DNA-mediated transformation of the filamentous fungus *Aspergillus nidulans*

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LANDBOUWCENTRUM
WAGENINGEN

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V

Bij transformatie van dierlijke cellen, plantecellen en schimmels, met uitzondering van gist, is integratie van het transformerende DNA in het genoom via niet-homologe recombinatie veeleer regel dan uitzondering.

VI

Het isoleren van pyrroloquinoline quinon als groei-stimulerende factor uit cultuurmedium van Escherichia coli is niet toe te schrijven aan productie van PQQ door bovengenoemde bacterie doch aan het gebruik van onzorgvuldig gewassen glaswerk door de auteurs.

Ameyama M et al. (1984) Agric Biol Chem 48: 3099-3107.

VII

Het verwerven van goedkeuring voor het gebruik van Aspergillus nidulans als industrieel productie-organisme zou een alternatief kunnen zijn voor het m.b.v. recombinant DNA technieken "veredelen" van reeds in gebruik zijnde Aspergillus productiestammen.

VIII

Het aanstellen van afgestudeerden met a.i.o. (achterstand in onderwijs) in de functie van a.i.o. (assistent in opleiding) zal bij het huidige universitaire beleid leiden tot a.i.o. (achterstand in onderzoek).

Stellingen bij het proefschrift van K. Wernars: "DNA-mediated transformation of the filamentous fungus Aspergillus nidulans". Wageningen, 2 december 1986.

STELLINGEN

I

De toepasbaarheid van ansl bevattende vectoren voor de constructie van genenbanken in Aspergillus nidulans is minder algemeen dan verondersteld door Ballance en Turner.

Ballance D J, Turner G (1985) Gene 36: 321-331.
Dit proefschrift.

II

Het verlies van het transformante fenotype tijdens de parasexuele analyse van Aspergillus nidulans Pyr^+ transformanten had waarschijnlijk voorkomen kunnen worden door gebruik te maken van een Pyr^- testerstam.

Ballance D J, Turner G (1985) Gene 36: 321-331.
Dit proefschrift.

III

De resultaten van Barnes en MacDonald pleiten eerder tegen dan vóór de aanwezigheid van autonoom replicerende vectoren in Aspergillus nidulans transformanten.

Barnes D E, MacDonald D W (1986) Curr Genet 10: 767-775.

IV

Het causale verband tussen U.V.-bestraling van muizecellen en amplificatie van een daarin aanwezig viraal TK gen wordt door de experimenten van de Vries et al. niet aangetoond.

de Vries P J et al. (1984) Somat Cell Mol Genet 10: 625-632.

Aan Rita, Kareltje en Elize

Aan mijn ouders

Voorwoord

Hoewel er slechts een naam voorop dit proefschrift staat is het een werkstuk waaraan velen hun medewerking hebben gegeven en diegenen wil ik hier graag noemen.

Henk van den Broek droeg zorg voor de dagelijkse begeleiding van het werk. Ik ben je zeer erkentelijk voor de prettige manier waarop je dat gedaan hebt en voor de steun die je mij hebt gegeven bij het oplossen van de wetenschappelijke problemen.

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Een woord van dank aan de vele doctoraal studenten die de afgelopen jaren hebben meegedraaid in het onderzoek is zeker op zijn plaats.

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Alle leden van de vakgroep Erfelijkheidsleer dank ik voor hun hulp en de prettige contacten die mijn promotietijd maakten tot een periode waarop ik met plezier terug kijk.

Het beschreven onderzoek kon tot stand komen mede door financiele steun van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO) te Den Haag.

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CHAPTER 1

General introduction

The genus Aspergillus

General aspects

Aspergilli, are a group of fungi named after the resemblance of their vegetative spore-forming structures to an aspergillum (a holy-water sprinkler). These fungi develop branched filamentous structures called hyphae, consisting of multinucleate cells with a rigid cell wall. Pored septa (cross-walls between the cells) allow migration of nuclei and cytoplasm. Formation of conidiospores (vegetative spores) takes place on top of conidiophores bearing specialized cells. The Aspergillus species share mutual morphological characteristics, but great differences in life cycle and physiology are observed. Some Aspergillus species (e.g. A. nidulans) have a known sexual stage and belong to the Ascomycetes. The official genus name is Emericella. Other Aspergilli (e.g. A. niger, A. oryzae) have only a vegetative life cycle and belong to the Fungi imperfecti. Because the Aspergilli are well known, the imperfect genus names are still used for species with a perfect stage.

From a human point of view some Aspergillus species possess disagreeable properties whereas others can be very advantageous. A. fumigatus for example is notorious for causing aspergillosis whereas A. flavus is feared for its excretion of very harmful toxins (Bennett 1980; Edwards and Al-Zubaidy 1977). On the other hand, A. niger and A. oryzae are of economic importance for their ability to produce several organic acids and enzymes on industrial scale (Berry et al. 1977; Jakubowska 1977; Barbesgaard 1977). Applications of recent developments like specific hydroxylation of

progesterone by A. ochraceus (Madyastha et al. 1984) or biodegradation of halogenated aromatic hydrocarbons by A. niger (Shailubhai et al. 1984) are very promising.

Genetics of Aspergillus nidulans

a) Life cycle

Aspergillus nidulans (Eidam) Winter is an ascomycete, capable of reproducing both vegetatively and sexually (Figure 1). In the vegetative growth cycle, the mycelium forms vertical branches (conidiophores) on which long chains of uninucleate asexual spores (conidia) develop, each from one uninucleate sterigma. Each conidium can give rise to a new colony, thus completing the cycle. Under optimized conditions the vegetative cycle takes only two days. Conidia have shown to be convenient starting material to study mutations per se and to obtain mutant strains by using irradiation (X-rays, U.V.) or chemical mutagens (Pontecorvo et al. 1953; Alderson and Heartly 1969; Bos 1985).

Sexual reproduction takes place within specialized structures. It starts with the differentiation of mycelium into a branched ascogonium, which consists of binucleate cells. The tissue is surrounded by a dense hyphal structure and the ascocarp is called a cleistothecium. In the top cells of the ascogonium karyogamy (nuclear fusion) takes place yielding diploid zygotes, which enter meiosis followed by a mitotic division. Thus each zygote produces eight haploid cells. After another mitosis the ascus contains eight binucleate cells which develop into ascospores. Germination of these spores results in the formation of a normal haploid mycelium. A. nidulans can complete the generative cycle in about 10 days.

Figure 1: Life cycle of Aspergillus nidulans (Eidam) Winter.
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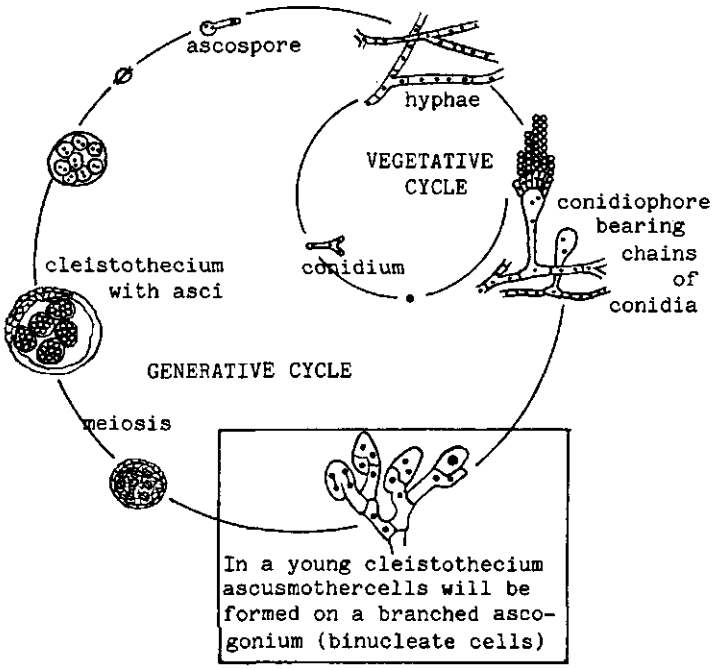


b) Heterokaryosis

Between the hyphae of A. nidulans strains anastomoses (fusions) can occur allowing exchange of nuclei. By this process mycelium containing nuclei of different genotype (heterokaryon) can arise. Heterokaryons can be selected and maintained if selective pressure is applied. For two strains with complementary auxotrophic properties the heterokaryotic association can be maintained by culturing on medium lacking the supplements required by the parental strain. Since is general conidiospores of A. nidulans are mononucleate they cannot be used for the propagation of heterokaryons.

Laboratory strains of A. nidulans usually form heterokaryons readily; in contrast between wild type strains heterokaryon incompatibility can be encountered (Grindle 1963a, b).

c) Sexual analysis

Since A. nidulans is homothallic (no mating types) strains can easily be crossed by the isolation of cleistothecia from a heterokaryon. Since cleistothecia are formed in general as the result of a single fertilization event, all asci within it will usually be of the same origin. Thus a cleistothecium of a heterokaryon will either contain ascospores of one parental type if "selfing" has occurred, or will segregate for different alleles of genes in both parents if "cross fertilization" has taken place. Using strains with a different colour of the conidiospores these hybrid cleistothecia can be recognized easily from the resulting colonies (Pontecorvo et al. 1953).

In the diploid zygotes normal meiotic crossing-over takes place. From the intrachromosomal recombination frequencies gene maps for each of the A. nidulans chromosomes have been constructed. Recently, an updated description of known loci and a linkage map was published by Clutterbuck (1984).

d) Parasexual analysis

Occasional fusion of two nuclei in vegetative mycelial cells can result in the formation of diploid nuclei and a sterigma containing such a nucleus will yield a chain of diploid conidia. Conidia heterozygous for different auxotrophic markers can be identified by the ability of the resulting colonies to grow on a medium lacking the supplements required by either parent. The frequency of these heterozygous diploid conidia on a heterokaryon is about 10^{-6}.

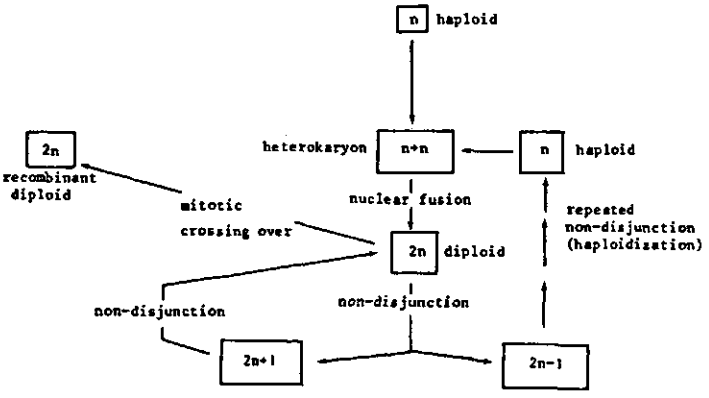
A. nidulans diploids are not completely stable and can "break up" to give haploid strains (Pontecorvo et al. 1954). This phenomenon is based on irregular segregation (non-disjunction) of chromatids during mitosis, estimated to occur about once in 50 nuclear

divisions (Käfer 1977). The resulting daughter cells are aneuploid (2n+1 and 2n-1) and develop abnormally due to genome imbalance. The 2n+1 aneuploid can regain normal growth by loss of a single chromosome to give the original diploid number (2n). In the 2n-1 strain sequential loss of other chromosomes by non-disjunction will produce the normal haploid chromosome number (n); this process is known as haploidization. The frequency of haploidization can be enhanced by arsenate (van Arkel 1963), p-fluorphenylalanine (McCully and Forbes 1965), benlate (Hastie 1970) or chloralhydrate (Singh and Sinha 1976).

In diploid nuclei mitotic crossing-over may occur, however its frequency is low and consequently, following haploidization, in general not more than one cross-over event is detected in about 200 haploids (Pontecorvo and Käfer 1958).

The alternation of haploid and diploid stages in the absence of meiosis is known as the parasexual cycle (Pontecorvo 1956). Features of this cycle, which are summarized in Figure 2, have been found in many other fungi besides A. nidulans (Roper 1966).

Figure 2: The para-sexual cycle



The parasexual cycle is a convenient tool for genetic analysis. Compared to the meiotic process mitotic recombination between homologous chromosomes occurs with low frequency, i.e. during haploidization preponderantly non-recombinant chromosomes reassort. Haploidization of a diploid obtained by combination of a strain carrying an unmapped mutation and a master strain (i.e. a strain having each chromosome marked with a mutant gene) allows assignment of this mutation to a particular chromosome (Pontecorvo 1956). Parasexual analysis also allows the detection of chromosomal translocations. A translocation in one of the component strains of a diploid will cause linked segregation of the two chromosomes involved during haploidization due to inviability of segregants carrying duplications or deletions (Käfer 1962). Other applications of the parasexual cycle are the study of mitotic recombination per se, gene mapping and centromere localization (for a review see Clutterbuck 1974).

Genetic transformation

a) History

In 1928 Griffith observed that mice, injected with a mixture of heat-killed cells of a virulent Diplococcus pneumoniae strain and living non-virulent cells of the same bacterial species, frequently succumbed to infection, and that the blood of these animals contained living virulent Diplococcus cells. The phenomenon was not observed after injection with either heat-killed cells of the virulent strain or living non-virulent cells. This observation may be considered as the discovery of genetic transformation although the driving force behind the change of non-virulent to virulent

cells remained elusive. A few years later Dawson and Sia (1931) succeeded in inducing this transformation in vitro.

Although in the fourties already valid experimental evidence was given that DNA is the substance that brings about genetic transformation in bacteria (Avery et al 1943; McCarty and Avery 1946) many scientists at that time were reluctant to accept the view that DNA is the carrier of genetic transformation until Hershey and Chase (1952) demonstrated that when a bacterial virus infects a bacterium the DNA enters the cell whereas most of the protein remains outside. Genetic transformation was then defined as the transfer of a heritable character by purified DNA.

Since then, further development of the chemistry of DNA, its molecular structure and the unraveling of genetic processes (for an overview see Portugal and Cohen 1977) have established the process of genetic transformation as a method to transfer genetic information, at first only among bacteria.

The first evidence for genetic transformation in eukaryotic organisms was provided by McBride and Ozer (1973). From that time on genetic transformation has been applied to animal cells, plant cells and fungi. Some of these eukaryotic systems for gene transfer will be briefly reviewed here.

b) Gene transfer to animal cells.

Several procedures are available now for the introduction of transforming DNA into animal host cells. Recombinant DNA molecules of viral origin can enter cultured host cells by either infection (using an infectious particle) or by transformation/transfection using naked DNA molecules. Transformation occurs at very low frequencies but it can be increased drastically by coprecipitating

the cells and the transforming DNA with calcium phosphate (Graham and van der Eb 1973), resulting in the uptake of exogenous DNA by about 20% of the cells (Chu and Sharp 1981). Klebe et al. (1981) demonstrated that polyethylene glycol can induce a similar effect.

Introduction of exogenous DNA by micro-injection into the nucleus of cultured cells is also possible (e.g. Kondoh et al. 1983). This technique can in addition be used for gene transfer to undifferentiated cells like oocytes, eggs and embryos. A well known result using this mode of gene transfer is the generation of "super mice" from fertilized egg cells micro-injected with an, in vitro altered, rat growth hormone gene (Palmiter et al. 1982).

Another method of DNA transfer makes use of electropermeabilization or electroporation. This technique applies an electrical pulse field to reversibly open up the cell membrane, allowing the transforming DNA to enter (Stopper et al. 1985; Toneguzzo et al. 1986). A method using a fine laser beam for the introduction of foreign DNA has recently been described by Kurata et al. (1986).

For the introduction of DNA into mammalian cells several selectable markers are available. The first marker to be used was the thymidine kinase gene of Herpes simplex virus. Upon transformation, TK^+ cells can be selected on a suitable medium (Wigler et al. 1977), however the use of TK^- recipient cells is a prerequisite. Other cloned genes, being used as selectable marker, have either a (semi) dominant character or are hemizygous: the dihydrofolate reductase (DHFR) gene (Wigler et al. 1980; O'Hare et al. 1981), the CAD gene coding for the multifunctional enzyme carbamyl phosphate synthetase: aspartate transcarbamylase: dihydroorotase (de Saint Vincent et al. 1981), the xantine-guanine phosphoribosyl transferase (XGPRT) gene (Mulligan and Berg 1981), the neomycine phosphotransferase gene (Colbère-Garapin 1981) and the hygromycin

B phospotransferase gene (Blochhinger and Diggelmann 1984).

An important observation from Wigler et al. (1979) was that DNA without a selectable marker can be introduced into mammalian cells by cotransformation with a selectable vector.

Cloned genes not linked to a functional (eukaryotic) origin of replication, will integrate into the genomic DNA of the recipient without showing any site specificity (Robbins et al. 1981, Steele et al. 1984). Rubnitz and Subramani (1984) have shown that the process of homologous recombination in mammalian cells requires only very short stretches of sequence homology.

