR mRNA levels not altered in amdR mutants). Additionally, Adrianopolous and Hynes (1988) showed that multiple copies of amdR can reverse the titration effect of multiple copies of the amdS gene (antititration).

Another method of analysis can be seen in the investigation of the trpC promoter. Here, lacZ fusions have been employed in order to determine the transcriptional signals for trpC (Van Gorcom et al.

1985 and 1986). Subsequent deletion analysis of the 5' region by Hamer and Timberlake (1987) has delineated a region -142 to -11 required for gene expression. The region -67 to -11 does not affect the level of gene expression but causes the formation of mRNAs with incorrectly initiated ends.

Investigation at the functional level of promoter sequences of the qa cluster (N. crassa) has centred on a number of approaches. Firstly, characterization at the sequence level of revertants of qa-2^{+},qa-1F^{-},arom-9^{-} revealed that all mutations occur in a region 5' to the qa-2 gene (Geever et al. 1986). Thus, these mutants which result from a variety of DNA sequence alterations, exhibit constitutive expression of qa-2 independent of the qa-1F gene product. Transcription initiation analysis of these qa-2^{ai} mutants (activator independent) and qa-1F^{ts} mutants indicated that only some of the initiation sites are used compared to the wild-type (termed type II promoters). The activation of these promoters is independent of orientation, position, and precise separation of the mutations with respect to the promoters, suggesting that the various qa-2^{ai} mutations have created enhancer like elements in the -200 to -88 bp region 5' to qa-2. Construction of a qa-2^{ai},qa-1F^{+} strain and analysis of transcription initiation revealed transcripts starting from type II promoters in the absence of the inducer, but on addition of quinic acid, expression switched to initiation from the type I

promoter. DNase I hypersensitive sites in chromatin within the qa gene cluster have also provided support for the interpretations based on qa mRNA studies of qa-2^{ai} and qa-1F^{ts} mutants. The DNase I hypersensitive sites of qa-x and qa-2 chromatin have been studied in the greatest detail, and both qa-1F^{ts} and qa-2^{ai} mutations have been examined for an alteration on these sites (Baum and Giles 1985 and 1986). Although uninduced wild-type chromatin contains hypersensitive sites in the common 5' flanking region of qa-x and qa-2, additional sites can be detected upon induction. These changes in sensitivity to DNase I appear dependent on qa-1F^{+} and seem to occur in the -200 to -88 bp region upstream of qa-2. A computer search of DNA sequences in the qa gene cluster revealed an apparently conserved sequence that is present with slight variations, one or more times upstream of each qa gene. The 16 bp consensus sequence GGATAANNNNTTATCC exhibits dyad symmetry extending for six nucleotides, and the sequence is closely associated with the DNase I hypersensitive sites. Expression of the qa-1F activator protein in vitro using a Baculovirus expression vector in Lepidopteran cell culture has identified the consensus sequence as the upstream binding site and has enabled the DNA-binding domain of the qa-1F protein to be determined (Baum et al. 1987). Thus the components of the Britten and Davidson model (1969) can be observed in the qa system and a regulatory protein has been identified as a DNA-binding protein with site specificity.

I.6 Cloning and Characterization Strategy

Using the method of DNA mediated homologous transformation approximately 10,000 transformants from the gene bank constructed by Johnstone et el. (1985) were screened, one of which complemented the double mutant argB2,niiA4. The total genomic DNA from this transformant was isolated and used to transform E. coli to ampicillin resistance. This plasmid DNA was amplified in E. coli and then used to retransform the original recipient strain (argB2,niiA4). Complementation of both mutations was observed at high frequencies. The plasmid DNA also complemented niaD17 strains but at a lower frequency. Initial studies by Johnstone (unpublished) suggested that the plasmid had probably integrated at the nitrate gene cluster site.

The nitrate gene cluster, like that of the qa gene cluster, offers good prospects for investigation at the molecular level of the structural genes. Cloning of the regulatory genes such as areA (Caddick et al. 1986) and nirA (Scazzocchio pers. comm.) offers possibilities for studying a complete system of regulatory and structural components. With this in mind, characterization of the clone(s) with respect to the organization of the genes and their control regions was undertaken. Also, required was conclusive proof that the clone isolated was indeed that encompassing the structural genes and not some suppressor type loci.

MATERIALS AND METHODS

II.1 Plasmids and Strains

II.1.1 Plasmids: (see figs. 7-10). The recombinant molecule pNiiA contains an 11.1 Kb partial Sau3A insert cloned into the BamHI site (now inactivated) of the vector pILJ16 (Johnstone et al. 1985 and unpublished). The pNiiA plasmid was isolated by homologous transformation and complementation of the niiA4 and argB2 mutations. This recombinant plasmid was further subcloned in later experiments (see section III.2). Overlapping clones were isolated by Johnstone (pILJ141) and Harford (λAN8a). These plasmids contained 4.6 Kb and 15 Kb inserts of A. nidulans DNA detected by Southern hybridization using probes constructed from pNiiA (Johnstone and Harford, unpublished). For a more detailed map of the A. nidulans DNA see fig. 21.

The pAN923- series of lacZ vectors (Van Gorcom et al. 1986) were a gift from Dr. Cees van den Hondel. These vectors contain a unique BamHI site in three different translational phases in front of the truncated lacZ gene from E. coli (first nine codons deleted) and were used for lacZ fusion constructions (section IV.1). The pAN923-21B plasmid was a gift from Dr. Cees van den Hondel and this contains the lacZ gene fused in translational phase to the promoter and N-terminus of the trpC gene (Van Gorcom et al. 1986).

II.1.2 Bacterial strains: DH1 (Hanahan 1983),

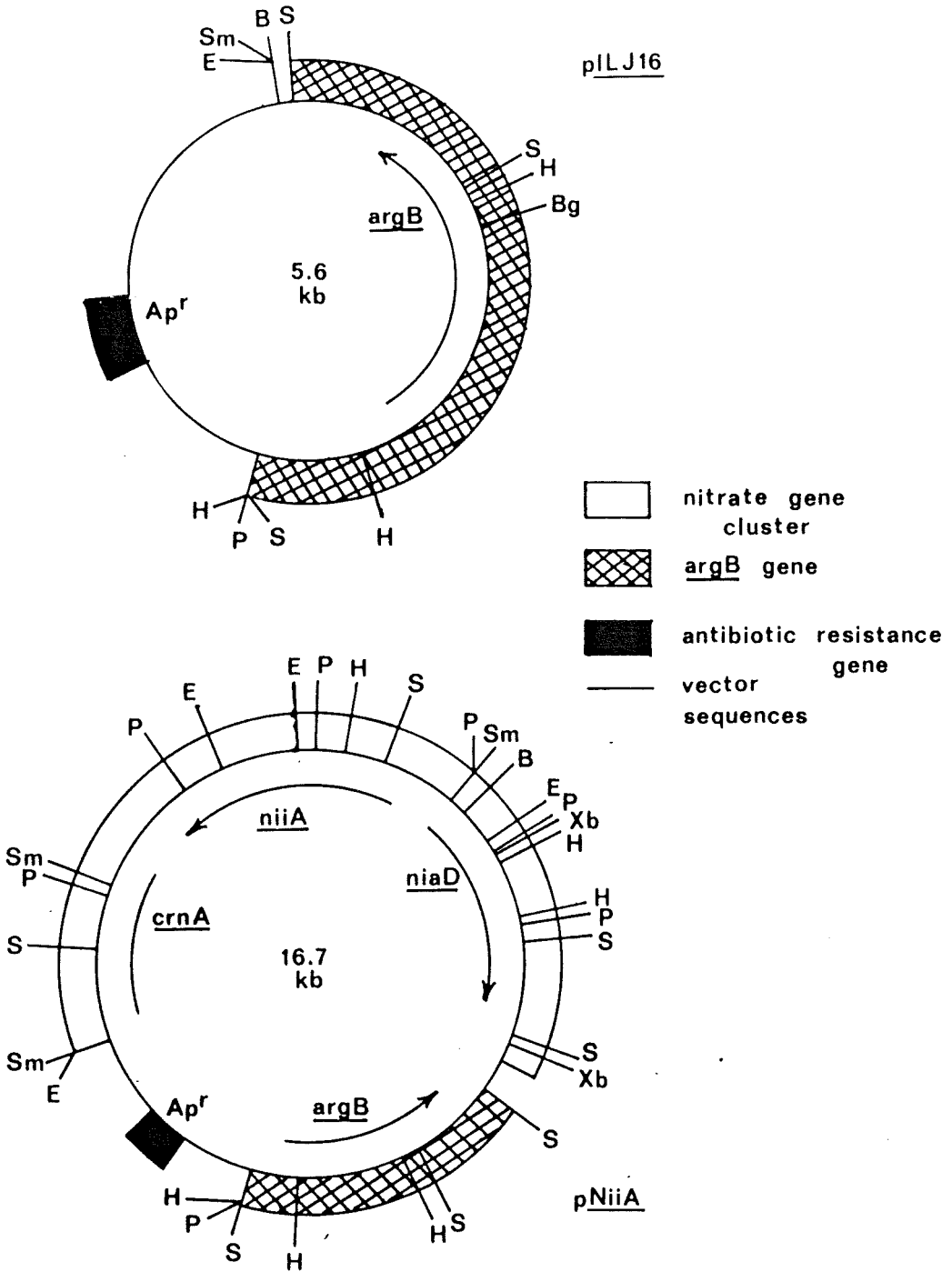

pILJ16
B S
Sm
E
S
H
Bg
argB
5.6
kb
Ap^r
H
P S
H
nitrate gene cluster
argB gene
antibiotic resistance gene
vector sequences
E P H
S
E
P
P Sm
B
E P
Xb
H
niiA
niaD
Sm
P
H
P
S
crnA
16.7
kb
S
Sm
E
S
Xb
Ap^r
argB
S
H
P
S
H
H S
pNiiA

Fig. 7. The pILJ16 vector and pNiiA, the recombinant clone which complements the *niiA4* mutation isolated from the pILJ16 bank.

Top. The vector pILJ16 containing the 2.9 Kb double *Sal*I fragment encoding the *A. nidulans argB* gene cloned into the pUC8 plasmid which has the ampicillin resistance gene.

Below. The recombinant plasmid pNiiA was isolated from a partial *Sau*3A bank constructed with pILJ16 (Johnstone et al. 1985). The pNiiA plasmid contains an 11.1 Kb insert with the *Bam*HI site of the vector now inactivated.

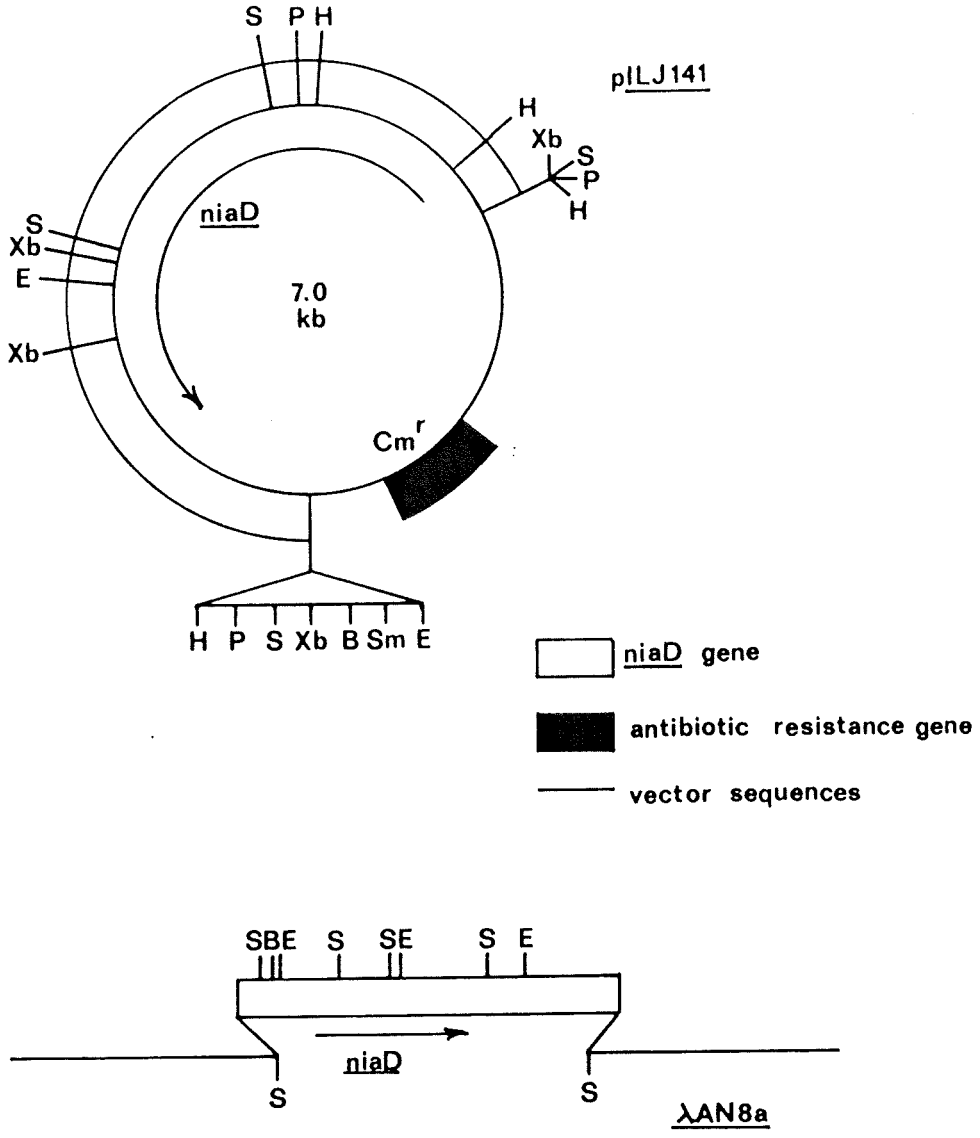


Fig. 8. Overlapping clones isolated by hybridization and complementing distal niaD sequences.

Top. The pILJ141 plasmid contains the 3.5 Kb HindIII fragment isolated from a bank by Southern hybridization (with additional pUC19 polylinker sequences) cloned into a pUC13 vector containing a 5' region of the niaD gene and the chloramphenicol resistance gene (Johnstone, unpublished).

Below. The λAN8a clone was isolated from a partial Sau3A bank in the EMBL3 replacement vector by Southern hybridization (Harford, unpublished).

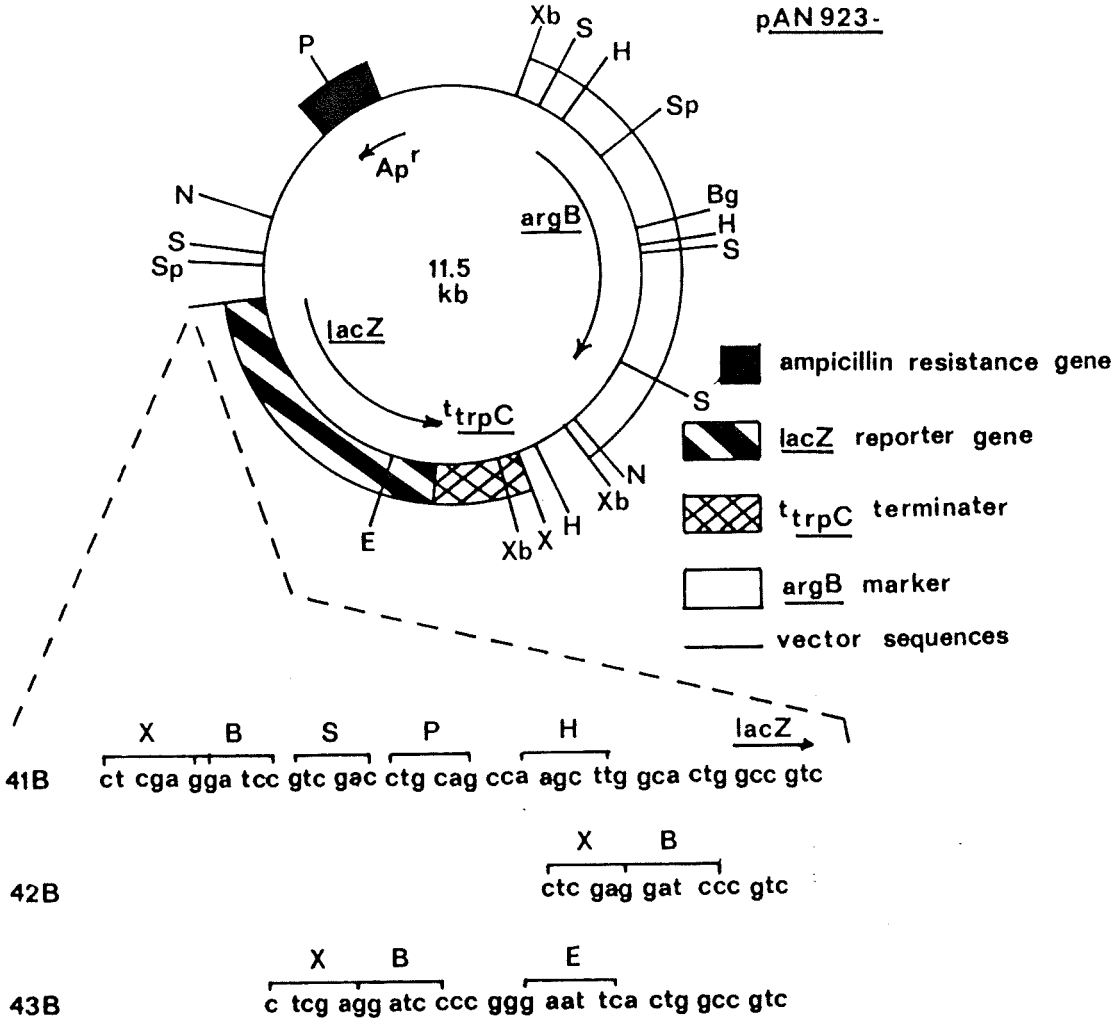


Fig. 9. The pAN923- series of lacZ reporter vectors. For the construction of the pAN923- series of lacZ vectors see Van Gorcom et al. (1986). These plasmids have the A. nidulans argB gene on a 3.5 Kb XbaI fragment inserted into the pBR329 plasmid with the ampicillin resistance gene. The lacZ gene is from E. coli and has the first nine codons deleted and contains a unique BamHI site in three different translational phases. The 3' of lacZ is fused to the A. nidulans trpC terminator.

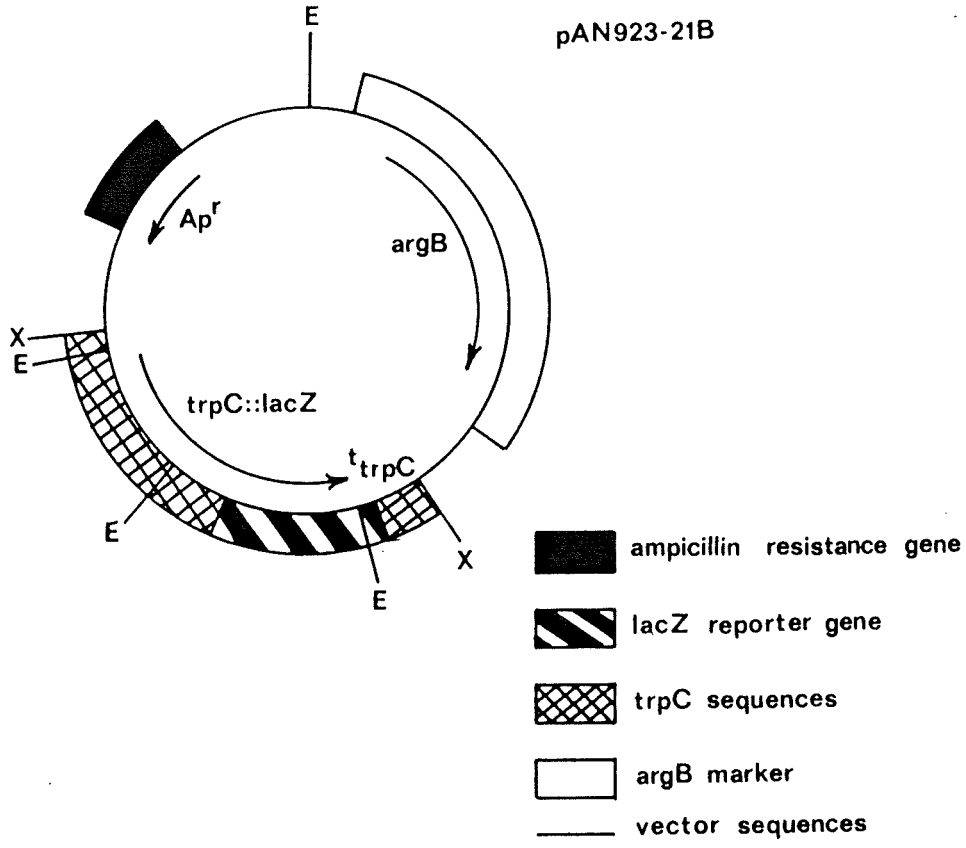


Fig. 10. The pAN923-21B trpC::lacZ fusion plasmid. The pAN923-21B plasmid contains the A. nidulans argB gene on the 3.5 Kb XbaI fragment cloned into pBR329 (containing the ampicillin resistance gene). Also, on a 7.1 Kb XhoI fragment is the lacZ gene of E. coli (promoterless) inserted into the A. nidulans trpC gene in translational phase so that lacZ is under control of the trpC promoter.

HB101 (Boyer and Rowland-Dussoix 1969) and JA221 (Clarke and Carbon 1978) all of which are recA$^-$ strains, sensitive to the antibiotics ampicillin and chloramphenicol.

II.1.3 Fungal strains: Gene symbols have their usual meaning as defined in Clutterbuck (1984) and the conventions of Clutterbuck (1973) are followed (also cited in Bennett and Lasure 1985).

Glasgow strains: (From the Glasgow strain collection and Dr. John Clutterbuck.)

AJC473.14	yA2,pyroA4,argB2,niaD17
AJC9.4	brlA42,pabaA1,argB2,ivoA63
GO34	biA1,argB2
G34	yA2,methH2,argB2
G324	wA3,yA2,methH2,argB2,galA1,sC12,ivoA1
GO125	biA1,niaD15
GO252	yA1,pabaA1,trpC801
833	yA2,pyroA4,niiA4
918	sulA1,adE20,biA1 / acrA1,wA3 / actA1 / pyroA4 / facA303 / lacA1,sB3 / oliC2, choA1 / riboB2,chaA1

(918 is a master strain with linkage groups I-VIII indicated by a /).

Liverpool strains: (From Dr. Brian Tomsett. The deletions (Δ) are all in the nitrate gene cluster).

B5	biA1,Δ506
B72	fwA1,pabaA1,Δ509
B125	biA1,niaD26
B256	biA1,niaD169
B286	yA1,biA1,niaD5
B350	biA1,niaD18
B367	biA1,niiA18
B466	yA1,puA2,Δ507

Newcastle strains: (From Professor Herb Arst.)

1458	yA2,biA1,crnA1

Markers/phenotypes - see below, but for more information see Clutterbuck (1984) and references cited therein. For culture conditions see section II.5.

Antibiotic resistance markers: acrA1, acraflavine resistance; actA1, actidione (cyclohexamide) resistance; oliC2, oligomycin resistance; sulA1, sulphanilamide resistance.

Auxotrophic markers: adE20, requires adenine; argB2, requires arginine; biA1, requires biotin (leaky mutant); choA1, requires choline; methH2, requires methionine; pabaA1, requires p-aminobenzoic acid; puA2, requires putrescine; pyroA4, requires pyrodoxine (leaky mutant); riboB2, requires riboflavin; trpC801, requires tryptophan.

Carbon metabolism mutants: facA303, unable to grow on acetate (Fluoroacetate resistant); galA1, unable to grow on galactose; lacA1, unable to grow on lactose.

Morphological markers: brlA42, temperature sensitive allele that is unable to conidiate at 37°C (appears as white mycelia which on transferring to 25°C forms green conidiospores); chaA1, chartreuse coloured conidia; fwA1, fawn coloured conidia; ivoA1 and ivoB63, ivory coloured conidia; wA3, white coloured conidia; yA1 and yA2, yellow conidia.

Sulphur metabolism mutants: sB3 and sC12, both mutants affected in sulphate utilization; sB3 is additionally chromate resistant.

II.2 Chemicals and Equipment

Chemicals were analar grade or better, except for media components where metabolites were purchased from

Sigma, glucose from Boots, salts as general purpose reagents from BDH, and agar from BDH/Oxoid. Novozyme 234 was purchased from Novobiolabs (Novo Industri, A/S DK 2880 Bgsvaerd, Denmark). These preparations were variable in enzymatic activity but the latter preparations, batches No. 1906 and 1961, gave the best protoplast yields. Restriction enzymes and DNA modifying enzymes were purchased from Pharmacia (except for Calf intestinal phosphatase, which was obtained from BCL).

Centrifugation: For protoplast manipulations MSE benchtop centrifuges were used. For DNA isolation and preparation of enzyme extracts etc. a Sorvall RC-5B refrigerated superspeed centrifuge was used with either fixed-angle rotors SA600 (12 x 50 ml), GS3 (6 x 300 ml) or the swing-out rotor HB4 (4 x 50 ml). Cesium chloride gradients were done using the benchtop Beckman TL-100 ultracentrifuge and the fixed-angle TLA100-2 rotor (10 x 2 ml). Small scale centrifugation (less than 1.5 ml) was performed using a MSE microcentaur microfuge.

Shaking cultures: Conical flasks (unbaffled) from Gibco were used in a New Brunswick Scientific controlled enviroment shaker (model G25). The liquid media used and time of incubation and temperature were varied according to the type of experiment (transformation or enzyme assay etc.), see sections II.3-II.7.

Absorbance readings: Absorbance was measured with a Pye Unicam SP6-500 UV/vis spectrophotometer.

Electrophoresis was carried out in horizontal gel

rigs (models H4-H6) from BRL. Ethidium bromide stained gels were viewed above a transilluminator (Ultra Violet Products Inc. model TM40) and photographs taken from a Polaroid MP4 Land Camera using dark slides (exposure 20 sec at F 5.6) or Polaroid film 57 (exposure 0.5 sec at F 5.6).

Blotting: Agarose gels were depurinated etc. with appropriate solutions (section II.6.3) by gentle shaking with a Rotatest shaker (Luckham, model R100) and DNA was transferred to nitrocellulose (Hybond C from Amersham). Autoradiographs were performed using Fuji RX X-ray film and films were processed by a Fuji (RG II) X-ray film processor.

II.3 E. coli Techniques

II.3.1. Media: (Maniatis et al. 1982).

Luria Broth:
10g Tryptone
5g Yeast extract
10g NaCl
made up to 1 l and adjusted to pH 7.5 with NaOH.

SOB:
20g Tryptone
5g Yeast extract
1.0ml 100mM $MgCl_2$
1.0ml 100mM $MgSO_4$
0.5ml 100mM NaCl
0.5ml 2mM KCl
made up to 1 l and adjusted to pH 7.5 with NaOH.

SOC: as SOB plus 20mM glucose (filter sterilized) added separately.

Agar, where necessary, was added at 1.5 % w/v and media was autoclaved at 120°C for 20 min. Antibiotics were made up as stock solutions as follows: ampicillin (sodium salt) was made up at 60 mg/ml in sterile

distilled water and filter sterilized. Chloramphenicol was made up at 34 mg/ml in absolute alcohol. The antibiotics were aliquoted into 1 ml volumes and stored in eppendorfs at -20°C. Ampicillin was used at a final concentration of 60 µg/ml and chloramphenicol at 17 µg/ml.

II.3.2 Preparation of competent cells and DNA transformation: Two methods were used, the first for general plasmid transformation and the second for plasmid rescue experiments (transformation with total A. nidulans DNA).

(1). (Maniatis et al 1982). Using a toothpick, a single colony of E. coli was inoculated into a 5 ml (universal) culture and grown at 37°C, 200 rpm overnight. Then 100 ml of Luria broth (in a 500 ml conical flask) was inoculated with 5 ml of the overnight culture and grown as before, for approximately 1.5 h, until cell density at $O.D._{550}$ = 0.5 (this for $recA^-$ strains corresponds to 5×10^7 cells/ml). Cells were then chilled on ice for 10 min and pelleted at 8K rpm, 4°C, 5 min in the Sorvall GS3 rotor. The supernatant was discarded and the cells were resuspended in 25 ml of ice cold 100mM $MgCl_2$. The cells were again pelleted at 8K rpm, 4°C, 5 min in the Sorvall SA600 rotor and the supernatant discarded, while the cells were resuspended in 25 ml ice cold 100mM $CaCl_2$ and kept on ice for at least 20 min. Cells were pelleted as before, the supernatant discarded and the cells resuspended in 5 ml ice cold 100mM $CaCl_2$, 14% v/v

glycerol solution. The cell suspension was then aliquoted in 200 µl volumes into precooled eppendorfs. These were stored at -80°C for cryogenic storage and were viable for at least one month. For transformation, an aliquot of cells were thawed in an ice/water bath and added to a precooled eppendorf containing the DNA sample (no more than 1 µg). After mixing gently, the cells and DNA were left on ice for 20 min. Cells/DNA were then transferred to a heating block at 42°C for 2-3 min after which 800 µl of Luria broth was added and the cells transferred to a heating block at 37°C for 1 h to allow full expression of antibiotic resistance. Cells were serially diluted in Luria and spread (maximum volume of 200 µl) on 90 mm plates containg Luria agar or Luria agar plus antibiotic to estimate cell viabilities and DNA transformation efficiences. Similar treatment for cells with no DNA was also performed as a control. Normally a 200 µl aliquot yielded about 5×10^7 viable cells and typical frequencies by this method ranged between 10^5-10^7 transformants per µg of pUC8 DNA.