Many integrating vectors harbouring a drug resistance gene, can be amplified by gradual increase of the selective pressure giving concomitant amplification of unselected vector sequences (for a review see Stark and Wahl 1984). Using viral origins of replication from SV40 and bovine papilloma virus (BPV), (shuttle) vectors have been constructed which are able to replicate autonomously in mammalian cells (e.g. Lusky and Botchan 1981; Law et al. 1983).

c) Gene transfer to plant cells.

For the introduction of exogenous DNA into plant cells several techniques can be used now. The most advanced system employs the soil bacterium Agrobacterium tumefaciens. This prokaryote has evolved a natural system for the genetic engeneering of plant cells. Virulent A. tumefaciens strains harbour large plasmids (Ti-plasmids). Upon infection a section of these plasmids (T-DNA) is transferred to the plant cell and becomes integrated in the nuclear DNA. This gives the cell tumorous properties: a callus-like growth and production of opines. These opines can be used by the A. tumefaciens bacteria as sole carbon- and nitrogen source (for

review see van Montagu and Schell 1982).

For the transfer of foreign DNA into plant cells with the help of A. tumefaciens bacteria, vectors can be used which are derived from these Ti-plasmids. Genes cloned within the T-DNA region integrate into the plant genome together with this region (de Framond et al. 1983; Hoekema et al. 1983; Herrera-Estrella et al. 1983).

A. tumefaciens provides one way for overcoming the cell wall as an obstacle for the delivery of DNA into the nucleus. Another way to circumvent the cell wall barrier is the use of protoplasts which often appear to be totipotent (Davey 1983). An in vitro system for transforming these protoplasts with A. tumefaciens has been developed (Marton et al. 1979). Protoplasts can also be transformed by coprecipitation with the naked Ti-plasmid DNA (Krens et al. 1982).

The Ti-system can now be used for routine integration of foreign genes into plant nuclear DNA and their transfer to sexual offspring (Zambryski et al. 1984). A general drawback of this system is its host range limitation.

Another method for gene transfer into plants makes use of DNA plant virusses as vectors. Brisson et al. (1984) have described such a system using the Cauliflower Mosaic Virus as a cloning vector for the bacterial DHFR-gene. This system has also a limited host range. Furthermore, it is hampered by restrictions of the size of the cloned gene and the lack of transfer of the cloned sequence to sexual offspring.

Plant protoplasts can be used as recipient for direct gene transfer via naked DNA which subsequently integrates into the genomic DNA. This DNA can be introduced either by micro-injection (Crossway et al. 1986) or by a calciumphosphate/PEG treatment (Paszkowski et al. 1984). Transformants cannot be selected by

their ability to grow *in vitro* in the absence of growth hormones as in the case of Ti-transformed cells. Recently, selectable genes have been constructed by linking plant promotors to bacterial genes such as the neomycine phosphotransferase gene, the dihydrofolate reductase gene and the chloramphemicol acetyltransferase gene, all conferring resistance when expressed in plants (Bevan et al. 1983; Herrera-Estrella et al. 1983; Horsch et al. 1984). The integration of the DNA appears to be not site-directed; it usually occurs in a tandem array of several copies, which sometimes show mitotic and/or meiotic instability (Paszkowski et al. 1984; Potrykus et al. 1985 a, b and c; Hain et al. 1985; Jongsma et al. 1986). A great advantage of the direct gene transfer method is the absence of host range limitations (Potrykus et al. 1985 b; Lörz et al. 1985). However, the latter procedure depends on the production of regenerable protoplasts which can sometimes be problematic.

d) Transformation of fungi

- *Saccharomyces cerevisiae*

In 1978 Hinnen et al. successfully transformed the yeast *Saccharomyces cerevisiae* with a chimaeric plasmid. Protoplasts of a $Leu2^-$ strain were incubated with an *E. coli* vector containing the cloned yeast *leu2* gene in the presence of PEG and $CaCl_2$ and regenerated on medium lacking leucine. The transformants were shown to have integrated the transforming vector into the genomic DNA, mostly one copy at the homologous *leu2* locus. The discovery that many yeast genes are able to complement mutations in *E. coli* (e.g. *trp1*, *his3*, *arg4*, *ura3*) subsequently increased the number of marker genes for yeast transformation (Clarke and Carbon 1978; Davis et al. 1979; Petes et al. 1978). The type of vector used by

Hinnen et al. (1978) belongs to the class of YIp-vectors (yeast integrative plasmid), which are unable to replicate autonomously in yeast, can only be maintained by integration into the yeast genomic DNA and give rise to 1-10 transformants/µg DNA. Once integrated the cloned gene is transmitted stably and shows Mendelian segregation.

Integration of transforming plasmid DNA into the yeast genomic DNA almost exclusively takes place by homologous recombination (Szostak and Wu 1979; Orr-Weaver et al 1981). This phenomenon allows precise targeting of integration events and can therefore be used to introduce deletions, gene disruptions and gene replacements by transformation (Scherer and Davis 1979; Botstein and Davis 1982; Stuhl 1983 a; Rudolf et al. 1985).

Soon after the development of YIp-vectors chimaeric plasmids were constructed which were able to replicate both in E. coli and in yeast and they were called YEp's (yeast episomal plasmids). These are derived from the 2 µ plasmid, naturally occurring in many Saccharomyces strains and replicating under nuclear control (Broach 1982). Combination of 2 µ DNA and E. coli plasmids yielded vectors which had high copy numbers in yeast. They transformed at fairly high frequencies (10^3-10^5 yeast transformants/µg DNA) (Beggs 1978, Gerbaud et al. 1979, Struhl et al. 1979), thus allowing direct selection of cloned genes in yeast. A disadvantage of these vectors is their tendency to give new recombinants by in vivo recombination with endogenous 2 µ plasmids in the recipient Saccharomyces strains.

Another class of autonomously replicating vectors are YRp's (yeast replicator plasmids), containing a fragment of chromosomal yeast DNA conferring autonomous vector replication (ars) (Struhl et al. 1979; Kingsman et al. 1979). Although these plasmids transform yeast very efficiently and are present in 5-40 copies/cell,

the resulting transformants are rather unstable. With a low frequency stable transformants arise (10^{-5}/generation) due to homologous integration of the YRp-vector into the genomic DNA (Stinchcomb et al. 1979; Nasmyth and Reed 1980).

By insertion of a functional centromere region (CEN) of yeast into an YRp-vector, plasmids can be obtained with behave very simular to yeast chromosomes (Clarke and Carbon 1980; Hsiao and Carbon 1981; Stinchcomb et al 1982; Fitzgerald-Hayes et al 1982). These circular mini-chromosomes, which are present in one copy per nucleus, are lost at a frequency of only 10^{-2} per generation and about 90% of the cells entering meiosis with the correct number of plasmids, show proper disjunction (Clarke and Carbon 1980; Fitzgerald-Hayes et al 1982 a). By addition of telomeric sequences such a vector is able to replicate in a linear form (Szostak and Blackburn 1982; Murray and Szostak 1983). Small constructs were found to be rather unstable but larger ones (50-150 kb) behaved like naturally occurring chromosomes.

So a wide variety of yeast vector classes, each with specific properties concerning mode of replication, copy number, transforming frequency etc. is available for yeast transformation. They are useful to study very different topics, e.g. gene structure and function, effects of gene dosage, gene regulation and expression of foreign cloned genes. These applications and several others are reviewed by Mishra (1985).

The discovery that several bacterial genes conferring antibiotic resistance, e.g. chloramphenicol acetyl transferase and aminoglycoside phosphotransferase, are expressed in yeast (Cohen et al. 1980; Jiminez and Davies 1980) has allowed the construction of vectors carrying these genes as dominant selection markers for transformation especially of non-mutant strains. The transformation procedure has been simplified by the observation that intact

yeast cells can be transformed with exogenous DNA after treatment with lithium acetate (Ito et al. 1983) making the use of (osmotically labile) protoplasts unnecessary. At present the yeast transformation system is the most advanced one among all eukaryotic transformation systems developed; it has evolved into a cloning system suitable for the study of many basic and applied problems in molecular genetics.

- Neurospora crassa

N. crassa was the first filamentous fungus serving as a recipient for transformation using a chimaeric plasmid. Case et al. (1979) were able to transform a qa2 deficient strain with a vector containing the wild type gene.

All of the transformants examined showed integration of the vector into the genomic DNA, about 40% at loci different from the resident qa2 locus. Others (e.g. Bull and Wootton 1984) have found that integration of transforming plasmids into the genome can occur in long arrays of tandemly repeated vector copies.

Meanwhile, some other cloned genes like am (Kinsey and Rambosek, 1984) and pyr4 (Buxton and Radford, 1983) have been used as selection markers for N. crassa transformation. As reported for yeast (Ito et al. 1983) intact Neurospora cells i.e. germinating conidiospores, can efficiently be transformed after treatment with lithium acetate (Dhawale et al. 1984), which simplifies Neurospora transformation.

In 1983 Stohl and Lambowitz have constructed a vector consisting of plasmid pBR325, the qa2 gene and a mitochondrial plasmid. This construct could replicate in E. coli and could be reisolated via E. coli from undigested DNA of N. crassa $qa2^{+}$ transformants. Further analysis showed that loss of the mitochondrial sequences from this plasmid did not abolish the presence of free

circular vector molecules in N. crassa transformants (Stohl et al. 1984). These authors favour the idea that these free plasmids are maintained in N. crassa by autonomous replication. Recombinant plasmids containing the am gene were also claimed to replicate autonomously (Grant et al. 1984). Other attempts to construct vectors, autonomously replicating in N. crassa were made by Buxton and Radford (1984) and Paietta and Marzluf (1985).

All Neurospora vectors developed as yet, even the ones claimed to be autonomously replicating, are able to integrate into the genomic DNA and frequently do so. Therefore conclusive evidence for autonomous replication of plasmids in N. crassa has not yet been given.

- Other filamentous fungi

Concomitantly with the developments in the field of transformation of A. nidulans (subject of this thesis) and in part thanks to them, direct gene transfer has been achieved recently in several other filamentous fungi. In Podospora anserina transformation has been demonstrated using a chimaeric plasmid consisting of bacterial DNA and Podospora mitochondrial DNA. This plasmid replicated autonomously in this fungus and expressed the Podospora senescence trait (Stahl et al. 1982). A chromosomally integrating vector containing the ura5 gene as selection marker was applied by Begueret and coworkers (Begueret et al. 1984; Razanamparany and Begueret 1986). A very sophisticated system was recently developed by Brygoo and Debuchy (1985); these authors selected P. anserina transformants using chimaeric vectors containing cloned tRNA suppressors.

The cloned amdS gene from A. nidulans (Hynes et al. 1983) can serve as a selection marker in other fungi as was demonstrated in Cochliobolus heterostrophus (Turgeon et al. 1985). Moreover this

gene can be used to transform the industrially important A. niger (Kelly and Hynes 1985). Recently another A. nidulans gene, argB, was shown to be useful as selection marker in A. niger too (Buxton et al. 1985).

Aim and outline of our investigations

The aim of the work presented here was to develop a genetically well-marked, preferentially homologous DNA vector system for the fungus Aspergillus. Such a system should provide means for the genetic manipulation of this fungus using recombinant DNA techniques.

The species A. nidulans has been a subject of genetic studies for thirty years and thus has become a genetically well-characterized eukaryotic organism. In the sixties A. nidulans was also recognized as convenient host for biochemical and developmental investigations at the molecular level. For these reasons the species A. nidulans was chosen for the transformation studies described here and the results obtained should be the basis for the use of recombinant DNA techniques in the genetic manipulation of other, biotechnologically important Aspergillus species like A. niger.

At the time these studies were started the possibility of DNA mediated genetic transformation of A. nidulans was yet to be demonstrated. Although protoplasts could be prepared in fairly high amounts and regenerated on osmotically stabilized media, the

absence of useful selection markers hampered the transformation studies. Efforts initiated by us and by others to select A. nidulans genes in E. coli via complementation were, at that time, unsuccessful.

Extensive screening of wild type Aspergillus strains did not result in the detection of free endogenous plasmid structures to be used in vector constructions. Due to the fact that A. nidulans in general is insensitive to antibiotics, even at high concentrations, bacterial vectors containing drug resistance genes could not be used as selection markers in A. nidulans transformation.

However, the cloning by other groups of the A. nidulans amdS and trpC genes and the N. crassa pyr4 gene in 1982 and 1983 has eventually allowed successful transformation of A. nidulans. The intensive and competitive efforts of several Aspergillus research teams around the world have now made A. nidulans to an attractive eukaryotic host for the study of transformation and for performing a variety of gene cloning experiments.

The following chapters describe the experiments we have performed in this respect, partly in close collaboration with the Recombinant DNA Research Group of the Medical Biological Laboratory TNO at Rijswijk (The Netherlands).

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CHAPTER 2

Gene amplification in *Aspergillus nidulans* by transformation with vectors containing the *amdS* gene

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Summary. Conidial protoplasts of an *A. nidulans amdS* deletion strain (MH1277) have been transformed to the AmdS$^+$ phenotype with a plasmid carrying the wild type gene (p3SR2). Optimalisation of transformation and plating conditions now has resulted in frequencies of 300–400 transformants per μg of DNA.

Analysis of DNA from AmdS$^+$ transformants of MH1277 showed that transformation had occurred by integration of vector DNA sequences into the genome. In virtually all these transformants multiple copies of the vector were present in a tandemly repeated fashion, not preferentially at the resident, partially deleted *amdS* gene. It is suggested that the observed integration phenomena are dependent on the genetic background of the *A. nidulans* strain, used for transformation. A model to explain the tandem type of integration is proposed.

Key words: Transformation of Aspergillus – Conidial protoplasts – Multicopy/tandem integration – Gene amplification

Introduction

Fungi play an important role in chemical industry. They are used in fermentation processes as well as for the production of antibiotics and different metabolites. Several of the biotechnologically important species belong to the genus *Aspergillus*. Well known examples are *A. niger* and *A. oryzae* which are used for the production of organic acids and enzymes (Berry et al. 1977; Jakubowska 1977; Barbesgaard 1977). The development of methods for in vitro genetic manipulations might be exploited for a more direct and efficient improvement of Aspergilli. For the cloning and expression of eukaryotic genes in *Aspergillus* a suitable DNA vector system has to be developed. In this context we have studied the aspects of transformation, using the genetically well marked, but industrially unimportant species *A. nidulans* as a model system.

For this organism effective methods have been developed in our laboratory for the production of large quantities of protoplasts from both hyphae and conidiospores (van den Broek et al. 1979; Bos and Slakhorst 1981). Especially the conidial protoplasts were thought to be a very useful starting material for transformation experiments.

A. nidulans is insensitive to most antibiotics. Even the aminoglycoside G418 and hygromycin B, which is applicable to yeast and higher eukaryotes (Jimenez and Davies 1980; Colbère-Garapin et al. 1981; Gritz and Davies 1983) does not sufficiently inhibit growth of *A. nidulans*. We therefore were not able to use resistance genes of bacterial origin for the selection of transformants. Instead we used the *A. nidulans amdS* gene as a selection marker in our transformation studies. The *amdS* gene has been studied by Hynes and coworkers (Hynes and Pateman, 1970; Hynes 1982) and recently the gene has been isolated, cloned and characterised (Hynes et al. 1983). The successful use of this gene as a selection marker in the transformation of mycelial protoplasts, was recently reported by Tilburn et al. (1983).

In this paper we describe the transformation of conidial protoplasts of *amdS* deletion strains using various plasmids with the cloned wild type *amdS* gene. A model explaining the results is presented.

Abbrevations: bp, base pairs; kb, 1,000 bp; EtBr, ethidiumbromide; PEG, polyethyleneglycol; r-DNA, ribosomal DNA; c.f.u., colony forming units

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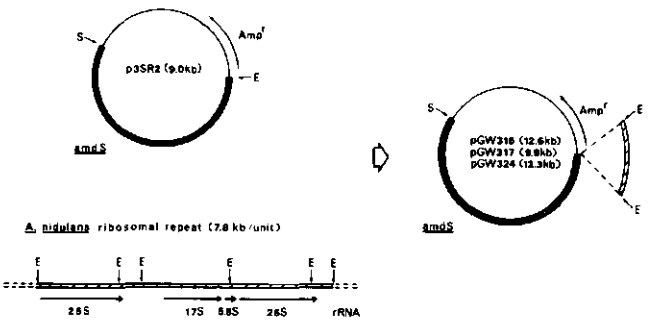


Fig. 1. Schematic drawing of the plasmids p3SR2, pGW315, pGW317, pGW324 and the *A. nidulans* ribosomal repeat unit. Plasmid p3SR2 is a pBR322 derivative (Bolivar et al. 1977) in which the smaller *Eco*RI-*Sal*I fragment has been replaced by a 5.2 kb *A. nidulans* DNA fragment, containing the complete wild type *amdS* gene (Hynes et al. 1983). Plasmids pGW315, pGW317 and pGW324 were constructed by inserting three *Eco*RI fragments (3.6, 0.9 and 3.3 kb respectively), constituting the *A. nidulans* ribosomal repeat unit, into the *Eco*RI restriction site of p3SR2. These r-DNA fragments were derived from plasmid pMN1 (Borsuk et al. 1982). *E* = *Eco*RI restriction site, *S* = *Sal*I restriction site

Materials and methods

Chemicals and enzymes. Acetamide and L-proline were purchased from Sigma, acrylamide (gel electrophoresis quality) from Serva. Polyethyleneglycol (PEG) was from Merck, KochLight and BDH. For selective medium Oxoid purified agar was washed with cold, distilled water followed by washing in 96% ethanol and subsequent drying at 65 °C before use. [α-^{32}P]dATP was from New England Nuclear. Blots were made on Schleicher & Schüll BA85 nitrocellulose. Restriction endonucleases were purchased from Boehringer Mannheim, T4-DNA ligase and DNA polymerase from New England Biolabs. All other chemicals used were of analytical grade.

Strains. As recipients for transformation the *A. nidulans* strains MH1277 (*biA*1, *amdS*320, *amdI*18, *amdA*7, *niiA*4; Hynes et al. 1983), WG290 (a *yA*2, *pantoB*100-derivative of MH1277; Tilburn et al. 1983) and MH1354 (*biA*1, *amdS*368, *amdA*7, *niiA*4; Hynes et al. 1983) were used.

Plasmids were propagated in *E. coli* K12 strain JA221.

Media and growth conditions. For the preparation of conidiospores, the *A. nidulans* recipients were grown on agar-solidified complete medium (Pontecorvo et al. 1953) without nitrate, containing 3.7 g NH_4 Cl/l as nitrogen source, supplemented with 15 nM D(+)biotin and 8 μM pantothenic acid. Plates were inoculated with approximately 1×10^3 conidiospores and incubated for 3 days at 37 °C.

AmdS$^+$ transformants were selected on minimal medium (Pontecorve et al. 1953), in which nitrate was replaced by 10 mM acetamide as sole nitrogen source, 15 mM CsCl, 1.0 M sucrose, 15 nM D(+)biotin and 8 μM pantothenic acid, solidified with 1.2% agar. In non-selective medium the acetamide/CsCl was replaced by 10 mM L-proline. Transformants were propagated on the selective medium described above, in which 1.0 M sucrose was replaced by 50 mM glucose.

For the preparation of mycelium *A. nidulans* strains were grown in non-selective minimal medium containing 50 mM glucose. One liter flasks, containing 250 ml of medium were inoculated with 10^6 conidiospores/ml and incubated for 18 h in a New Brunswick G25 orbital shaker at 37 °C (300 rpm). Mycelium was harvested by filtration, washed with distilled water, blotted dry and frozen immediately in liquid nitrogen. It was either used directly for DNA isolation or stored at −80 °C until use.

E. coli was grown in L-broth medium supplemented with the appropriate antibiotics.

Plasmids. Plasmid p3SR2 is described in Fig. 1. The plasmids pGW315, pGW317 and pGW324 were constructed as explained in the legend of Fig. 1.

Plasmid DNA was extracted from *E. coli* cells using standard procedures (Birnboim and Doly 1979) and purified by two successive isopycnic centrifugations in CsCl/ethidiumbromide followed by Sepharose 6B column chromatography.

Preparation of protoplasts. For the preparation of conidial protoplasts the procedure described by Bos and Slakhorst (1981) was scaled up to convert 2×10^9 conidiospores into $1-1.5 \times 10^9$ viable protoplasts; the residual intact conidiospores (1–30%) were not removed. The conidial protoplast suspension was stored overnight in the lytic mixture at 4 °C.

Mycelial protoplasts were prepared according to van den Broek et al. (1979). Cellular debris was removed by centrifugation for 1 min at 1,000 x *g*. These protoplasts were used for transformation immediately after preparation.

Protoplasts were collected by centrifugation, washed twice with 1.0 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris (pH 7.5) and resuspended in the same buffer at a density of $0.5-1.0 \times 10^9$ protoplasts/ml.

Transformation of A. nidulans protoplasts. Protoplasts were transformed essentially as described by Tilburn et al. (1983) with minor modifications. A 100 μl aliquot of the protoplast suspension was mixed with an equal volume of the same buffer containing the vector DNA. Immediately thereafter the suspension was thoroughly mixed with 1 ml of a solution containing 60% (w/v) PEG 6000, 10 mM $CaCl_2$, 10 mM Tris (pH 7.5). After 20 min incubation at room temperature the protoplasts were collected by centrifugation (5 min, 12,000 x g) and resuspended in 400 μl 1.0 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris (pH 7.5). Aliquots of 100 μl were plated on selective medium using a 2 ml 0.25% agar overlay. The viability of the protoplasts was determined by plating appropriate dilutions on non-selective medium. Transformant colonies appeared after 24–36 h of incubation at 37 °C.

Isolation of A. nidulans DNA. DNA was isolated from 0.5–1.0 g liquid N_2 frozen mycelium as described by Yelton et al. (1984).

DNA manipulations. DNA was digested with restriction endonucleases according to the manufacturers instructions. Ligations were performed with T4-DNA ligase using standard procedures. DNA was fractionated on 0.6% agarose gels and transferred from the gels onto nitrocellulose according to Southern (1975).

Specific DNA fragments were isolated from gels using a glass powder-binding procedure (Vogelstein and Gillespie 1979).

Hybridisation probes were labelled by nick translation (Rigby et al. 1977) and hybridised with the blots at 68 °C for at least 24 h as described by Maniatis et al. (1982). After washing the blots were autoradiographed on Sakura X-ray film at −80 °C using Tungsten intensifying screens.

Results

Properties of AmdS$^+$ transformants

Transformants appearing on selective medium after transformation of MH1277 with p3SR2 show a wide variation in growth rate. Two classes of colonies can be distinguished:

- Type I, showing continuous growth and normal sporulation on acetamide,
- Type II, which are extremely small, never sporulate and do not show further development upon prolonged incubation.

In control experiments, in which no transforming DNA is added, colonies are never found. The Type II transformants cannot be considered to be abortive, as they can be rescued by conversion into Type I. This may be achieved by transferring Type II colonies to non-selective medium, followed by replating of conidiospores on selective medium. With at least 50% of the Type II colonies this results in the appearance of Type I, whereas Type II is no longer found. Although the Type I colonies, found after transformation, have a fairly homogeneous appearance (no sectors), initially a large variation is found in the fraction of conidiospores, possessing the AmdS$^+$ property; values ranging from 0.1% to virtually 100% are found. However, after this first subculturing step colonies are all homogeneous in the AmdS$^+$ property.

The individual Type I transformants differ greatly in growth rate on medium containing the non-inducing substrate acrylamide as sole nitrogen source and several (about 0.1%) of the AmdS$^+$ transformants have a colony morphology which differs from the parental type suggesting that transformation to the AmdS$^+$ phenotype is not an identical event in all individual transformants.

The stability of the AmdS$^+$ property through mitosis in two p3SR2-derived transformants of MH1277, ht6 and ht7, was investigated during subculturing for five successive growth cycles on nonselective medium. From each cycle none of the 400 colonies tested had lost the AmdS$^+$ property, suggesting that these p3SR2-derived transformants were mitotically stable. The stability of the same two transformants through meiosis was investigated by analysing selfed fruiting bodies. A relatively low viability of the ascospores was observed for both transformants and MH1277 (about 10% compared to a wild type strain). From both transformants 350 individual colonies, arisen from ascospores, were tested; all still contained the AmdS$^+$ property.

Transformation frequency

With 10 μg of p3SR2 in general 10–100 transformants of Type I and 10–500 of Type II were obtained, using conidial protoplasts. This frequency (1–50/μg) is comparable to that reported for *S. cerevisiae* or *N. crassa* using integrating vectors (Hinnen et al. 1978; Case et al. 1979). In the system used, the viability of the protoplasts after transformation is relatively high (40–50%). Both the transformation frequency and the ratio of Type I/Type II colonies varies between individual experiments. However, with aliquots of one batch of protoplasts always reproducible results have been obtained.

In contrast to the results for *S. cerevisiae* with linearised vectors (Orr-Weaver et al. 1981), linearisation of the vector p3SR2, either within or outside the *amdS* coding sequence, does not alter the transformation frequency.

Since the possibilities for direct cloning and selection of genes in *Aspergillus* depends on a high transformation frequency, many parameters which might affect this frequency (e.g. carrier DNA, incubation period, incubation temperature, PEG molecular weight, PEG concentration, addition of cations, purity of transforming DNA) have been studied. Only the purity of the vector DNA was a factor of great importance for efficient transformation. Best results were obtained with DNA, extensively purified by two cycles of CsCl/ethidiumbromide centrifugation, followed by Sepharose 6B column chromatography.

A non-linear relationship was observed between the number of transformants and the amount of p3SR2 used for transformation. A minimum of 2 μg of vector DNA was needed to obtain any transformant at all. The number of transformants increased almost exponentially with an increasing amount of p3SR2 DNA (up to 10 μg) and at higher concentrations this relation became linear. With more than 50 μg the frequency dropped, possibly caused by impurities in the DNA preparations (results not shown).

For our transformation system recently a correlation was found between transformation frequency and density of plating. In the standard procedure 10^8 protoplasts are transformed and plated at a density of 2.5×10^7 c.f.u./plate, normally resulting in a frequency of 50–70 transformants/μg of DNA. When 10^7 protoplasts are used for transformation and plated at 2.5×10^6 c.f.u./plate, the frequency drops about one order of magnitude. If, however, 2.5×10^6 incubated protoplasts are plated in the presence of 2.5×10^7 untreated conidiospores from the *amdS* deletion strain, the transformation frequency is at least 5 times higher than under the stan-

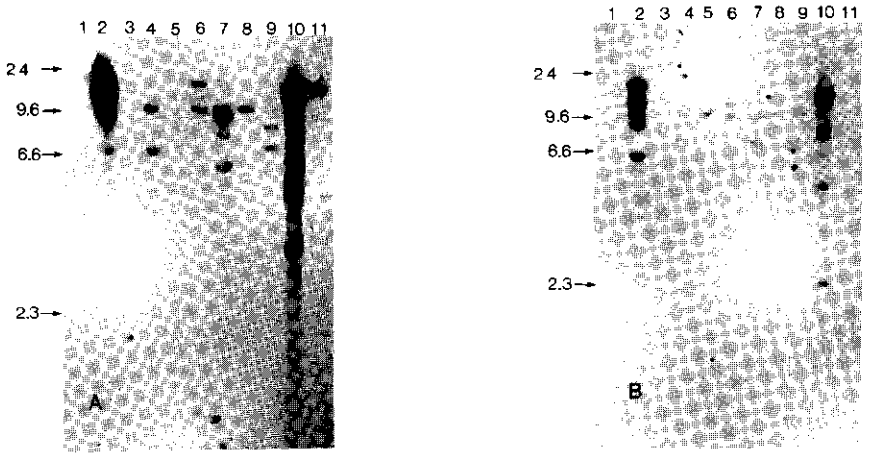


Fig. 2A, B. Analysis of DNA from AmdS$^+$ transformants obtained with conidial protoplasts of MH1277 and plasmid p3SR2. *A. nidulans* DNA (2–5 μg) was digested with *Eco*RI, fractionated on an agarose gel and transferred to nitrocellulose as described in Materials and methods. Blots were probed with ^{32}P-labelled *amdS*-fragment (i.e. the large *Eco*RI-*Sal*I fragment of p3SR2) (panel A) or ^{32}P-labelled plasmid pBR322 (panel B). *Lanes 1* and *11* contain DNA from the untransformed MH1277 strain and a wild type strain respectively; *lanes 2–4*, DNA from ct1–3 and *lanes 6–10*, DNA from ct4–8. *Lane 5* contains linearised p3SR2. *Arrows* indicate the position of molecular weight markers (kb)

dard condition. Under these optimised conditions frequencies of 300–400 transformants/μg of DNA can routinely be obtained.

Vector modifications

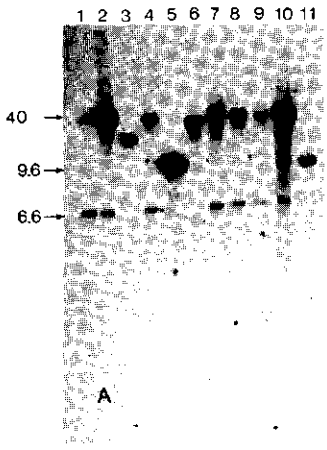
For *S. cerevisiae* it has been reported that an increased homology between the integrating vector and the chromosome results in an increase in transformation frequency (Szostak and Wu 1979). To increase homology of the *amdS* containing vector and the *A. nidulans* genome the three *Eco*RI fragments, together constituting the ribosomal repeat unit of *A. nidulans*, were cloned in p3SR2 (Fig. 1). The resulting plasmids were then used to transform *A. nidulans*. Irrespective the r-DNA fragment inserted, the transformation frequency was similar to that obtained with p3SR2. Also for these modified vectors, linearisation did not influence the frequency of transformation.

Biochemical analysis of AmdS$^+$ transformants

To investigate the state and location of the vector sequences used for transformation, DNA of a number of AmdS$^+$ transformants, obtained with p3SR2, has been analysed by Southern blotting and hybridisation with specific ^{32}P-labelled probes.

When Southern blots of undigested DNA from different transformants were probed with ^{32}P-labelled p3SR2, strong hybridisation signals were found in the region of the chromosomal DNA (results not shown). Even under conditions where 1 copy/10 *A. nidulans* nuclei could be detected, no indications for the presence of free plasmid DNA sequences were obtained from these experiments. This indicates that transformation probably has occurred by integration of the vector DNA into the *A. nidulans* genome.

To analyse the integrated vector sequences, DNA of eight AmdS$^+$ transformants (ct1–ct8) obtained with conidial MH1277 protoplasts and p3SR2, was digested with *Eco*RI prior to gel electrophoresis and blotting. The autoradiographs after hybridisation with the ^{32}P-labelled *amdS* fragment (i.e. the large *Eco*RI-*Sal*I fragment of p3SR2; Fig. 1) or pBR322 are shown in Fig. 2. The hybridisation patterns observed are, in general, very complex, with up to 21 distinct bands (ct8) hybridising to pBR322, the *amdS* fragment or both. Furthermore each individual transformant shows a different pattern. This may indicate multiple insertions of vector DNA into the genome. From this experiment it could not be established whether or not integration had occurred at the site of the resident, partially deleted, *amdS* gene since the *Eco*RI fragments from the wild type and the deletion strain, hybridising to the *amdS* probe, migrate very similarly on the gel (Fig. 3A, lanes 1 and 11).