(2). (Hanahan 1983). Again, a single colony of E. coli was inoculated into a 5 ml (universal) culture and grown overnight at 37°C, 200 rpm. From this overnight culture, 1 ml was taken and used to inoculate 50 ml (in a 500 ml flask) of SOB media. The culture was grown at 37°C, 200 rpm for approximately 2 h ($O.D._{550} = 0.5$) and was then chilled on ice for 10 min. The cells were pelleted in the Sorvall GS3 rotor at 8K rpm, 4°C, 5 min; the

supernatant discarded and the pellet gently resuspended in 10 ml TFB solution (10mM K-MES pH 6.2, 100mM RbCl (or KCl), 45mM $MnCl_2$, 10mM $CaCl_2$ and 3mM hexamine $CoCl_2$). This suspension was left on ice for 10 min before the cells were again pelleted at 8K rpm, 4°C, 5 min in the Sorvall SA600 rotor and the supernatant discarded. The cells were gently resuspended in 2 ml of TFB solution, to which 70 µl fresh DMSO was added. This was gently mixed and left on ice for 5 min. Then 70 µl of a 2.25M DTT, 40mM KAc ph 6.0 solution was added, gently mixed and left on ice for 10 min. Finally, a further 70 µl DMSO was added and the mixture was left on ice for 5 min before aliquoting 210 µl into precooled eppendorfs. To the chilled cells (used fresh), DNA (<10 µl) was added, gently mixed and left on ice for 30 min. Then the cells/DNA were transferred to a heating block at 44°C for 1-2 min, after which time the mixture was placed back in the ice for a further 2 min and 800 µl of SOC media was added before incubation at 37°C for 1 h to allow full expression of antibiotic resistance. Similar experiments using cells and no DNA were performed as a control. Cells were then serially diluted and plated out (plating <200 µl with a flamed glass spreader) on the media with or without antibiotic to estimate cell viabilities and transformation efficiencies. Typical frequencies with plasmid DNA were greater than 10^6 transformants/µg DNA and with 10 µg of *A. nidulans* transformant DNA, 1-10 colonies of *E. coli* emerged with antibiotic resistance.

II.3.3 Plasmid isolation and purification:

(Maniatis et al. 1982). A 5 ml sample of E. coli containing plasmid (all high copy number), was taken from an overnight culture (as before) and used to inoculate 500 ml of Luria broth plus antibiotic (in 2 l flasks). Cells were grown overnight (about 16 h) at 37°C, 200 rpm and were harvested by centrifugation in the Sorvall GS3 rotor at 8K rpm, 4°C, 5 min. The supernatant was discarded and the cell pellet resuspended in 50 ml of solution I (50mM glucose, 25mM Tris.HCl pH 8, 10mM EDTA) per 1 l of culture. To the turbid solution 80 ml (per 1 l of culture) of solution II (0.2M NaOH, 1% SDS - freshly made) was added producing a cleared lysate. This was incubated on ice for 5 min before adding 40 ml (per 1 l of culture) of solution III (3M KAc, pH to 4.8 with glacial acetic acid). The suspension was mixed gently, and was then spun in the Sorvall GS3 rotor at 8K rpm, 4°C, 5 min to pellet the precipitated cell debris. The supernatant (containing plasmid DNA) was poured through muslin into another sterile centrifuge tube, while the pellet was discarded. To the supernatant 0.6 volumes of cold iso-propanol was added, mixed, and the precipitated nucleic acid was pelleted at 8K rpm, 4°C, 10 min in the Sorvall GS3 rotor. The supernatant was discarded, and after air-drying, the pellet was taken up in an appropriate volume of TE (10mM Tris.HCl pH 7.5, 1mM EDTA).

A similar treatment was used for small scale

plasmid isolation (minipreps) where 1.5 ml of an overnight culture was pelleted and treated with 100 µl solution I, 200 µl solution II, 150 µl solution III and the nucleic acid was precipitated with 2 volumes of cold ethanol.

DNA was purified by either CsCl gradients or phenol/chloroform extraction and RNase treatment.

CsCl gradient: For every 1 ml of DNA solution, 1.1 g of CsCl was added, followed by 75 µl of a 20 mg/ml EtBr solution. At this point the suspension could be transferred to eppendorf tubes and microfuged for 2-3 min, pelleting a significant amount of protein material. The superntant was then transferred to a Beckman, quick seal tube (polyallomer belltop - 2 ml), filling the tubes to the base of the neck. Tubes were weighed and balanced to two decimal places and then heat sealed until the metal cap was flush with the shoulder. It was important that few or no air bubbles were present and if necessary liquid paraffin was used to fill the tube. Tubes were then checked for flaws before inserting in the TLA100-2 rotor with the rubber caps in place. The gradients were run overnight at 80K rpm, 20°C and the supercoiled plasmid band (lower) was harvested using needle and syringe. The EtBr was removed by extracting with n-butanol 3-4 times (butanol upper phase) and the DNA dialysed against TE buffer overnight at 4°C (dialysis tubing purchased from BRL).

Phenol extraction: Phenol (crystalline) was melted

at 68°C and redistilled at 160°C (keeping the condenser warm to prevent the phenol from crystallizing out). To the distilled phenol, 8-hydroxyquinoline was added to a final concentration of 0.1% w/v. The distilled phenol was saturated with an equal volume of 1.0M Tris.HCl pH 8.0, followed by 0.1M Tris.HCl pH 8.0 and 0.2% v/v β-mercaptoethanol. After checking the pH (>7.5) the phenol could be stored in the dark at 4°C for up to 1 month.

The plasmid DNA solution was extracted with an equal volume of this Tris-saturated phenol and spun at 4K rpm, 4°C, 5 min in the Sorvall SA600 rotor (or microfuged if the volumes were suitable). The uppermost aqueous phase (containing DNA) was taken and could be repeatedly phenol extracted in this way until a clear interface was obtained. The DNA phase was then chloroform extracted (chloroform:iso-amyl alcohol at 24:1) twice to remove traces of the phenol. The nucleic acid (from the uppermost aqueous phase) was precipitated by the addition of 2 volumes of cold ethanol and 0.1 volumes of 3M NaAc pH 5.6, mixing before placing at -80°C for 30 min (or -20°C overnight) and pelleting the nucleic acid at >8K rpm, 4°C, 15 min in the Sorvall SA600 rotor (or microfuging for 10-15 min). To remove RNA from the DNA, the pellet was resuspended in an appropriate volume of TE and DNase-free RNase (prepared by boiling a stock solution at 10 mg/ml in 10mM EDTA for 10 min) to a final concentration of 20 µg/ml. The solution was incubated at 37°C for 1-2 h and the RNase

removed by phenol/chloroform extraction as previously described.

II.3.4 Spectrophotometric analysis of DNA: The resultant DNA solution can then be checked for DNA concentration and purity by measuring absorbance at 260 nm and then 280 nm, a pure DNA solution having a ratio of 1.8 (260:280). DNA concentration was calculated using the relationship that a solution of 50 µg/ml has an absorbance at 260 nm of 1.0. Typically, a 500 ml culture would yield about 500 µg - 1 mg of DNA. All solutions for DNA manipulations were routinely sterilized, either by autoclaving or filtration, to remove nuclease contamination.

II.4 Molecular Biology Tecniques

II.4.1 Restriction enzyme digests: Assay buffers for the restriction enzymes were made up from sterile solutions as below:

assay buffer (x1)	Tris.HCl (mM)	pH	NaCl (mM)	$MgCl_2$ (mM)	β-ME (mM)	temp. °C
BamHI	10	8.0	100	10	1	37
BglII	10	8.0	100*	10	10	37
EcoRI	10	7.5	100	10	1	37
HindIII	10	8.0	60	10	1	37
KpnI	10	7.5	10	10	10	37
PstI	10	8.0	50	10	1	37
PvuII	10	8.0	60*	10	10	37
SalI	10	8.0	150	10	1	37
SmaI	10	8.0	20*	10	10	30
XbaI	10	8.0	100	10	1	37
XhoI	10	8.0	150	10	10	37

*KCl substituted for NaCl.

Assay buffers were made up as 10 x stock solutions and stored at -20°C. Although the addition of 100 µg/ml

nuclease-free BSA is generally recommended, it was found for most enzymes not to be necessary.

Analytical digests were typically done in a reaction volume of 20 µl with 1 µg of DNA and 5 units of enzyme (where 1 unit is sufficient to digest 1 µg of DNA in 1 h). The addition of RNase to the reaction was also possible. Digests were performed at the assay temperature for 2-4 h. For restriction mapping purposes a combination of single and double digests were performed in a common buffer. If this was not possible a sequential digest was used i.e. a low salt enzyme (e.g. KpnI) was used first and then salt was added with the second enzyme. Alternatively the first digest was ethanol precipitated and the pellet resuspended in the second buffer. Large scale digests, e.g. isolation of fragments or digestion of A. nidulans chromosomal DNA, involved 10-50 µg of DNA in a final volume of 20-200 µl. A tenfold excess of enzyme units was used, but keeping the volume added less than a tenth of the final volume, in order to avoid inhibition by the glycerol present in the storage buffer. These digests were performed at the assay temperature for at least 3 h. At the end of the incubation period restriction digests were terminated by the addition of one sixth volume of loading buffer (0.25% w/v bromophenol blue, 40% w/v sucrose, 100mM EDTA pH 8.0).

II.4.2 Electrophoresis: Buffers were made up as 10 x concentrated stock solutions which were kept at room

temperature. Stock solution compositions are given below:

Buffer (10 x)	add per 1 l dH_2O
Tris.acetate (TAE)	48.4 g Tris 11.4 ml glacial acetic acid 20.0 ml 0.5M EDTA (pH 8.0)
Tris.borate (TBE)	108.0 g Tris 55.0 g boric acid 40.0 ml 0.5M EDTA (pH 8.0)

For analytical and chromosomal digests (for blotting) the DNA was electrophoresed through a horizontal agarose gel. Typically, for resolution of fragments, 0.8% w/v agarose (ultra-pure, BRL) was used, made up in either TAE or TBE (1 x). For analytical gels the running buffer (the same as that used to make the gel) was stained with EtBr (stock solution at 10 mg/ml) to a final concentration of 0.5 µg/ml. In preparing a gel, a suitable tray and comb was chosen (BRL models H4-H6), the ends of the tray were taped up and the comb inserted at one end. The agarose was fully dissolved in TBE (TAE) by heating over a bunsen (or in a microwave oven) and allowed to cool to about 50°C before pouring. Typically, the gel was left at 4°C for 1 h in order to set before removing the comb/tape, placing in the gel rig and loading samples into the wells. Usually, such gels were run overnight at 75 V for H4 gels (300 ml capacity); 25 V for H5 gels (100 ml); while minigels (H6 - 30 ml) were run at 50 V for 2-3 h if a quick result was required. DNA digests and fragments etc. were detected by placing the gel on the UV transilluminator.

II.4.3 Recovery of DNA fragments: Two methods of preparative gel electrophoresis were used. In the first, electroelution, dialysis tubing (from BRL) was prewashed with 50% ethanol and then heated to 100°C in 10mM EDTA for 10 min to remove any nucleases. The tubing was rinsed in sterile dH_2O and stored in 50% ethanol before use. Digested DNA was electrophoresed as before, with some of the comb's teeth taped together, to form a trough in place of individual wells and omitting the EtBr stain. Once the bromophenol blue had reached the bottom of the gel, the gel was removed from the gel rig and stained in 0.5 µg/ml EtBr for 30 min. The gel was washed in running buffer before viewing under UV light on the transilluminator. Using a sterile scalpel blade the band required was cut out of the gel as quickly as possible in order to reduce nicking of the DNA. The gel slice was transferred to dialysis tubing and one end of the tubing was clamped or knotted. Excluding air-bubbles, an appropriate volume of electroelution buffer (5mM Tris, 2.5mM glacial acetic acid), was added before similarly clamping the other end. The dialysis bag containing the gel slice was returned to the gel rig and electrophoresed for a further 2 h in the same direction as before. After this time the current was reversed for 30 sec, to remove any DNA adhering to the dialysis tube wall. By this time any UV stain should be in the electroelution buffer and not in the gel slice. The buffer was then removed from the dialysis tube and

transferred to eppendorfs. To remove the EtBr, the electroelution buffer/DNA was extracted 3-4 times with an equal volume of n-butanol (butanol upper phase). After this, the lower aqueous phases were pooled and ethanol precipitated. In the second method, low melting point agarose (where hydroxyethyl groups have been introduced into the agarose molecule) was used. Gels were made up as previously but were allowed to set at 4°C for an extended period. Again, DNA samples were electrophoresed as above, omitting the EtBr stain in the running buffer. After electrophoresis, the gel was stained with 0.2 µg/ml EtBr for 10 min before visualizing under UV. The fragment band was excised as before and transferred to a sterile universal. Five times the volume (of the gel slice) of TE was added and the low melting point agarose was melted at 65°C (well below the Tm for most DNAs) for 5 min. The agarose was extracted with phenol at 37°C and spun at 4K rpm, 5 min in the Sorvall SA600 rotor. The aqueous phase (upper) was re-extracted with phenol at room temperature until the interface was clear. The sample was chloroform extracted and if necessary volume was reduced by n-butanol extraction before ethanol precipitation.

II.4.4 Blunt-ending: A 10 x DNA polymerase buffer was made up as 70mM Tris.HCl pH 7.4, 500mM NaCl, 70mM $MgCl_2$ in dH_2O and stored at 4°C. Typically, a blunt-ending reaction was performed using the Klenow fragment of DNA polymerase as follows:

DNA sample	:	20 µl
10 x Polymerase buffer	:	3 µl
10mM DTT	:	3 µl
0.5mM dNTPs	:	3 µl
4.5 units/µl Klenow	:	1 µl

(Where 1 unit catalyzes the incorporation of 10 nmoles of dNTPs in 30 min at 37°C.) The reaction mix was incubated at room temperature for 1 h, and the sample diluted to 400 µl with dH_2O before removing the enzyme by phenol and chloroform extractions. The fragment was ethanol precipitated as before, air-dried, and taken up in 20 µl of TE solution.

II.4.5 Phosphatasing: Calf intestinal phosphatase was used and a 10 x buffer was made consisting of 0.5M Tris.HCl pH 9.0, 10mM $MgCl_2$, 1mM $ZnCl_2$. A reaction was set up as follows:

DNA sample	:	45 µl
10 x Phosphatase buffer	:	5 µl
CIP (1 unit/µl)	:	0.2 µl

The sample was phosphatased at 37°C for 1 h after which time the enzyme was heat-killed (5 min at 70°C) and then removed by phenol and chloroform extraction followed by ethanol precipitation as in the Klenow step.

II.4.6 Ligations: A theoretical analysis of ligations was performed by Dugaiczyk et al. (1975) based on studies of linear 'phage lambda DNA. Briefly, they obtained the following equation:

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

where Mw is the molecular weight of the DNA sample (N.B. 660 Kdal = 1 Kb), j is the effective concentration of

one end of a molecule relative to the other end, $\underline{i}$ is the total concentration of ends and $\underline{(DNA)}$ is the DNA concentration in g/l. A graph of this equation can be used to predict the type of products expected during ligation of a specific DNA fragment at various concentrations (fig. 11). When j = i, the probability of the two ends of the same molecule finding each other should be the same probability of finding the end of a different molecule. When j > i, the formation of circular molecules is favoured and when j < i, the formation of linear concatamers during ligation will be favoured. When two fragments of different molecular weights are being ligated, a sufficiently accurate approximation can be made by averaging the molecular weights. Also, when ligating a mixture of DNA fragments, it is possible to affect the ligation products by manipulating the ratio of the fragments in the ligation reaction.

Hence, in setting up the ligations, sufficient DNA concentrations were used to favour the formation of recombinant molecules. For an intramolecular ligation of a linear DNA molecule a much lower concentration of DNA could be used. In general, two classes of ligation events could be described; one, sticky-end ligation and two, blunt-end ligation. High concentrations of ATP in the ligation mix inhibit blunt-end ligation, and the Km of T_4 DNA ligase for blunt-end fragments is much higher than that of sticky-ends. Accordingly two different

ligation conditions were used for sticky-end and blunt-end ligation:

Sticky-ends:	DNA fragments	: 7 µl
	10 x Ligation buffer I	: 1 µl
	10mM ATP	: 1 µl
	T_4 DNA ligase (4 units/µl)	: 1 µl
Blunt-ends:	DNA fragments	: 7 µl
	10 x Ligation buffer II	: 1 µl
	5mM ATP	: 1 µl
	T_4 DNA ligase (40 units/µl)	: 1 µl

Where buffer I consists of 500mM Tris.HCl pH 7.5, 100mM $MgCl_2$ and 5mM DTT. Buffer II consists of 250mM Tris.HCl pH 7.5, 50mM $MgCl_2$ and 5mM DTT. One unit of T_4 DNA ligase activity is defined as that sufficient to catalyze 50% ligation of λDNA HindII fragments in 1 h. Both ligation reactions were allowed to proceed at 14°C overnight.

After the ligation reaction had been performed, the mix was transformed into competent E. coli. Controls also performed included digested vector which had been phosphatased and ligated, digested vector religated, and uncut vector all transformed into E. coli (section II.3.2). If the phosphatase had been successful then no transformants of the E. coli/digested vector which had been phosphatased and ligated should be observed whereas the other controls (digested vector religated and uncut vector) should all produce E. coli transformants if the ligase and the competent cells were satisfactory. Colonies from the vector plus insert ligation transformation were grown up in universal cultures, overnight and then miniprepped (section II.3.3) and

electrophoresed (section II.4.2). Successful ligations with phosphatased vector resulted in about 50% recombinant molecules as determined by analysis of restriction digests.

II.4.7 Labelling of DNA with ^{32}P: (Feinberg and Vogelstein 1983, 1984). DNA was labelled using an Amersham Multiprime kit (RPN 1601) as follows:

DNA fragment (200 ng)	:	10 µl
dH_2O	:	18 µl
Buffer I	:	10 µl
Primer II	:	5 µl
Enzyme III	:	2 µl
^{32}P-dCTP (50 µCi)	:	5 µl

The DNA and dH_2O were boiled for 2 min before ice-cooling to allow strand separation. Buffer I consists of dNTPs in Tris.HCl pH 7.8, $MgCl_2$ and β-ME buffer; Primer II being random hexanucleotides (primers) with nuclease-free BSA; and enzyme III consists of Klenow (1 unit/µl) in phosphate buffer pH 6.5, 10mM β-ME and 50% v/v glycerol. The reaction mix was gently mixed by pipetting up and down before incubating at room temperature for 3-18 h.

II.4.8 Removal of unincorporated nucleotides: A commercial Nick^{TM} column (from Pharmacia) was used, but alternatively a G50 Sephadex column may be used. The column is first equilibrated with approximately 10 ml (3 x volume of the column) of TE before loading the 50 µl labelling reaction followed by 400 µl of TE. The column was periodically loaded with 100 µl aliquots of TE to prevent drying out and to elute the labelled

fragment. Roughly 100 µl (2 drops) were collected dropwise for 20 fractions. These fractions were then monitored using a Geiger counter and 2 peaks of activity were obtained. The fractions in the first eluted peak were pooled and used later in hybridization experiments (section II.6.5).

II.5 A. nidulans Techniques

II.5.1 Media: Modified from Cove (1966) and Clutterbuck (1974).

Salt solution:
- 26 g KCl
- 26 g $MgSO_4.7H_2O$
- 76 g KH_2PO_4

add dH_2O to 1 l.

Hunter's Trace element solution (Hunter et al. 1950)
- 1.5 g $Na_2MoO_4.2H_2O$
- 11.0 g H_3BO_3
- 1.6 g $CoCl.\ 6H_2O$
- 1.6 g $CuSO_4.5H_2O$
- 50.0 g EDTA
- 5.0 g $FeSO_4.7H_2O$
- 5.0 g $MnCl_3.4H_2O$
- 22.0 g $ZnCl_2.7H_2O$

made up to 1 l with dH_2O, heated to boiling, cooled to 60°C and pH adjusted to 6.5-6.8 with KOH.

Minimal media:
- 10 g D-glucose
- 20 ml Salt solution
- 1 ml Trace element solution

add dH_2O to 1 l and adjust pH to 6.5 with NaOH. Agar may be added at a final concentration of 1.5% w/v.

Complete media supplement:
- 7.5 g Adenine
- 5.0 g L-methionine
- 36.5 g Lysine.HCl
- 0.5 g Riboflavin

made up to 1 l with dH_2O.

Vitamin solution:	1.5 g	Aneurine (thiamine)
	2.5 g	Biotin
	2.5 g	Nicotinic acid
	20.0 g	Choline.HCl
	0.8 g	PABA
	1.0 g	Pyrodoxine.HCl
	2.5 g	Riboflavin
	2.0 g	Pantothenate (Ca salt)

made up to 1 l with dH_2O.

Complete media:	10 g	D-glucose
	2 g	Mycological peptone
	1 g	Yeast extract
	1 g	Casein hydrolysate
	20 ml	Salt solution
	10 ml	Complete media supplement
	1 ml	Vitamin solution
	1 ml	Trace element solution

add dH_2O to 1 l and adjust pH to 6.0 with NaOH. Agar may be added at a final concentration of 1.5% w/v.

All the above solutions can be autoclaved at 120°C for 20 min.

In addition to the above solutions, various supplement stock solutions were made up at 100 x final concentrates as follows (g per 100 ml): adenine, 7.5 g; arginine.HCl, 4.2 g; biotin, 0.1 g; choline.HCl, 2.0 g; methionine, 0.5 g; putrescine, 0.2 g; pyrodoxine.HCl, 1.0 g; riboflavin, 1.0 g; tryptophan, 6.0 g. With the exception of putrescine and tryptophan (both filter sterilized) all these supplements were sterilized by autoclaving. In addition stock solutions of 1.0M $NaNO_3$, 1.0M $NaNO_2$, 1.0M sodium glutamate, 0.5M ammonium tartrate, and 0.1M adenine were made up for the various nitrogen sources; being added at 1% v/v to the media. If glucose was to be replaced by acetate or lactose then MM was made up with the desired carbon source, acetate at a final concentration of 100mM and lactose at 1% w/v.

The antibiotics acraflavin and actidione (cyclohexamide) were used at final concentrations of 0.005% w/v and 0.0025% w/v respectively, while benlate (for diploid haploidization) was used at a final concentration of 0.0019% w/v.

II.5.2 Storage of strains: From plate subcultures, conidia from the strain were scraped off, using a wire-loop, into a suspension of 5% w/v Marvel (Cadbury's). Approximately 500 µl of this suspension was transferred to a small vial containing sufficient silica (mesh 60-120) to remain dry (previously sterilized by baking at 180°C). Strains could then be stored at 4°C for 2 or more years and still remain viable.

II.5.3 Sexual crosses: (Clutterbuck 1974). Strains to be crossed were chosen so that they differed for both auxotrophic markers and spore colour. These strains were subcultured on CM plates by transferring conidia via a glass needle and scoring the agar down the middle. Plates inoculated in this way were incubated at 37°C for 5-7 days, after which time the strains had usually covered most of the agar plate. For the argB2, trpC801, and puA2 strains the media was supplemented with the appropriate requirement (see section II.5.1). From these subcultures, a flamed wire-loop dipped in a sterile saline-Tween solution (0.9% NaCl, 0.01% Tween 80) transferred 2-3 loopfuls of conidia from each strain onto a MM plate, where the conidia of both strains were well mixed. To the surface of the agar, 50 µl of CM

(plus any additional growth requirement) was added, mixed with the conidia and the agar was cut up to induce anaerobiosis. The plates were placed in the 37°C incubator and after 1 days growth were sealed with parafilm before further incubation for 7-10 days. It was found that the addition of arginine at the initial stages was essential for a successful cross involving argB2 to result, as the argB2 allele is an extremely tight mutation preventing any germination of the strain in selective conditions. After 8-10 days growth, the black cleistothecia were picked off from areas of mixed conidia (A. nidulans is homothallic and cleistothecia from one area of similar (colour) conidia usually turn out to be selfed) and the adjoining Hülle cells were removed by rolling the cleistothecia on a 3% agar plate, using a glass needle, until the cleistothecia appeared relatively shiny. The cleaned cleistothecia were then burst between two glass needles to produce a purple spore suspension. A sample (tip of the needle) of the spores was then stabbed onto a non-selective CM (plus any additional requirement) plate. This was repeated for 6-10 cleistothecia per cross, after which the CM+ plate was incubated at 37°C for 2-3 days and the 3% agar plate containing the remainder of the ascospores was stored at 4°C. A hybrid cleistothecium (usually larger than selfed cleistothecia) was identified by mixed spore colours originating from a single stab, and spores from the original cleistothecium (on a 3% agar plate) were taken

up to 1 ml of saline-Tween and plated in aliquots (over a dilution range) onto CM+ plus sodium deoxycholate (at 400 µg/ml), spreading with a flamed glass spreader. The sodium deoxycholate induces the formation of micro-colonies (MacKintosh and Pritchard 1963). From the micro-colonies produced, about 50-100 were randomly selected and stabbed to form a master plate (CM+), where the progeny were arranged in a 5 x 5 array with one asymmetric point. Then using a 25 steel pin replicator (similar array to the master plate), the colonies from the master plate were replicated onto various test-media (MM plus different supplements) in order to determine the genotype of the progeny. To avoid spore scatter the inoculating steel pins of the replicator were always stabbed upwards into the medium of an inverted petri dish. On all master plates were the two parental strains so that growth of the progeny could always be compared to that of the parents. Also a MM plus all possible supplements was included in the test-media to ensure spore transfer (and thus indicate possible false negatives).

II.5.4 Parasexual analysis: (Clutterbuck 1974). To do this the first step is to construct a diploid between the strain and a master strain (e.g. strain 918, section II.1.3) via a heterokaryon stage. As heterokaryons of A. nidulans are unstable unless forced by balanced nutritional requirements, the two strains have to differ in auxotrophic markers. It is also useful in visual

selection of diploids to have strains differing in spore colour. A heterokaryon can be constructed by inoculating a 4 ml CM+ broth, in a universal container, with the two strains and incubating the culture on a slant overnight at 37°C without shaking. The resulting mycelial mat was vortexed in fresh sterile saline-Tween solution several times and pieces of mycelia were transferred to a MM plate. The MM plate was incubated at 37°C for 3-5 days, after which time a fan shape of rapidly growing mycelia (containing both conidial types) was observed. Such a heterokaryon formed between a yellow transformant and the white master strain allowed visual selection of the green diploid conidia (section III.5.2). On subculturing the heterokaryon, several diploid conidia were observed per plate (a frequency of about 1×10^{-7}). The diploids were then subcultured and stored on silica gel as before (section II.5.2). The diploids were haploidized on CM+ containing benlate (at a final concentration of 0.0019%) by point inoculating diploid conidia at a density of 16 stabs a plate. The diploids were incubated at 37°C for 3-4 days. As benlate restricts diploid growth, but not haploid growth at this concentration, haploid sectors could easily be distinguished from the moribund diploid centre as rapidly growing sectors (Hastie 1970). The haploid sectors were picked off and transferred to master plates as before (section II.5.3). After incubating the master plates for 2-3 days at 37°C, the haploid segregents can be screened for their genotype by

replica plating as before (section II.5.3).

The heterokaryon stage may also be used as the inoculum for a sexual cross, the cleistothecia being induced by sealing the plate and allowing 8-10 days growth at 37°C. The cleistothecia may be analysed as in section II.5.3.

II.5.5 Transformation of A. nidulans: (Johnstone et al. 1985). Mycelia for protoplasting was obtained either from cellophane culture (Ballance and Turner 1983), or from liquid culture. In each case, inocula used was from a fresh plate (5-7 days old). For cellophanes, a 90 mm diameter cellophane was placed onto a CM plus arginine plate and a sterile cotton wool swab was used to transfer a dilute conidial suspension (10^3-10^4 conidia/ml) to the cellophane, dabbing at 10 mm intervals. The plates were then incubated at 37°C for 14-15 h. Alternatively, a whole plate of conidia (10^8-10^9 coidiospores) was used to inoculate 200 ml MM plus supplements with 20mM nitrogen source in a 1 l unbaffled flask. This culture was incubated in a shaking culture at either 13-14 h at 37°C or 16-18 h at 30°C, 200 rpm. The mycelia was harvested aseptically by filtration through 2 layers of muslin placed in a Buchner funnel.