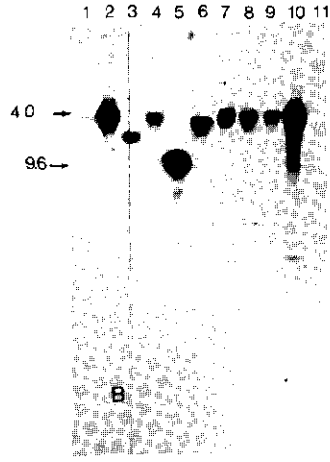
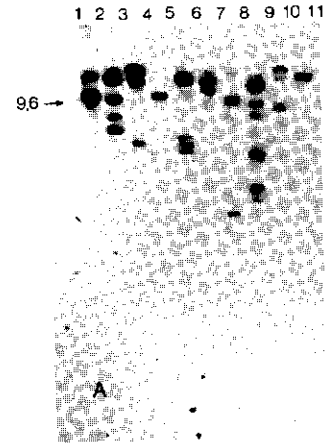


Fig. 3A, B. Analysis of DNA from AmdS$^+$ transformants obtained with conidial protoplasts of MH1277 and plasmid p3SR2. *A. nidulans* DNA (2–5 μg) was digested with *Xho*1, fractionated by agarose gel electrophoresis and transferred to nitrocellulose. Blots were probed with ^{32}P-labelled *amdS* fragment (panel A) or ^{32}P-labelled plasmid pBR322 (panel B). *Lanes 1* and *11* contain DNA from the untransformed MH1277 strain and a wild type strain respectively; *lanes 2–4*, DNA from ct1–3 and *lanes 6–10*, DNA from ct4–8. *Lane 5* contains linearised p3SR2. *Arrows* indicate the position of molecular weight markers (kb)



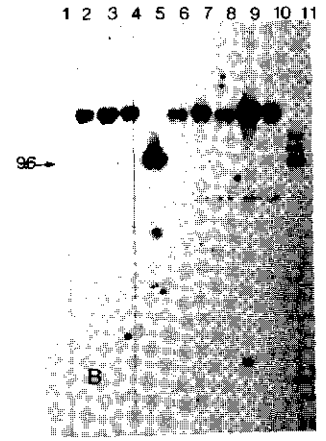


Fig. 4A, B. Analysis of DNA from AmdS$^+$ transformants obtained with mycelial protoplasts of MH1277 and plasmid p3SR2. *A. nidulans* DNA (2–5 μg) was digested with either *Eco*RI (panel A) or *Xho*1 (panel B), fractionated on an agarose gel and transferred to nitrocellulose. Blots were probed with ^{32}P-labelled *amdS* fragment. *Lanes 1* and *11* contain DNA from the untransformed MH1277 strain and a wild type strain respectively; *lanes 2–4*, DNA from ht1–3 and *lanes 6–10*, DNA from ht4–8. *Lane 5* contains linearised p3SR2. *Arrow* indicates the position of molecular weight maker (kb)

Blots of *Xho*I digested DNA of the same set of transformants showed much simpler hybridisation patterns with both pBR322 and the *amdS* fragment (Fig. 3B). In all transformants, except ct2, a fragment of at least 40 kb can be observed, hybridising strongly to both probes. Since no *Xho*I restriction site is present in p3SR2, this result suggests that the integration of the vector in most transformants must have occurred in a tandemly repeated fashion. However, the possibility of a free multimeric form of vector DNA cannot be completely excluded (see

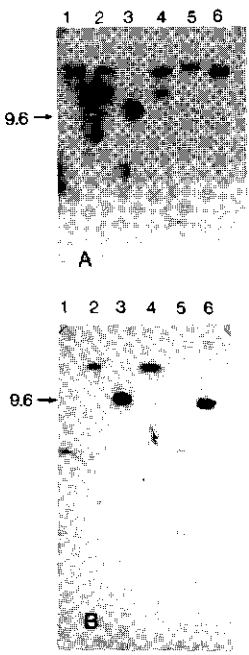


Fig. 5A, B. Analysis of DNA from AmdS$^+$ transformants (yt1–3) obtained with conidial protoplasts of WG290 and p3SR2. *A. nidulans* DNA (2–5 μg) was digested with either *Eco*RI (panel **A**) or *Xho*I (panel **B**), fractionated on an agarose gel and transferred to nitrocellulose. Blots were probed with ^{32}P-labelled *amdS* fragment. *Lanes 1* and *6* contain DNA from the untransformed WG290 strain and a wild type strain respectively; *lane 2* DNA from yt1 and *lanes 4–5*, DNA from yt2–3. *Lane 3* contains linearised p3SR2. *Arrow* indicates the position of molecular weight marker (kb)

Discussion). Based on this observation, the complex hybridisation patterns, obtained with *Eco*RI digested DNA, can only be explained if sequence rearrangements have taken place within the individual copies in the tandem. In addition to the large *Xho*I fragment in most transformants a 7 kb fragment, hybridising only with the *amdS* probe and corresponding to the *amdS* deletion fragment in MH1277, is present. If integration of the vector has occurred, in most transformants this has not taken place at the resident *amdS* locus.

Over 30 conidial p3SR2-derived transformants of MH1277 have been analysed as described before. In only one case (ct2) a hybridisation pattern was found which could be explained as being the result of integrative recombination of one single copy of p3SR2 at the resident *amdS* locus.

These results are completely different from those reported by Tilburn et al. (1983), who observed predominantly, as in ct2, integration of one copy of p3SR2 at the resident *amdS* locus with transformants obtained from mycelial protoplasts of WG290, a *yA2*, *pantoB100*-derivative of MH1277. Since the results obtained by us with mycelial protoplasts of MH1277 (Fig. 4) are completely equivalent to the ones found for conidial protoplasts, it is suggested that the tandem integration of multiple copies of p3SR2 is not influenced by the physiological differences between the protoplasts.

Considering that differences between the acceptor strains might be involved in the observed discrepancies we then analysed the DNA of several AmdS$^+$ transformants obtained with p3SR2 and WG290. Examples of the resulting hybridisation patterns are shown in Fig. 5. In most WG290 transformants the pattern is consistent with the integration of one copy of the vector at the partially deleted *amdS* gene (yt1 and yt2). Although in very few cases different hybridisation patterns were observed (yt3), tandemly repeated, multiple copy integrations were never found. These results strongly suggest that the type of integration is dependent on the strain used for transformation.

The influence of additional *A. nidulans* ribosomal DNA sequences in the vector on the hybridisation pattern, was examined by analysing MH1277 transformants, obtained with the plasmids pGW315, pGW317 and pGW324. The results invariably show complex hybridisation patterns indicating that multicopy tandem integration is not influenced by extensive homology between the vector and the chromosome (not shown). Whether or not integration had taken place at the ribosomal repeat could not be determined by these standard blotting procedures.

Stability of the tandemly repeated inserts

The correlation between mitotic and meiotic stability of the AmdS$^+$ phenotype and the stability of the integration patterns derived from Southern analysis has been investigated with transformants ht6 and ht7. The hybridisation patterns of *Eco*RI digested DNA from the extensively subcultured transformants remained completely unchanged. This indicates that not only the AmdS property, but also the integration of the tandemly arranged vector DNA sequences is inherited in a stable manner during mitosis.

Meiotic stability was investigated with DNA from colonies, arisen from ascospores of selfed cleistothecia from the same set of transformants. The results (Fig. 6) show that in some cases alterations in the hybridisation patterns have occurred. This indicates that in spite of the

Fig. 6. Meiotic stability of the hybridisation pattern of AmdS$^+$ transformants. DNA from two progeny colonies of selfed cleistothecia of ht7 was digested with *Eco*RI and probed with ^{32}P-labelled *amdS* fragment. The *arrow* indicates the difference between the two hybridisation patterns. *Lane 2* is identical to the parental pattern

apparent phenotypic stability of the AmdS$^+$ property, the tandemly repeated inserts are not always meiotically stable.

Discussion

Transformation of *A. nidulans* mycelial protoplasts has been reported now by a few research groups using different selection markers (Ballance et al. 1983; Tilburn et al. 1983; Yelton et al. 1984; J. L. Johnstone, University of Glasgow, pers. comm.). In general, one copy of vector DNA becomes integrated into the genome at the homologous site.

In this paper we show that also conidial protoplasts of *A. nidulans* can be used for transformation. Conidial protoplasts have the advantage that they can be prepared and purified easily in large amounts (Bos and Slakhorst 1981) and give viability values after handling, which are much higher than those reported for mycelial protoplasts (Yelton et al. 1984). Using the conidial protoplasts, transformation of *amdS* deletion strains results in the formation of growing colonies (Type I), but also in a large fraction of transformant colonies, which fail to develop into mature ones (Type II). Others (Tilburn et al. 1983; Yelton et al. 1984) have also observed such type of colonies, which they called "abortives". Our observations support the assumption that this description is not adequate since the majority still contains the vector DNA sequences; as indicated by their capacity to be converted into well growing (Type I) transformants. We would like to speculate that, once the vector DNA has entered the cell, integration into the genome is limited in time. The Type II colonies would then have stopped growing by lack of sufficient nitrogen, before integration has taken place. Upon transfer to non-selective medium, growth is resumed, thus providing new opportunities for the vector to integrate. The large variation in the fraction of AmdS$^+$ conidiospores from the initial Type I transformants might reflect the same time limitation: an early integration event will lead to a large fraction of AmdS$^+$ conidiospores, whereas later events will result in an increased number of cells which do not contain the AmdS$^+$ property. That such colonies nevertheless grow and sporulate on selective medium can be explained by cross-feeding of untransformed cells by AmdS$^+$ cells.

In contrast to other organisms, homology between the vector and the genome of the acceptor does not seem to have great impact on the transformation frequency. Neither an increased homology by adding r-DNA fragments to the vector, nor a decreased homology by using acceptor strains, in which all *amdS* sequences are deleted (e.g. MH1354, unpublished results) has an effect on transformation frequency.

Under optimised transformation conditions we now are able to obtain 300–400 transformants/μg DNA. However, since this frequency is still rather low for direct selection of cloned genes in *A. nidulans* we continue our search for elements and conditions which will improve the transformation frequency.

Southern blotting experiments with DNA from transformants of MH1277 revealed a type of gene amplification: multiple copies of complete and incomplete vectors arranged in a tandemly repeated fashion. Although the results shown here do not fully exclude the possibility of free multimeric forms of the vector DNA (cf. Grant et al. 1984), results on the genetic analysis of these transformants (Wernars et al. to be published) unambiguously indicate chromosome-linked inheritance of the AmdS$^+$ property, thus strongly favouring the idea of integrated vector DNA copies. In *A. nidulans* occasionally integratin of more than one copy of the vector has been observed (Tilburn et al. 1983; Yelton et al. 1984), but amplified, integrated vector DNA as a result of transformation is very unusually and may even be unique. Amplification of integrated sequences in eukaryotes has been reported, but only as the result of the application of selective pressure (see for review: Stark and Wahl 1984).

The hybridisation results with the *A. nidulans* strains MH1277 and WG290 strongly suggest that the type of integration is strain dependent. It can be speculated that a cryptic mutation, present in MH1277 but not in WG290 is responsible for this. In fact, strains with different *amdS* deletions, e.g. MH1354 (Hynes et al. 1983) but sharing the same genetic background as MH1277 exhibit similar integration phenomena upon transformation (unpublished results). At present we are trying to map the putative mutation on the *A. nidulans* genome by genetic analysis, in order to exploit it also in combination with

other selection markers. The blotting data anyhow clearly indicate that the integration is not preferentially at the resident, partially deleted, *amdS* locus. Together with the occurrence of *A. nidulans* transformants, which show altered colony morphology this could signify that integration of transforming sequences might lead to gene disruption. If integration was to be completely at random, this would provide novel means of isolating *A. nidulans* genes. Detailed analysis of a number of MH1277 transformants should elucidate whether integration shows any site specificity.

To explain the observed multicopy type of integration we propose the following model. An efficient non-homologous recombination system is assumed, which is repressed in WG290 but derepressed in MH1277, due to the cryptic mutation. This system efficiently recombines a number of plasmid molecules, which have entered the protoplast, in a non-homologous fashion. The resulting "scrambled" cointegrate subsequently is integrated into the genome. Since apart from this non-homologous recombination system, homologous recombination still occurs, integration into the genome can take place either at the resident, partially deleted *amdS* locus or at random locations. The presence of a "scrambled" cointegrate as an intermediate in the integration process not only explains the formation of tandem vector repeats, but also the sequence rearrangements observed within these tandems.

The transformation system described here seems to be a promising one; it combines fairly high transformation frequencies with high stability and amplifications of the integrated vector DNA sequences.

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CHAPTER 3

Genetic Analysis of *Aspergillus nidulans* AmdS$^+$ transformants.

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Summary

To correlate the genetic background of an A. nidulans amdS deletion strain MH1277 with the integrational behaviour of transforming vectors, classical genetic methods were used to construct AmdS$^-$ strains in which whole chromosomes were exchanged with those of a master strain. Progeny strains were transformed to the AmdS$^+$ phenotype with vector p3SR2. From Southern analysis it was concluded that transformants from all constructs contained tandemly repeated, multiple copy inserts of vector DNA as found for MH1277 derived AmdS$^+$ transformants.

AmdS$^+$ transformants of MH1277 were analyzed genetically to prove that the transformant phenotype is genome-linked and that transformation by integration can take place at various chromosomes. In one case the AmdS$^+$ property showed linkage to both chromosome II and IV, due to a chromosomal translocation.

Sexual analysis of two transformants with AmdS$^+$ insertions on the same chromosome revealed a considerable instability of the AmdS$^+$ phenotype in one of the strains upon selfing. Due to this instability no decisive answer could be given for the degree of linkage between the AmdS$^+$ insertions in these transformants.

Key words: Transformation, Aspergillus, amdS, genetic analysis.

Introduction

Since Pontecorvo et al. (1953) established techniques for the genetic analysis of *Aspergillus nidulans*, many studies (for a review see Smith and Pateman 1977) have contributed to a better understanding of genetic processes in lower eukaryotes.

The elegance with which genetic analysis of *A. nidulans* can be performed is due to features of its life cycle. Besides an asexual reproduction cycle yielding the vegetative conidia, efficient sexual reproduction by means of ascospores can occur. This allows easy crossing of *A. nidulans* strains. In contrast to many other eukaryotes *A. nidulans* can alternate between diploid and haploid stages without going through meiosis. This process, the so called parasexual cycle allows rapid reassortment of whole chromosomes (Pontecorvo et al. 1953; Roper 1966). These studies have resulted in a linkage map for *A. nidulans*, comprising over five hundred different loci, distributed over eight linkage groups (Clutterbuck 1984).

As was concluded from DNA analysis, transformation of the *amdS* deletion strains MH1277 and WG290 with vectors containing the wild type *amdS* gene resulted in integration of the transforming sequences into the genomic DNA of the recipient (Tilburn et al. 1983; Wernars et al. 1985). Our studies also revealed that in nearly all MH1277 derived $AmdS^+$ transformants integration was not at the resident *amdS* locus whereas in WG290 derived transformants it was (Wernars et al. 1985). For other *A. nidulans* transformation systems (Yelton et al. 1984; Ballance et al. 1983; John and Peberdy 1984) non-homologous integrations have been reported in some cases but no evidence was found that the genetic background of the strains used for transformation could affect the behaviour of the transforming vector upon integration.

We proposed a model to explain the difference between recipients MH1277 and WG290 with respect to integration, assuming a cryptic mutation in the genome of MH1277 which leads to multiple copy, tandemly repeated integration (Wernars et al. 1985). In this paper experiments are described which aimed at providing a genetic basis for the model by mapping the mutation.

Although Southern analysis of MH1277 AmdS$^+$ transformants showed that vector integration usually is not at the homologous locus, no indication was obtained about the site(s) where integration did take place. We therefore analysed genetically a number of such transformants to locate the AmdS$^+$ property with respect to linkage group.

Materials and Methods

Strains. The following A. nidulans strains were used

- WG201: suA1adE20, yA2, adE20, ; acrA1; meaB6; pyroA4; pA1; sB3; nicB8; riboB2 (strain collection Dept. of Genetics, Agricultural University)
- WG314: yA2; acrA1; amdI18, amdS320; pyroA4; pA1; sB3; pantoB100; riboB2 (strain collection Dept. of Genetics, Agricultural University)
- MH1277: biA1; amdI18, amdS320; amdA7; niiA4 (Hynes et al. 1983)
- MH1277 derived AmdS$^+$ transformants: ht6, ht7, ct2, ct3, ct7 and ct8 (Wernars et al. 1985).

Plasmids were propagated in E. coli K-12 strain MH1 (Goddard et al. 1983).

Media. The media used for A. nidulans were essentially as described by Pontecorvo et al. (1953). Their composition per liter

of deionized water was:

MM: glucose 10 g, KCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, KH_2PO_4 1.5 g, of each $FeSO_4$, $ZnCl_2$, $MnCl_2$ and $CuSO_4$ 1 mg. One of the following nitrogen sources was added: $NaNO_3$ 70 mM, L-proline 10 mM, acetamide 10 mM or methylammoniumchloride 100 mM.

ME: glucose 5 g, malt extract 20 g, trypticase pepton 1 g, vitamin free casamino acids 1 g, yeast extract 1 g, yeast ribonucleic acid hydrolysate 0.2 g.

Before autoclaving the pH was adjusted to 6.0 with NaOH. If appropriate, MM was solidified with 1.2% Oxoid agar No. 1 and ME with 1.5% Oxoid technical agar No. 3. For growth of auxotrophs the medium was supplemented with the required amino acids (2 mg/ml) and vitamins (D(+)biotin 1 µg/ml, nicotinamide 1 µg/ml, pantothenic acid 0.2 mg/ml, pyridoxin 1 µg/ml, riboflavin 10 µg/ml).

Acriflavin resistance was tested on ME containing this compound at a concentration of 50 µg/ml.

E. coli was grown as described earlier (Wernars et al. 1985).

Manipulation techniques for A. nidulans. All techniques for in vivo manipulation of A. nidulans including sexual crosses, parasexual cycle with benlate induced haploidisation, collection of conidia, colony replication and testing were carried out using established procedures (Pontecorvo et al. 1953; Hastie 1970; Rowlands and Turner 1973; Upshall et al. 1977; Bos 1985). Selection of heterokaryons and diploids from $AmdS^+$ transformants was performed on MM containing acetamide. Sexual crosses were performed on MM containing proline, selfings on ME.

Results

Is a genetic factor responsible for tandem integration in A. nidulans?

To map the putative mutation responsible for the multicopy, tandem integration (Wernars et al. 1985) a diploid of MH1277 and master strain WG201 was constructed. Haploid segregants were isolated and analysed for their phenotype. To facilitate the distinction between green diploids and haploids, in this experiment only segregants with yellow coloured conidia were taken. From a total of 250 haploid segregants, eight were chosen with different combinations of MH1277 and WG201 markers (Table 1), but all with yA2 from WG201 and amdS320 from MH1277 in common. To examine the pattern of vector integration, these strains were transformed with p3SR2 and from each, two AmdS$^+$ transformants were randomly taken and their DNA was subjected to Southern blotting analysis. In all cases this revealed integration of many vector copies in a tandemly repeated fashion (results not shown) as found previously with MH1277 derived AmdS$^+$ transformants (Wernars et al. 1985). Therefore it can be concluded that exchange of chromosomes between MH1277 and WG201 by means of the parasexual cycle did not result in loss of the multicopy, tandem integration phenomenon.

Parasexual analysis of AmdS$^+$ transformants

Six p3SR2-derived transformants of MH1277, i.e. ht6, ht7, ct2, ct3, ct7 and ct8 (Wernars et al. 1985), were subjected to genetic analysis. Diploids were constructed between these transformants and the AmdS$^-$ master strain WG314. Since the use of a non-selective nitrogen source occasionally resulted in the isolation of heterozygous diploids with an AmdS$^-$ phenotype, selection of di-

Table 1: Marker composition of eight haploid segregants from diploid MH1277/WG201.

Segregant	Marker mutations from WG201 and linkage group							
	yA2 (I)	acrA1 (II)	meaB6 (III)	pyroA4 (IV)	pA1 (V)	sB3 (VI)	nicB8 (VII)	riboB2 (VIII)
WG338	*	*						
WG339	*			*				
WG340	*	*			*			
WG341	*			*	*			
WG342	*					*		
WG343	*						*	
WG344	*						*	*
WG345	*							

The presence of a genetic marker originating from parent WG201 is designated by "*". All strains denoted carry amdS320 from parent MH1277.

ploids was carried out on MM containing acetamide. Haploid segregants were isolated and tested for the presence of genetic markers and the transformed AmdS$^+$ property. The results are summarized in Table 2.

It is to be noticed that the AmdS$^-$ phenotype predominated among the segregants of three diploids (i.e. those derived from ct3, ht7 and ct8). This might suggest a disadvantage for the chromosome carrying the AmdS$^+$ insertion.

In all groups, with the exception of ct2, strong linkage is found between the AmdS$^+$ phenotype and at least one marker of WG314, confirming the previous conclusion from the biochemical analysis (Wernars et al. 1985) that the transforming sequences are

Table 2. Marker distribution of haploid segregation from diploids of strain WG314 and the AmdS$^+$ transformants.

marker from WG314	L.G.	MH1277	ct2		ct3		ht7		ct8		ht6		ct7	
			-	+	-	+	-	+	-	+	-	+	-	+
yA2	I		82	85	158	1	55	18	98	57	50	39	99	102
		w.t.	31	37	0	63	48	20	39	31	41	42	74	85
acrA1	II		42	55	73	25	103	4	137	1	33	37	170	6
		w.t.	71	67	85	39	0	34	0	87	58	44	3	181
pyroA4	IV		42	70	84	44	49	15	62	42	44	36	169	0
		w.t.	71	52	74	20	54	23	75	46	47	45	4	187
pA1	V		23	24	81	29	22	2	27	26	14	13	59	70
		w.t.	90	98	77	35	81	36	110	62	77	68	114	117
sB3	VI		61	68	85	32	18	10	84	46	42	35	74	81
		w.t.	52	54	73	32	85	28	53	42	49	46	99	106
pantoB100	VII		64	65	84	29	22	7	70	35	19	10	73	100
		w.t.	49	57	74	35	81	31	67	53	72	71	100	87
riboB2	VIII		56	61	107	46	54	22	66	55	91	0	121	124
		w.t.	57	61	51	18	49	16	71	33	0	81	52	63
w.t.	VIII		60	66	110	48	62	24	70	56	91	5	124	123
		niiA4	53	56	48	16	41	14	67	32	0	76	49	64
		Total	113	122	158	64	103	38	137	88	91	81	173	187

Only the tested markers are listed. The segregants able to grow on acetamide as sole nitrogen source are designated "+", those unable by "-". The boxes denote linkage between a marker and the AmdS$^+$ property. Both strain WG314 and the AmdS$^+$ transformants carry the *amdS320* mutation on linkage group III. L.G.=linkage group; w.t.=wild type gene.

integrated into the genomic DNA.

For ct2 no linkage is observed to any of the tested markers (Table 2, first column). By elimination, the AmdS$^+$ insertion can only be assigned to linkage group III, which carries the amdS320 mutation, but no other genetic marker. This again is in accordance with the results of the biochemical analysis which showed integration of a single vector copy at the homologous, partially deleted amdS locus.

In four other transformants the AmdS$^+$ property maps on one chromosome only: for ct3 and ht6 on chromosome I and VIII respectively, for both ht7 and ct8 on chromosome II. From the results in Table 2 however, it cannot be concluded whether integration in ht7 and ct8 has occurred at the same chromosomal location.

In contrast to the transformants mentioned above, the AmdS$^+$ property in ct7 shows linkage to two markers: to acrA1 on chromosome II and pyroA4 on IV. At first sight this would be in

Table 3. Distribution of the markers acrA1 and pyroA4 and the transformed AmdS$^+$ property, among haploid segregants from the diploid WG314//ct7.

Number of haploid segregants	Phenotype
169	AcrA$^-$, PyroA$^-$, AmdS$^-$: parental (WG314)
181	AcrA$^+$, PyroA$^+$, AmdS$^+$: parental (ct7)
6	AcrA$^-$, PyroA$^+$, AmdS$^+$: recombinant
3	AcrA$^+$, PyroA$^+$, AmdS$^-$: recombinant
1	AcrA$^-$, PyroA$^+$, AmdS$^-$: recombinant
0	: other recombinants

accordance with integration on both chromosomes. However, in addition the two markers failed to segregate independently from each other: among the segregants, only 2% had separated the acrA1 and pyroA4 marker instead of 50% (Table 3). This led to the conclusion that a translocation between chromosomes II and IV is involved (see Discussion).

As the integration of the transforming vector into the chromosomal DNA is the result of a recombination event it is worthwhile to investigate mitotic recombination in our material. From the segregation experiments mentioned before the frequency of mitotic recombination for chromosome VIII could be estimated (Table 4). Although the distribution of the segregants is distorted at the expense of the $NiiA^-$ mutants in general and the $NiiA^-$, $RiboB^-$ recombinants in particular, the recombination frequency for these two markers is at least 3%; this is an order of magnitude higher than 0.1-0.3% per chromosome arm, as estimated by Käfer (1977).

Table 4. Distribution of the niiA4 and riboB2 markers among haploid segregants from the diploids of strain WG314 and $AmdS^+$ transformants

Phenotype of segregant	Transformant in diploid					
	ct2	ct3	ht7	ct8	ht6	ct7
$Nii^-, Ribo^+$	107	64	55	99	76	113
$Nii^+, Ribo^-$	115	153	76	121	91	245
$Nii^-, Ribo^-$	2	0	0	0	0	0
$Nii^+, Ribo^+$	11	5	10	5	5	2

Sexual analysis of transformants ht7 and ct8

To determine whether the AmdS$^+$ sequences had integrated at identical chromosomal locations the transformants ht7 and ct8 with the AmdS$^+$ insertions mapping on the same chromosome (i.e. II), were further analysed by sexual crossing. Since both transformants have similar phenotypes direct crossing could not be performed. Therefore derivatives of ht7 and ct8, which combine the AmdS$^+$ phenotype and additional markers, were selected from the previous experiment. Thus crosses were made between ht7 and ct8-pyro (yA2; AmdS$^+$; amdI18, amdS320; pyroA4; amdA7; niiA4), ht7 and ct8-ribo (yA2; AmdS$^+$; amdI18, amdS320; amdA7; riboB2). As a control selfings of the individual partners were performed. Progeny from crossed cleistothecia and selfings was analysed and the results are summarized in Table 5A.

Selfings of ct8-pyro and ct8-ribo showed a fairly stable meiotic transmission of the AmdS$^+$ phenotype: only one single AmdS$^-$ colony was obtained. Strain ht7 however behaved completely different. Of all progeny tested over 40% had an AmdS$^-$ phenotype and large differences in AmdS$^+$/AmdS$^-$ ratio were found for the individual cleistothecia.

Surprisingly the crosses ht7 x ct8-pyro and ht7 x ct8-ribo yielded a rather small number of AmdS$^-$ progeny. In only one cleistothecium a 1:1 ratio of AmdS$^+$ and AmdS$^-$ ascospores was found. The high meiotic instability in the parental strain makes it difficult to draw conclusions about the relative distance between the AmdS$^+$ insertions in both parents.

In order to test the possibility that in contrast to ht7 itself derivatives of ht7 would display stable meiotic transmission of the AmdS$^+$ property, these were used in combination with ct8 (Table 5B.). Like ht7, its derivatives ht7-pyro (yA2; AmdS$^+$;

Table 5. Sexual analysis of transformants ht7 and ct8

A)

cleisto-thecium	ct8-pyro selfing		ct8-ribo selfing		ht7 selfing		ht7 x ct8-pyro		ht7 x ct8-ribo	
	+	-	+	-	+	-	+	-	+	-
1	49	0	17	0	16	24	100	0	98	2
2	48	0	27	0	17	13	50	0	38	1
3	12	1	42	0	12	38	49	1	155	1
4	52	0	48	0	36	14			41	49
5	26	0	36	0	32	18				
6	12	0	20	0	13	5				
7			12	0	27	7				
8			9	0	32	18				
9			26	0	32	18				
10			43	0	37	13				
11			26	0	28	0				
12					18	30				
Total	199	1	306	0	286	212	199	1	332	54

B)

cleisto-thecium	ht7-pyro selfing		ht7-ribo selfing		ct8 selfing		ct8 x ht7-pyro		ct8 x ht7-ribo	
	+	-	+	-	+	-	+	-	+	-
1	28	12	39	0	30	0	98	2	100	0
2	33	25	32	11	98	2	100	0	99	1
3	52	48	34	17	68	0	100	0	94	6
4	32	2	24	10	26	2				
5	32	30	25	8	68	1				
6			22	6						
Total	177	117	176	52	290	5	298	2	293	7

Table 5: Selfed or crossed cleistothecia were isolated and from each a number of progeny colonies was tested for the presence (+) or absence (-) of the AmdS$^+$ phenotype. Crossed cleistothecia contained YA$^+$ and YA$^-$ progeny in a 1:1 ratio.
A) ht7 with ct8 derivatives
B) ct8 crossed with ht7 derivatives
For further explanation see text.

amdI18, amdS320; pyroA4; amdA7; niiA4) and ht7-ribo (yA2; AmdS$^+$; amdI18, amdS320; amdA7; riboB2) frequently lost the AmdS$^+$ phenotype upon selfing but the crosses only yielded again small numbers of AmdS$^-$ progeny.

The possibility that the observed instability in ht7 and its derivatives was mitotic rather than meiotic was ruled out by analysis of their conidiospores: among 300 tested none was AmdS$^-$.

Discussion

In the experiments described here classical genetic methods were applied. Firstly we tried to find support for our hypothesis (Wernars et al. 1985) that a cryptic mutation in the genome of MH1277 is responsible for the multiple copy, tandemly repeated type of integration of the transforming vector. However, none of the constructed strains with exchanged chromosomes from MH1277 and WG201 predominantly integrated one copy of the vector DNA at the homologous locus. Since chromosome III contains the indispensible amdS deletion, this chromosome could not be tested in this way, leaving the possibility that the presumed mutation might be pre-

sent on chromosome III. On the other hand master strain WG201 might contain the same mutation. However, the latter possibility cannot be verified since no pedigrees are available for both strains.

Recent reports involving transformation of A. nidulans (John and Peberdy 1984;Ballance and Turner 1985; Durrens et al. 1985; Miller et al. 1985) indicate that tandem integration, although less pronounced than in MH1277, also occurs in other strains. Therefore our model (Wernars et al. 1985) might need revision by the assumption of the presence of a cryptic mutation in WG290, resulting in the absence of multiple copy integration.

Based on data, obtained from Southern analysis (Wernars et al. 1985) we already concluded that AmdS$^+$ transformants of MH1277 have integrated the transforming DNA into the genome. Transformation by integration of the vector into the genomic DNA has been reported so far for all A. nidulans transformation systems (Tilburn et al. 1983; Ballance et al. 1983; John and Peberdy 1984; Yelton et al. 1984). Genetic analysis can be employed to verify a conclusion like this. Mitotic haploidisation was successfully used by Johnstone et al. (1985) for the genetic analysis of an ArgB$^+$ transformant. However, the method failed in the analysis of Pyr$^+$ transformants, due to loss of the transformant phenotype (Ballance and Turner 1985). This loss could be due to the fact that the master strain used was a uridine prototroph, resulting in the absence of selective pressure necessary to maintain the transforming DNA in the diploid. Our observation that AmdS$^-$ diploids will arise in the absence of selective pressure supports this assumption.

The genetic analysis of six MH1277 AmdS$^+$ transformants unambiguously shows a genome linked inheritance of the transformant phenotype. There seems to be no preference for the integration into one of the eight chromosomes: in six transformants the AmdS$^+$

property mapped on five different chromosomes. With the exception of ct2 the transformant phenotype mapped outside linkage group III containing the resident amdS region. For ct2 the result is in agreement with the conclusion from the biochemical analysis (Wernars et al. 1985). From all transformants tested, ct7 is the only one that shows linkage of the AmdS$^+$ property to two markers on different chromosomes. This situation however cannot be simply explained by assuming that the AmdS$^+$ property is present on two linkage groups, in view of the observation that the pyroA4 and acrA1 markers fail to segregate independently. Linkage of two, previously unlinked markers can occur as a result of a translocation event (Bainbridge and Roper 1966). Such an event cannot have occurred in WG314 since this marker linkage was absent in the other segregant pools (result not shown). When however, a translocation was present in the transformant this could also lead to apparent linkage of markers from the master strain (Käfer 1962, 1965; Bainbridge 1980) due to inviability or poor growth of segregants with an imbalanced chromosome complement. Therefore it is very likely that ct7 contains a translocation involving linkage groups II and IV. Integration of the amdS vector in one or both of these chromosomes will then result in the segregation of mainly AmdS$^-$, AcrA$^-$, PyroB$^-$ and AmdS$^+$, AcrA$^+$, PyroB$^+$ haploids from ct7//WG314. Another option might be that translocation occurred as the result of vector integration, e.g. by homologous recombination between vector inserts on both chromosomes.

The observation from Table 2, that segregation of AmdS$^-$ and AmdS$^+$ phenotype is strongly biassed in some segregant pools may reflect a disadvantage during reassortment of chromosomes carrying a large insert. The fact that this bias is not found for all transformants might be connected with length and/or location of the insert.

As a result of mitotic crossing-over in a heterozygous diploid haploid segregants can be obtained in which recombination has taken place between markers on the same chromosome. This process has been studied extensively (Roper and Pritchard 1955; Pontecorvo and Käfer 1958) and Käfer (1977) estimates the frequency of mitotic crossing-over to be 0.1 to 0.3% per chromosome arm. In the diploid combinations of WG314 and the MH1277 AmdS$^+$ transformants this frequency is at least an order of magnitude higher, considering the 3% recombinants between niiA4 and riboB2 on chromosome VIII. De Bertoldi et al. (1980) have found that the use of benlate does not lead to an increase of mitotic recombination. One could speculate that this unusually high level of recombination is the basis of a peculiar integration mechanism in MH1277.

The sexual analysis of ht7 and ct8 revealed a high instability of the AmdS$^+$ phenotype upon selfing in the former transformant. For A. nidulans transformants meiotic instability has been reported, as well as stable transmission of the transformant phenotype (Ballance and Turner 1986; Ward et al. 1986; Johnstone et al. 1985). Tandem repeats can easily undergo rearrangements through meiosis (Miller et al. 1985; Durrens et al. 1985). In previous experiments (Wernars et al. 1985) this was also found for ht7. In that experiment however, AmdS$^-$ ascospores as a result of selfing were not observed.In the course of the experiments described here, no obvious explanation was found for this discrepancy. Possibly transformant ht7 underwent genetic change during storage or handling.

Considering the results obtained with the ht7 derivatives (Table 5) it seems obvious that the instability of the AmdS$^+$ phenotype in ht7 is correlated with the chromosome containing the vector insert. Apparently, loss of the transformant phenotype depends on factors that can differ for each individual cleisto-

thecium as no fixed AmdS$^+$: AmdS$^-$ ratio was found. Since crosses of ht7 and ct8 (or their derivatives) mainly yielded AmdS$^+$ progeny, presence of chromosome II from ct8 during meiosis seems to have a stabilising effect on the inheritance of the AmdS$^+$ phenotype. About the mode of action of this stabilisation one might speculate. If integration of vector DNA in chromosome II of ht7 occurred at a location disadvantageous for maintenance during meiosis, a strong selective pressure will be present for loss of this drawback. Upon selfing of ht7 this might occur by elimination of the vector insert whereas in a cross the whole chromosome II of ht7 could be discarded. This hypothesis implies that the majority of the AmdS$^+$ progeny from the crosses ht7 x ct8 will possess chromosome II from ct8. However, the results described here do not allow a conclusion about this. Due to the meiotic instability of the AmdS$^+$ phenotype in ht7 no decisive answer can be given on how closely or not the AmdS$^+$ insertions in both transformants are linked. If the observed instability results from the location of the vector integration, the site in which this occurred is probably different for ht7 and ct8, since the latter exhibits a stable meiotic inheritance of the transformant phenotype.