To protoplast the mycelia (from liquid or cellophane culture), the mycelia was resuspended in 30-50 ml of protoplast buffer (0.8M $MgSO_4$, 10mM phosphate buffer pH 5.8, 0.01% v/v β-ME and 10 mg/ml Novozyme 234). Initial studies used 1.2M $MgSO_4$ in the protoplast

buffer but some of the later Novozyme batches were not fully soluble at this concentration. The mycelia and protoplast buffer were incubated in a 250 ml conical flask at 30°C with occasional shaking, for approximately 2 h. After this time, the contents were transferred to 25 ml sterile universal tubes and centrifuged in the benchtop MSE centrifuge in a swing-out rotor at 4 x g, 20 min at room temperature. The 0.6M sorbitol overlay used by Johnstone et al. (1985) was omitted. In $MgSO_4$, the protoplasts formed tend to vacuolate and become less dense than the protoplast buffer and hence can be separated from the mycelial debris by centrifugation. In 1.2M $MgSO_4$ the protoplasts typically formed a layer at the top of the protoplast buffer following centrifugation, but sometimes and more frequently in 0.8M $MgSO_4$, the protoplasts produced were at the same density as the buffer and, while not pelleting with the mycelial debris, were present throughout the supernatant. The supernatant, containing the protoplasts was transferred using a Gilson pipette to other sterile universal tubes and was diluted at least 4-fold in transformation buffer (1.2M sorbitol, 10mM $CaCl_2$, 10mM Tris.HCl pH 7.5) and the contents recentrifuged at 4 x g for 5 min at room temperature in a fixed-angle rotor of the MSE benchtop centrifuge. The supernatant was discarded and the protoplast pellets were pooled. In this way, the protoplasts were washed several times by centrifugation and resuspension of pellets in fresh

transformation buffer. After the final wash, the protoplasts were taken up in a fixed volume (usually 10 ml) and a small sample was diluted tenfold in order to count the protoplasts under a microscope using a haemocytometer. Typically, 10^8-10^9 total protoplasts per experiment were obtained as estimated from the haemocytometer count. These were then pelleted and resuspended in transformation buffer to a density of 3×10^8 protoplasts/ml and small volumes of DNA (<50 µl/ml) were added to aliquots of the protoplast suspension (<1 ml). The DNA and protoplasts were gently mixed before incubating the suspension at room temperature for 20 min, after which time a 10 x volume of a PEG solution (50% w/v PEG4000, 10mM $CaCl_2$, 10mM Tris.HCl pH 7.5) was added to each aliquot, mixed gently and incubated at room temperature for a further 15 min. At this point fusion of protoplasts could be observed under the microscope. The transformation mix was then diluted at least three fold in transformation buffer before plating. Although it was possible to pellet the protoplasts at this stage, it was found invariably to reduce protoplast viability, presumably by loss of protoplasts to the supernatant. The transformed protoplasts were plated directly into molten selection medium containing 1.0M sorbitol or sucrose as osmotic stabilizer (temperature below 50°C). Controls performed for each transformation included a protoplast and zero DNA control, and protoplasts plated on non-selective

media (plus osmotic stabilizer) at a range of dilutions (usually 10^{-4} to 10^{-6}) in order to estimate protoplast viabilities. In addition, another possible control was to plate the protoplasts on non-selective but osmotically unbuffered media in order to estimate the number of osmotically insensitive protoplasts (i.e. an estimate of cell wall material). Plates were incubated for 3-4 days at 37°C.

Transformation efficiences were variable and ranged from 1-100 transformants/µg DNA depending on protoplast purity (lack of cell wall material) and quality of DNA preparation (CsCl gradient purified was at least an order of magnitude better than phenol, chloroformed DNA). Any strain differences were slight. Protoplast variabilities ranged from about 1-30% of the counted protoplasts (estimated from the haemocytometer count). In the PEG solution, no difference was found between using PEG 6000 or 8000 molecular weights.

II.5.6 Stability of transformants: Conidial suspensions of niaD transformants and wild-type in saline-Tween were made and conidia concentration was estimated using a haemocytometer. Spore suspensions were plated in molten MM, supplements plus 100mM chlorate, 10mM arginine and 800 µg/ml deoxycholate; at dilutions of 10^{-6}-10^{-8} spores per plate. Colonies resistant to chlorate were replica plated onto MM plus supplements but no arginine to determine if there were any arginine auxotrophs generated by this selection. The relative

rates of mutation were compared with the wild-type strain to see if there was any mitotic instability present in the transformant strains.

II.6 Molecular Genetic Techniques

II.6.1 Isolation of A. nidulans chromosomal DNA: (Baum and Giles 1985). Strains/transformants were grown up overnight at 37°C, 200 rpm in MM plus supplements for 18 h. Mycelia was harvested through 2 layers of muslin in a Buchner funnel and then blotted dry before being weighed. Typically, 2-4 g of mycelia were obtained from a culture inoculated with a fresh plateful of conidia. About 2 g of mycelium was ground with a mortar and pestle in liquid nitrogen to a very fine powder before being resuspended in 10 ml of buffer A (1M sorbitol, 7% w/v ficoll, 20% v/v glycerol, 5mM MgAc, 5mM EGTA, 3mM DTT, 3mM $CaCl_2$ and 50mM Tris.HCl pH 7.5; the DTT being filter sterilized as a stock solution and added after autoclaving the other components). Then 20 ml of buffer B (10% v/v glycerol, 5mM MgAc, 5mM EGTA and 25mM Tris. HCl pH 7.5) was slowly added, mixing gently all the time. This suspension was layered onto 8.1 ml of a mixture of buffer A:B at 1:1.7, in 30 ml sterile Sorvall tubes. The samples were centrifuged in a HB4 (swing-out) rotor at 4K rpm, 7 min, 4°C and the supernatant gently decanted into another sterile Sorvall tube, to which 3 ml of step gradient buffer (1M sucrose, 10% v/v glycerol, 5mM MgAc, 1mM DTT and 25mM Tris.HCl pH 7.5)

was added by displacement. The contents were centrifuged using the HB4 rotor (in the Sorvall) at 7K rpm, 4°C, 15 min to give a crude nuclear pellet (appears as a fairy ring at the bottom of the tube). The supernatant was carefully discarded and the pellet quickly resuspended in 1 ml storage buffer (25% v/v glycerol, 5mM MgAc, 3mM DTT, 0.1mM EDTA and 25mM Tris.HCl pH 7.5) and then aliquoted into 250 µl volumes in eppendorf tubes for storage at -70°C. To release the DNA from the nuclei, 100 µl of a 2% w/v sarkosyl, 100mM EDTA pH 8.0 solution was added to a 250 µl aliquot of nuclei/storage buffer. This lysed the nuclei, releasing the DNA. The volume was made up to 500 µl with TE before extracting the DNA with phenol (at least 3 times) and chloroform. The genomic DNA was ethanol precipitated overnight at -70°C and for 2 g starting material, approximately 200 µg of genomic DNA was obtained. The purity and concentration of DNA was checked by spectrophotometric measurements and gel electrophoresis as before (sections II.3.4 and II.4.2). Although yields of DNA are less than 10% of other methods (e.g. Tilburn et al. 1983), the quality and size of undigested DNA was significantly better.

II.6.2 Southern transfer: (Maniatis et al. 1982). Genomic DNA which had been digested and electrophoresed as described previously (sections II.4.1 and II.4.2). The gel was transferred to a 0.25M HCl depurinating solution, sufficient to cover the gel, and gently shaken on a Rotatest shaker. Two such, 15 min washes in the

depurinating solution were performed, after which the gel was rinsed in dH_2O. The gel was then denatured in 0.5M NaOH, 1.5M NaCl by two, 15 min washes as before, and rinsed in dH_2O before being neutralized by two, 30 min washes in 0.5M Tris.HCl pH 7.5, 3M NaCl. The gel was then placed upside down on a Whatman 3MM paper wick soaked in 20 x SSC (consists of 3M NaCl, 0.3M sodium citrate pH 7.0). Then an exact size of nitrocellulose (Hybond C from Amersham) filter was cut out (wearing gloves to avoid handling the nitrocellulose) and prewet in dH_2O, then in 2 x SSC before placing onto the gel. Two to four Whatman 3MM sheets were placed over the nitrocellulose filter, on top of which a stack of paper towels was placed. The stack of paper towels was weighed down by a heavy weight placed on top of a glass plate. The tray containing the 20 x SSC in which the gel and filters was set up was sealed with cling-film to reduce evaporation of the SSC solution directly to the air (but allow passage through the gel, filter and stack of paper towels). Transfer was carried out overnight, after which the gel (now dried) was checked for EtBr stain to ensure complete transfer had occurred. Normally a corner of the nitrocellulose filter was cut to determine the orientation of the filter. Again handling with gloves, the filter was placed in between two Whatman 3MM sheets and baked in vacuo for 2 h at 80°C.

II.6.3 Dot blotting: (Mason and Williams 1985). An exact size of Whatman 3MM paper was cut out to match the

size of the BRL Hybri-dot manifold. The paper was punctured to allow the screws to pass through, and aligned in the manifold after prewetting in 2 x SSC (fitted in between the top two layers). A nitrocellulose filter (Hybond C), prewet in 2 x SSC, was placed onto the 3MM paper and a corner cut for orientation. The top layer of the manifold was placed over the filter and the screws tightened by hand. Any unused wells were covered with parafilm before adjusting the suction to about 500 µl of TE through the filter in 5 min (water pump at low speed). After setting up the manifold, 25 µl of DNA (to be loaded) was boiled for 10 min then ice-cooled, the DNA was further denatured by the addition of 25 µl of 0.5M NaOH, 1.5M NaCl and incubating at room temperature for 20 min. The samples were transferred to ice and 200 µl of neutralization buffer (0.5M Tris.HCl pH 7.5, 3M NaCl) was added. The samples were mixed and pipetted into the manifold with the water pump running. After loading, the apparatus was dismantled and the filter air-dried for 1 h at room temperature before baking the filter at 80°C, in vacuo for 2 h. The advantage of using a manifold is that it is able to concentrate the DNA sample to a small area and thus give a sharper signal on hybridization.

II.6.4 Prehybridization of filters: (Maniatis et al. 1982). Filters were placed in a polythene bag (open on 3 sides) after being prewet in dH_2O and then 2 x SSC (both sides). The bag was double sealed (with a bag

sealer) on 2 sides before adding the prehybridization solution, made up as follows:

Deionized formamide	: 2.5 ml
20 x SSC	: 1.5 ml
50 x Denhardts	: 0.5 ml
1M Phosphate buffer pH 6.5	: 250 µl
5 mg/ml sheared Herring sperm DNA *	: 250 µl

(* The DNA was boiled for 10 min and then ice-cooled before addition to the rest of the mix). Air bubbles were removed as far as possible and the bag double sealed, leaving a 10 mm gap above the filter. The bag and contents were incubated at 42°C for a minimum of 6 h in a sealed container placed in a shaking water bath at gentle speed. Deionized formamide was prepared by adding 10-20 % w/v mixed bed resin (AG 501-X8(D), 20-50 mesh from Bio-Rad) and stirring for 1 h at room temperature. The mixture was filtered through Whatman number 1 filter paper in a Buchnar funnel and aliquoted into 20 ml volumes before storing in the dark at -20°C. The 50 x Denhardts solution consists of 1 g BSA fraction V, 1 g ficoll and 1 g polyvinylpyrrolidone made up to 100 ml in dH_2O. Herring sperm DNA may be sheared by passage through a needle (23 guage) several times.

II.6.5 Hybridization: The prehybridization mix was removed as far as possible before adding the hybridization solution as follows:

Deionized formamide	: 2.5 ml
20 x SSC	: 1.5 ml
50 x Denhardts	: 100 µl
1M Phosphate buffer pH 6.5	: 250 µl
5 mg/ml Herring sperm DNA *	: 100 µl
Labelled probe *	: 300 µl
dH_2O *	: 250 µl

(* The DNAs and dH_2O were boiled for 10 min then ice-cooled and the components of the hybridization solution were mixed before addition to the filter (in the bag).) The bag was double sealed and placed in another polythene bag (which was then sealed) and hybridization was carried out at 42°C for at least 12 h with gentle shaking in a water bath as before.

II.6.6 Washing filter conditions: The polythene bag containing the hybridization mix was opened and the solution carefully decanted. The filters were washed initially in 2 x SSC at room temperature to remove excess label. The filters were then washed twice in 2 x SSC, 0.1% SDS at 68°C for 30 min before more stringent washes (twice) in 0.1 x SSC, 0.1% SDS at 68°C for 30 min were performed. The filters were rinsed once in 0.1 x SSC to remove the SDS, air-dried and placed in a polythene bag. At this stage, filters may be monitored using a hand held Geiger counter.

II.6.7 Autoradiography: Autoradiograph (X-ray) film from Fuji (RX film) was placed over the filters (under a safe light) in a Kodak film cassette between two intensifying screens. The cassette was stored at -70°C before developing. Films were developed, after thawing, using a Fuji X-ray film processor.

II.7 Enzyme Assays

II.7.1 β-galactosidase: (Van Gorcom et al. 1985). Mycelia were grown in MM plus different nitrogen regimes

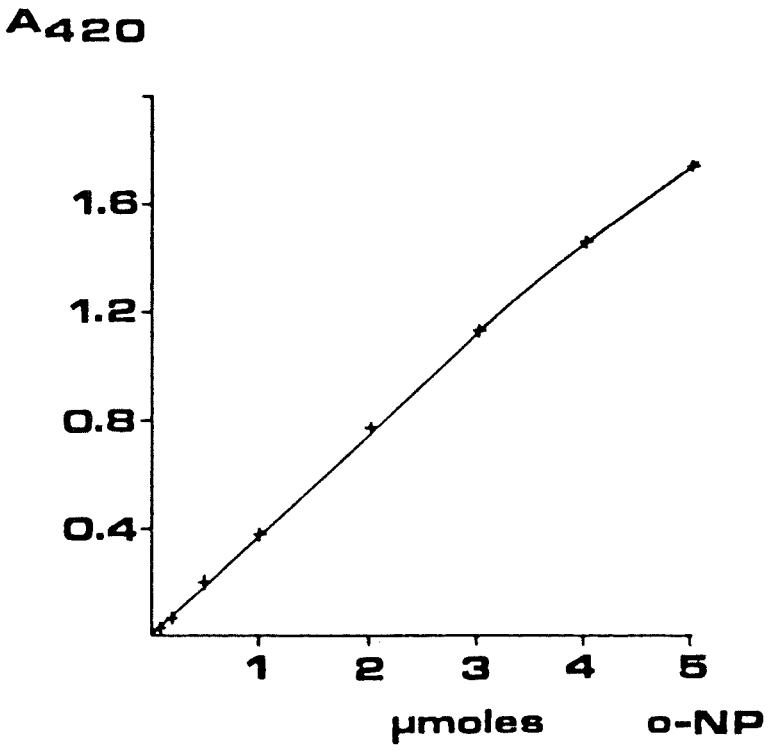


Fig. 12. Standard curve of the amount of o-NP (in reaction buffer) against the absorbance at 420 nm. All values were determined in triplicate. Preliminary experiments were performed in order to determine conditions in which the reaction rate of the assay was linear with respect to time of incubation and to volume of extract used. This allows the standard curve to be used to estimate the amount of o-NP produced.

for 18 h at 37°C, 200 rpm in 1 l unbaffled flasks. The mycelia was harvested through two layers of muslin, blotted dry and placed on ice (wrapped in tin foil). The mycelium was ground with a mortar and pestle in liquid nitrogen; and the fine powder was resuspended in five volumes of 50mM sodium pyrophosphate buffer pH 7.0, 20µM PMSF (a serine protease inhibitor). The suspension was placed on ice for 15 min before centrifuging at 15K rpm, 4°C in the Sorvall SA600 rotor. The supernatant was carefully decanted into a fresh tube and stored in ice. The extract was assayed in a final volume of 500 µl as follows:

10 mg/ml ONPG	: 0.1 ml
Extract/50mM Phosphate buffer pH 7.0	: 0.4 ml

This reaction mix was incubated at 37°C for a fixed time period (typically 10 min) before stopping the reaction with the addition of 500 µl of 1M Na_2CO_3. Zero time controls (blanks) were prepared by adding the Na_2CO_3 to the ONPG before the enzyme extract. The absorbance of any yellow colour produced, was measured at 420 nm against the blank for that particular reaction. All assays were performed in duplicate and the µmoles o-NP produced was estimated from a standard curve of the amount of o-NP against absorbance at 420 nm (fig. 12).

To determine the β-galactosidase activity of A. nidulans strains/transformants in vivo, the procedure of Van Gorcom et al. (1985) was followed. MM with various nitrogen sources and appropriate supplements was

buffered to pH 7.0 with sodium phosphate buffer before adding X-gal to a final concentration of 40 µg/ml. The X-gal was made up as a stock solution of 4 mg/ml in dimethylformanide. Plates were inoculated with stabs of various strains/transformants and then incubated for 2 days at 37°C. After this time, the β-galactosidase activity was detected by a blue colouration. The glucose present in MM is sufficient to prevent any endogenous β-galactosidase activity (Fantes and Roberts 1973).

II.7.2 Nitrate reductase: (Modified from Cove (1966)). Mycelia were prepared from switch experiments, where initially a 1 l unbaffled flask containing 200 ml MM plus 10mM glutamate had been incubated with 10^8-10^9 conidia and grown at 37°C, 200 rpm for 16 h. The mycelia were harvested aseptically by filtering the medium through two layers of muslin and were resuspended in a fresh 1 l flask containing 200 ml of MM plus 10mM of various nitrogen sources. This second culture was incubated for a further 4 h at 37°C, 200 rpm before being harvested. Mycelia were routinely frozen in liquid nitrogen and stored at -20°C for up to two weeks before the assay for nitrate reductase was performed.

For the assay, 1-2 g of tissue was ground in liquid nitrogen to a fine powder and 5 ml of 100mM sodium phosphate buffer pH 7.2 was added. The suspension was mixed and kept on ice before pelleting the debris at 15K rpm, 4°C, 15 min using the Sorvall SA600 rotor. The supernatant was decanted and kept on ice. The assay mix

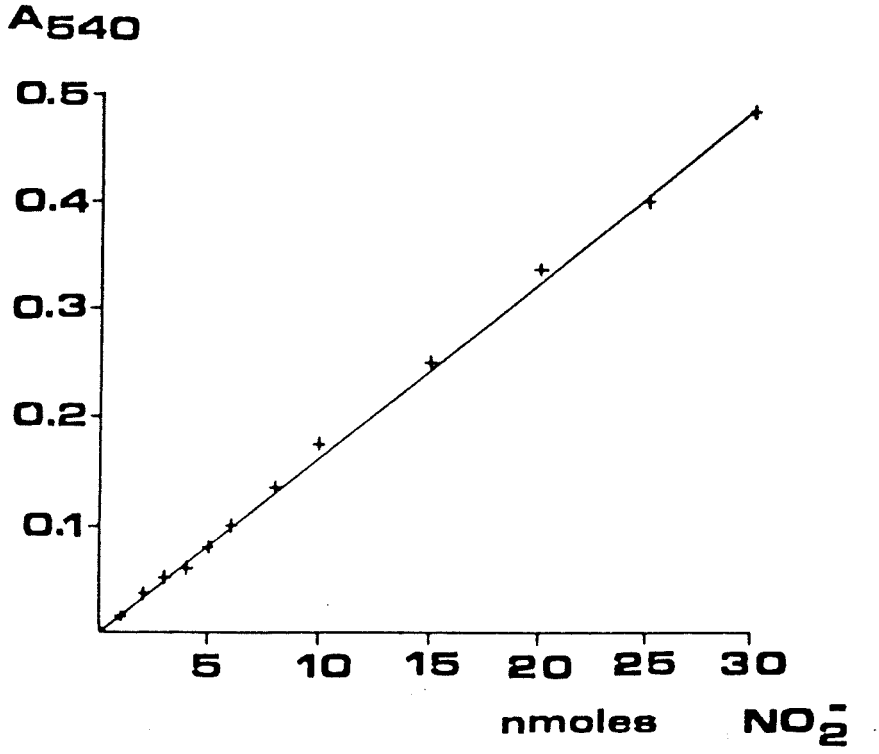


Fig. 13. Standard curve of the amount of nitrite (in reaction buffer) against the absorbance at 540 nm. All values were determined in triplicate. Preliminary experiments were performed in order to determine conditions in which the reaction rate of the assay was linear with respect to time of incubation and to volume of extract used. This allows the standard curve to be used to estimate the amount of nitrite produced.

for nitrate reductase was made up as follows:

100µM FAD	: 0.1 ml
2mM NADPH	: 0.1 ml
100mM $NaNO_3$	: 0.1 ml
50mM $NaSO_2$	: 0.1 ml
Extract/100mM Phosphate buffer pH 7.75	: 0.6 ml

The sodium sulphite was added to inhibit any nitrite reductase activity. The reaction was mixed and incubated at 25°C for a fixed time period (usually 10 min) before adding 1.0 ml of a 1% w/v sulphanilamide in 3N HCl solution, to stop the reaction; and then adding 1.0 ml of a 0.02% w/v NED aqueous solution to develop the (pink) colour. Blanks were prepared for each assay, as above, except the sulphanilamide was added before the enzyme extract (Lewis and Fincham 1970). After 10 min, the resultant colour was measured spectrophotometrically at 540 nm against the blank control. Again, all the assays were performed in duplicate and the nmoles of nitrite produced was estimated from a standard curve of the amount of nitrite against absorbance at 540 nm (fig. 13).

II.7.3 Acetamidase: (Hynes 1972). The growth conditions used were the same as that for the nitrate reductase assay (except acetamide at 10mM was used for induction) and the mycelium was harvested and frozen as for nitrate reductase determinations (section II.7.2). For the assay two reagents were prepared as follows: Reagent I was a 20% w/v of phenol dissolved in ethanol. Reagent II was prepared by dissolving 25 g calcium

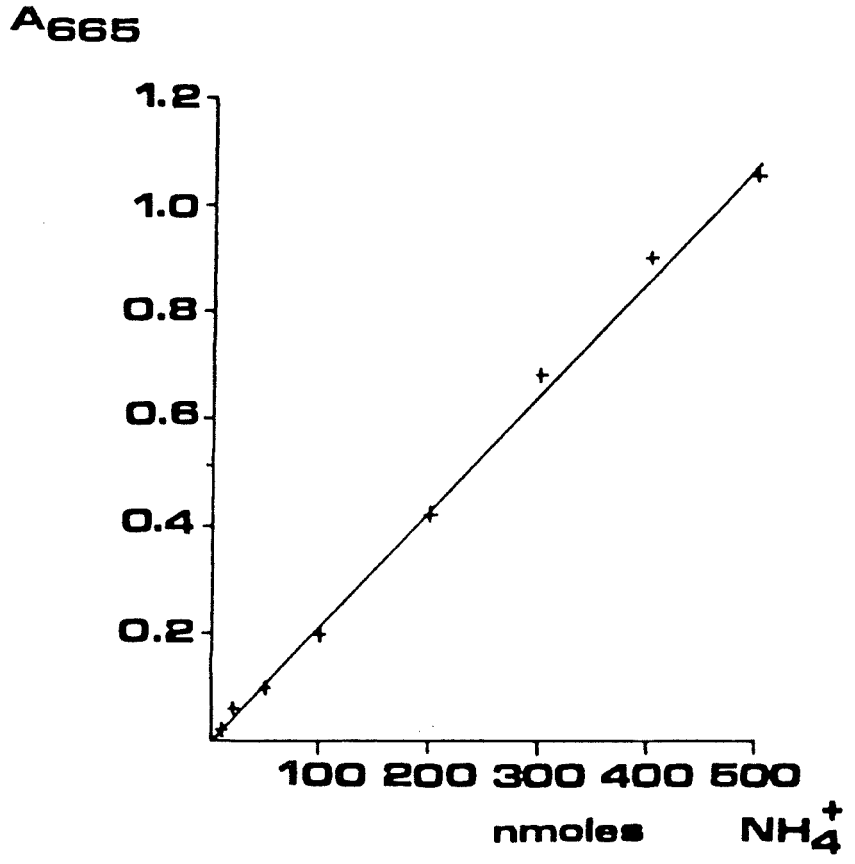


Fig. 14. Standard curve of the amount of ammonium (in reaction buffer) against the absorbance at 655 nm. All values were determined in triplicate. Preliminary experiments were performed in order to determine conditions in which the reaction rate of the assay was linear with respect to time of incubation and to volume of extract used. This allows the standard curve to be used to estimate the amount of ammonium produced.

hypochlorite in 300 ml hot distilled water and adding 135 ml of a 20% w/v K_2CO_3 aqueous solution, whilst keeping the mixture stirred. The suspension produced was mixed throughly, heated and diluted to 500 ml before filtering through Whatman number 1 filter paper. The mixture (reagent II) was stored in the dark at 4°C.

For the assay, the mycelium was ground in liquid nitrogen with a mortar and pestle, and then resuspended in five volumes of sodium phosphate buffer at pH 7.2. The resulting suspension was then spun in the Sorvall SA600 rotor at 15K rpm, 4°C, 15 min and the supernatant carefully decanted and stored on ice. Typically, 100-200 µl of extract was added to a 0.1M acetamide solution to a final volume of 1 ml. The assay mix was then incubated at 37°C for a fixed time period (usually 10 min), after which 0.5 ml of reagent I was added to stop the reaction. Then 1.0 ml of reagent II was added and the blue colour allowed to develop for 5-10 min. Blanks were prepared for each assay by the addition of reagent I to the acetamide solution before the enzyme extract. The absorbance of the final assay mix was measured at 655 nm against that of the blank. All assays were performed in duplicate and the nmoles of ammonium produced was estimated from a standard curve of the amount of ammonium against absorbance at 655 nm (fig. 14).

II.7.4 Protease: (Cohen 1972). For the in vivo determination of protease activity of A. nidulans strains/transformants a plate assay was used. MM with

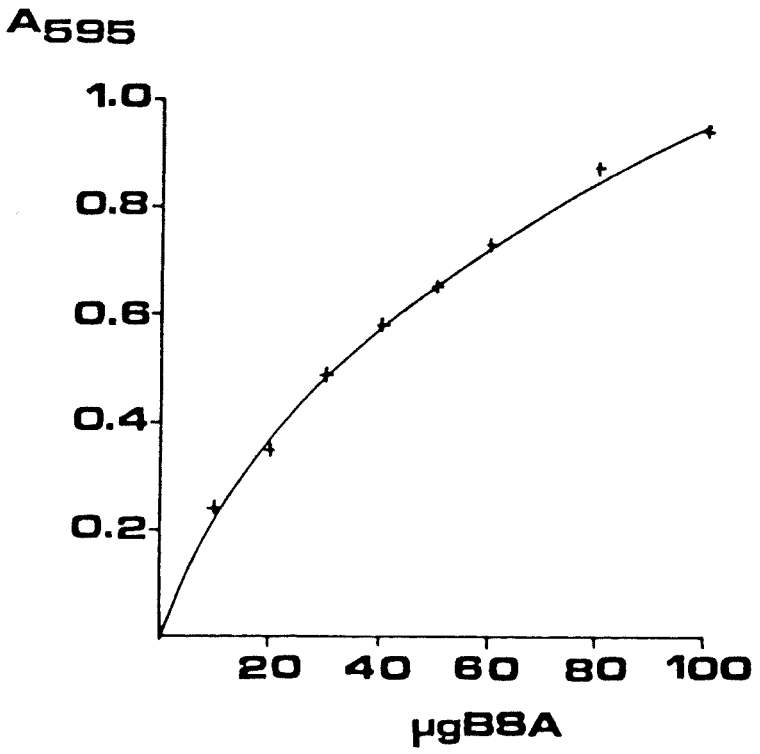


Fig. 15. Standard curve of the amount of protein (in extraction buffer) against absorbance at 595 nm. All values were determined in triplicate. The amount of protein in the enzyme extract can be estimated from the standard curve and thus specific enzyme activities of the enzyme extracts can be calculated.

appropriate supplements but minus a nitrogen source was used. To this molten medium, a 10% w/v Marvel (Cadbury) solution (autoclaved at 120°C for 10 min) was added to a final concentration of 0.3% w/v. Plates were inoculated via stabs and then incubated at 37°C for 2 days. Protease production was demonstrated by the formation of a halo of milk clearing around the fungus, the addition of ammonium completely repressing this protease activity (performed as a control).

II.7.5 Protein determinations: (Bradford 1976). To normalize all quantitative enzyme activity data, the protein concentration of the enzyme extracts was determined. For this a protein-binding dye was used and prepared as follows: 100 mg of Coomassie Brilliant blue G was dissolved in 55 ml of 95% ethanol and stirred for 30 min or until the dye had completely dissolved. Then 100 ml of orthophosphoric acid was added and the solution was diluted to 1 l with sodium phosphate buffer pH 7.5. This suspension was filtered through Whatman number 1 filter paper and stored in the dark at room temperature for up to two weeks.

To determine protein concentrations, 10 µl from the enzyme extract was taken and made up to 100 µl with dH_2O. To this, 5 ml of the Bradford's reagent (above) was added, the contents of the tube mixed and the blue colour allowed to develop for 10 min before measuring the absorbance at 595 nm against a suitable blank (dH_2O and reagent). Protein amounts were estimated from a

standard curve of the amount of BSA against absorbance at 595 nm (fig. 15). All protein determinations of extracts were carried out in duplicate. This allowed all enzyme activities to be calculated in units of product formed/min/mg protein, i.e. as specific activities.

RESULTS AND DISCUSSION
A. CHARACTERIZATION AND TRANSFORMATION OF pNiiA AND SUBCLONES

III.1 Restriction Mapping

Restriction mapping of the clones pNiiA and λAN8a (figs. 7-8) was performed by Brian Anderson and Sarah Gurr, while I determined the restriction map of pILJ141.

Single digests: Samples of pILJ141 were digested with particular enzymes and electrophoresed, along with λ*Hin*dIII/*Eco*RI size markers (see fig. 16). The mobility (mm) of the lambda fragments were plotted against the $\log_{10}$Kb of the particular fragment size, producing a standard curve (fig. 17). The mobilities of the various fragments produced by the digests were measured and the size of the fragment estimated from the standard curve. The following sizes from the single digests were obtained (sizes given in Kb):

*Bam*HI	6.9
*Bgl*II	6.9
*Eco*RI	5.0, 1.9
*Hin*dIII	3.6, 2.6, 0.9
*Kpn*I	4.6, 2.4
*Pst*I	3.5, 2.6, 0.8
*Pvu*II	2.5, 2.3, 1.8, 0.7
*Sal*I	2.6, 2.0, 1.5, 1.1
*Sma*I	6.9
*Xba*I	2.6, 2.4, 1.6, 0.7
*Xho*I	6.3, 0.9

Double digests: Samples of pILJ141 were digested with a combination of enzymes and electrophoresed, along with size markers (fig. 18). A standard curve of the lambda fragments was produced (fig. 19) and the sizes of the fragments estimated as before. The following sizes

Fig. 16. Single restriction enzyme digests of pILJ141 electrophoresed in a 0.8% agarose gel. Digests are as follows: lane A, λ*Hin*dIII/*Eco*RI size markers; B, *Bam*HI; C, *Bgl*II; D, *Eco*RI; E, *Hin*dIII; F, *Kpn*I; G, *Pst*I; H, *Pvu*II; I, *Sal*I; J, *Sma*I; K, *Xba*I; L, *Xho*I; M, undigested pILJ141.

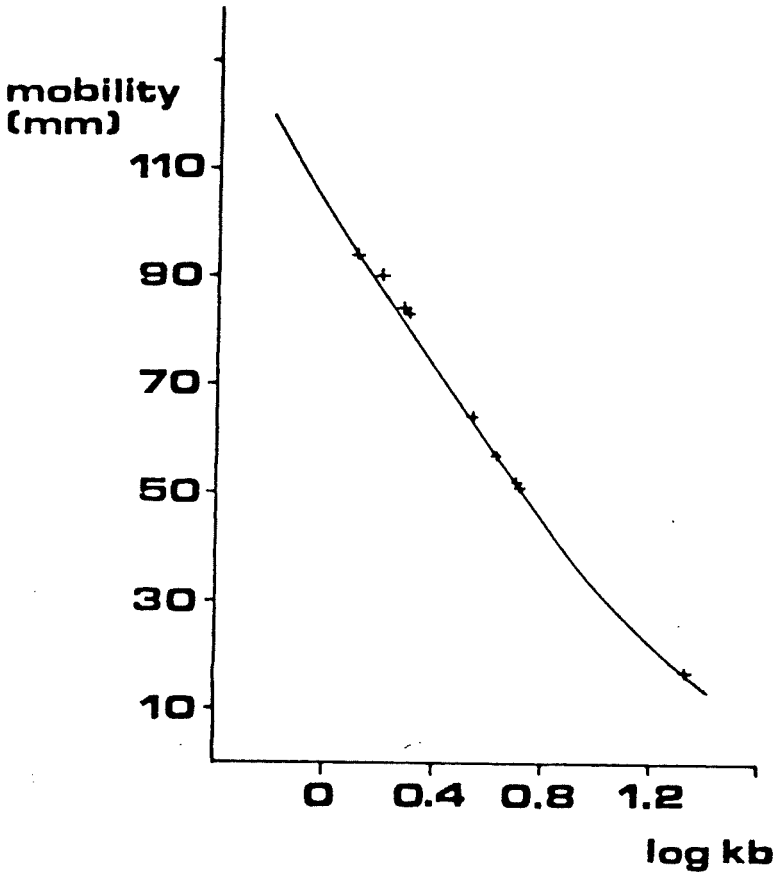


Fig. 17. Relationship between molecular weight size of lambda DNA size markers and mobility in 0.8% agarose gel (fig. 16). From this standard curve the mobility of differently sized fragments can be used to determine the size of the fragment.

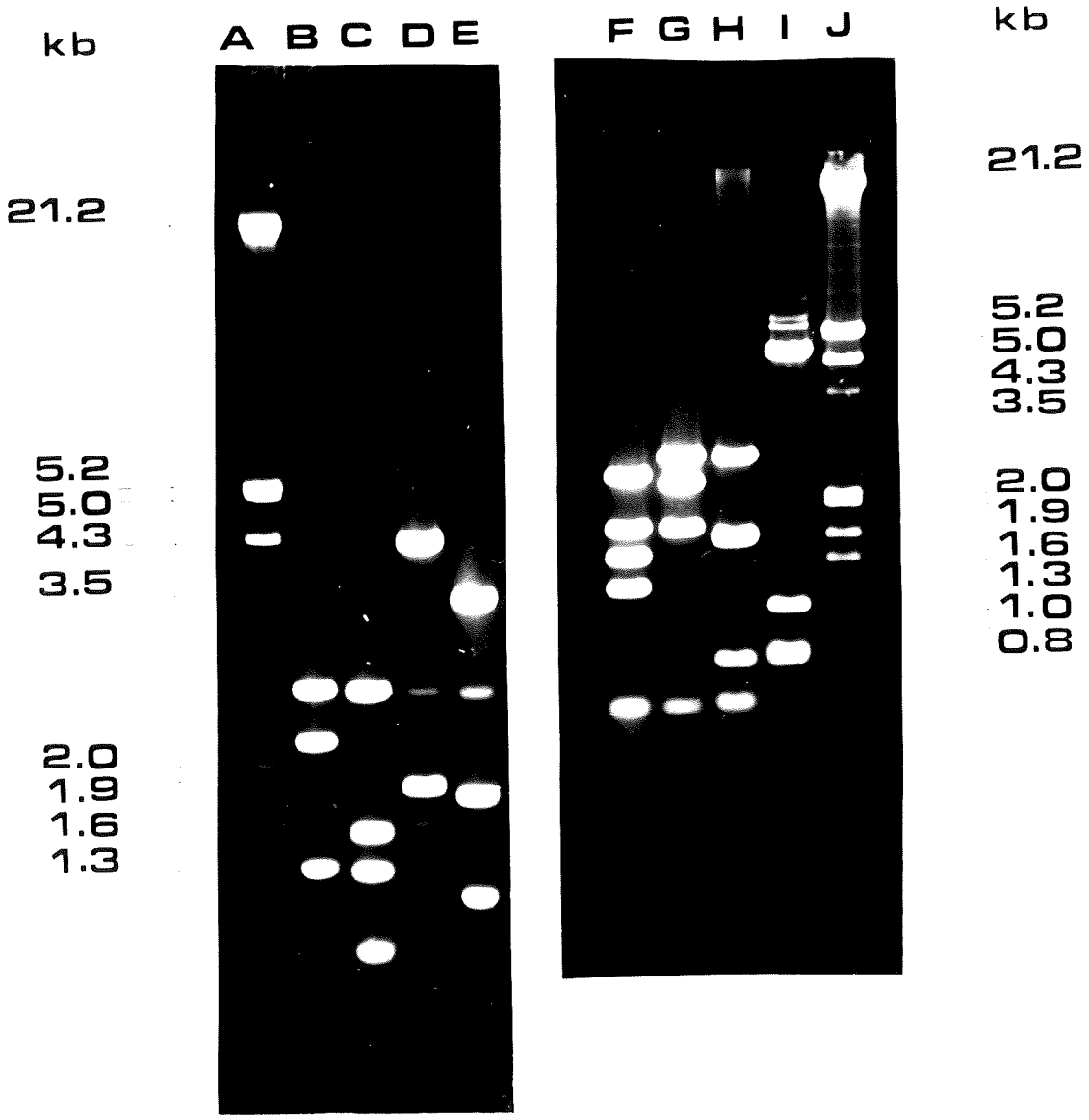


Fig. 18. Double restriction enzyme digests of pILJ141 electrophoresed in a 0.8% agarose gel. Digests are as follows: lane A, λ*Hin*dIII/*Eco*RI size markers; B, *Xho*I/*Sal*I; C, *Xba*I/*Sal*I; D, *Xho*I/*Eco*RI; E, *Kpn*I/*Eco*RI; F, *Pvu*II/*Bgl*II; G, *Pvu*II/*Sma*I; H, *Xba*I/*Xho*I; I, *Kpn*I/*Xho*I; J, λ*Hin*dIII/*Eco*RI size markers.

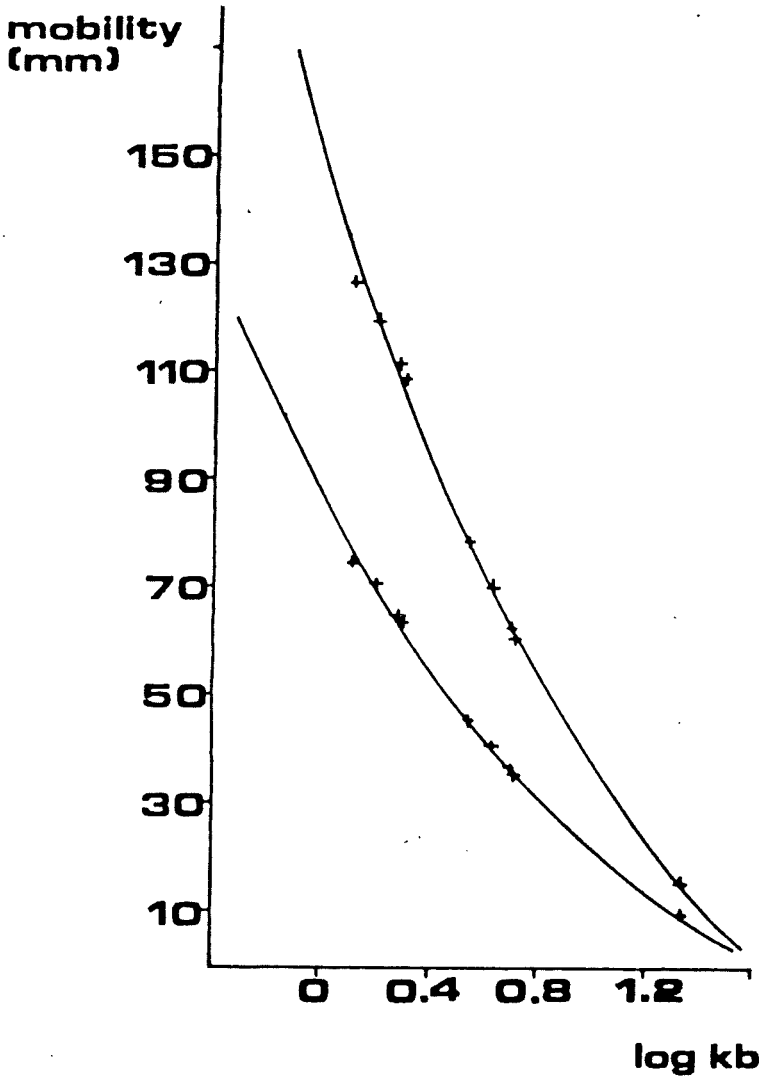


Fig. 19. Relationship between molecular weight size of lambda DNA size markers and mobility in a 0.8% agarose gel (fig. 18). Lower curve is taken from the markers on the right of fig. 18 and the upper curve is taken from the markers on the left of fig. 18. From the standard curves the size of the fragments can be estimated from the mobility of the fragment in the gel.

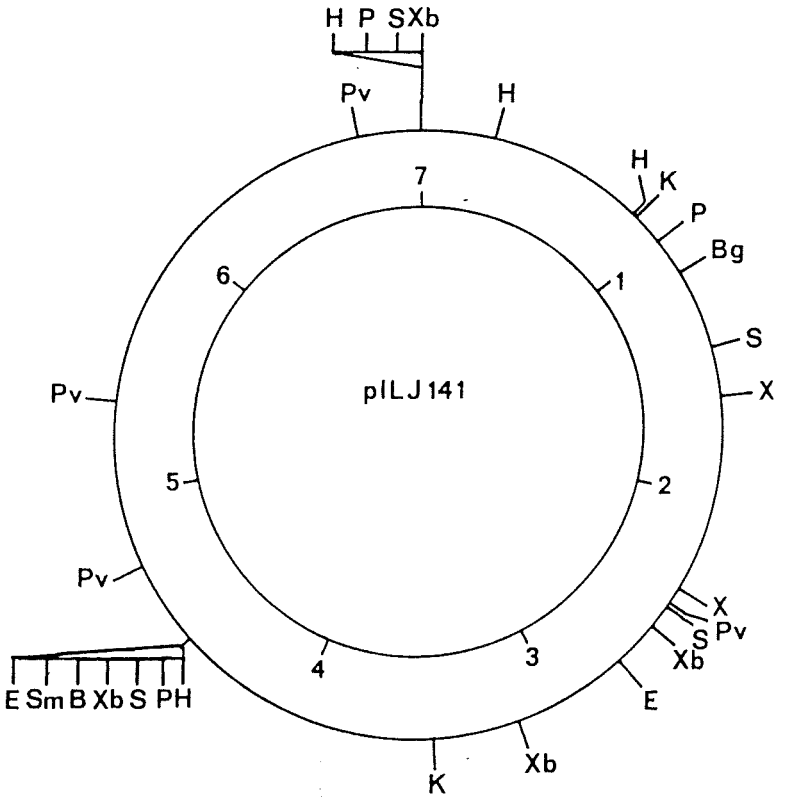


Fig. 20. Circular restriction map of pILJ141 determined from the combination of single and double digests of the plasmid. The scale is indicated by the inner circle which shows size in Kb.

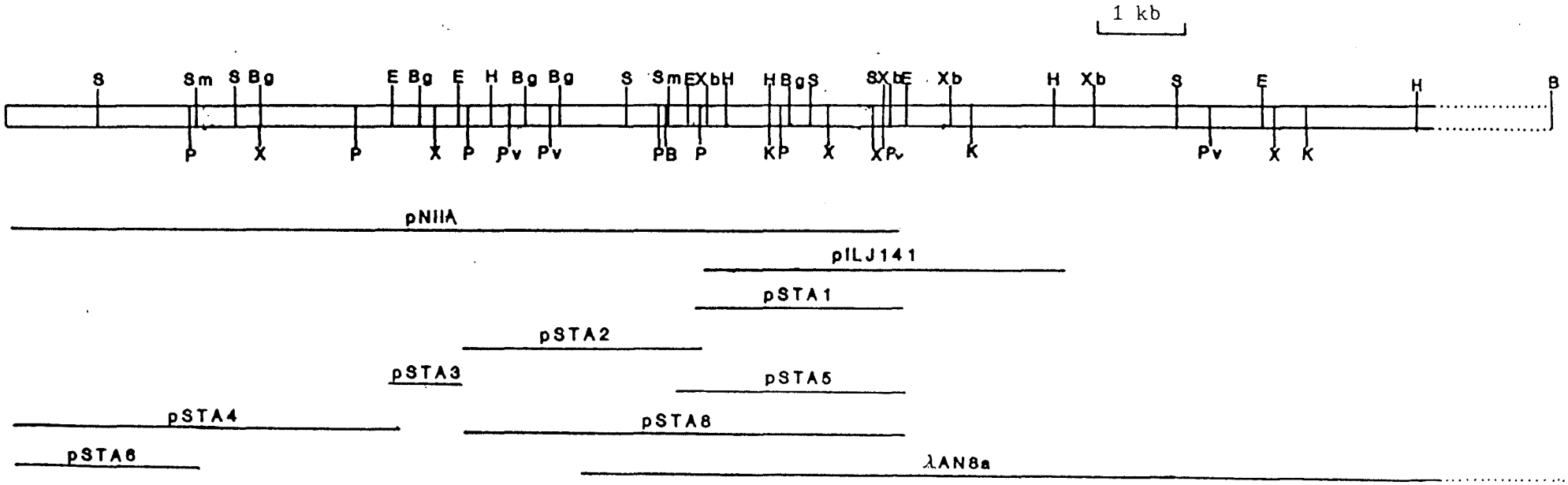
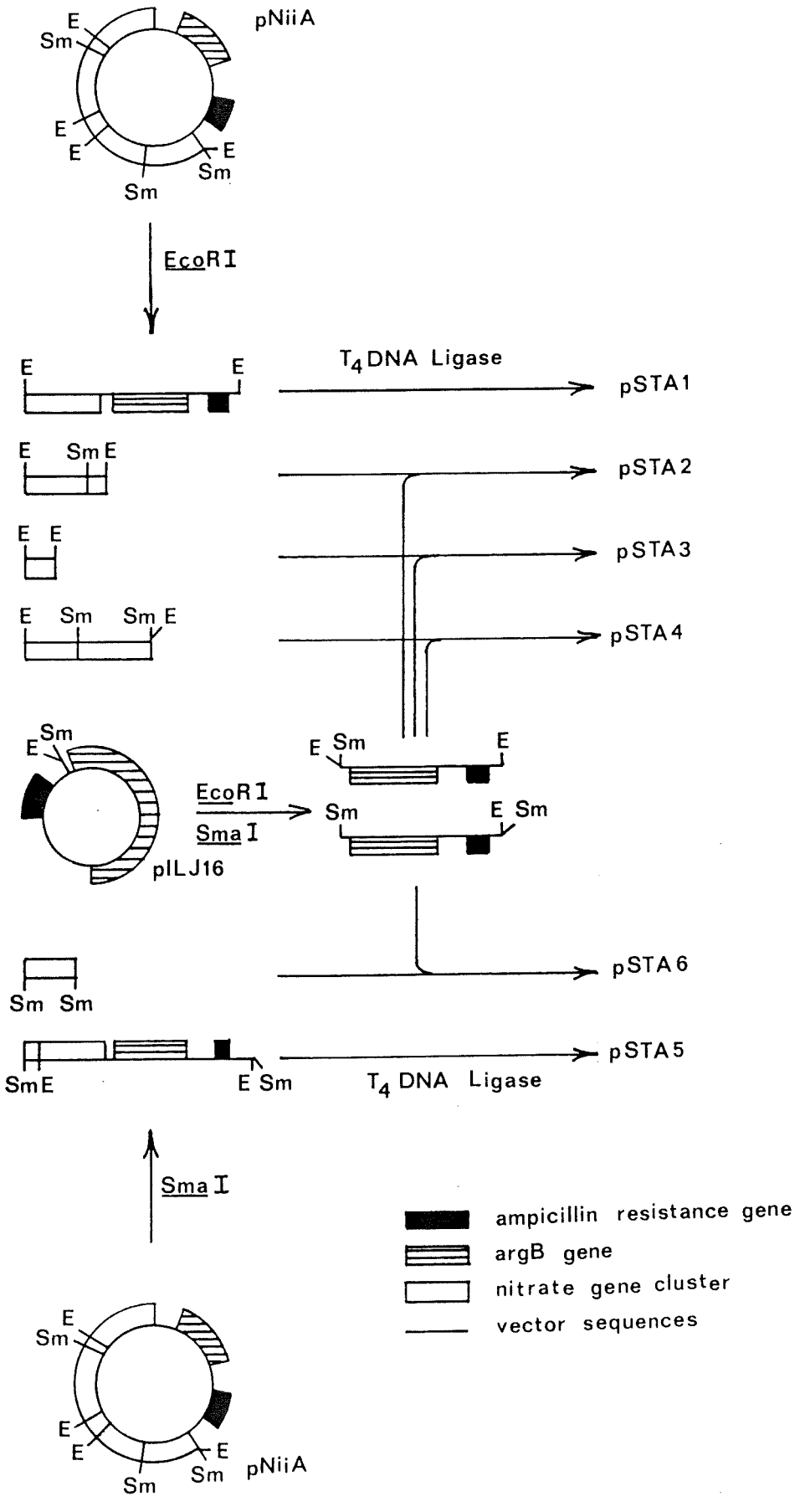


Fig. 21. Linear restriction map of the nitrate gene cluster determined from the analysis of the plasmids pNiiA, pILJ141 and the lambda clone λAN8a. Also shown are the extent of the inserts present in each of the clones/subclones below the restriction map. For details of pNiiA, pILJ141 and λAN8a see figs. 7-8; and for the pSTA subclones see fig. 22.


pNiiA
E
Sm
E
E
E
Sm
Sm
EcoRI
T4 DNA Ligase
E
E
pSTA1
E Sm E
pSTA2
E E
pSTA3
E Sm Sm E
pSTA4
Sm
E
pILJ16
EcoRI
SmaI
E Sm
E
Sm
E Sm
pSTA6
Sm Sm
pSTA5
SmE
E Sm
T4 DNA Ligase
SmaI
ampicillin resistance gene
argB gene
nitrate gene cluster
vector sequences
pNiiA

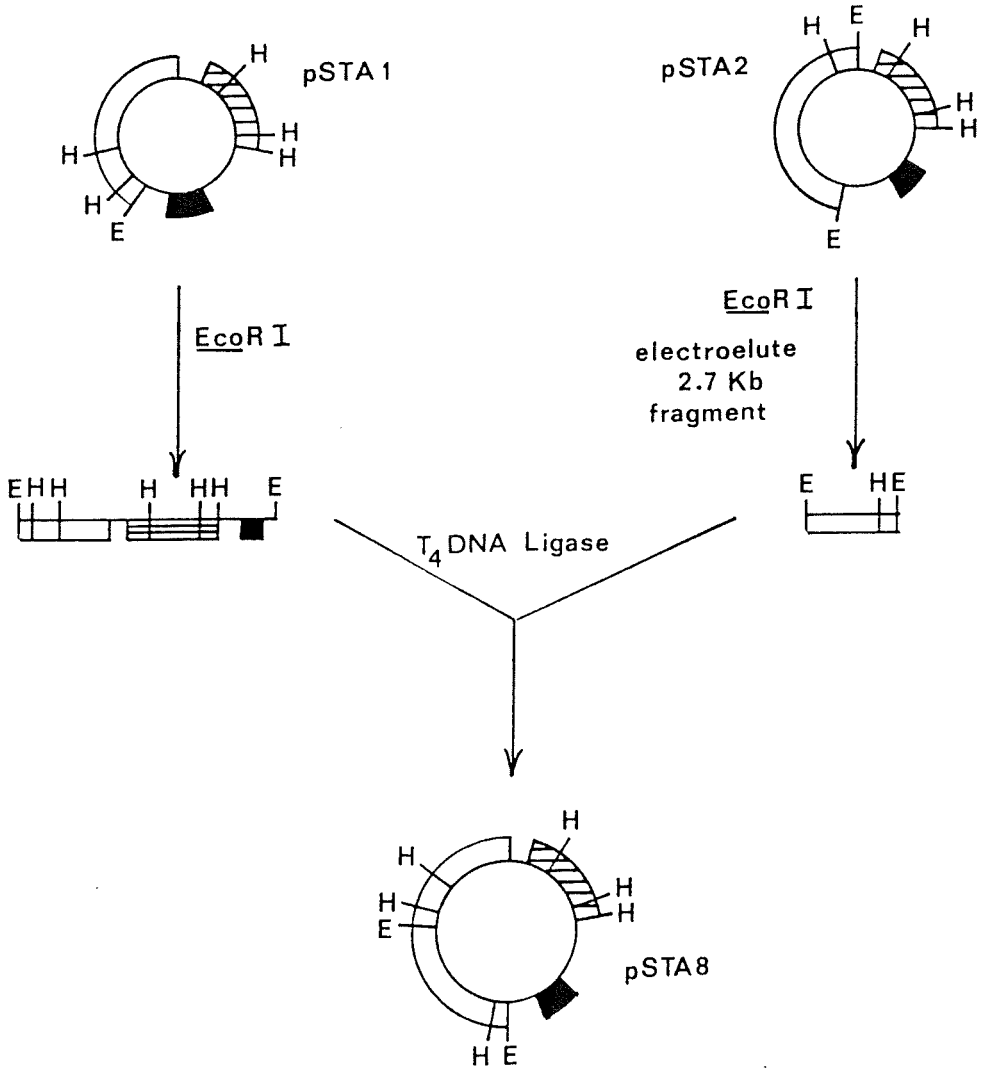


Fig. 22. Subcloning of pNiiA. Facing page, pNiiA was digested with EcoRI and the 8.3 Kb fragment was religated, while the smaller EcoRI fragments were ligated into the EcoRI site of pILJ16; thus producing subclones pSTA1-4. pNiiA was also digested with SmaI, the 8.5 Kb fragment religated and the 2.2 Kb fragment was ligated into the SmaI site of pILJ16; thus producing subclones pSTA5-6 (Brian Anderson, unpublished). Above, subclone pSTA8 was constructed by ligating the 2.7 Kb EcoRI fragment from pSTA2 into the EcoRI site of pSTA1 (keeping the same orientation as in pNiiA - determined by a HindIII digest). For details of pNiiA and pILJ16 see fig. 7.

from the double digests were obtained (sizes given in Kb):

PvuII/BglII	2.2, 1.6, 1.4, 1.1, 0.6
PvuII/SmaI	2.5, 2.1, 1.6, 0.6
XbaI/XhoI	2.5, 1.6, 0.8, 0.6, 0.5, 0.4
KpnI/XhoI	4.3, 1.0, 0.8, 0.8
XhoI/SalI	2.6, 2.2, 1.4, 0.8
XbaI/SalI	2.6, 1.7, 1.4, 1.1, 0.7
XhoI/EcoRI	4.4, 1.9, 0.8
KpnI/EcoRI	3.6, 1.9, 1.3, 0.8

From this information and the details of construction of pILJ141 (Johnstone unpublished) a circular restriction map of the pILJ141 plasmid could be determined (fig. 20). This restriction map could be compared to the restriction maps obtained from the pNiiA and λAN8a clones. The restriction map of the nitrate gene cluster is given in fig. 21.

III.2 Subcloning pNiiA

The pNiiA plasmid (fig. 7) had been mapped by restriction enzyme sites (section III.1 and fig. 21) but in order to localize complementation ability of a particular mutant to a more defined stretch of DNA it was decided to subclone the various EcoRI and SmaI fragments of pNiiA (figs. 21 and 22). Thus pNiiA was digested with EcoRI and the 4.8 Kb, 2.7 Kb and 0.8 Kb fragments were isolated from a gel and then ligated into the EcoRI site of pILJ16 (the pILJ16 plasmid can be seen in fig. 7) to give pSTA4, pSTA2 and pSTA3 respectively. The 8.3 Kb EcoRI fragment, containing all the vector sequences and 2.6 Kb of insert DNA (from the nitrate

gene cluster), was simply religated to give pSTA1. Also an overlapping subclone pSTA8, which encompasses both the inserts of pSTA1 and pSTA2, was constructed by inserting the 2.7 Kb EcoRI fragment from pSTA2 (isolated from a gel) into the EcoRI site of pSTA1. The orientation of the 2.7 Kb EcoRI fragment was then determined by a HindIII digest to ensure the insert was orientated as in pNiiA. Further overlapping subclones were constructed from some of the SmaI fragments (Brian Anderson unpublished). The 8.6 Kb SmaI fragment, containing virtually all the vector sequences (except the EcoRI site of the pUC8 polylinker) was simply religated to form pSTA5. The 2.2 Kb SmaI fragment was inserted into the SmaI site of pILJ16 to form pSTA6.

III.3 Construction of Double Mutant Strains

For the complementation analysis using the pNiiA clone and its subclones, various alleles of niiA and niaD were introduced into an argB2 background by sexual crosses. As it is useful to choose strains with different conidial colour markers, three argB2 strains were used, namely: G324 (white), G34 (yellow) and G034 (green). With this in mind, crosses between the nitrate assimilation mutant and one of the argB2 mutants were set up. Cleistothecia were analyzed for mixed spore colours and spores from hybrid cleistothecia were isolated, a master plate formed and progeny analyzed for their genotype (as section II.5.3). One or two mutants

TABLE 1

Construction of Double Mutants

Cross[1]	Mutant Selected	Genotype of Double Mutant[2]
B5 x G34	SAA1010	yA2,methH2,argB2,Δ506.
B72 x G34	SAA1012	fwA1,yA2,pabaA1,methH2,argB2, Δ509.
B72 x G34	SAA1014	yA2,argB2,Δ509.
G0125 x G34	SAA1019	biA1,methH2,argB2,niaD15.
1458 x G324	SAA1020	yA2,biA1,methH2,argB2,crnA1.[3]
1458 x G324	SAA1021	wA3,yA2,methH2,argB2,crnA1.[3]
B350 x G34	SAA1023	yA2,biA1,methH2,argB2,niaD18.
B256 x G34	SAA1024	biA1,methH2,argB2,niaD169.
B466 x G034	SAA1028	yA1,puA2,argB2,Δ507.
B367 x G34	SAA1045	yA2,methH2,argB2,niiA18.
B286 x G034	SAA1049	yA2,biA1,argB2,niaD5.
B125 x G0252	SAA1051	yA1,pabaA1,trpC801,niaD26.
833 x G034	SAA2002	yA2,biA1,argB2,niiA4.