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CHAPTER 4

Isolation of transforming DNA sequences from *Aspergillus nidulans* AmdS$^+$ transformants in *E. coli*.

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Summary

Digestion of DNA from Aspergillus nidulans MH1277-derived AmdS$^+$ transformants with the restriction enzyme EcoRI, followed by ligation and transformation to E. coli, resulted in ampicillin resistant colonies bearing plasmids which were either identical or strongly related to the amdS vector used originally to transform A. nidulans. Similar plasmids could also be selected in E. coli using undigested DNA from the same AmdS$^+$ transformants. Evidence is presented that these transformants also contain free circular plasmid DNA molecules, be it at very low copy number, which arise in Aspergillus by excision through in vivo recombination between individual copies within integrated tandem repeats.

A detailed analysis of reisolated vector sequences is presented.

Introduction

Analysis of Aspergillus nidulans transformants by Southern blotting (Wernars et al. 1985; Yelton et al. 1984) or genetic analysis (Wernars et al. 1986; Johnstone et al. 1985) invariably shows that the transforming DNA sequences are integrated in the genome. For manipulation and analysis of cloned genes it is highly desirable to possess simple procedures for reintroducing the transformed DNA sequences into E. coli.

For the lower eukaryote Saccharomyces cerevisiae this was achieved by the construction of shuttle vectors, able to replicate autonomously in both E. coli and S. cerevisiae (Struhl 1983). Similar vectors have also been developed for animal cells (e.g. Lusky and Botchan 1981). In some systems the use of vectors of

viral origin has enabled the isolation of transforming sequences by *in vivo* packaging into viral particles (Cone and Mulligan 1984; Brisson et al. 1984).

As all efforts to construct vectors able to replicate autonomously in *A. nidulans* (van Gorcom, pers. comm.; Goosen et al. 1985) have been unsuccessful, isolation of transforming sequences from Aspergillus would require *in vitro* circularization prior to transformation into *E. coli*.

We here describe such re-isolation experiments with DNA from *A. nidulans* AmdS$^+$ transformants, which either contain one or multiple copies of the transforming *amdS* gene (Wernars et al. 1985). These experiments also indicate that circularization can occur spontaneously *in vivo* in these *A. nidulans* transformants. We present a detailed analysis of several reisolated plasmids.

Materials and Methods

A. nidulans strains. MH1277-derived AmdS$^+$ transformants ht1, ht2, ht3, ht4, ht7, ct2, ct5 and ct8 were described previously (Wernars et al. 1985). The transformants r10t1, r12t1 and r13t1 were obtained by transformation of *A. nidulans* strain MH1277 to the AmdS$^+$ phenotype using vectors pGW315, pGW317 and pGW324 respectively (Wernars et al. 1985). Southern blotting analysis showed that they contain multiple copies of the vector inserted in a tandemly repeated fashion (unpublished results).

E. coli strains. Strain JA221 (Δ *trpE* 5, *leuB*, *recA*, *hsdR*$^-$, HsdM$^+$; Clarke and Carbon 1978) and KMBL1164 (*thi*, Δ *lac-pro*, *supE*; Giphart-Gassler and van der Putte 1979) were used for transformation.

Media and growth. All media and growth conditions have been described before (Wernars et al. 1985).

Transformation of E. coli. Transformation was carried out using the RbCl procedure described by Maniatis et al. (1982). Frequencies were determined using 20pg of pBR322 DNA.

All other procedures were as described previously (Wernars et al. 1985).

Results

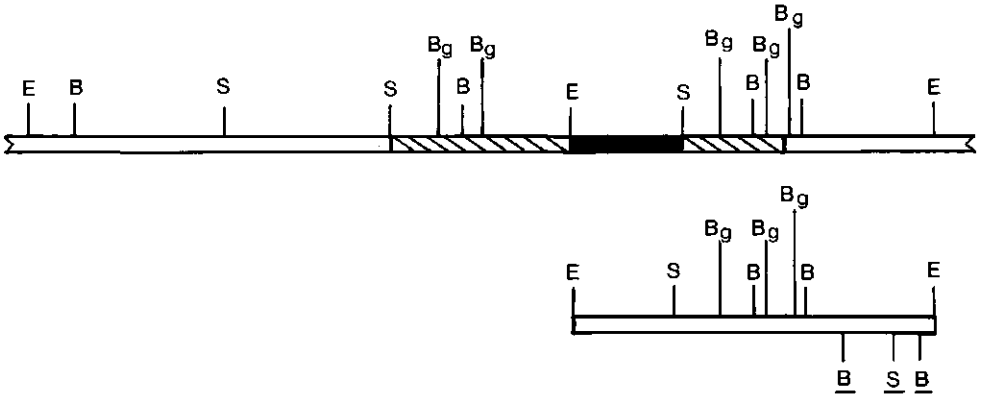
Isolation of transforming sequences from A. nidulans by in vitro circularization.

Re-isolating transforming sequences by in vitro circularization was initially performed with DNA from strain ct2, which contains only one copy of the vector p3SR2. The DNA of this strain was digested to completion with EcoRI, phenol extracted and precipitated. Then a 0.15 µg aliquot of this digested DNA was ligated in a volume of 150 µl overnight at 16 °C and subsequently used to transform E. coli strain JA221.

This yielded six ampicillin resistant E. coli transformants. Five of these clones contained an identical 11.4kb plasmid and the remaining colony had a 9.0kb plasmid.

The restriction map of the larger plasmid was in agreement with that predicted for a homologous vector integration at the amdS locus (fig. 1). Compared to the restriction map of the amdS region, reported by Hynes et al. (1983), we found additional SalI and BamHI sites. The 9.0kb plasmid was indistinguishable from the parental vector p3SR2. It is obvious from fig. 1 that this 9.0kb

Figure 1: Physical map of the amdS locus in AmdS$^+$ transformant ct2 and re-isolated plasmids



Underlined restriction sites were not represented in the previously published physical map of the amdS genomic region (Hynes et al. 1983).

E= EcoRI, B = BamHI, S = SalI, Bg = BglII.

■: bacterial DNA, □: A. nidulans DNA,

▨: A. nidulans amdS DNA.

Top: transformant ct2. Bottom: re-isolated plasmid.

plasmid can not have arisen from an in vitro circularization event.

A similar re-isolation experiment was carried out with DNA from ht7, ct5 and ct8, all containing tandemly repeated, multiple copies of the vector p3SR2 (Wernars et al. 1985). The results (table 1) show that the frequencies with which plasmids could be re-isolated from these transformants, exceeded by far that of ct2. The frequency in which plasmid molecules are re-isolated is more or less proportional to the estimated copy number of the vector

Table 1: Ampicillin resistant *E. coli* transformants obtained with *EcoRI* digested and religated *A. nidulans* AmdS$^+$ DNA

AmdS$^+$ transformant	*E. coli* transformants per 1 µg DNA	copy number
ct2	37	1
ht7	140	29
ct5	45	7
ct8	250	36

Transformant DNA (100 ng) was digested with *EcoRI*, self-ligated and used to transform *E. coli* strain JA221 to ampicilline resistance. The transformation frequency of pBR322 DNA was 2×10^7 transformants/µg DNA. The number of vector copies in AmdS$^+$ transformants was determined by DNA dot blotting (results not shown).

present in the genome of the AmdS$^+$ transformants. The plasmids obtained had various sizes, ranging from approximately 7 to 15kb.

From the results presented here we conclude that the re-isolation of transforming sequences from MH1277 derived AmdS$^+$ transformants by *in vitro* circularization is possible, even when only one single copy is present in the genome.

Re-isolation of transforming sequences from undigested transformant DNA.

As mentioned in the previous section, a 9.0 kb plasmid indistinguishable from p3SR2 was re-isolated from ct2 DNA. Since the in vitro circularization procedure could not have yielded this plasmid we examined the possibility that free circular vector molecules are present in undigested DNA from MH1277 derived AmdS$^+$ transformants. From a number of such transformants DNA was isolated and without further treatment transformed to the non-

Table 2: Ampicillin resistant E. coli transformants obtained with undigested A. nidulans AmdS$^+$ DNA

AmdS$^+$ transformant	E. coli transformants/µg DNA	
	JA221(r^-m^+)	KMBL1164(r^+m^+)
ht1	16	0
ht2	2	0
ht3	4	0
r10t1	3	0
r12t1	4	0
r13t1	17	0

Of each A. nidulans AmdS$^+$ transformant ten aliquots of 100 ng undigested DNA were used to transform each of the E. coli strains JA221 and KMBL1164. Transformation frequency of pBR322 DNA was $5x10^6$ transformants/µg DNA.

restricting *E. coli* strain JA221. This indeed yielded a number of ampicillin resistant colonies (Table 2), all containing plasmids. To ascertain that these plasmids really originate from Aspergillus, the DNA preparations were simultaneously transformed to the restricting host KMBL1164. Although modified pBR322 transformed both strains with equal frequencies, Aspergillus DNA did not give transformants in KMBL1164 (Table 2).

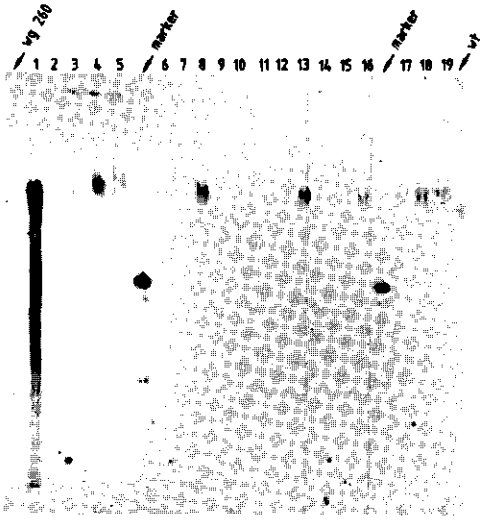
These results indicate that free circular vector sequences might be present in *A. nidulans* AmdS$^+$ transformants.

Are free plasmids present in AmdS$^+$ transformants?

Undigested DNA preparations of nineteen transformants were fractionated on a 0.4% agarose gel, blotted onto a nitrocellulose membrane and probed with ^{32}P-labelled p3SR2 DNA. On the resulting autoradiograms (fig. 2) no hybridisation signals corresponding to free plasmids could be detected even after prolonged exposure, whereas 0.1 ng of p3SR2 gave a strong signal. Since the haploid *A. nidulans* genome has about 3×10^3 the size of p3SR2 (Timberlake 1978) we estimate that the supposed free plasmid molecules are present in a ratio of less than 1 copy per 50 to 100 *A. nidulans* genome equivalents.

One could assume that no free plasmids are present at all in the AmdS$^+$ transformants, but that integrated copies of the transforming vector are circularized in *E. coli*. We therefore subjected DNA from four AmdS$^+$ transformants to centrifugation in CsCl/EtBr gradients. From these gradients, in which only a chromosomal DNA band was visible, fractions of chromosomal and plasmid density were collected, purified and aliquots were transformed to *E. coli*. The results are shown in Table 3.

Figure 2: Southern analysis of undigested DNA from AmdS$^+$ transformants



A. nidulans DNA 3 µg/lane) was fractionated by electrophoresis on a 0.3% agarose gel, transferred onto a nitrocellulose membrane and hybridized with ^{32}P-labelled p3SR2. Lanes 1-19: AmdS$^+$ transformants, obtained by transformation of strain MH1277 with plasmid p3SR2; wt = wild type A. nidulans; WG260 = A. nidulans strain MH1277. The marker lanes contain 0.1 ng p3SR2 DNA.

Although in the fractions of plasmid density no DNA could be detected by physical means they yielded ampicillin resistant E. coli JA221 transformants. A number of transformants in the same order of magnitude could be obtained upon using 1 µg of DNA from

the chromosomal fraction for transformation. The results demonstrate that indeed low numbers of free circular plasmids are present in AmdS$^+$ transformants of A. nidulans strain MH1277.

Table 3: Ampicillin resistant E. coli transformants obtained with fractionated undigested A. nidulans AmdS$^+$ DNA

AmdS$^+$ transformant	Number of E. coli transformants with:	
	"plasmid band"	chromosomal band
ht2	6	1
ht4	2	11
ht7	1	1
r13t1	5	23

Transformant DNA was subjected to CsCl/EtBr gradient centrifugation. Fractions of chromosomal and plasmid density were collected, the DNA was purified and dissolved in equal volumes. These preparations were used to transform E. coli strain JA221. Of the chromosomal fractions 1 µg of DNA was transformed, which equals about 1% of the DNA recovered. Of the plasmid fraction, which contained no visible amount of DNA, one half was taken for transformation. The transformation frequency of pBR322 DNA was 5×10^6 transformants/µg.

Analysis of re-isolated plasmids.

Plasmid DNA was extracted from ampicillin resistant E. coli transformants, obtained with undigested DNA from A. nidulans AmdS$^+$ transformant r13t1, purified, digested with both EcoRI and SalI and fractionated on a 0.6% agarose gel. Of 12 plasmids isolated 5 showed restriction patterns identical to pGW324, the plasmid originally used to transform A. nidulans. The other 7 plasmids were markedly different. These, together with one of the former plasmids (p21-6) were further analyzed.

EcoRI and SalI digested DNA was blotted from gels onto nitrocellulose membranes in triplicate and hybridized to ^{32}P-labelled pGW324-fragments (fig. 3).

The resulting autoradiograms showed that despite different restriction pattern all plasmids were reminiscent to pGW324 as they hybridized to the amdS fragment (Fig. 3B), pBR322 (fig. 3C) and the 3.3kb EcoRI fragment of A. nidulans ribosomal DNA (Fig. 3D).

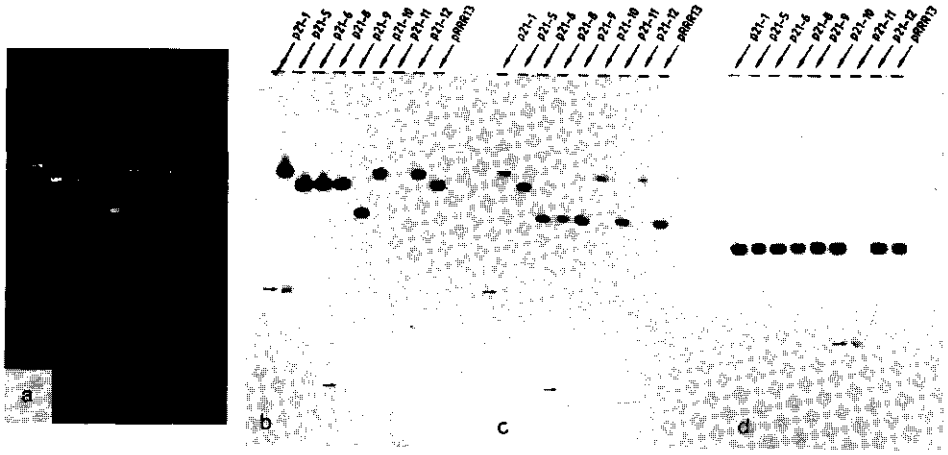
In HaeIII digests of the plasmids this relation was even more obvious. Some HaeIII fragments of re-isolated plasmids appeared to be doublets, where pGW324 showed singular bands (Fig. 4A). Probing with ^{32}P-labelled amdS fragment also indicated that in some re-isolated plasmids sequence duplications had occurred (Fig. 4B).

To establish which A. nidulans DNA sequences were present in the plasmids re-isolated from r13t1, three of these, p21-1, p21-10 and p21-11 were labelled by nick translation and used as hybridization probes for blots of restriction digests of chromosomal A. nidulans DNA (Fig. 5).

With all three plasmids only the expected ribosomal DNA fragments (strong signals) and amdS containing fragments (weak signals) were visible. This means that the re-isolated plasmids

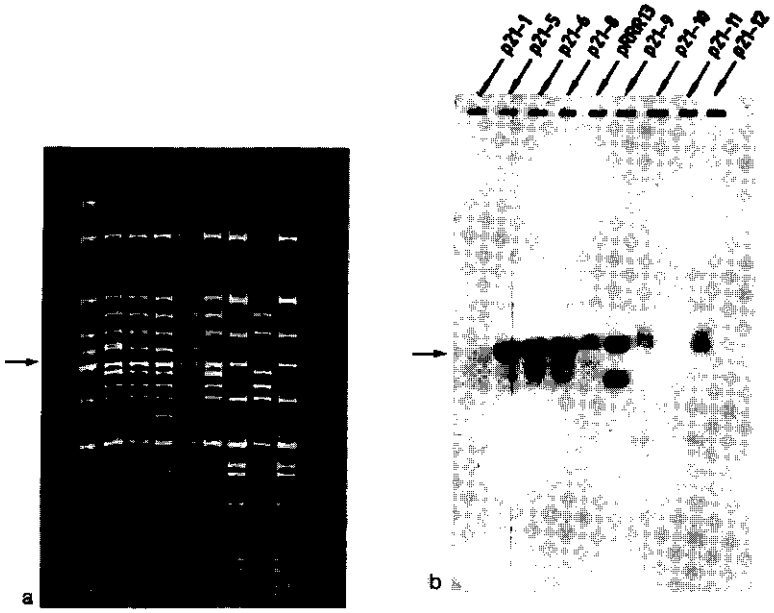
solely consisted of fragments, present in the parental plasmid pGW324.

Figure 3. Southern analysis of re-isolated plasmids: EcoRI - SalI double digests



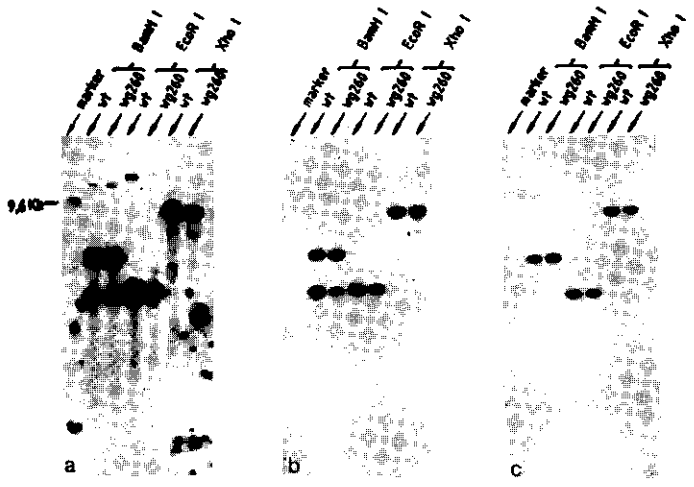
Plasmids re-isolated from undigested DNA of AmdS$^+$ transformant r13t1 were digested with EcoRI and SalI and submitted to gel electrophoresis on 0.6% agarose. The DNA was transferred to nitrocellulose membranes in triplicate and hybridized with ^{32}P-labelled DNA fragments. The arrows indicate the positions of minor hybridization signals visible on the original autoradiograms.
Panel A: EtBr-stained gel. Panel B: blot hybridized with EcoRI - SalI amdS fragment. Panel C: blot hybridized with pBR322 DNA. Panel D: blot hybridized with the 3.3kb EcoRI fragment of A. nidulans rDNA.
Fragments were isolated from pGW324 (Wernars et al. 1985) which is identical to pRRR13.

Figure 4: Southern analysis of re-isolated plasmids: HaeIII digests



Plasmids re-isolated from undigested DNA of AmdS$^+$ transformant r13t1 were digested with HaeIII and submitted to gel electrophoresis in 1.2% agarose. The DNA was transferred to a nitrocellulose membrane and hybridized with ^{32}P-labelled amdS fragment.
Panel A: EtBr-stained gel. Panel B: autoradiogram. pRRR13 is identical to pGW324. The arrow indicates the position of doublet bands.

Figure 5: Hybridization of re-isolated plasmids to chromosomal *A. nidulans* DNA



Chromosomal *A. nidulans* DNA from a wild type strain (wt) and strain MH1277 (WG260) was digested with *Bam*HI, *Eco*RI or *Xho*I, fractionated by gel electrophoresis and transferred to nitrocellulose membranes (in triplicate). Re-isolated plasmids were ^{32}P-labelled and used as hybridization probes.
Panel A: blot hybridized with p21-1. Panel B: blot hybridized with p21-10. Panel C: blot hybridized with p21-11. Marker lane contains linearized p3SR2 DNA (1 ng).

Discussion

Our results show that transforming vector DNA sequences can be re-isolated from AmdS$^+$ transformants of *A. nidulans* strain MH1277 - even in the most unfavourable situation. When only one copy of the vector had integrated into the genomic DNA, isolation by *in vitro* circularization was possible. Resulting plasmids revealed incompleteness of the physical map of the chromosomal *amdS* region, previously published by Hynes et al. (1983). Since this map was based on data from genomic blotting experiments, being more prone to errors than plasmid mapping, Hynes et al. probably overlooked these sites.

From AmdS$^+$ transformants, carrying multiple copies of the inserted vector, plasmids could be recloned at a considerably higher frequency. These showed a similar length polymorphism as found in Southern blots of *EcoRI* digested chromosomal DNA (Wernars et al. 1985). Some of these *EcoRI* fragments have not been found among the re-cloned plasmids, probably due to the absence of a pBR322 origin of replication and/or β-lactamase gene.

Furthermore, a clear correlation was present between the number of integrated vector copies in each *A. nidulans* transformant and the frequency in which plasmids could be recloned.

One plasmid re-isolated from transformant ct2 could not have been formed by *in vitro* circularization. Extraction of this plasmid as a closed circular molecule from *A. nidulans* would be a plausible explanation for its isolation. Indeed vector molecules could also be isolated in *E. coli* using undigested AmdS$^+$ transformant DNA although even thorough analysis by Southern blotting had not revealed any sign of free plasmids in this material. The presence of very large free multimeric vector co-integrates could not have been detected in this way. However, upon transformation into

E. coli such co-integrates would resolve, yielding a heterogeneous colony. Since these were never found (not shown) we consider this explanation unlikely.

An other possibility could be that the plasmids have arisen by recombination dependent circularization of tandem vector repeats in E. coli, which can occur even in a recombination deficient strain (Conley and Saunders 1984). The CsCl/EtBr centrifugations however, demonstrated the presence of free covalently closed circular vector molecules in the A. nidulans DNA preparations. Moreover the use of a recombination proficient E. coli as recipient strain did not increase the transformation frequency (results not shown). We therefore conclude that recombination dependent circularization does not play an important role in the formation of re-isolated plasmids.

Like the recloned plasmids, also plasmids isolated from undigested DNA show a wide range of sizes and differences in restriction patterns. Nevertheless, those examined all exclusively consisted of DNA sequences present in the parental vector whereby duplications and/or deletions had taken place. In view of the resemblance with the state of the integrated vector DNA, consisting of large, rearranged tandems, we propose that the free plasmid molecules arise by excision through in vivo recombination between individual copies within such a tandem repeat.

Also for A. nidulans transformants, obtained with other selection markers indications have been found for the presence of free vector molecules (Johnstone et al. 1985; Ballance and Turner 1985). Although this certainly needs further investigation, the presence of free vector molecules might be a common feature for many A. nidulans transformants.

The prospects of gene cloning in Aspergillus using the amdS selection marker are promising. It offers fairly high transforma-

tion frequencies, amplification of the cloned sequences, a stable mitotic transmission and, as shown here, a simple re-isolation procedure.

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CHAPTER 5

One-step gene replacement in *Aspergillus nidulans* by cotransformation.

Karel Wernars, Theo Goosen, Bert M.J. Wennekes, Klaas Swart, Cees A.M.J.J. van den Hondel and Henk W.J. van den Broek

Summary

When during the transformation of amdS320 deletion strains of A. nidulans with a vector containing the wild type amdS gene an non-selected DNA sequence was added, the AmdS$^+$ transformants were cotransformed at high frequency. Cotransformation with an amdS320, trpC801 double mutant strain showed that both the molar ratio of the two vectors and the concentration of the cotransforming vector affect the cotransformation frequency. The maximum frequency obtained, was defined by the gene chosen as selection marker for transformation.

Cotransformation was applied to induce a gene replacement in A. nidulans. An amdS320 strain was transformed to AmdS$^+$ and cotransformed with a DNA fragment containing a fusion between a non-functional A. nidulans trpC gene and the E.coli lacZ gene. Ten AmdS$^+$, LacZ$^+$ transformants with a Trp$^-$ mutant phenotype were selected. All of these strains could be transformed with a functional copy of the A. nidulans trpC gene, but only two strains yielded TrpC$^+$ transformants which, with a low frequency, had a LacZ$^-$ phenotype. These latter transformants had also lost the AmdS$^+$ phenotype.

Southern blotting analysis of DNA from these transformants confirmed the inactivation of the wild type trpC gene, but revealed that also amdS vector sequences were involved in the gene replacement events.

Introduction

Intensive research in the last few years has resulted in the development of methods for genetic manipulation of fungi at the

molecular level (for a review see Mishra 1985). For Aspergillus nidulans transformation procedures have been developed based on complementation of mutant strains with cloned genes (Ballance et al. 1983; Tilburn et al. 1983; Yelton et al. 1984; John and Peederby 1984). In all reports on transformation of A. nidulans so far this complemetation is effectuated by integrated copies of the transforming vector in the genomic DNA of the recipient.

Plasmids carrying a selectable marker gene have successfully been used as vectors to clone genes in A. nidulans e.g. yA (Yelton et al. 1985) and β-tubulin genes (May et al. 1985). Also the introduction of an A. nidulans trpC-E. coli lacZ hybrid gene into A. nidulans (van Gorcom et al. 1985) was based on such a vector. A disadvantage of this procedure is the need to link the DNA of interest covalently with the vector containing the selectable gene. In bacteria (Kretschmer et al. 1975), yeasts (Hicks et al. 1978; Gaillardin et al. 1985) and mammalian cells (Wigler et al. 1979) introduction of non-selected DNA sequences can be accomplished by cotransformation with a selectable gene without prior covalent linkage of both sequences.

In this report we have employed the amdS gene (Hynes et al. 1983), the trpC gene (Yelton et al. 1983) and the A. nidulans trpC-E. coli lacZ hybrid gene (van Gorcom et al. 1985) to demonstrate that in A. nidulans too, cotransformation can be used as a practical tool for the introduction of non-selected DNA sequences.

Development of transformation systems for A. nidulans has also provided the opportunity to study gene expression and regulation at the molecular level. Cloned genes can be mutated in vitro and subsequently reintroduced into the A. nidulans genome. In such studies it is essential to direct the mutated gene to a predetermined chromosomal location. In the yeast Saccharomyces cerevisiae homologous recombination between a transforming DNA se-

quence and its counterpart on the genome of the recipient has been used to obtain transformants in which the former had replaced the latter in either one-step or two-step procedures (Scherer and Davis 1979; Rothstein 1983) and even methods have been developed which directly select for this event (Struhl 1983).

Also from the fungus Neurospora crassa, in which integration of transforming sequences at non-homologous loci is far more frequent than in S. cerevisiae (Dhwale and Marzluf 1985), transformants have been isolated which had undergone replacement of a resident gene by an in vitro disrupted copy (Paietta and Marzluf 1985). Although non-homologous integration in A. nidulans is in some cases very high (Wernars et al. 1985), at least with some genes site directed replacement can be realized (Miller et al. 1985).

Here we report the use of cotransformation to generate TrpC$^-$ mutants in which the wild type trpC gene has been replaced. The nature of these transformants is analyzed and discussed.

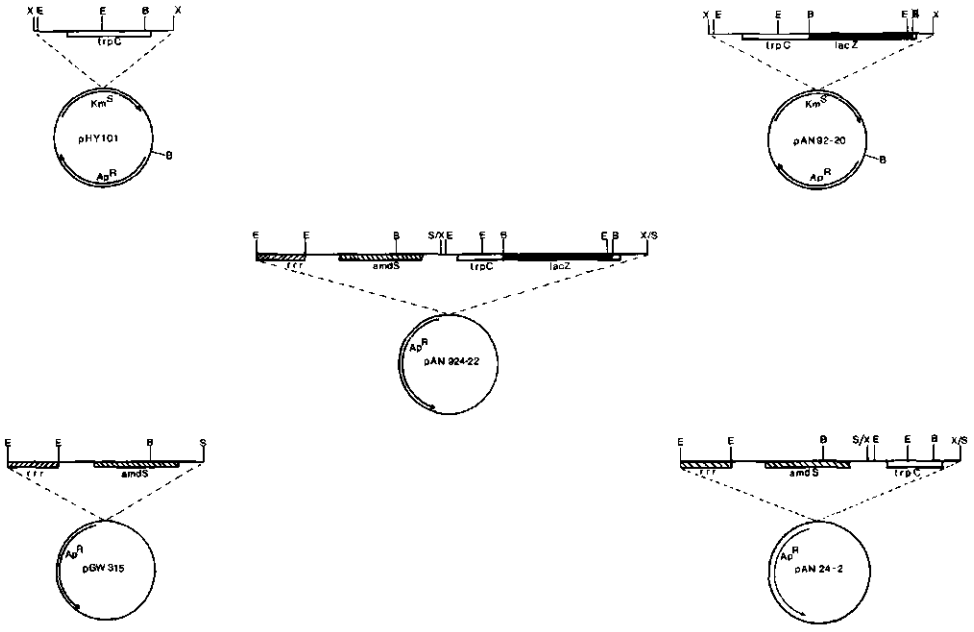
Materials and Methods

Strains. A. nidulans strains MH1277, WG290 and FGSC237 were described previously (Hynes et al. 1983; Tilburn et al. 1983; Wernars et al. 1985; this thesis; Yelton et al. 1984). Strain WG316 (yA1, pabaA1; amdI18, amdS320; trpC801) was constructed by somatic recombination (Bos and Kobus, unpublished).

All plasmids were propagated in E. coli strain MH1 (Goddard et al. 1983).

DNA isolation and manipulation. Isolation of plasmids and A. nidulans DNA was described previously (Wernars et al. 1985). The plasmids used in this study are described in fig. 1.

Figure 1: Plasmids used in this study.



- pGW315: amdS containing vector with a fragment from the A. nidulans ribosomal repeat (Wernars et al, 1985).
- pHY101: trpC containing vector (Yelton et al, 1984).
- pAN24-2: pGW315-derivative containing the A. nidulans trpC gene (van Gorcom, unpubl. results).
- pAN92-20: constructed from pAN92-2 (van Gorcom et al. 1985) by removal of the BamHI site distal to the lacZ sequence.
- pAN924-22: pGW315-derivative containing the A. nidulans trpC - E. coli lacZ hybrid gene from pAN92-2 (van Gorcom et al. 1985).

DNA was manipulated according to standard procedures described previously (Wernars et al. 1985).

Media, growth and transformation of A. nidulans. Media and growth conditions have been described previously (Wernars et al. 1985; this thesis). For growth of tryptophan auxotrophs all media were supplemented with 1 mg/ml L-tryptophan.

Transformations were carried out with conidial protoplasts (Bos and Slakhorst 1985) using the following method: A maximum of 10 µl DNA solution in sterile bidest was mixed with 2.10^7 protoplasts in 0.2 ml sorbitol solution (1.0 M sorbitol, 50 mM $CaCl_2$). After addition of 50 µl PEG-buffer (25% w/v polyethyleneglycol 6000, 50 mM $CaCl_2$, 10 mM Tris pH 7.5) the mixture was incubated on ice for 20 minutes. Then 2 ml PEG buffer was added and after a 5 minutes interval 4 ml sorbitol solution both at room temperature. Aliquots were plated on MMS using a toplayer of 4 ml MMS containing 1.3% agar. To test A. nidulans transformants for the expression of bacterial β-galactosidase activity they were screened for blue colour on solidified M9 medium (pH 7.5), containing 2% glucose and 40 µg/ml X-gal (van Gorcom et al. 1985).

Enrichment for Trp^- mutants. Trp^- mutants were isolated by filtration enrichment as used for the isolation of auxotrophic mutants and recombinants (Bos et al. 1981; Bos 1985). Twenty ml of liquid MM, supplemented with 8 µM pantothenic acid, was inoculated with $2x10^7$ conidiospores and incubated for 14 hours at 37 °C under vigorous agitation. Then the suspension was filtered through a sterile funnel containing a glasswool plug to remove germinated conidiospores. The material that passed the filter was pelleted by centrifugation, resuspended in 20 ml fresh medium and incubated for another 5 hours. Filtration was then repeated and aliquots of

the resulting suspension were plated on ME containing tryptophan. Such a treatment resulted in a reduction of colony forming units to $0.5-1.0 \times 10^4$.

Results

Cotransformation. As shown previously (van Gorcom et al. 1985), approximately 85% of the AmdS$^+$ transformants from strain MH1277, obtained with a vector containing both the cloned amdS gene and the trpC-lacZ hybrid gene, exhibited expression of a β-galactosidase fusion protein.

In a similar experiment this strain was transformed with equimolar amounts of pGW315 (amdS) and pAN92-20 (trpC-lacZ). When tested for lacZ gene expression a large proportion of the AmdS$^+$ transformants turned blue on X-gal medium (Table I). In fact the percentage of β-galactosidase expressing transformants was as high as that obtained with both genes on one composite vector.