[1] For details of parental genotypes see section II.1.3.

[2] The markers puA2, argB2 and methH2 seemed to be selected against in these crosses (reduction in viability?).

[3] The markers ivoA1, galA1 and sC12 for these strains were not determined.

[4] Set up by Adrian Butt (unpublished).

TABLE 2

Complementation of Deletions in the Nitrate Gene Cluster upon Transformation with pNiiA, Subclones and Overlapping Clones.

Plasmid[1]	Deletion[2]	No. of *argB*$^+$ transformants obtained	No. of *argB*$^+$ transformants that complement:-		
			niaD[3]	*niiA*	*crnA*[4]
pNiiA	Δ*506*	3	0	3	-
pNiiA	Δ*509*	392	2 (14)	41	-
pSTA2	Δ*509*	66	0	2	-
pSTA3	Δ*509*	70	0	0	-
pNiiA	Δ*507*	455	2 (1)	10	1
pILJ141	*niaD26*[5]	-	72	-	-
λAN8a	*niaD26*[5]	-	16	-	-

[1] For details of plasmids see figs. 7,8 and 21.

[2] The extent of the deletions can be seen in fig. 5.

[3] The numbers in brackets represent partial complementation (see text).

[4] Complementation of *crnA* can only be seen in *niaD*$^+$ transformants.

[5] These strains do not carry the *argB2* mutation, so complementation of the *niaD* deletion was selected for directly.

of the desired genotype (i.e. argB2 and a nitrate assimilation mutant) were selected and stored on plates and silicas (section II.5.2). The crnA phenotype was determined by using 10mM methylammonium in addition to nitrate; the mutant phenotype showing a hypersensitive reaction in the presence of methylammonium (Brownlee and Arst 1983). In scoring the nitrate assimilation phenotypes, supplementation of arginine (for argB2 mutants) was reduced tenfold as high levels of arginine (a good nitrogen source) obscured the nitrate assimilation phenotype. A summary of the crosses performed and the genotypes of the selected double mutants is given in table 1.

III.4 Transformation Results

Initial experiments were designed to see if the recombinant clone pNiiA was able to complement various deletions present within the nitrate gene cluster. As deletions are non-revertable strains, complementation by pNiiA would rule out the possibility that a suppressor had been cloned. From table 2 it can be seen that pNiiA was able, albeit at low frequencies, to complement the niiA deleted region in strains with the Δ506, Δ507, Δ509 deletions. On rare occasions pNiiA was able to complement the niaD deleted regions of Δ507 and Δ509 strains. Regarding the Δ507 strain, where the deletion is thought to extend at least some way into the crnA gene (Tomsett and Cove 1979), one transformant was

TABLE 3

Complementation of Point Mutations in the Nitrate Gene Cluster upon Transformation with pNiiA and the pSTA Subclones.

Plasmid[1]	Mutation[2]	No. of *argB*$^+$ transformants obtained	No. of *argB*$^+$ transformants that complement:		
			niaD [3]	*niiA* [3]	*crnA*
pNiiA	*crnA1*	100			51
pSTA4	*crnA1*	100			43
pSTA6	*crnA1*	69			0
pNiiA	*niiA18*	216		129	
pSTA3	*niiA18*	71		0	
pSTA4	*niiA18*	350		24(25)	
pSTA6	*niiA18*	96		0	
pNiiA	*niiA4*	4000		1600	
pSTA1	*niiA4*	23		0	
pSTA2	*niiA4*	17		0	
pSTA3	*niiA4*	106		0	
pSTA4	*niiA4*	61		0	
pSTA8	*niiA4*	190		0	
pSTA2	*niaD5*	1000	0		
pSTA8	*niaD5*	80	15(7)		
pSTA1	*niaD17*	176	0(33)		
pSTA2	*niaD17*	160	0		
pSTA3	*niaD17*	23	0		
pSTA4	*niaD17*	14	0		
pSTA5	*niaD17*	1000	37(4)		
pSTA8	*niaD17*	69	4(12)		
pSTA8	*niaD18*	69	3(4)		
pSTA8	*niaD169*	300	11(15)		
pSTA8	*niaD15*	69	3(8)		

[1] For details of the plasmids see fig. 21.

[2] Relative position of point mutations can be seen in fig. 5.

[3] Numbers in brackets represent partial complementation (see text).

obtained which complemented the entire deletion. From these experiments it is clear that the recombinant plasmid pNiiA contained at least some of the DNA encoding the three structural genes (*crnA*, *niiA*, *niaD*) of the nitrate gene cluster. Also, because *niiA* from the deletion map (fig. 5) is located between the other genes *crnA* and *niaD*, it may be assumed (in the absence of DNA rearrangements in the isolated clone) that the complete *niiA* gene is present on the recombinant pNiiA clone. Southern blotting of wild-type genomic DNA and probing with the recombinant plasmid (Sarah Gurr, unpublished) indicated that no DNA rearrangements in the isolated clone had occurred.

A further series of experiments was designed to determine the approximate positions of the three genes with respect to the restriction map and whether the complete *crnA* and *niaD* genes were present on the recombinant plasmid pNiiA. Various subclones were used to transform several alleles within the nitrate gene cluster, screening initially on the basis of arginine complementation. Transformants, thus obtained, were then screened by replica plating for complementation of the particular allele within the nitrate gene cluster (table 3).

Using the plasmids pNiiA and pSTA4 (fig. 21), complementation of the *crnA1* mutation is able to occur at a high frequency - some 50% of the *argB*$^{+}$ transformants (fig. 23). This frequency may represent

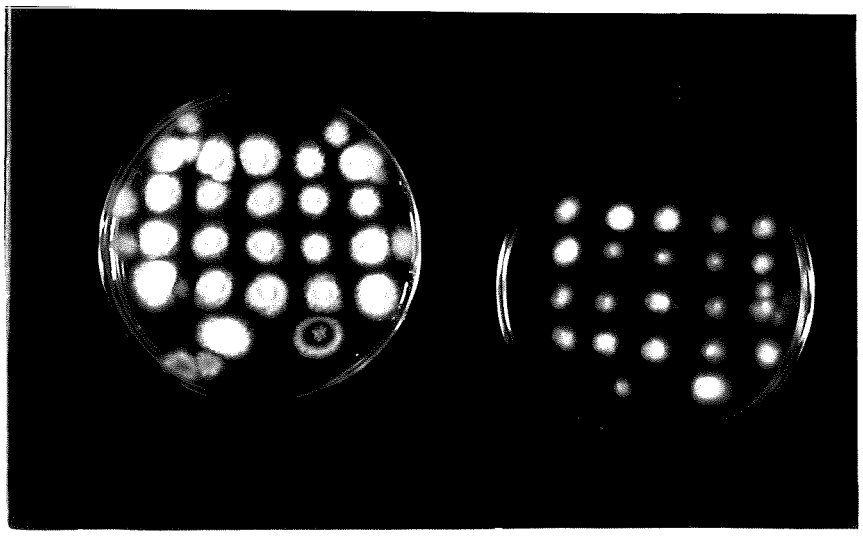


Fig. 23. Phenotype of pSTA4 *argB*$^+$ transformants screened for *crnA1* complementation. On the left, the transformants on MM + 10mM nitrate. On the right, the transformants on MM + 10mM methylammonium and nitrate. Controls (bottom two colonies) include T56 (a SAA1020 (*crnA1*) strain transformed with pILJ16; on the left) and a wild-type (*biA1*; on the right). On the methylammonium plate, the stronger growing colonies are *crnA*$^+$. For details of pSTA4, pILJ16 see figs. 21 and 7.

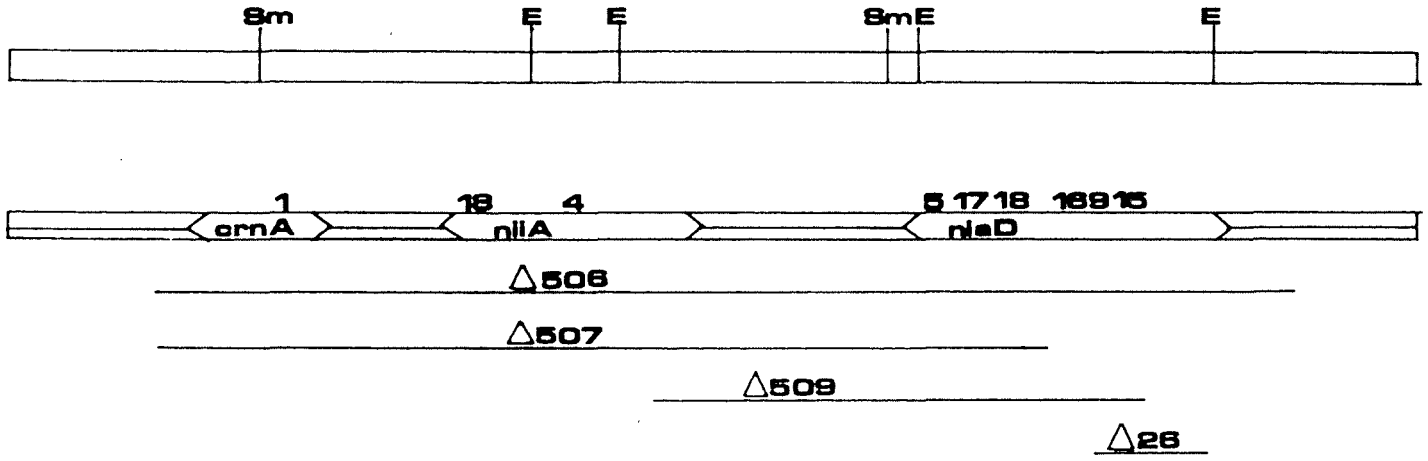


Fig. 24. Schematic representation of the approximate positions of the genes of the nitrate gene cluster with respect to the restriction map. Top, the restriction map with the EcoRI and SmaI sites indicated (marking the extent of the pSTA subclones - see fig. 21). Below, part of the genetic map of the nitrate gene cluster (fig. 5) indicating the point mutations (numbers above) and deletions (lines below) used in the transformations (see tables 2 and 3).

the relative balance between type I and II integration events compared to that of type III where just the argB2 gene is restored (see fig. 6). Alternatively, the frequency may reflect differences in transformants in which the crnA^{+} gene may be expressed or silent. The plasmid pSTA6 (fig. 21), however, was unable to complement the crnA1 mutation. These results would suggest that the whole crnA gene is present within the 4.8 Kb EcoRI fragment of the pSTA4 subclone. That the pSTA6 subclone is unable to complement would suggest that the crnA1 DNA mutation, or its wild-type DNA counterpart, is not present within the 2.2 Kb SmaI fragment of pSTA6, but is within the 2.6 Kb SmaI-EcoRI fragment of pSTA4. A schematic representation of these (and the following) results can be seen in fig. 24. Note that it is entirely possible that remaining crnA sequences may extend into the pSTA6 SmaI fragment.

When pNiiA was introduced into the argB2 strain with the niiA4 mutation, a high frequency (again about 50%) of complementation of the niiA gene was observed. However, when any of the pSTA1-4 subclones (fig. 21) were introduced into the same strain, although argB^{+} transformants were obtained, none appeared to complement the niiA4 mutation. This would suggest that none of these EcoRI fragments encode for the entire niiA gene. Transformation of the argB2 strain carrying the niiA18 allele with pSTA4 (but not pSTA6) did result in a low frequency of nitrite prototrophy; and transformation of

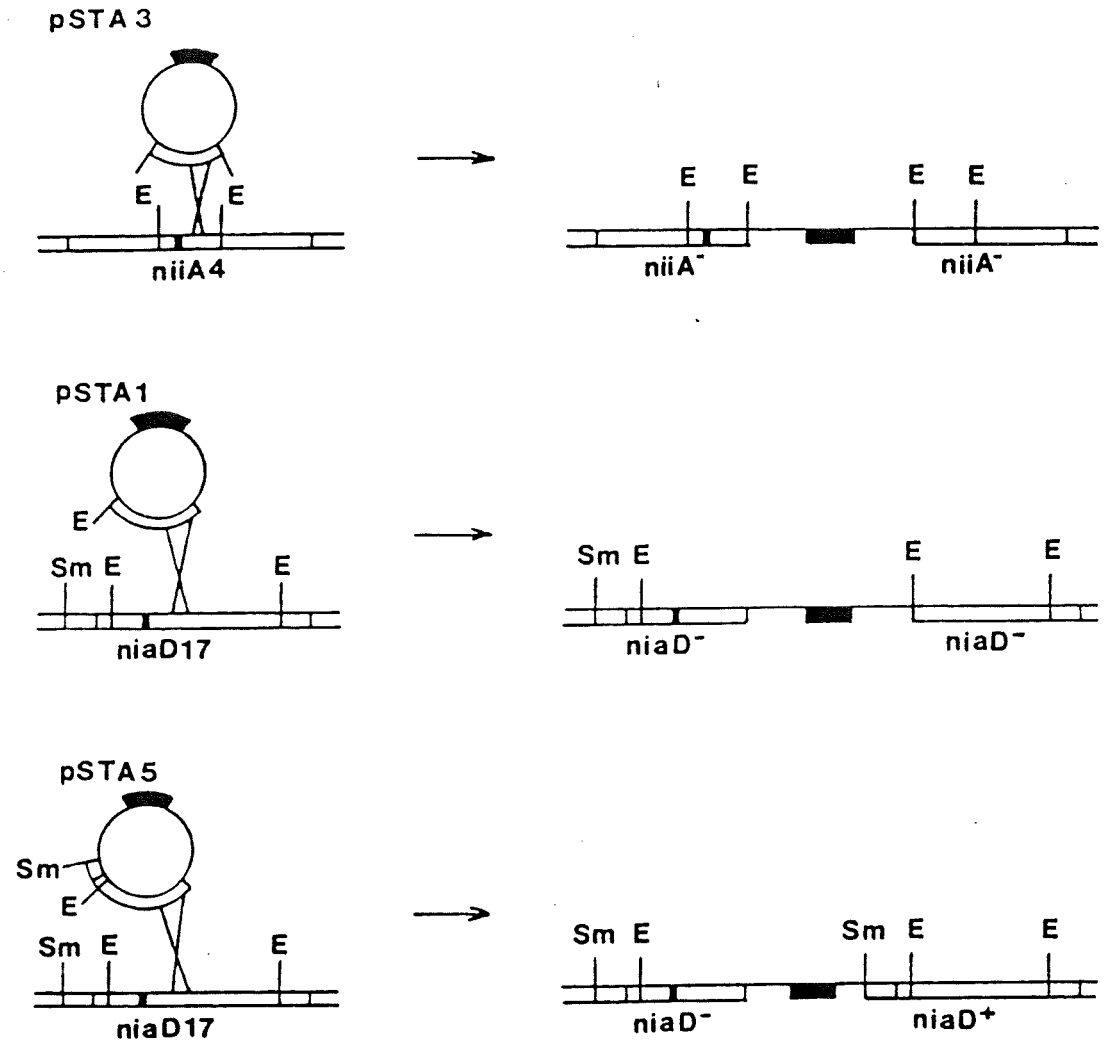


Fig. 25. How transformation of incomplete clones/ subclones may or may not complement various point mutations. Top, pSTA3 containing an internal segment of niiA (lacking both 3' and 5' ends) transformed into a niiA4 strain. Integration at the niiA gene still produces two null alleles. Middle, pSTA1 containing an internal segment of niaD (lacking both 3' and 5' ends) transformed into a niaD17 strain. Integration at the niaD gene still produces two null alleles. Bottom, pSTA5 (lacking only the 3' end) transformed into a niaD17 strain. Integration at the niaD gene (distal to the point mutation) is able to reconstitute a wild-type niaD. Note that in this last case if integration is proximal to the point mutation then complementation does not occur (this may account for the lower frequencies of complementation with incomplete genes).

the argB2 strain carrying the Δ509 deletion by pSTA2 resulted in the rare complementation of the deleted niiA sequences. This would suggest that the niiA gene extends from the pSTA4 SmaI site, through the pSTA3 and into the pSTA2 EcoRI fragments (fig. 24). One possible explanation of why no complementation of the niiA4 allele with any of the subclones occurred, could be that the corresponding wild-type DNA to this mutation lies within the pSTA3 0.8 Kb EcoRI fragment. As this fragment would appear to contain an internal section of the niiA gene, integration by a single cross-over into the niiA region would still result in two mutant alleles (fig. 25).

Transformation of argB2 strains carrying niaD mutations with pNiiA only resulted in a low level of complementation of the niaD alleles (Johnstone, unpublished). Of the subclones used to transform niaD mutant alleles, only pSTA5 and pSTA8 (fig. 21) were able to complement the niaD allele at low frequency. This might suggest that the complete niaD gene is not present on the pNiiA plasmid. The fact that pSTA5 but not pSTA1 is able to complement niaD17 suggests that pSTA1 encodes an internal segment of the niaD gene (on a similar argument to niiA4 complementation) and further might indicate that one end of the niaD gene is within the 400 bp SmaI-EcoRI fragment of pSTA2 (fig. 24). That pSTA2 is unable to complement even the most niiA-proximal niaD mutation, suggests that the corresponding wild-type DNA

is present within the pSTA1 insert. The complementation of *niaD15* by pSTA8 and the rare complementation of *Δ509* (*niiA* and *niaD*) by pNiiA might indicate that a considerable part of the *niaD* gene, if not the complete gene, is present within this original recombinant clone. Subsequent to this work, sequence data (Johnstone, unpublished) has now confirmed these relative positions of the *niiA* and *niaD* genes (but see section VI).

Later, as clones pILJ141 (Johnstone, unpublished) and λAN8a (Harford, unpublished) became available, these overlapping clones were used to transform *niaD* internal deletions (table 2). Unfortunately no other marker (e.g. *argB*) was present on these constructs and so direct selection for complementation of the deletion had to be used. Being deletion strains however, the strains were unable to revert and so any *niaD*$^+$ colonies were assumed to be genuine transformants. The plasmid pILJ141 (figs. 8 and 20-21) was able to complement the *niaD26* mutation at a low frequency despite missing the *niaD* sequences in the 600 bp *Sma*I-*Xba*I fragment of pSTA5 (fig. 21). Thus it is reasonable to assume that the complete *niaD* gene is present between the pNiiA (5' region) and pILJ141 (3' region) clones. The lambda λAN8a clone (fig. 8) which on the restriction map encompassed both these these plasmid inserts (fig. 21), not surprisingly was able to complement the *niaD10* deletion. However, the frequency of transformation was low; whether this was due to poor protoplasts/impure DNA or due to the size of

TABLE 4

Transformants used in Genetic Studies

Transformant No.	Plasmid/Strain[1]		Linkage group[2]	Phenotype[3]
T1	pNiiA	SAA1010	VIII	$niiA^+$,$niaD^-$
T2	pNiiA	SAA1010	II	$niiA^+$,$niaD^-$
T5-T9	pNiiA	SAA1012	VIII	$niiA^+$,$niaD^-$
T21-T22	pSTA2	SAA1014	VIII	$niiA^+$,$niaD^-$
T24+T26	pSTA8	AJC473.14	VIII	$niaD^+$
T28-T29	pSTA8	SAA1023	VIII	$niaD^+$
T30+T32	pSTA8	SAA1024	VIII	$niaD^+$
T33-T34	pSTA8	SAA1019	VIII	$niaD^+$
T36-T37	pNiiA	SAA1014	(VIII)	$niiA^+$,$niaD^+$
T38	pNiiA	SAA1020	VIII	$crnA^+$
T40	pSTA4	SAA1021	III	$crnA^+$
T41	pNiiA	SAA1028	?	$niiA^+$,$niaD^-$
T42	pNiiA	SAA1028	II	$niiA^+$,$niaD^-$
T44	pNiiA	SAA1028	VIII	$niiA^+$,$niaD^-$
T51	pNiiA	SAA1028	VIII	$niiA^+$,$niaD^+$,$crnA^+$
T52	pSTA8	AJC473.14	VIII	partial $niaD^+$
T53	pSTA8	SAA1023	VIII	partial $niaD^+$
T54	pSTA8	SAA1024	VIII	partial $niaD^+$

[1] For plasmids see fig. 21, and genotypes of recipient strains are given in section II.1.3 and table 1.

[2] Determination of site of integration. Details can be found in tables 5-11 and section III.5. Integration at chromosome VIII is without exception at the nitrate gene cluster; and integration on chromosome III is at the *argB* site.

[3] Phenotype that the transformant was selected as. Details of specific transformations can be found in tables 2 and 3.

TABLE 5

Linkage Analysis of SAA1010 Transformants

Progeny from the cross: Transformant ($niiA^+$,$niaD^-$,$argB^+$,$brlA^+$,$methH^-$) [1,2,3,4]
x AJC9.4 ($niiA^+$,$niaD^+$,$argB^-$,$brlA^-$,$methH^+$).

AJC9.4	++--+	+-++-	++---	+-+++	+--++	+++++	-----	+++-+	+++--	---+-
x T1	15	7	23	7	1	0	0	0	3	18
(x T1)	-	18	-	26	-	1	-	1	0	-
x T2	10	2	2	3	6	7	2	10	5	5

AJC9.4	+--+-	++-++	----+	----+	+---+	+-+-+	--+++	--++-	++++-	+-+--
x T1	2	2	22	0	0	0	0	0	0	0
(x T1)	-	-	-	-	-	4	0	0	0	1
x T2	2	1	8	2	1	1	12	15	4	0

[1] Progeny are given a +/- for niiA,niaD,argB,brlA and methH phenotypes respectively.

[2] (x T1) refers to additional $argB^+$ progeny (under-represented in this cross) scored for the respective phenotypes. For further details of T1 and T2 see table 4.

[3] Allele ratios (mutant/wild-type allele):

	niiA	niaD	argB	brlA	methH
AJC9.4 x T1:	0.66	1.33	4.88	0.69	1.13
AJC9.4 x T2:	0.79	1.44	0.64	0.49	0.59

[4] % Recombination values for $argB^+$ with:

	niaD	brlA	methH	no. progeny	%niiA
AJC9.4 x T1:	18	18	41	100	17
(AJC9.4 x T1):	4	12	63	51	0
AJC9.4 x T2:	45	27	56	98	59

TABLE 6

Linkage Analysis of SAA1012 Transformants

Progeny from the cross: Transformant ($niiA^+$,$niaD^-$,$argB^+$,$brlA^+$,$methH^-$) [1,2,3]
x AJC9.4 ($niiA^+$,$niaD^+$,$argB^-$,$brlA^-$,$methH^+$).

AJC9.4	++--+	+-++-	++---	+-+++	+--+-	++++-	--++-	+--++	---+-	+++++
x T5	7	26	3	29	7	0	0	4	1	0
x T6	8	27	8	24	4	1	0	4	4	0
x T7	12	22	3	25	4	0	1	7	3	0
x T8	8	19	4	27	5	1	1	8	7	1
x T9	17	13	5	19	4	0	1	4	11	0

AJC9.4	++-++	---++	+-+--	++-+-	--+-+	+----	----+	+-+-+	+---+
x T5	4	3	0	1	0	0	0	4	4
x T6	5	2	3	3	0	0	2	1	0
x T7	3	0	1	0	0	1	0	0	2
x T8	1	3	1	0	0	2	1	1	4
x T9	1	5	1	3	1	2	4	0	5

[1] Progeny are given a +/- for niiA,niaD,argB,brlA and methH phenotypes respectively. Details of the transformants are given in table 4.

[2] Allele ratios (mutant/wild-type alleles):

	niiA	niaD	argB	brlA	methH
mean:	0.12	3.68	0.85	0.33	0.78

[3] % Recombination values for $argB^+$ with:

	niaD	brlA	methH	no. progeny	%niiA
mean:	1	5	53	463	11

TABLE 7

Linkage Analysis of SAA1014 Transformants

Progeny from the cross: Transformant ($brlA^+$,$argB^+$,$niaD^-$,$niiA^+$) [1,2,3]
x AJC9.4 ($brlA^-$,$argB^-$,$niaD^+$,$niiA^+$).

AJC9.4	++-	--+	+-+	-+-	+--	-++	+++	---	all $niiA^+$
x T21	47	34	8	10	0	1	0	0	
x T22	42	41	8	8	0	1	0	0	

[1] Progeny are given a +/- for brlA, argB and niaD phenotypes respectively. All the progeny were wild-type for the niiA allele. Details of T21 and T22 are given in table 4.

[2] Allele ratios (mutant/wild-type allele): brlA argB niaD
mean for T21+T22 x AJC9.4: 0.91 0.84 1.16

[3] % Recombination values for $argB^+$ with: brlA niaD | no. progeny %$niiA^-$
mean for T21+T22 x AJC9.4: 18 2 | 200 0

TABLE 8

Linkage Analysis of SAA1028 Transformants

Progeny from the cross: Transformant ($brlA^+$,$argB^+$,$niaD^{-/+}$,$niiA^+$) [1,2,3,4]
x AJC9.4 ($brlA^-$,$argB^-$,$niaD^+$,$niiA^+$).

AJC9.4	---+++	++-+	----	+++-	---+	--+-	+-++	-+--
x T41	16	7	10	0	2	0	4	2
x T42	12	4	1	0	2	0	0	6
x T44	41	32	0	0	0	0	3	0
x T51	22	10	3	0	1	0	6	0

AJC9.4	++++	-+++	+---	++--	+-+-	-+-+	+--+	-++-
x T41	0	14	20	21	0	4	0	0
x T42	2	28	7	32	0	5	0	0
x T44	0	0	6	0	0	6	11	0
x T51	35	2	5	16	0	0	0	0

[1] Progeny are given a +/- for brlA, argB, niaD and niiA phenotypes respectively.

[2] Allele ratios (mutant/wild-type alleles):

AJC9.4	brlA	argB	niaD	niiA
x T41:	0.92	1.08	1.94	1.13
x T42:	1.20	0.28	1.36	0.87
x T44:	0.90	1.60	1.00	0.06
x T51:	0.39	1.13	0.54	0.32

[3] % Recombination values for $argB^+$ with:

AJC9.4	brlA	niaD	no. progeny	%$niiA^-$
x T41:	42	29	100	53
x T42:	51	39	99	46
x T44:	16	0	99	6
x T51:	3	-	100	24

[4] Details of the transformants are given in table 4.

TABLE 9

Linkage Analysis of *niaD* Transformants

Progeny from the cross: Transformant ($brlA^+$, $methH^{-/+}$, $argB^+$, $niaD^+$) [1,2,3]
x AJC9.4 ($brlA^-$, $methH^+$, $argB^-$, $niaD^+$).

AJC9.4	+-+	-+-	+++	---	++-	--+	-++	+--
x T24	-	35	48	-	5	-	13	-
x T26	-	51	40	-	3	-	6	-
x T28	13	31	32	15	0	1	7	0
x T29	19	28	26	28	1	1	3	2
x T30	11	29	42	19	1	1	12	1
x T32	19	28	31	13	0	0	3	1
x T33	15	21	34	15	4	2	4	5
x T34	20	25	24	21	4	3	1	2

[1] Progeny are given a +/- for brlA, methH and argB phenotypes respectively. All the progeny were wild-type for niaD. Details of T24 - T34 are given in table 4.

[2] Allele ratios (mutant/wild-type alleles):

	brlA	methH	argB
mean:	1.11	0.60	0.92

[3] % Recombination values for $argB^+$ with:

	brlA	methH	no. progeny	%$niaD^-$
mean:	13	68	819	0

TABLE 10

Linkage Analysis of Partial *niaD* Transformants

Progeny from the cross: Transformant ($brlA^+$, $argB^+$, $methH^-$, $niaD^{\pm}$) [1,2,3]
x AJC9.4 ($brlA^-$, $argB^-$, $methH^+$, $niaD^+$).

AJC9.4	--++	++-±	---+	+++±	+---	++++	+++-	--+-	--+±
x T52	28	-	-	32	-	7	11	6	2
x T53	28	17	19	24	2	1	1	0	0
x T54	28	21	15	22	1	0	0	1	1

AJC9.4	+-++	+-+-	-+++	-++-	-+-±	++--	-++±	----
x T52	0	10	1	1	-	-	0	-
x T53	1	3	2	2	0	0	0	0
x T54	2	2	0	0	1	1	3	2

[1] Progeny are given a +/- for brlA, argB, methH and niaD phenotypes respectively. Details of the partial transformants are given in table 4. ± is a partial phenotype.

[2] Allele ratios (mutant/wild-type alleles);

	brlA	argB	methH	niaD
mean:	0.92	0.99	0.64	1.33

[3] % Recombination values for $argB^+$ with:

	brlA	methH	no. progeny	%$niaD^-$
mean:	4	58	298	14

TABLE 11

Linkage Analysis of *crnA* Transformants

Progeny from the cross: Transformant ($brlA^+$,$methH^-$,$argB^+$,$crnA^+$) [1,2,3,4]
x AJC9.4 ($brlA^-$,$methH^+$,$argB^-$,$crnA^+$).

AJC9.4	+-++	-+-+	++++	+++-	+-+-	---+	----	-+--
x T38	6	46	10	.	.	32	.	.
(x T38)	3	-	25	22	30	-	-	-
x T40	72	28	2	0	0	3	1	4
AJC9.4	++-+	-+++	++--	-++-	--+-	+---	--++	+--+
x T38	65	3	.	.	.	.	3	33
(x T38)	-	5	-	8	4	-	1	-
x T40	6	9	25	0	1	9	17	2

[1] Progeny are given a +/- for brlA, methH, argB and crnA phenotypes respectively. For details of the transformants see table 4.

[2] For the T38 cross the crnA phenotype was not determined (difficult to determine and in the table all progeny assumed to be wild-type). On screening argB prototrophs in the progeny (seen in the (x T38) on the second line) the crnA phenotype was able to be determined.

[3]

Allele ratios:	brlA	methH	argB	crnA
AJC9.4 x T38:	0.74	0.60	8.00	-
(AJC9.4 x T38):	0.22	0.63	-	1.65
AJC9.4 x T40:	0.54	1.42	0.77	0.29

[4] % Recombination values for $argB^+$ with:

AJC9.4	brlA	methH	no. progeny	%$crnA^-$
x T38:	27	59	198	?
(x T38):	18	61	98	65
x T40:	27	11	179	22

the lambda DNA and the fact that it is linear is uncertain, although transformation of protoplasts using cosmids has been established (Yelton et al. 1985).

It can be seen that in a number of transformations using subclones (when the entire gene is not present on the plasmid), partial complementation of the particular allele results (table 2 and 3). Such transformants appear to be frequently sectoring and show a reduced growth on selective plates (intermediate between mutant and wild-type phenotypes). The basis of this phenomenon at present is unknown and further investigation is required.

III.5 Genetic Analysis of Transformants

Transformants (listed in table 4) were crossed to the AJC9.4 strain carrying the brlA42 and argB2 alleles (section II.1.3). The brlA locus is closely linked to the nitrate gene cluster (10 %) on chromosome VIII; and the argB2 allele allows the linkage of the wild-type allele from the plasmid to be followed. In some crosses the additional markers of methH2 (7 % from argB on chromosome III) and niaD (<1 % from niiA) were also present. Typically 100 progeny were analyzed for the various markers within the nitrate gene cluster and the argB, brlA and methH (where appropriate) phenotypes were determined (see tables 5-11). Allele ratios were calculated (mutant/wild-type alleles) to see if there was any selection for a particular allele and percentage

recobination was calculated for the *argB*$^+$ allele (simplest) against the various markers available in the cross. The percentage recombination being used to determine the site of integration, as tight linkage (a low value) of *argB*$^+$ with a marker indicating that integration had occurred at a closely linked site.

The pNiiA/Δ*506* transformants T1 and T2 were crossed to the AJC9.4 strain and the progeny analyzed (table 5). The linkage values obtained from the two crosses seemed to suggest different sites of integration for the pNiiA plasmid in the two transformants. The recombination frequencies calculated from the T1 progeny while possibly suggesting tighter linkage of *argB*$^+$ to the *niaD* and *brlA* genes than to *methH*, were by no means conclusive due to the small numbers of *argB*$^+$ phenotypes. However, on returning to the same hybrid cleistothecium and screening directly for more *argB*$^+$ progeny and analysis of the other markers did reveal a tight linkage to the *niaD* (4%) and *brlA* (12%) genes, while segregating freely with the *methH* gene. This would suggest that pNiiA in T1 has integrated into, or very near to, the nitrate gene cluster. At first sight this is perhaps surprising given the extensive Δ*506* deletion (fig. 5), as homology of the plasmid insert to the chromosomal DNA of this strain is considerably reduced. It might be possible that some homology exists adjacent to the *crnA* gene (the transformation results in section III.4 indicate that only part of the *niaD* gene is present on

pNiiA) into which pNiiA may have recombined. Given integration of pNiiA into the nitrate gene cluster, the T1 progeny showed a high proportion of the argB2 recombinant classes and a large number of niiA$^-$argB$^-$ phenotypes. This may indicate a considerable meiotic instability of the integrated plasmid. In the analysis of the T2 progeny no discernable linkage of the argB$^+$ allele with the other markers in the cross was noted and a higher frequency of niiA$^-$ phenotypes was present than expected for a type II integration event (fig. 6) suggesting some plasmid meiotic instability.

Analysis of the pNiiA/Δ509 transformants by the sexual cross to the brlA42 strain (table 6) is hampered by the selection against the brlA$^-$ allele and the niaD$^+$ allele from the AJC9.4 strain. That aside, it would appear that the argB$^+$ allele is linked to the niaD (1%) and brlA (5%) genes; again indicating that integration has occurred into the nitrate gene cluster. Although the Δ509 deletion is by no means as extensive as that of Δ506, it is perhaps still surprising that all the transformants T5-T9 have integration into the nitrate gene cluster. For the two pSTA2/Δ509 transformants (table 7), integration would clearly have occurred into the niiA gene (in order to produce niiA$^+$) and as expected the argB$^+$ allele is linked with brlA (18%) and niaD (2%) genes.

The pNiiA/Δ507 transformants (table 8) can be catagorized into three different classes on genetic

mapping data. Two transformants, T41 and T42 show no linkage of the *argB*$^+$ allele with *brlA* or *niaD* (*methH* was not available in this cross); and show a high frequency of *niiA*$^-$ phenotypes in the progeny, greater than that expected if integration had occurred on a chromosome away from the nitrate gene cluster suggesting meiotic instability. It is possible that integration had occurred on chromosome III (resident *argB* gene) but this cannot be determined from this data (for T42 this was resolved later). Both T44 and T51 appear to have integrated into the nitrate gene cluster as *argB*$^+$ was tightly linked to *brlA* (16%) and *niaD* (no recombinants). As the deletion Δ*507* extends from the *crnA* gene, through *niiA* and into the *niaD* gene (fig. 5), such an integration event may seem surprising. Integration of plasmid sequences by a type I integration event (fig. 6) into the *niaD* sequences available in Δ*507* should result in a wild-type *niaD*$^+$ gene. This event is probably seen in T51, although the presence of *niiA*$^-$ and *niaD*$^-$ phenotypes in the progeny may suggest some plasmid meiotic instability. In T44, integration is likely to have occurred on the other side of the Δ*507* deletion as T44 has a *niaD*$^-$ phenotype. Given that in T51 the plasmid has integrated into the remaining *niaD* gene and that T51 is wild-type for the *crnA* phenotype, this would indicate that the complete *crnA* gene is present as complementation of the deleted *crnA* gene is in *trans*.

All the *niaD* point mutation/*argB*$^+$ transformants

(table 9) look to have plasmid sequences integrated into the nitrate gene cluster since the *argB*$^+$ allele shows tight linkage to *brlA* (mean = 13%) and is unlinked to *methH*. This would be expected as transformation with the incomplete gene requires a type I integration event into the *niaD* gene in order to produce a *niaD*$^+$ phenotype (fig. 25). Interestingly, none of these transformants seem to show any meiotic instability as compared to the previous deletion transformants. In addition, a number of partial *niaD*$^+$ complementing *argB*$^+$ transformants were analyzed by crossing to the AJC9.4 strain (table 10). Recombination results of the *argB*$^+$ allele would suggest that integration has occurred into the nitrate gene cluster as linkage to *brlA* (mean = 4 %), but not *methH* is observed. Also, seen in these crosses is a high frequency of full *niaD*$^-$ phenotypes. Whether this reflects meiotic instability of the plasmid in these transformants or somehow relates to the very nature of partial complementation is unclear.

The *crnA* phenotype proved difficult to determine in the *argB*$^-$ background and analysis of the *argB*$^+$ allele in the linkage data of T38 (table 11) appeared inconclusive. Although analysis of selected *argB*$^+$ progeny indicated possible integration at a site linked to *brlA* (18%), there seemed to be a high proportion of *crnA*$^-$ phenotypes. The latter might indicate meiotic instability of the plasmid or may reflect integration at a site other than the resident *crnA* gene. In T40, which

TABLE 12

Summary of Genetic Analysis of Transformants

Class [1]	Linkage of *argB*$^+$ to:- [2] *niaD*	*brlA*	*methH*	Transformants [3]
A: Integration at the nitrate gene cluster.	1.8 ± 1.7 (9)	11.1 ± 6.3 (22)	60.6 ± 5.6 (15)	1, 5-9, 21-22, 24, 26, 28-30, 32-34, 38, 44, 51-54.
B: Integration at the *argB* gene.	-	27 (1)	11 (1)	40.
C: Integration at some other site.	37.7 ± 8.1 (3)	40.0 ± 12.1 (3)	56 (1)	2, 41-42.

[1] Transformants are divided into three different classes, according to the site of integration.

[2] Linkage of the *argB*$^+$ gene to the other genes is indicated as a mean ± standard error with the sample size given in brackets below. Note that not all the markers are available for analysis of each transformant so sample size varies.

[3] For details of specific transformants see table 4. Details of the crosses can be seen in tables 5-11.

shows possible linkage of the *argB*$^+$ allele to the *methH* gene (11%) and appears unlinked to *brlA*, a similar proportion of *crnA*$^-$ to *crnA*$^+$ phenotypes are seen amongst the progeny. This would suggest integration into the *argB* site, and thus complementation of *crnA* in *trans* would indicate that the complete *crnA* gene is present on the plasmid pSTA4.

A summary of *argB*$^+$ linkages is presented in table 12. From this it can be clearly seen that many of the transformants have plasmid preferentially integrated into the nitrate gene cluster. If integration does occur through sites of homology to the plasmid, this then would present good evidence that the structural genes of the nitrate gene cluster have been cloned. A few of the transformants have been shown to have plasmid DNA mapping away from the nitrate gene cluster, and here complementation of *crnA*/*niiA* is still observed; suggesting that the entire genes of *crnA* and *niiA* are present on the plasmid.

Two transformants, T2 and T42, had plasmid DNA mapping away from the nitrate gene cluster and these transformants were investigated further by means of the parasexual cycle. By constructing a diploid strain between the transformant and the master strain 918 (see section II.1.3) and subsequent haploidization, the chromosome in which the plasmid had integrated into could be determined. The haploid segregants were screened for the *argB*$^-$ phenotype and these were then

TABLE 13

Parasexual Analysis of T2 and T42

argB⁻ [1] segregant No.	Chromosomal markers I to VIII [2]							
	adE	wA	actA	pyroA	facA	lacA	choA	riboB
T2/1	+	-	-	-	+	+	+	+
T2/2	-	-	-	-	-	-	-	+
T2/3	+	-	-	-	-	-	-	-
T2/4	+	-	-	+	+	+	+	+
T2/5	-	-	-	+	-	+	+	-
T2/6	-	-	-	-	-	+	+	+
T2/7	+	-	-	+	-	+	+	+
T2/8	-	-	-	-	-	-	+	-
T42/1	+	-	-	-	+	-	+	+
T42/2	+	-	-	-	+	-	+	+
T42/3	+	-	-	+	+	+	-	+
T42/4	+	-	-	+	-	+	+	+
T42/5	-	-	-	-	+	-	-	-
T42/6	+	-	-	+	-	-	+	+
T42/7	-	-	-	+	+	-	+	-
T42/8	+	-	-	-	+	-	-	-
T42/9	-	-	-	-	-	-	+	+
T42/10	-	-	-	-	+	-	-	+
T42/11	-	-	-	+	-	-	+	+
T42/12	+	-	-	-	+	+	-	+
T42/13	-	-	-	-	+	-	+	+
T42/14	-	-	-	+	-	+	+	+
T42/15	+	-	-	+	-	-	+	-
T42/16	+	-	-	-	-	+	+	+
T42/17	-	-	-	+	+	+	+	-
T42/18	+	-	-	+	+	+	+	+
T42/19	+	-	-	-	+	+	+	-

[1] The *argB*⁻ haploid segregants were obtained on haploidizing the diploid between the transformant and the master strain 918. Details of the transformant can be found in table 4 and the genotype of the master strain in section II.1.3.

[2] The *argB*⁻ haploid segregants were screened for the various markers present on each chromosome: a + indicating wild-type (chromosome from the transformant) and a - indicating mutant (chromosome from strain 918). For *actA* a + represents the resistance gene from strain 918 and a - represents the wild-type sensitive gene from the transformant.

TABLE 14

Summary of the Parasexual Analysis of T2 and T42

Transformant	No. *argB*⁻ auxotrophs [1]	Chromosome [2]							
		I adE	II wA	III actA	IV pyroA	V facA	VI lacA	VII choA	VIII riboB
T2	8	4	8	0	5	6	3	2	3
T42	19	8	19	0	10	7	11	5	6

[1] Details of specific *argB*⁻ haploid segregants (from the diploid between the transformant and master strain 918) are given in table 13. Origin of the transformant is given in table 4 and the genotype of the master strain can be found in section II.1.3.

[2] Here the number of *argB*⁻ haploid segregants has been pooled and the number of those segregants carrying the master strain (918) chromosome (I to VIII) is given.

subcultured onto a master plate and by replica plating onto various test media, the phenotype of the eight chromosomal markers were determined. Although over 300 haploid sectors were screened only a small number (for T2, 8; and for T42, 19) of argB$^-$ phenotypes were recovered; possibly indicating selection against this phenotype. Clearly, out of the markers investigated (table 13), those on chromosomes I, IV, V, VI, VII, VIII were segregating while those on chromosomes II and III were not (table 14). Obviously, to be argB2 these strains require to have inherited the argB2 allele from the transformant chrmosome III (the master strain's is argB$^+$) but not the transformant chromosome where the plasmid argB$^+$ has integrated. The argB$^-$ haploid segregants would all appear to have inherited the master strain's chromosome II (and not the transformants) and this would indicate that the site of integration for both these transformants is on chromosome II.

III.6 Mitotic Stability of Transformants

As can be seen from the genetic analysis of the transformants, a number (particularly deletion transformants) exhibit meiotic instability of plasmid sequences. Previous reports by Yelton et al. (1985) also indicated meiotic instability associated with integrated cosmid DNA. On repeated subculturing of the transformant strains (table 4), one or two transformants did not retain some of the plasmid encoded phenotypes. Such

TABLE 15

Mitotic Stability of Transformants

Strain [1]	Conidia Screened [2]	No. Chlorate Resistant	No. *argB* Auxotrophs [3]
wild-type	10^9	290	0
T26	5×10^6	269	194
T36	5×10^6	338	129

[1] The wild-type was the *biA1* strain. For details of the transformants see table 4.

[2] The conidia of the various strains were plated onto MM plus 10mM arginine, 100mM potassium chlorate and supplements. Conidia concentrations had been previously determined by counting under a microsope using a haemocytometer.

[3] The chlorate resistant colonies obtained in the third column were then replica plated onto MM plus 10mM ammonium and supplements (no arginine) and the number of arginine auxotrophs was determined.

strains included T2 and T42 (table 4) which initially were selected as *argB*$^+$,*niiA*$^+$; but after subculturing on CM many times had the phenotype *argB*$^+$,*niiA*$^-$. The transformants T8, T9 and T41 (table 4) were also initially selected as *argB*$^+$,*niiA*$^+$; but similarly after repeated subculturing on CM plus arginine had the phenotype of the original recipient strain *argB*$^-$,*niiA*$^-$. The T1 transformant (table 4) exhibited extreme sectoring of *argB*$^+$/*argB*$^-$ phenotypes even on repeated subculturing. The basis of this sectoring is at present unknown and requires further investigation. To see if such strains had lost the phenotype through excision of plasmid sequences, an attempt was made to revert the T2 and T42 strains back to wild-type *niiA*$^+$. Approximately 1×10^8 conidia were screened on MM plus nitrite and supplements. After 4 days at 37°C, the T42 strain did not show any revertants but T2 had about 100 colonies growing as wild-type. The revertants still carried the *methH2* marker and thus presumably were not contaminants. This phenomenon requires further investigation.

Most of the point mutation transformants did appear to be stable on repeated subculturing, but in order to obtain more quantitative results two *niaD*$^+$ transformants were screened on chlorate (in the presence of arginine) for the reversion frequencies back to the mutant phenotype. As a control a wild-type strain was similarly treated (table 15). The high frequency of chlorate resistant revertants from the wild-type strain

reflects the fact that chlorate resistance can be affected by mutation in many genes. In the transformants the frequency of reversion is approximately 200 times greater than that of the wild-type. On screening for the arginine phenotype, some of the transformant revertants showed concomitant loss of the *argB*$^{+}$ allele (not seen in the wild-type). Whether there is any significance in the differences between *argB* prototroph and auxotroph ratios between the transformant strains is difficult to estimate from such limited data. Loss of the *argB*$^{+}$ allele and the reversion back to *niaD*$^{-}$ almost certainly has resulted from excision of plasmid sequences by a single cross-over between the direct repeats (reversal of integration). In other cases the *niaD*$^{-}$ reversion might have arisen through a gene conversion event between the repeated sequences. Such a phenomenon has also been reported by Ward and Turner (1986) with the cloned *oliC*^{R} gene (oligomycin resistance) and in yeast (Jackson and Fink 1981).

RESULTS AND DISCUSSION
B. STUDY OF *niaD* AND *niiA* EXPRESSION USING *lacZ* GENE FUSIONS

IV.1 Fusion of the *niaD* and *niiA* Promoters to *lacZ*

From the complementation analysis in section III.4 (summarized in tables 2 and 3) it appears that the 2.7 Kb *Eco*RI fragment of pSTA2 probably contains the 5' ends of both *niiA* and *niaD* (evidence from Arst et al. (1979) would suggest at least the *niiA* promoter is present within the intergenic region). Accordingly, the 2.7 Kb *Eco*RI fragment was isolated from a gel, blunt-ended using the Klenow enzyme and ligated into the blunt-ended (phosphatased) *Bam*HI site of the *lacZ* vectors pAN923-41B, 42B and 43B (fig. 9) as shown in fig. 26. The orientation of the insert of the recombinant plasmids was then determined using *Bam*HI/*Xho*I double digests (the *Bam*HI site of the vector has now been inactivated by the blunt-end ligation and the 2.7 Kb *Eco*RI insert contains a single asymmetric *Bam*HI site). The direction of the insert, once determined, indicates whether the *niaD* promoter or the *niiA* is fused to the *lacZ* gene of the vector (fig. 26).

IV.2 Transformation with the *lacZ* Fusion Plasmids

Of the fusion plasmids generated by insertion of the 2.7 Kb *Eco*RI fragment containing the intergenic region (and the 5' ends of both structural genes - fig. 24), two, pSTA20 (nitrate promoter in phase with *lacZ* of

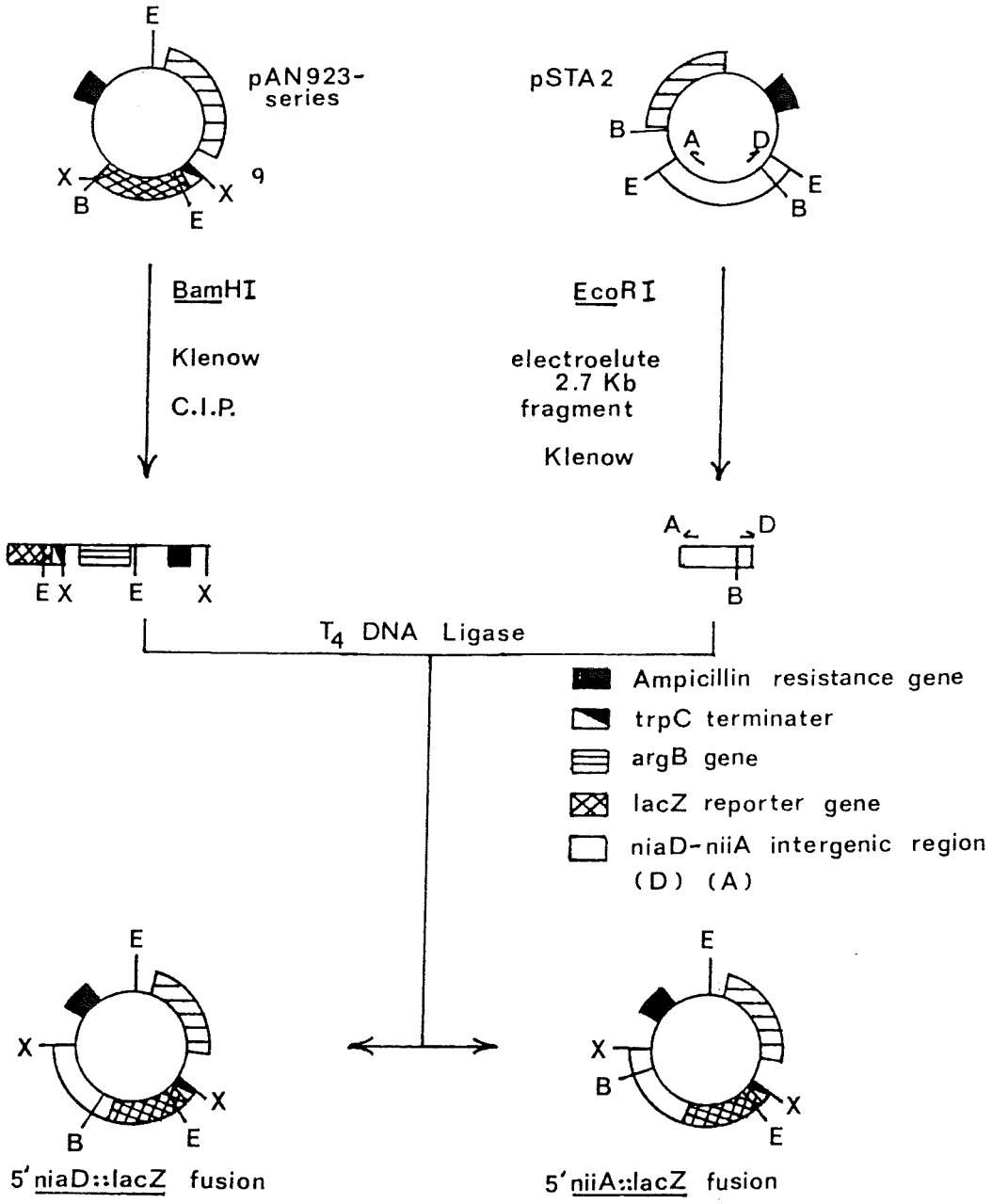


Fig. 26. Subcloning strategy for lacZ fusions. The 2.7 Kb EcoRI fragment was blunt-ended using the Klenow enzyme and ligated with the phosphatased, blunt-ended (at the BamHI site) lacZ vectors (see fig. 9). The 2.7 Kb fragment in one orientation places the niaD promoter next to the lacZ gene, while in the opposite direction the niiA promoter is adjacent to the lacZ gene. The two orientations being distinguished by a BamHI/XhoI double digest. Details of pSTA2 are given in fig. 22.

TABLE 16

Transformantion with the *lacZ* Fusion Plasmids

Plasmid[1]	Recipient	Strain[2]	No. of *argB*$^+$ transformants obtained	No. of *argB*$^+$ transformants that are *lacZ*$^+$	No. of *argB*$^+$ transformants that are *niiA*$^+$	No. of *argB*$^+$,*niiA*$^+$ transformants that are *lacZ*$^+$
pSTA20	G324	*niaD*$^+$	114	70		
pSTA21	G324	*niaD*$^+$	35	24		
42.10A	G324	*niaD*$^+$	72	0		
43.16D	G324	*niaD*$^+$	90	0		
pAN923-21B	G324	*niaD*$^+$	40	22		
pSTA20	SAA1012	Δ509	125	22	2	2
pSTA21	SAA1012	Δ509	125	17	2	0

[1] Details of pAN923-21B can be found in fig. 10, pSTA20 is a *niaD::lacZ* fusion in phase, pSTA21 is a *niiA::lacZ* fusion in phase, plasmids 42.10A and 43.16D are *lacZ* fusion plasmids with the *niiA* and *niaD* promoters (respectively) in fusion (different phase) to *lacZ*. (pAN923-21B is a *trpC::lacZ* fusion.)

[2] Both recipient strains carry the *argB2* marker. For details of strain genotypes see see section II.1.3 (G324) and table 1 (SAA1012). In transforming the SAA1012 strain, complementation of the Δ509 deletion (the *niiA* part) could also occur and such transformants were then screened for expression of *lacZ*.

pAN923-42B) and pSTA21 (nitrite promoter in phase with lacZ of pAN923-43B) were able to show β-galactosidase activity in A. nidulans argB$^+$ transformants on MM plus nitrate and X-gal plates (table 16 and fig. 39). Approximately 60% of the G324 argB$^+$ transformants (both plasmids) had β-galactosidase activity and this probably is a reflection of the balance between the different types of integration events. The control plasmid pAN923-21B (containing the trpC::lacZ fusion (fig. 10) similarly had about 50% of the argB$^+$ transformants with β-galactosidase activity (table 16). Two other fusion constructs (nitrate promoter with lacZ of pAN923-43B and nitrite promoter with lacZ of pAN923-42B) did not show any β-galactosidase activity in the argB$^+$ transformants, presumably because the galactosidase protein was not in translational phase; and these plasmids were not used further. Transformation of pSTA20 and pSTA21 into the SAA1012 strain carrying the Δ509 deletion, again allowed β-galactosidase expression in some of the argB$^+$ transformants although the numbers were somewhat reduced for some unexplained reason (only one experiment performed). The Δ509,argB$^+$ transformants were also screened for niiA complementation, and 4 complementors using pSTA20 (2) and pSTA21 (2) were found. On screening these niiA$^+$ transformants, the pSTA20 transformants showed β-galactosidase activity whereas the pSTA21 transformants did not. By analogy with previous transformation results, it seemed likely that these

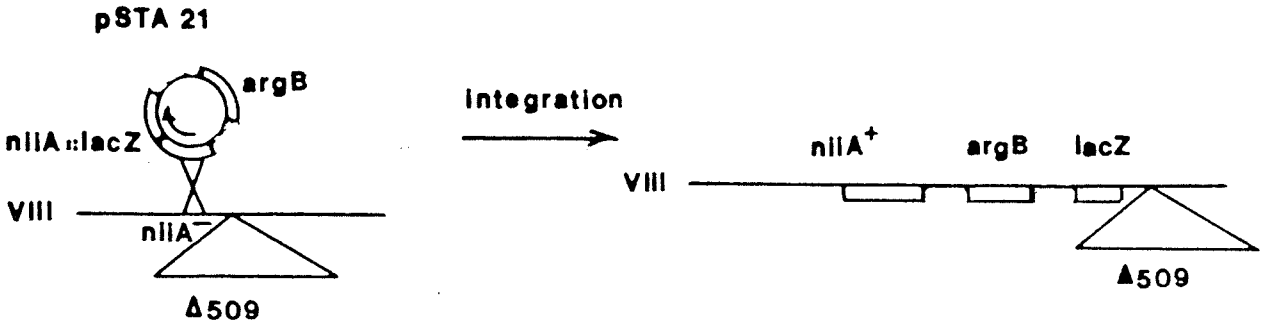


Fig. 27. Schematic representation of integration of of pSTA21 (fig. 26) into the chromosome at the niiA gene (Δ509 strain). The resultant cross-over fuses the niiA promoter and N-terminal sequences to the niiA gene (3') while the lacZ is now left adjacent to the Δ509 deletion. This represents an example of gene disruption by transformation.

TABLE 17

Transformants Used in *lacZ* Studies

Transformant[1]	Plasmid[2]	Recipient[3]	Linkage[4]	Comments[5]
Tnap2	pSTA20	G324	?	
Tnap3	pSTA20	G324	?	multi-copy
Tnap4-6	pSTA20	G324	VIII	
Tnap11-12	pSTA20	SAA1012	VIII	*niiA*$^+$, *lacZ*$^+$
Tnap13	pSTA20	SAA1012	?	
Tnap14	pSTA20	SAA1012	III?	
Tnip5	pSTA21	G324	VIII	
Tnip6-7	pSTA21	G324	VIII	*Eco*RI site
Tnip9	pSTA21	G324	VIII	missing?
Tnip11-12	pSTA21	SAA1012	VIII	*niiA*$^+$, *lacZ*$^-$
Tnip13	pSTA21	SAA1012	III?	
Tnip14	pSTA21	SAA1012	?	
Ttrp5	pAN923-21B	G324	?	

[1] Results of specific transformations can be seen in table 16.

[2] Details of plasmids can be found in fig. 26 (pSTA20 and pSTA21) and fig. 10 (pAN923-21B).

[3] Genotypes of recipient strains can be found in section II.1.3 (G324) and table 1 (SAA1012).

[4] Linkage of plasmid sequences is based on results from Southern blotting experiments (section IV.3).

[5] Tnap3 is a multi-copy transformant based on blotting experiments. Further details of some *niiA*-Δ*509* transformants are given. For Tnip6, 7 and 9 see the discussion in section IV.3.

plasmids had integrated into the niiA gene by a type I integration event (fig. 6), and in order to produce a wild-type copy of the niiA gene for pSTA21 this would inevitably mean that the niiA::lacZ fusion would be disrupted (fig. 27) and thus no β-galactosidase activity would be seen as the lacZ gene is now fused to a deleted promoter. These latter strains (Tnip11-12) without β-galactosidase activity were used as negative controls for later experiments.

IV.3 Southern Analysis of lacZ Fusion Transformants

As indicated in table 17, those transformants derived from pSTA20 transformations were designated Tnap (nitrate promoter), while those generated from pSTA21 were designated Tnip (nitrite promoter). Preliminary genetic mapping experiments by crossing the lacZ fusion transformants to AJC9.4 suggested that for Tnap2 and Tnip5, the plasmid had integrated into the nitrate gene cluster as the $argB^+$ allele was tightly linked to brlA (11% and 13% respectively).

Transformants with the lacZ constructs were analyzed by Southern blotting and hybridization experiments. Chromosomal DNA was isolated from crude nuclei preparations (section II.6.1) and then checked on an agarose gel against undigested lambda standard. The chromosomal DNA was found to be of high molecular weight (about 50 Kb in size) with only some degradation/ shearing (fig. 28). This chromosomal DNA was then

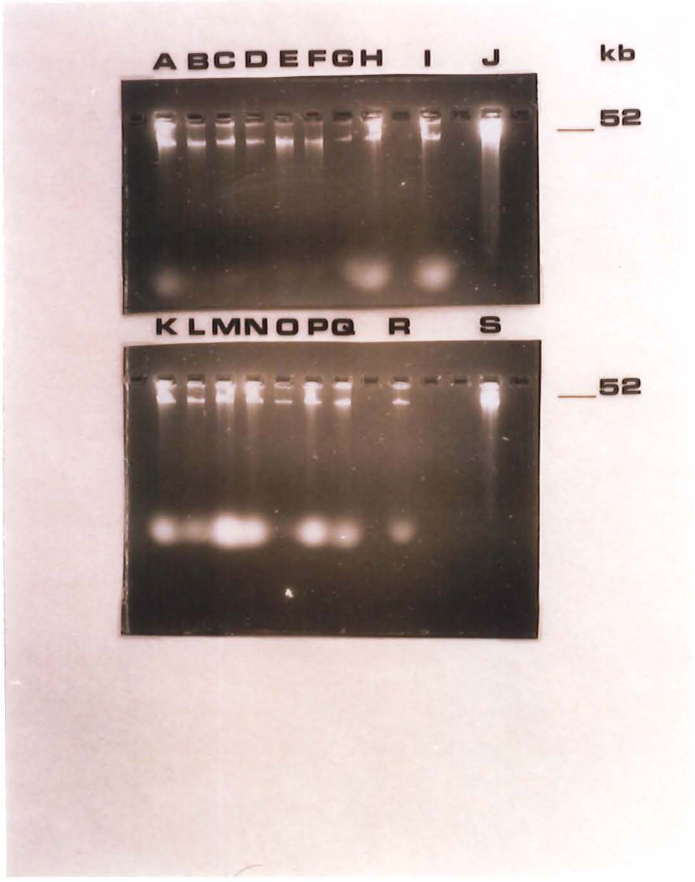


Fig. 28. Electrophoresis of chromosomal DNA isolated from crude nuclei preparations of the lacZ transformants. Undigested chromosomal DNAs were electrophoresed in a 0.8% agarose gel as follows: lane A, G324; B, Tnap2; C, Tnap3; D, Tnap4; E, Tnap5; F, Tnap6; G, Tnap11; H, Tnap12; I, Tnap14; J, λDNA; K, G324; L, Tnip5; M, Tnip6; N, Tnip7; O, Tnip9; P, Tnip13; Q, Tnip14; R, Tnap13; S, λDNA.

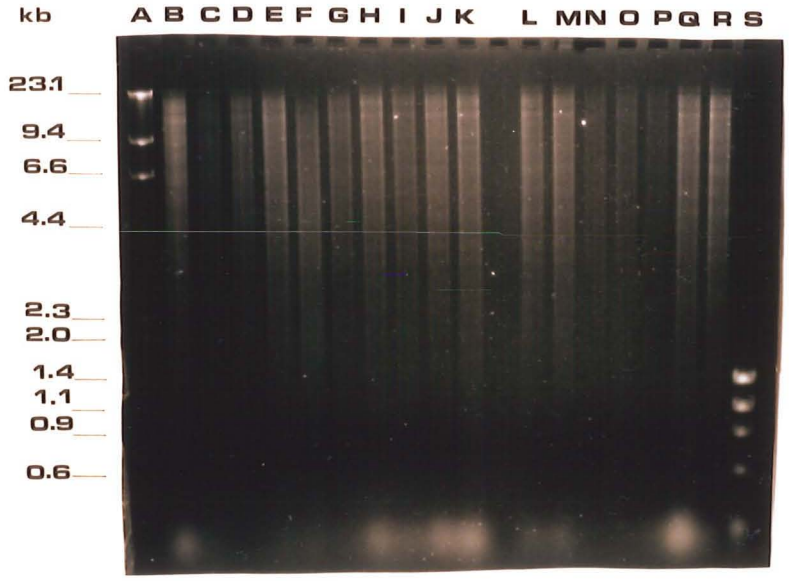


Fig. 29. Electrophoresis of EcoRI digested chromosomal DNA from the lacZ transformants. Chromosomal DNA samples were digested with EcoRI and electrophoresed in a 0.8% agarose gel as follows: lane A, λHindIII; B, G324; C, Tnap2; D, Tnap3; E, Tnap4; F, Tnap5; G, Tnap6; H, Tnap11; I, Tnap12; J, Tnap13; K, Tnap14; L, Tnip5; M, Tnip6; N, Tnip7; O, Tnip9; P, Tnip13; Q, Tnip14; R, G324; S, ΦX174 HaeIII.

Fig. 30. Genomic Southern blotting analysis of G324 (*niiA*$^+$,*niaD*$^+$) and SAA1012 (Δ*509*) pSTA20 transformants. *Eco*RI digested chromosomal DNAs were electrophoresed, blotted onto nitrocellulose and baked. The filter was hybridized to labelled pSTA20 in 50% formamide, 6 x SSC at 42°C overnight. The filter was washed at 68°C in 0.1 x SSC and X-ray film was exposed for 2 days at -70°C (further details can be found in section II.6). Lanes are as follows: A, λ*Hind*III; B, G324; C, Tnap2; D, Tnap3; E, Tnap4; F, Tnap5; G, Tnap6; H, Tnap11; I, Tnap12; J, Tnap13; K, Tnap14. Details of the transformants and G324 are given in table 17 and section II.1.3. Construction of pSTA20 is shown in fig. 26.

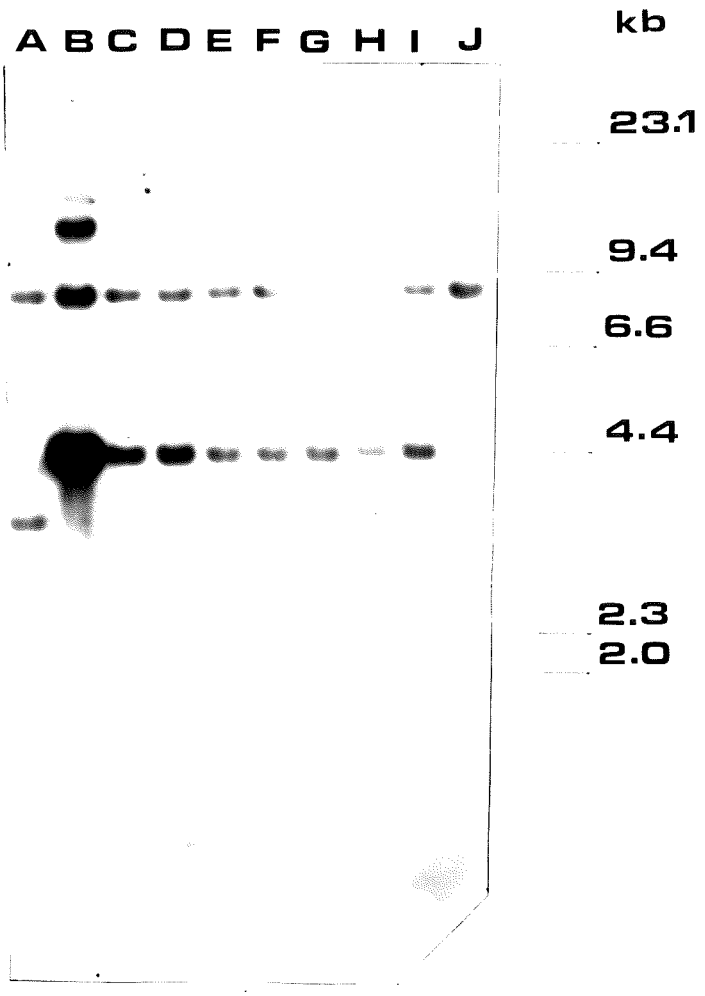


Fig. 32. Genomic Southern blotting analysis of G324 (*niiA*$^+$,*niaD*$^+$) *lacZ* transformants probed with *argB* sequences. *Eco*RI digested chromosomal DNAs were electrophoresed, blotted onto nitrocellulose and baked. The filter was hybridized to the labelled 1.9 Kb *Xba*I-*Bgl*II fragment of the *argB* gene in 50% formamide, 6 x SSC at 42°C overnight. The filter was washed at 68°C in 0.1 x SSC and X-ray film was exposed for 4 days at -70°C (further details can be found in section II.6). Lanes are as follows: A, Tnap2; B, Tnap3; C, Tnap4; D, Tnap5; E, Tnap6; F, Tnip5; G, Tnip6; H, Tnip7; I, Tnip9; J, G324. Details of the transformants and G324 are given in table 17 and section II.1.3.

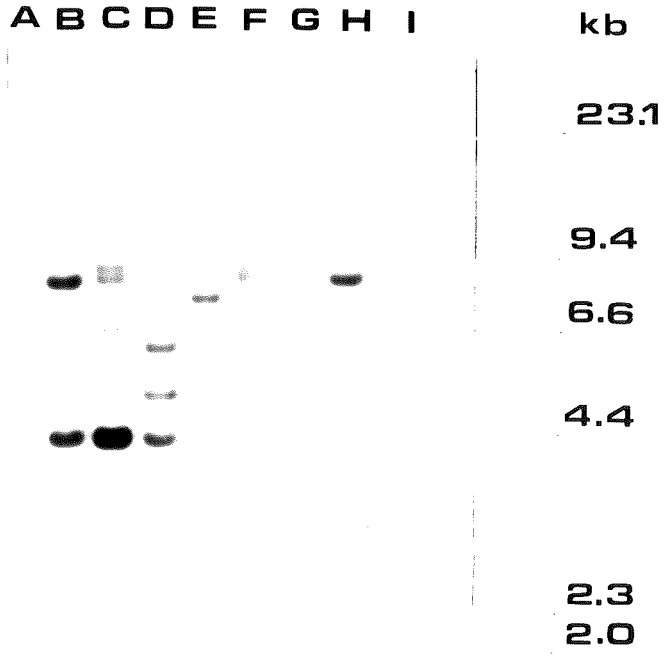


Fig. 33. Genomic Southern blotting analysis of SAA1012 (Δ509) lacZ transformants probed with argB sequences. EcoRI digested chromosomal DNAs were electrophoresed, blotted onto nitrocellulose and baked. The filter was hybridized to the labelled 1.9 Kb XbaI-BglII fragment of the argB gene in 50% formamide, 6 x SSC at 42°C overnight. The filter was washed at 68°C in 0.1 x SSC and X-ray film was exposed for 4 days at -70°C (further details can be found in section II.6). Lanes are as follows: A, Tnap11; B, Tnap12; C, Tnap13; D, Tnap14; E, Tnip13; F, Tnip14; G, SAA1012; H, G324; I, λHindIII. Details of the transformants, G324 and SAA1012 are given in table 17, 1 and section II.1.3.

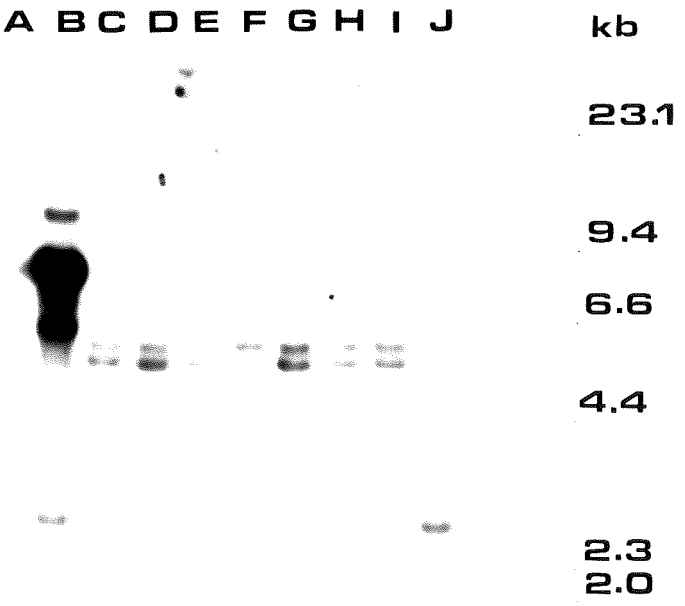


Fig. 34. Genomic Southern blotting analysis of G324 (*niiA*$^+$,*niaD*$^+$) *lacZ* transformants probed with the *niiA*-*niaD* intergenic region. *Eco*RI digested chromosomal DNAs were electrophoresed, blotted onto nitrocellulose and baked. The filter was hybridized to the labelled 2.7 Kb *Eco*RI fragment containing the *niiA*-*niaD* intergenic region, in 50% formamide, 6 x SSC at 42°C overnight. The filter was washed at 68°C in 0.1 x SSC and X-ray film was exposed for 7 days at -70°C (further details can be found in section II.6). Lanes are as follows: A, Tnap2; B, Tnap3; C, Tnap4; D, Tnap5; E, Tnap6; F, Tnip5; G, Tnip6; H, Tnip7; I, Tnip9; J, G324. Details of transformants and G324 are given in table 17 and section II.1.3.

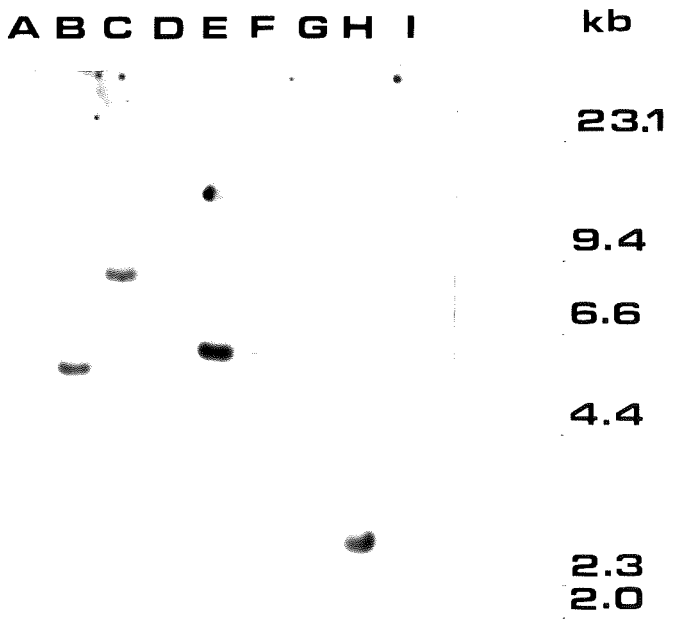


Fig. 35. Genomic Southern blotting analysis of SAA1012 (Δ509) *lacZ* transformants probed with the *niiA-niaD* intergenic region. *Eco*RI digested chromosomal DNAs were electrophoresed, blotted onto nitrocellulose and baked. The filter was hybridized to the labelled 2.7 Kb *Eco*RI fragment containing the *niiA-niaD* intergenic region, in 50% formamide, 6 x SSC at 42°C overnight. The filter was washed at 68°C in 0.1 x SSC and X-ray film was exposed for 7 days at -70°C (further details can be found in section II.6). Lanes are as follows: A, Tnap11; B, Tnap12; C, Tnap13; D, Tnap14; E, Tnip13; F, Tnip14; G, SAA1012; H, G324; I, λ*Hin*dIII. Details of the transformants, SAA1012 and G324 are given in tables 17, 1 and section II.1.3.

digested with EcoRI and electrophoresed along with size markers (fig. 29). Any hybridizing bands seen later on the autoradiograph could then have the size estimated by placing the autoradiograph over the photograph (x 1) of the gel (top of the filter being just below the wells).

Initially genomic EcoRI digests of Tnaps (fig. 30) and Tnips (fig. 31) with G324 (recipient strain for transformations) as a control were blotted and probed with pSTA20 and pSTA21 respectively. In all cases many bands were produced, presumably due to chromosomal sequences (8.3 and 2.7 Kb) and to integrated plasmid sequences. The weakly hybridizing 4.9 Kb band in G324 is possibly due to the trpC terminator present in the fusion plasmids (3' to lacZ) and is not necessarily seen in the other lanes. The 4.9 Kb EcoRI 3'-trpC band is approximately the expected size (Yelton et al. 1983). To resolve the situation further, blotted genomic EcoRI digests of G324 transformants (with G324 as a control - fig. 32) and blotted genomic EcoRI digests of SAA1012 transformants (with SAA1012 and G324 as controls - fig. 33) were probed with the 1.9 Kb XbaI-BglII fragment of the argB gene. Similar blots of the G324 (fig. 34) and SAA1012 (fig. 35) transformants were probed with the 2.7 Kb EcoRI fragment of the niaD-niiA intergenic region.

From the blots seen in figs. 32 and 33, the chromosomal argB EcoRI fragment appeared to be about 8.3 Kb in size (estimated from the G324 lane). In the G324 transformants (fig. 32) a 4.3 Kb EcoRI fragment

hybridizing to the argB probe was seen in most transformants except for Tnap2 where a 3.2 Kb EcoRI fragment was seen. These bands were not seen in the G324 control and presumably are the result of hybridization to plasmid sequences. The high molecular weight bands hybridizing in Tnap3 were probably the result of a partial digest (not seen in fig. 30). Digesting pSTA20 and pSTA21 (fig. 26) with EcoRI would produce a 4.3 Kb fragment containing the argB gene, and the presence of this and the chromosomal band (8.3 Kb) suggests that integration has occurred at a site other than the argB site. In the Tnap2 transformant the 4.3 Kb EcoRI fragment is reduced in size suggesting that during the integration event this fragment has been disrupted. However, the chromosomal 8.3 Kb EcoRI band is still present in Tnap2, therefore integration is at a site other than the argB gene. For the deletion transformants (fig. 33), the 8.3 Kb EcoRI chromosomal argB band is present in most strains except in Tnap14 and Tnip13 where smaller bands are present. This would indicate that in these transformants integration has occurred at the argB site. Although a 4.3 Kb EcoRI band is still present in these transformants it may be a result of new rearranged sequences.

When the intergenic niaD-niiA probe was used in the hybridization experiment with G324 strain (fig. 34) the genomic fragment was the expected 2.7 Kb EcoRI band whereas in the deletion strain SAA1012 (Δ509) this

appeared as a new 2.3 Kb EcoRI band (fig. 35). In the G324 transformants Tnap4-6 the 2.7 Kb EcoRI chromosomal band is missing, as is the 9.3 Kb EcoRI fragment from pSTA20 and two new bands are seen, suggesting that in these transformants integration has occurred into the niaD-niiA intergenic region. Tnap2 and Tnap3 still have the 2.7 Kb EcoRI genomic fragment present and both possess a 8.3 Kb EcoRI band (which in Tnap3 masks the chromosomal argB band in fig. 30). In addition Tnap3 has a 6.2 Kb EcoRI band. The site of integration for these two transformants is not clear from the molecular analysis, although by previous genetic mapping experiments, pSTA20 appeared to have integrated into the niaD-niiA intergenic region of G324. In transformants Tnip6, 7 and 9 the same blot pattern as Tnap4 (etc.) is seen, despite the fact that the construction of the pSTA21 plasmid (fig. 26) means that an extra EcoRI site is present in the linker adjacent to lacZ. The disappearance of the 2.7 Kb EcoRI chromosomal band would indicate that integration had occurred into the niaD-niiA intergenic region, but a serious doubt must remain as to the nature of these three transformants (Tnap contaminants or partial digests?). Tnip5, however gives the expected pattern for an integration event into the niaD-niiA intergenic region as the 2.7 Kb genomic band would be regenerated instead of the 5.4 Kb band seen in the other transformants. The EcoRI band of the lacZ gene appears to hybridize to pSTA21 (fig. 31 - a doublet

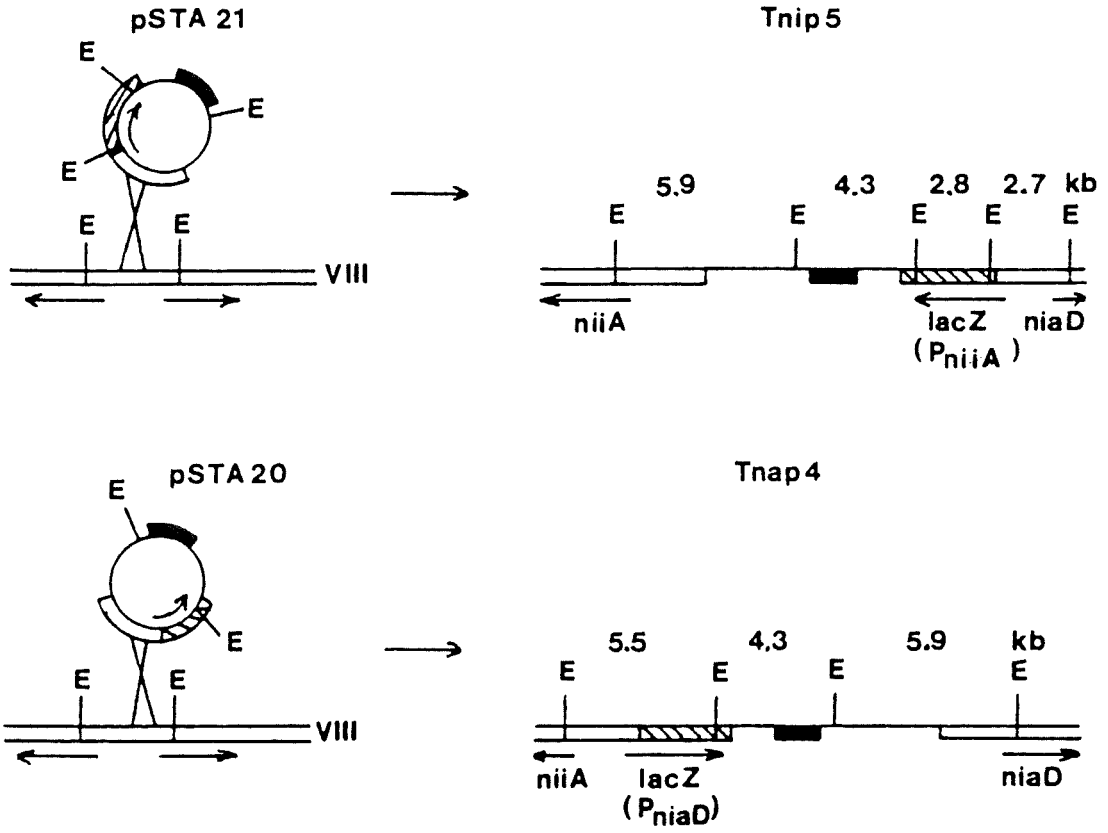


Fig. 36. Schematic diagram of the integration of pSTA20 (lower) and pSTA21 (top) into the niaD-niiA intergenic region. The EcoRI sites are indicated so that the expected EcoRI fragments on hybridization can be predicted.

about 2.7 Kb) but obviously does not hybridize to the niaD-niiA intergenic probe (fig. 34). The integration events in Tnap4-6 and Tnip5 are shown in fig. 36. Using the niaD-niiA intergenic probe against the deletion transformants (fig. 35) the resident 2.3 Kb EcoRI band of the Δ509 deletion is still present for the majority of the transformants. Hybridization of this band is less intense than the 2.7 Kb band of G324, presumably due to the reduced homology available to the probe. In Tnap11 and Tnap12, however, this 2.3 Kb band appears to be missing, suggesting that integration has occurred in the remaining niaD-niiA sequences. This would be in agreement with the fact that both these transformants complement niiA and hence have presumably integrated there. The integration site of the other transformants would be away from the remaining niaD-niiA sequences as this is still intact. (Tnap14 and Tnip13 have plasmid sequences probably integrated into the argB site.)

IV.4 Dot Blot Analysis

From the previous Southern blot analysis one particular feature observed was the multiple copies of the plasmid EcoRI bands in Tnap3. As only two such bands were produced this would suggest that a tandem array of copies is present in the transformant. In order to estimate the number of copies, dot blot analysis was employed.

Given that the genome size of A. nidulans is about

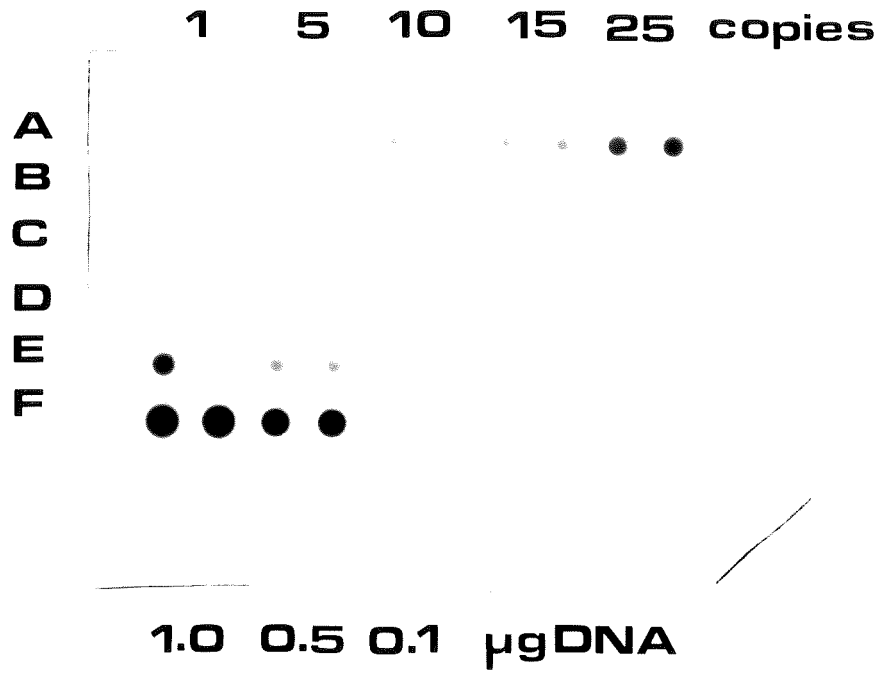


Fig. 37. DNA dot blotting analysis to determine the argB copy number in the Tnap3 transformant. Undigested DNAs dot blotted in duplicate as follows: row A, 530 pg of pSTA20; B, 265 pg of pSTA20; C, 53 pg of pSTA20; all in increasing amounts along the horizontal. Row D, G324; E, Tnap5; F, Tnap3; all in decreasing amounts along the horizontal. The nitrocellulose filter was hybridized in 50% formamide, 6 x SSC at 42°C with the labelled 1.9 Kb XbaI-BglII fragment of the argB gene, overnight. The filter was then washed at 68°C in 0.1 x SSC and the X-ray film was exposed at -70°C for 24 h. For details of G324 and the two transformants see section II.1.3 and table 17. Further details of hybridization and washing conditions can be found in section II.6.

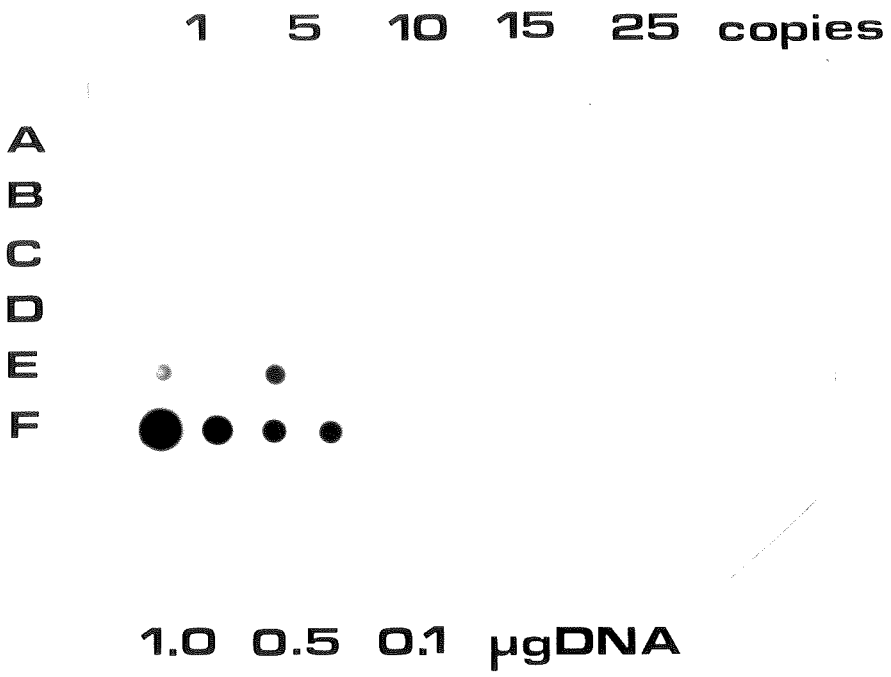


Fig. 38. DNA dot blotting analysis to determine the niiA-niaD intergenic region copy number in the Tnap3 transformant. Undigested DNAs dot blotted in duplicate as follows: row A, 530 pg of pSTA20; B, 265 pg of pSTA20; C, 53 pg of pSTA20; all in increasing amounts along the horizontal. Row D, G324; E, Tnap5; F, Tnap3; all in decreasing amounts along the horizontal. The nitrocellulose filter was hybridized in 50% formamide, 6 x SSC at 42°C with the labelled 1.6 Kb BglII-EcoRI fragment of the niiA-niaD intergenic region, overnight. The filter was then washed at 68°C in 0.1 x SSC and the X-ray film was exposed at -70°C for 24 h. For details of G324 and the two transformants see section II.1.3 and table 17. Further details of hybridization and washing conditions can be found in section II.6.

2.6×10^4 Kb (Timberlake 1978) and that the plasmid is about 13 Kb, a single copy of this integrated plasmid represents a fraction of 5×10^{-4} of the genome. Thus plasmid DNA was diluted appropriately and a single copy equivalent to 1.0 µg, 0.5 µg and 0.1 µg of genomic DNA was dot blotted. Further samples of plasmid DNA equivalent to increasing copy number of this sequence was dot blotted along the horizontal (figs. 37 and 38). Also blotted were the genomic DNAs of a wild-type strain (G324 recipient strain), single copy transformant (Tnap5) and the multiple copy transformant (Tnap3) in 1.0 µg, 0.5 µg and 0.1 µg amounts along the horizontal. The two nitrocellulose filters prepared in this way were then probed separately, one with the argB probe (1.9 Kb XbaI-BglII fragment - fig. 37) and the other with the niaD-niiA intergenic region probe (1.6 Kb BglII-EcoRI fragment - fig. 38). From the blots it would appear that at least ten copies of the plasmid are present in the multiple copy transformant (0.1 µg of Tnap3 gives the same signal as 1.0 µg of G324). The plasmid dilutions across the top appear inconsistent with the dilutions (particularly in fig. 38), but these would indicate greater than 25 copies of the plasmid present in Tnap3. The single copy transformant (Tnap5) appears to have two copies of the probed sequences (0.5 µg of Tnap5 gives approximately the same signal as 1.0 µg of G324). The integration of pSTA20 into the niaD-niiA intergenic region, as seen in Tnap5, would produce one copy of the

TABLE 18

Relative β-galactosidase Activities of *lacZ* Transformants

	Transformant[1]	Growth Conditions[2,3,4]				
		10mM nitrate	2mM adenine	10mM glutamate	10mM nitrate+ 10mM ammonium	10mM ammonium
	Tnap2	41	7	3	10	2
	Tnap3	527	122	99	136	37
	Tnap4	91	12	4	16	4
*	Tnap5	100	11	4	12	10
	Tnap6	84	13	4	11	3
	Tnap11	110	121	93	16	3
	Tnap12	132	78	120	18	6
	Tnap13	458	316	274	79	14
	Tnap14	37	16	42	5	1
*	Tnip5	100	3	5	20	1
	Tnip6	51	3	5	9	3
	Tnip7	40	6	5	9	3
	Tnip9	40	16	5	10	3
	Tnip11	<1	<1	<1	<1	<1
	Tnip12	<1	-	-	-	-
	Tnip13	1000	426	319	56	5
	Tnip14	203	105	13	7	1
*	Ttrp5	100	231	257	51	48

[1] For details of the transformants see table 17.

[2] For details of the growth conditions see section II.7.1.

[3] Relative β-galactosidase activities are presented in the table. For Tnap transformants activities are compared to that of Tnap5 grown in 10mM nitrate (100% = 1552.7 nmoles o-NP produced/min/mg protein at 37°C). For Tnip transformants activities are compared to that of 10mM grown Tnip5 (100% = 2072.8 nmoles o-NP produced/min/mg protein at 37°C), while for Ttrp5 100% activity is equal to 3579.9 nmoles o-NP produced/min/mg protein at 37°C (i.e. that activity on 10mM nitrate). Both Tnap5 and Tnip5 are single copy transformants wih plasmid integrated at the nitrate gene cluster.

[4] For all transformants activities were determined as the mean of duplicate assays from at least two independent experiments (except for the Tnip11-12 transformants).

promoter::lacZ fusion in tandem with the single copy of the wild-type promoter.

One possible reason for the apparently variable amounts of signal obtained is that unless the prepared DNA samples are blotted quickly, renaturation of the DNA will occur. This would be particularly marked with the plasmid DNA as the overall complexity of the DNA in solution is considerably less than that of the genomic samples.

IV.5 Enzyme Assays of lacZ Transformants

IV.5.1 β-galactosidase. Transformants obtained from the pSTA20 and pSTA21 plasmids (table 17) were grown in MM plus different nitrogen sources (for details of the growth conditions see section II.7.1), harvested and then assayed (section II.7.1). Enzyme activities were normalized to protein concentrations (determined by the Bradford's reagent, section II.7.5) and were then compared to those of the single copy transformants Tnap5 and Tnip5 (table 18). In the negative controls Tnip11-12 (section IV.3) endogenous β-galactosidase activity was negligible in the presence of 1% w/v glucose (table 18). Although there is considerable variation in the activities of induced β-galactosidase between the transformants, all show a maximal enzyme activity in conditions of nitrate induction/nitrogen metabolite derepression except for Ttrp5 which represents the trpC::lacZ fusion transformant. Additionally the SAA1012

(Δ509) transformants Tnap11-14 and Tnip13-14 show induced levels of β-galactosidase even in the absence of nitrate, although this activity is still repressed on the addition of ammonium. Thus the relative enzyme activities over the different nitrogen sources mirror that of nitrite reductase in the wild-type and certain mutant phenotypes. Perhaps a greater induction (or more consistent level between transformants) might have been obtained if growth in the nitrate medium had been for a shorter time (e.g. 9 h, or a switch experiment) but the experiment was designed to compare with previous results published with the trpC::lacZ fusion transformants (Van Gorcom et al. 1985, Hamer and Timberlake 1987). However, the transformant Ttrp5 exhibited a surprisingly high level of β-galactosidase activity compared with previous results obtained by Van Gorcom et al. (1985), perhaps as a result of a different integration event. Two strains which stand out with elevated levels of β-galactosidase activity are Tnap3 (5 x that of Tnap5, the single copy transformant with pSTA20 integrated in the niaD-niiA intergenic region) and Tnip13 (10 x that of Tnip5, the single copy transformant with pSTA21 integrated in the niaD-niiA intergenic region). The Tnap3 transformant appears to be a multiple copy transformant (with copies integrated in tandem, section IV.3), while the Tnip13 transformant seems to be a single copy transformant (section IV.3 and figs. 30-35). In the case of Tnip13, increased β-galactosidase activity could have resulted

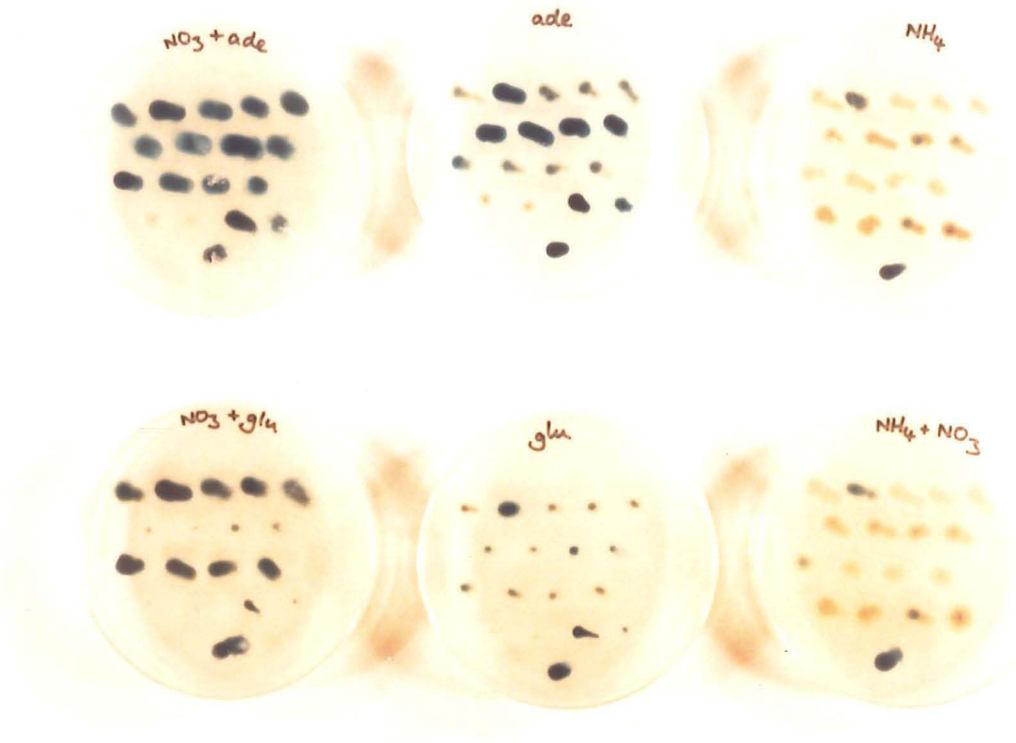


Fig. 39. In vivo β-galactosidase activities of the lacZ transformants indicating that the lacZ gene is under control of nitrate induction/nitrogen metabolite repression (similar to the niaD and niiA genes). Colonies are plated as follows:

top row: Tnap2, Tnap3, Tnap4, Tnap5, Tnap6;
row 2: Tnap11, Tnap12, Tnap13, Tnap14;
row 3: Tnip5, Tnip6, Tnip7, Tnip9;
row 4: Tnip11, Tnip12, Tnip13, Tnip14;
bottom: Ttrp5.

Details of the transformants are presented in table 17. Nitrogen sources used in the plates are as indicated (glu = glutamate, and ade = adenine; concentrations used are as in table 18).

from a fusion of the lacZ gene to a new promoter. The fact that the activity is still ammonium repressible, however, would suggest that the lacZ gene is still modulated by the nitrite reductase control signals. For Tnap3 the elevated β-galactosidase activity could be a result of either fusion to a new promoter, as above, or due to the presence of multiple copies producing more enzyme. The site of integration of pSTA20 in Tnap3 is uncertain and so both these possibilities are tenable. In vivo activities of all these transformants under the different nitrogen sources can be seen in fig. 39; and mirror the enzymatic activities obtained. Note, that for the Δ509 transformants glutamate and nitrate was used as nitrate inducing/nitrogen metabolite derepressing growth medium. Here it can be seen that the presence of nitrate reduces the utilization of glutamate, as observed by Cove (1979) and in fig. 39.

IV.5.2 Nitrate reductase. The presence of multiple copies in Tnap3 allows the possibility to determine whether the nitrate reductase activity is altered in this transformant. Previously Cove (1969) had suggested that the nirA gene product was present in limiting concentrations, thus multiple copies of the promoter sequence fused to the lacZ gene may titrate out the regulator, reducing the amount of regulator molecules available to activate transcription of the niaD gene (and hence synthesis of the nitrate reductase protein). In this regard Tnap3 (multiple copy transformant), Tnap5

TABLE 19

Relative Nitrate Reductase Activities of Selected *lacZ* Transformants

Strain[1]	Induction Medium[2,3]			
	10mM Nitrate	10mM Glutamate	10mM Nitrate+ Ammonium	10mM Ammonium
WT	100	1	1	<1
Tnap3	39	<1	<1	<1
Tnap5	106	1	1	<1

[1] Tnap3 is a multiple copy (of pSTA20) transformant and Tnap5 is a single copy (of pSTA20) transformant. The WT strain was a G324 (*argB*) transformant - Ttrp5. For further details of the strains see table 17.

[2] Relative nitrate reductase activities are presented where 100% = 150.9 nmoles nitrite produced/min/mg protein at 25°C. Activities are compared to the nitrate induced wild-type levels.

[3] Values are the mean of duplicate assays from at least two independent experiments. For details of the growth conditions and induction media used see section II.7.2.

(single copy transformant) and a wild-type strain (G324 *argB*$^+$ transformant) were analyzed for their nitrate reductase activities. To ensure maximal nitrate reductase activities (so any effect should be seen) a switch experiment was performed with mycelia induced with nitrate for 4 h (as Davis and Hynes (1987)). Similarly, mycelia were transferred to other nitrogen sources and nitrate reductase activities were determined (see table 19).

It can be seen that in Tnap3 the maximal nitrate reductase activity is only about 40% that of the wild-type and Tnap5. So it would appear that the *nirA* gene product is only titrated out by multiple copies of the fusion construct (no effect seen in Tnap5). Apart from this aspect, the nitrate reductase activity appears to be regulated normally in these strains.

IV.5.3 *Acetamidase*. In order to eliminate the possibility that the *areA* protein was limiting, the enzyme activity of another enzyme, acetamidase, under *areA* control was determined for the same three strains (wild-type, Tnap3, Tnap5). Again, the experiment was designed to produce maximal activities by using a switch experiment. Similarly, mycelia were transferred to various nitrogen regimes and the acetamidase activities determined (see table 20).

From this table, no differences between the three strains were seen, although the enzyme activities were uniformly low compared with those obtained by Hynes

TABLE 20

Relative Acetamidase Activities of Selected *lacZ* Transformants

Strain[1]	Induction Medium[2,3]		
	10mM Acetamide	10mM Glutamate	10mM Acetamide +Ammonium
WT	100	78	31
Tnap3	136	42	32
Tnap5	137	45	18

[1] Tnap3 is a multiple copy (of pSTA20) transformant and Tnap5 is a single copy (of pSTA20) transformant. The WT strain was a G324 (*argB*) transformant - Ttrp5. For further details of the strains see table 17.

[2] Relative acetamidase activities are presented where 100% = 16.3 nmoles ammonium produced/min/mg protein at 37°C. Activities are compared to the acetamide induced wild-type levels.

[3] Activities were the mean of duplicate assays from at least two independent experiments. For details of the growth conditions and induction media used see section II.7.3.

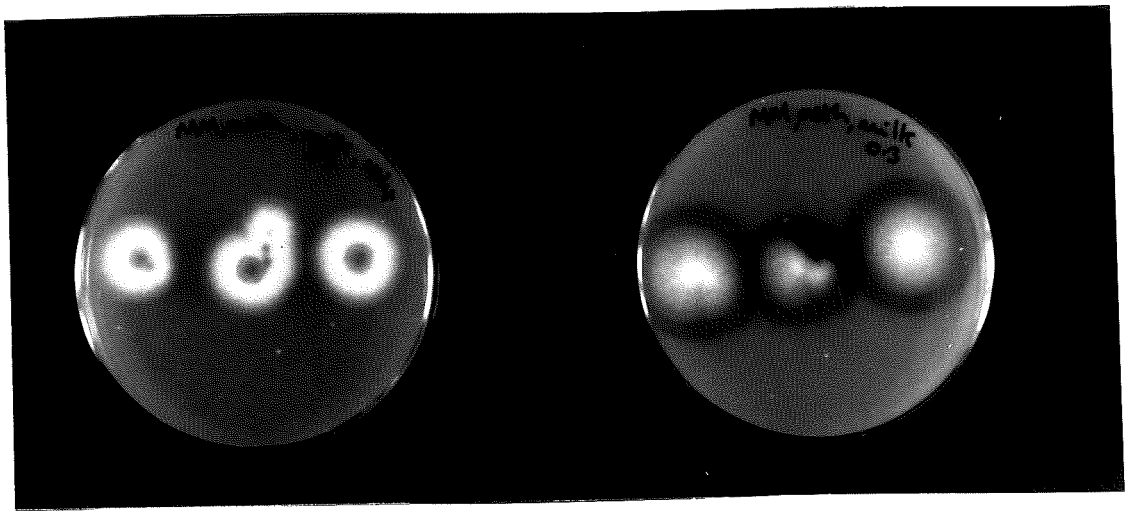


Fig. 40. In vivo protease activity of the lacZ transformants Ttrp5 (= WT, left), Tnap3 (centre) and Tnap5 (right). The left plate contains MM plus ammonium and milk whereas the right plate contains MM plus milk only. Protease activity is indicated by the size of the halo of milk clearing. Tnap3 is a multiple copy transformant while Tnap5 is a single copy transformant. For further details of the strains see table 17.

(1972). This would tentatively suggest that the areA gene product is not limiting in the multiple copy transformant Tnap3.

IV.5.4 Protease. Another enzyme activity under areA control is that of the extracellular protease (Cohen 1972). To determine whether this enzyme activity was altered in the three strains (wild-type, Tnap3 and Tnap5) plate assays were performed (fig. 40).

An ammonium plus milk (as nitrogen sources) plate was used as a negative control. As can be seen from fig. 40, the extent of the halo (and hence protease activity) in each case is approximately the same indicating that the protease levels produced by these three strains are equivalent, again ruling out the possibility that the areA protein is limiting in the multiple copy transformant Tnap3. This is in agreement with the results obtained by Kelly and Hynes (1987), who also suggested that areA levels were not limiting in an amdS multiple copy transformant.

CONCLUSION

V.1 Complementation and integration of pNiiA and subclones thereof.

Evidence has been presented to suggest that the nitrate gene cluster has been cloned. Complementation of the deletions Δ*507*, Δ*509*, *niaD10* and *niaD26* was observed, ruling out the possibility that a suppressor had been cloned. Complementation studies suggested that most of the nitrate gene cluster was present on the original recombinant plasmid pNiiA (see section II.1.1). However, transformation of a number of *niaD* alleles indicated that although extensive *niaD* sequences were present on the plasmid, part of the *niaD* gene was missing. Subsequent isolation of overlapping clones, pILJ141 and λAN8a (section II.1.1) to the right of the genetic map (centromere distal) and transformation experiments using these clones gave results consistent with the remaining *niaD* sequences having been cloned. Genetic mapping experiments from sexual crosses between the transformants and strain AJC9.4 (section II.1.3), indicated that the majority of the transformants had integrated into the nitrate gene cluster. This is consistent with the cloning of the nitrate gene cluster as integrative transformation in *A. nidulans* is postulated to occur at sites of homology between the plasmid and the genomic DNA. That the complete *crnA* and *niiA* genes are present on the original plasmid is supported by the fact that in a few rare transformants

the plasmid has integrated on chromosome III (T40) or chromosome II (T2 and T42) and hence complementation of the crnA/niiA allele is in trans. That the majority of transformants have the plasmid integrated into the nitrate gene cluster, even in extensive deletion strains was perhaps unexpected and might suggest a possible recombination "hot spot" within the nitrate gene cluster. The niaD and niiA genes recombine at about 1% (Tomsett and Cove 1979) and from the complementation analysis such a recombination fraction represents about 2 Kb (fig. 24). Other closely linked genes that have been cloned include alcA,alcR which are about 2 % apart on the genetic map and 2 Kb apart at the DNA level (Pateman et al. 1983; Arst and Scazzocchio 1985). The proline cluster and the qut cluster have also been cloned on single lambda fragments (cited in Arst and Scazzocchio 1985). This would indicate that such genes are tightly linked at the physical (DNA) level as well as at the genetic level. However, the closely linked genes acuA and acuD which recombine at about 1% are separated by greater than 30 Kb (Turner, unpublished). This implies that genetic distances do not necessarily correlate with physical distances at the DNA level. It was assumed in the linkage data that transformation had occurred by a single integration event, where the $argB^{+}$ was tightly linked to transforming sequences on the plasmid, when complementation of two alleles was observed. However, some of the crosses (particularly

those involving deletions) gave high niiA$^-$ allele ratios in a transformant (niiA$^+$,argB$^+$,niiA$^-$,argB$^-$) crossed to the AJC9.4 strain (niiA$^+$,argB$^-$). One would predict that any niiA$^-$ progeny present would also be argB$^-$, suggesting meiotic instability of the integrated plasmid and excision out of the chromosome. In the crosses involving T2, T38, T41 and T42 however, the niiA$^-$ allele seems to segregate freely with the argB$^+$ gene. This result seems difficult to explain, although it may be possible that multiple, independent integration events (i.e. not tandem arrays) can occur and such transformants would be difficult to analyze by genetic methods. Another possible explanation of the high frequency of null alleles appearing in the progeny might be gene conversion events between duplicated elements of the genome. Such a model has been suggested to occur in yeast by Jackson and Fink (1981).

If transformation has occurred by a single cross-over in a type I integration event, as would be suggested by most of the genetic data on the transformants analyzed, then this should result in direct repeats of cloned and chromosomal DNA. Such a genetic structure might be predicted to be less stable than the native chromosome and indeed results indicate a degree of both meiotic and mitotic instability of integrated plasmid sequences. The mitotic stability experiment resulted in a reversal of the integration event by excision of plasmid sequences but also

indicated that gene conversion events between the wild-type and the mutant copy of the niaD alleles may occur (as such revertants are still argB$^+$). This result may also support the idea of gene conversion producing niiA$^-$ progeny in the transformant crosses.

V.2 Expression of niaD and niiA in lacZ Transformants

In the lacZ fusion experiments, the 2.7 Kb EcoRI fragment was fused in both orientations in front of the E. coli β-galactosidase gene. This fragment encompasses the intergenic niaD-niiA region and it was not unexpected that this should have the regulatory signals present for expression of the niiA and niaD genes (but see section VI - Addendum). Previously, Arst et al. (1979) had suggested from observations with the nis-5 translocation, that the niiA promoter was present in the niaD-niiA intergenic region; and secondly the distribution of temperature sensitive alleles in the fine structure map of Tomsett and Cove (1979) might suggest that the protein encoding part of the niaD gene is centromere distal (see fig. 5). Certainly, the expression of β-galactosidase directed by the construct in A. nidulans strongly supports the idea of divergent transcription of these two genes. Although this 2.7 Kb EcoRI fragment contains sufficient control sequences to mediate the nitrate/ammonium regulation, it does not rule out the possibility of internal or downstream sequences having an additional role in maximal

expression of these genes (e.g. as in the PGK gene of yeast - Mellor et al. 1987). Again, blotting and hybridization results are consistent with many of the transformants containing plasmid integrated into the niaD-niiA intergenic region. Although such experiments are useful in determining the more complex type of integration events, such information as mapping of integrated sequences is more limited than genetic methods. One interesting transformant isolated was that of Tnap3, which appeared to have multiple tandem integrations of the fusion construct. This strain also had an elevated β-galactosidase activity, though it is uncertain whether this is due to the multiple copies or the site of integration. However, Tnap3 only had about 40% of the wild-type maximal nitrate reductase levels, while acetamidase and protease levels appeared unaltered. This would suggest that at the level of 10+ copies, the nirA gene product becomes limiting for maximum expression of the native niaD gene. In Tnap5, a single copy transformant, two copies of the promoter region are present (one in the lacZ fusion construct and one wild-type copy). Despite this duplication of the cis-acting regions no apparent titration of nitrate reductase was observed (table 19). Previously, it had been suggested that nirA was limiting by Cove (1969) on a more drastic level, such that niaD$^-$ strains only weakly complemented nirA$^-$ strains in heterokaryons. Diploids of the genotype nirA$^+$/nirA$^-$ genotype only had

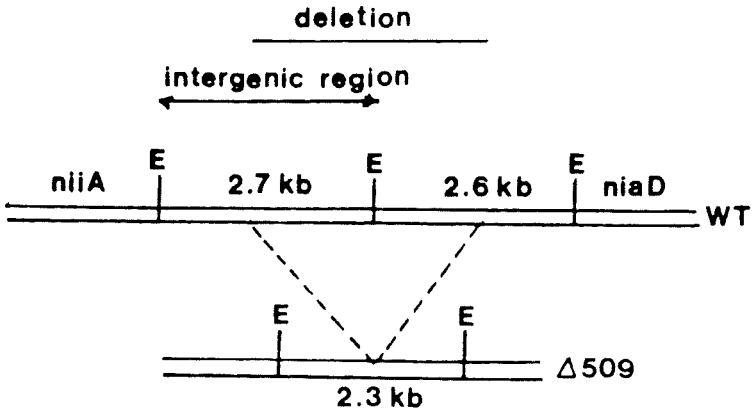


Fig. 41. Schematic representation of the hybridization results obtained with Δ509. Using the 2.7 Kb EcoRI fragment (wild-type niaD-niiA intergenic probe) a new 2.3 Kb band is observed on hybridization. Although this could be explained by a 400 bp deletion, this would be unlikely as the complementation analysis suggests a more extensive deletion. More likely is the 3.0 Kb deletion suggested above.

about 60% of the wild-type activity, leading to a gene dosage model proposed by Cove (1969). So while qualitatively a similar result has been obtained, *nirA* might not be as limiting as previously implied.

One other observation from the blotting and hybridization experiments with the *lacZ* fusion transformants is that the Δ*509* strain shows a 2.3 Kb *Eco*RI fragment compared to a 2.7 Kb *Eco*RI fragment of the wild-type *niiA*-*niaD* intergenic region. This fact and also from the complementation analysis suggesting that Δ*509* is an extensive deletion would indicate that Δ*509* has a 3.0 Kb deletion in the nitrate gene cluster (fig. 41).

ADDENDUM

VI.1 Sequence analysis of *niaD*

Since completion of this work, sequencing data and transcription initiation analysis of the *niaD* (and *niiA*) genes (Johnstone et al., unpublished) has revealed that the probable *niaD* translational start site (ATG) is outside the 2.7 Kb *Eco*RI fragment thought on the basis of complementation data (section III.4) and *lacZ* fusion studies (section IV.2) to contain the start of the *niaD* gene. The pSTA20 construct (fig. 26) is able to express the β-galactosidase protein (fig. 39) despite missing 51 bp immediately 5' of the *niaD* translational start site (also deleting the transcriptional initiation site). Sequence analysis of the junction site between *niaD* and *lacZ* of pSTA20 has indicated that an ATG at -70 bp from the real translational start site, and thus contained within the 2.7 Kb *Eco*RI fragment, is in translational phase with the β-galactosidase protein (Greaves, unpublished). Hamer and Timberlake (1987) have also reported that with their *trpC::lacZ* fusions, deletion of the first 67 bp immediately 5' of the ATG of *trpC* does not appear to affect *lacZ* expression, although the transcriptional start site is altered. In this regard it would be interesting to look at the transcriptional start site(s) in the Tnap transformants. That the ATG at -70 bp from the translational start site is not used in *vivo* is indicated by the presence of an in phase stop

codon, 30 bp downstream. It is possible that in the pSTA20 construct that the niiA promoter element(s) may direct the expression of the lacZ gene from the new start site. Formal proof of divergent transcription in the niaD-niiA intergenic region would require the two intact promoters to be fused to the lacZ gene (although, now, this is self evident from the sequence data). Ideally the titration effect of nirA seen in Tnap3 (section IV.5) should be repeated using an intact promoter (to show that titration of nirA is not an artifact of the promoter deriviative). Further functional analyses of the promoter region should reveal information of the structure and function of the niaD-niiA intergenic region.

VI.2 Future Prospects

With the cloning of the nitrate gene cluster and the areA gene and the nirA gene questions about the mechanism of gene regulation of the nitrate gene cluster can begin to be answered. Isolation of the regulatory proteins should be possible and their sites of action determined by gel retardation and DNaseI footprinting experiments. Complementary approaches such as deletion mapping the promoter regions of the structural genes for functional activity and constructing in vitro mutations may also be employed. At present DNaseI hypersensitivity experiments on the niiA-niaD intergenic region are being carried out by Paul Montague in this laboratory. From

sequence data (see section VI.1) comparison of the upstream regions of the structural genes in the nitrate gene cluster may reveal sites of homology. Whether gene regulation at a distance occurs in this system is unclear, although several models (albeit mainly prokaryotes) have been proposed (Ptashne 1986a). With all the molecular genetic techniques available, it should prove possible to determine the promoter structure at the functional level.

Particularly interesting would be to see if the nitrate reductase enzyme is able to bind the nirA gene product (and thus prevent niaD and niiA expression). The possibility of the nirA gene being regulated by the areA gene product can be tested by Northern blotting experiments. That a pathway specific regulatory gene may be regulated in turn by a wide domain regulatory gene has been suggested for the alcR gene under carbon catabolite repression (Lockington et al. 1987), and this might also explain the phenotype of the $nirA^{d}$ allele. Another problem to be solved is how the nirA and areA proteins are able to activate transcription. One might speculate that the nirA protein is able to interact with the RNA polymerase (or a transcription factor) in order to effect transcription of the structural genes. Experiments like those performed with the yeast GAL4 protein involving deletion mapping of the transcription activating domains may prove possible in this regard (Keegan et al. 1986; Brent and Ptashne 1985).

In conclusion, it can be seen that with the advent of gene cloning and recombinant DNA technology, the enormous wealth of A. nidulans genetics can be exploited to the full. The isolation of all the components of a regulatory circuit should enable gene expression in this fungus to be elucidated.

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