As strain MH1277 is known to integrate large numbers of vector DNA copies upon transformation (Wernars et al. 1985, chapter 2), this phenomenon could have accounted for the high cotransformation frequency. However with the amdS320 strain WG290 which in general does not exhibit multi-copy integration, similar cotransformation frequencies were obtained (Table 1). This indicates that cotransformation frequencies are high irrespective the acceptor strain used.

To correlate the cotransformation frequency and the concentrations of the cotransforming vectors, pGW315 (amdS) and pHY101 (trpC) were used in combination with the amdS320, trpC801 double mutant strain WG316. This strain was transformed with mixtures of both plasmids in various concentrations and either AmdS$^+$ or TrpC$^+$

Table 1: Transformation and cotransformation of A. nidulans (amdS320)

vector	AmdS$^+$ transf. (transf/µg DNA)		AmdS$^+$ LacZ$^+$ phenotype (%)	
	MH1277	WG290	MH1277	WG290
pGW315(amdS)	51	47	0	0
pAN924-22 (amdS, trpC-lacZ)	55	52	78	81
pGW315(amdS) + pAN92-20(trpC-lacZ)	47	53	83	85

A. nidulans strains MH1277 and WG290 were transformed with 5 µg of each plasmid. AmdS$^+$ transformants were selected and analyzed for expression of β-galactosidase. Transformation frequencies are per µg of amdS vector.

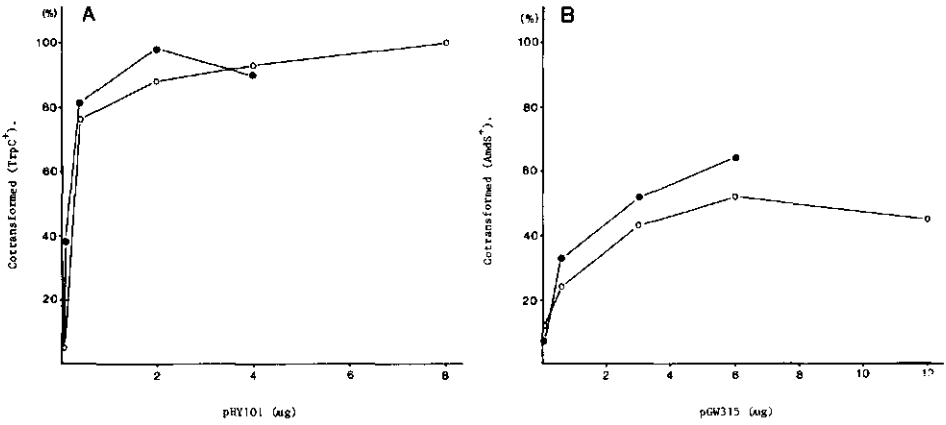
transformants were selected. These transformants were then tested for the expression of the second marker gene. As a control transformants obtained with plasmid pAN24-2 (amdS and trpC) were tested similarly. The results of this experiment are represented in Figure 2.

The graph in panel A of this figure represents a situation in which AmdS$^+$ transformants were selected upon transformation with a plasmid mixture containing a fixed concentration (0.6 µg or 6.0 µg) of the amdS vector and various amounts of cotransforming trpC vector). At low concentrations increase of the amount of the

cotransforming trpC vector results in a sharp increase of the cotransformation frequency whereupon both curves deflect and reach a maximum level of about 95%.

The effect of reversing this cotransformation experiment with respect to both markers is shown in panel B of Figure 2. Although the shape of the curves obtained is similar to that of panel A, the maximum cotransformation frequency reached is markedly lower:

Figure 2: Cotransformation of A. nidulans with amdS and trpC genes.



The A. nidulans amdS, trpC double mutant strain WG316 was cotransformed with various amounts of both vectors. Selected transformants were analyzed for the expression of the second marker gene.

A) Selection AmdS$^+$ (o——o: 6 µg pGW315; ●——●: 0.6 µg pGW315), cotransformation TrpC$^+$.

B) Selection TrpC$^+$ (o——o: 4 µg pHY101; ●——●: 0.4 µg pHY101), cotransformation AmdS$^+$.

only up to 50-60% of the TrpC$^+$ selected colonies possessed also the AmdS$^+$ phenotype.

The maximum cotransformation frequencies were similar to those found in the control experiments with pAN24-2: 95% of the AmdS$^+$ transformants was also TrpC$^+$ and 62% of the TrpC$^+$ transformants had an AmdS$^+$ phenotype.

Induction of Trp$^-$ mutants by cotransformation. In the previous section we have demonstrated that genes can efficiently be introduced into A. nidulans without selection, using cotransformation. To replace the wildtype trpC gene by the in vitro constructed A. nidulans trpC-E. coli lacZ hybrid gene, strain WG290 was transformed with a mixture of 2 µg pGW315 and 2 µg pAN92-20. Since in other organisms gene replacement appears to be favoured by the use of linear DNA (Orr-Weaver et al. 1981; Paietta and Marzluf 1985) the transformation was carried out with either XhoI-digested or undigested pAN92-20 DNA. AmdS$^+$ transformants were selected on medium supplemented with tryptophan and screened for LacZ$^+$ expression. With both digested and undigested cotransforming vector about 75% of the transformants exhibited bacterial β-galactosidase activity.

These transformants were then screened for Trp$^-$ mutants by stab inoculation of conidiospores on medium lacking tryptophan. Among 150 AmdS$^+$, LacZ$^+$ transformants, obtained with XhoI digested pAN92-20, two tryptophan auxotrophs were found. With the undigested DNA, 150 AmdS$^+$, LacZ$^+$ transformants yielded none. Both AmdS$^+$, LacZ$^+$, Trp$^-$ colonies obtained were designated Ta and Tb.

To obtain more Trp$^-$ mutants, transformation experiments were repeated using 5 µg of pGW315 and 7 µg of XhoI-digested pAN92-20. AmdS$^+$ transformants were selected at a density of 100 sporulating colonies per plate. Eighteen pools were prepared each containing

the conidiospores of two of these plates and this material was used for the selection of Trp$^-$ mutants. Dilutions were plated on ME containing tryptophan to obtain single colonies of which 200 were tested from each pool. In two pools (1 and 17) tryptophan auxotrophs were encountered (one and four, respectively), all showing LacZ$^+$ expression. From both pools one was taken for further analysis; they were designated T1 and T17, respectively.

Since the abundancy of Trp$^-$ conidia was apparently low, an enrichment procedure for these mutants was applied for eight pools

Table 2: Abundancy of Trp$^-$ and LacZ$^+$ colonies obtained from conidiospore pools.

pool number	not enriched		enriched for Trp$^-$	
	Trp$^-$ per 200 colonies tested	lacZ$^+$ (%)	Trp$^-$ per 100 colonies tested	LacZ$^+$ (%)
11	0	25	1	19
12	0	33	3	16
13	0	29	4	16
14	0	53	0	28
15	0	30	7	22
16	0	56	1	24
17	4	57	4	43
18	0	62	1	25

Single colonies were obtained on ME supplemented with L-tryptophan and these were tested for tryptophan requirement and expression of the trpC-lacZ hybrid gene.

as described in Materials and Methods. Aliquots of the treated suspensions were plated and for each pool 100 colonies were tested for tryptophan auxotrophy and expression of the β-galactosidase fusion protein. The results are shown in Table 2.

After enrichment, in seven out of eight pools one or more tryptophan requiring colonies, all expressing the *trpC-lacZ* hybrid gene were found. It should be noted that enrichment for Trp^- at the same time resulted in reduction of the percentage of colonies with a $LacZ^+$ phenotype.

From these Trp^- transformants six independent ones were taken and designated T11, T12, T13, T15, T16 and T18. The rate of spontaneous reversion of these strains to tryptophan prototrophy was determined to be below 10^{-8} per conidiospore.

Transformation of Trp^-,$LacZ^+$ *strains to tryptophan prototrophy.* To investigate the possibility of restoring tryptophan prototrophy by reintroduction of the intact *trpC* gene, all ten Trp^-, $LacZ^+$ mutant strains described above and the *trpC801* mutant FGSC237 were transformed with 10 μg *Xho*I-digested pHY101 DNA. Each of these strains could be transformed to $TrpC^+$, indicating that all induced Trp^- strains, were $TrpC^-$ indeed. Surprisingly however, whereas the classical *trpC801* mutant strain FGSC237 gave rise to about 40 transformants per μg vector DNA, the $TrpC^-$, $LacZ^+$ mutants transformed at frequencies of 1100-1400 per μg DNA.

We considered the possibility that these high transformation frequencies were correlated with the presence of bacterial sequences in the genome of the $TrpC^-$, $LacZ^+$ strains. Therefore the *amdS320*, *trpC801* double mutant strain WG316 was transformed with plasmids pGW315 (*amdS*) or pHY101 (*trpC*) to $AmdS^+$ or $TrpC^+$ phenotype, respectively. These transformants were then used as acceptor strain in a transformation experiment with the second marker. No

differences in transformation frequencies could be detected between the original double mutant acceptor strain and the strains that had already been transformed once, thus invalidating our supposition.

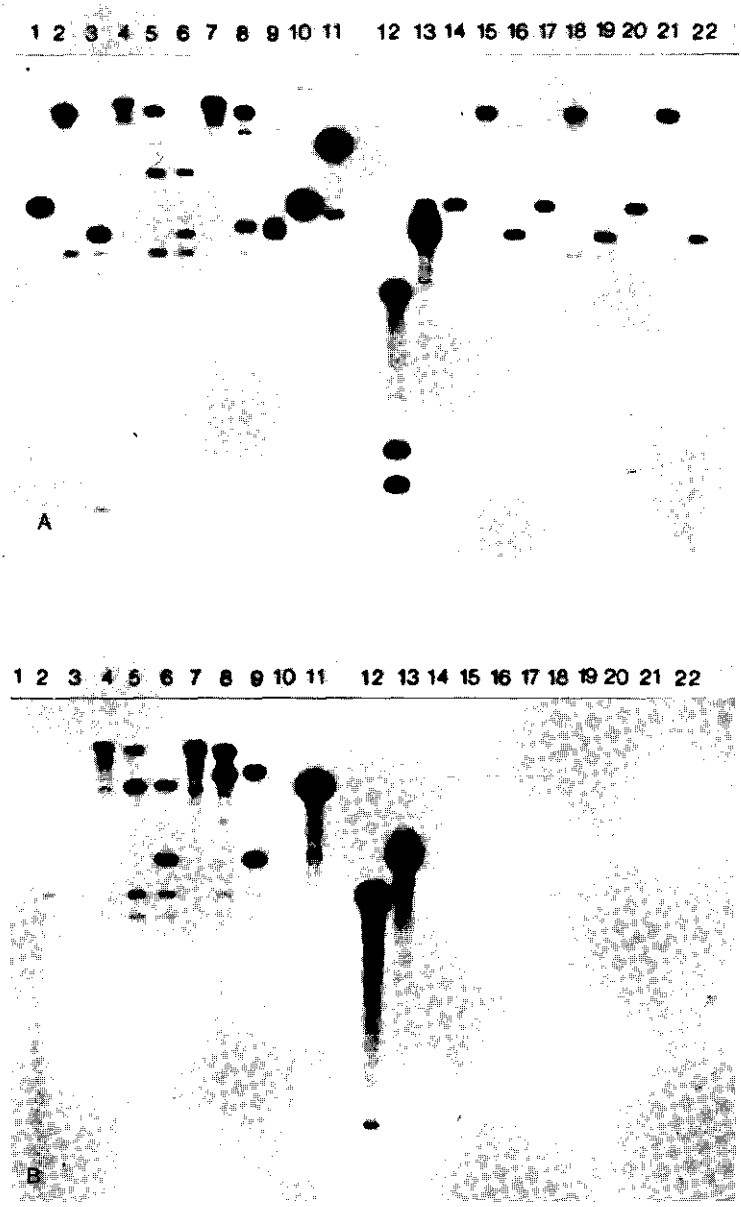
If transformation of a TrpC$^-$, LacZ$^+$ strain to tryptophan prototrophy was the result of replacement of the trpC-lacZ hybrid gene by a copy of the wild type trpC gene, then its LacZ$^+$ phenotype should be abolished provided that no more than one biologically active copy of the trpC-lacZ gene was present in the genome of that strain.

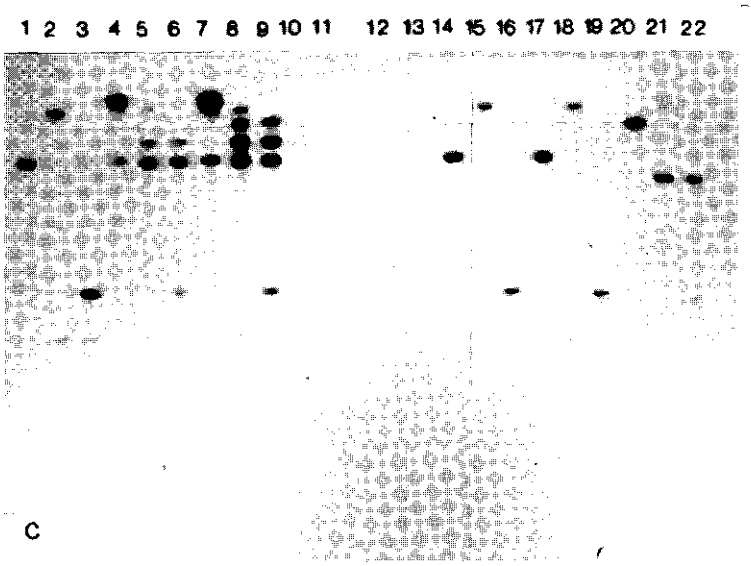
To isolate such TrpC$^+$, LacZ$^-$ colonies, transformants obtained with XhoI-digested pHY101 DNA were tested. Conidiospores from about 1200 TrpC$^+$ transformant colonies were collected and plated on X-gal medium. Only from TrpC$^-$, LacZ$^+$ strains T1 and T13 TrpC$^+$ transformants were found which did not express the β-galactosidase fusion protein. These LacZ$^-$ colonies occurred at a frequency of 10^{-2} per conidiospore. At the same time both strains had lost then AmdS$^+$ phenotype, suggesting that in T1 and T13 the amdS and trpC-lacZ genes are contiguous. One T1-derived and one T13-derived white TrpC$^+$ colony was taken for further analysis and designated T1W and T13W, respectively.

Biochemical analysis of TrpC$^-$,LacZ$^+$ and TrpC$^+$, LacZ$^-$ transformants. The structural rearrangements in the DNA of the transformants described above were visualized by Southern blotting analysis. DNA from these transformants was isolated, digested with various restriction endonucleases, fractionated by electrophoresis, blotted onto nitrocellulose and hybridized with ^{32}P-labelled probes (trpC, lacZ and amdS). The resulting autoradiograms are shown in Figure 3.

In the TrpC$^-$, LacZ$^+$ transformants T1 and T13, the 4.2 kb wild

Figure 3: Southern analysis of TrpC$^-$, LacZ$^+$ transformants T1 and T13 and TrpC$^+$, LacZ$^-$ derivatives T1W and T13W.





A. nidulans DNA was isolated, 2 µg was digested with restriction enzymes, fractionated on a 0.6% agarose gel and blotted to nitrocellulose in triplicate. Blots were hybridized with ^{32}P labelled probes.

Lanes 1, 2 and 3: WG290 DNA; lanes 4, 5 and 6: T1 DNA, lanes 7, 8 and 9: T13 DNA, lanes 14, 15 and 16 T1W DNA, lanes 17, 18 and 19: T13W DNA, lanes 20, 21 and 22: wild type A. nidulans DNA.

The DNA in lanes 1, 4, 7, 14, 17 and 20 was digested with XhoI, the DNA in lanes 2, 5, 8, 15, 18 and 21 with BamHI, the DNA in lanes 3, 6, 9, 16, 19 and 22 with XhoI and BamHI.

Lane 10: 3 ng pHY101 digested with XhoI, lane 11: 3 ng pAN92-20 digested with BamHI, lane 12: 3 ng pAN92-20 digested with XhoI, BamHI and EcoRI, lane 13: 3 ng pAN92-20 digested with XhoI and BamHI.

Panel A: Hybridized with labelled 4.2 kb trpC fragment

Panel B: Hybridized with labelled 3.1 kb lacZ fragment

Panel C: Hybridized with labelled 5.2 kb amdS fragment

type XhoI-fragment containing the resident trpC gene, was absent. However, in neither of these strains hybridisation signals corresponding to the 7.3kb XhoI-fragment carrying the trpC-lacZ hybrid gene were observed. Instead, only a band of high molecular weight (about 20-25 Kb), hybridizing to each of the trpC, lacZ and amdS probes was visible in these lanes. In the XhoI digests of T1 and T13 also the 6.6kb fragment was detected, corresponding to the XhoI amdS320 deletion fragment of WG290. This indicates that the transforming amdS vector had not integrated at the homologous, partially deleted amdS locus. Digestion with BamHI or double digestion with BamHI/XhoI revealed that for none of the probes used. T1 and T13 yielded identical hybridization patterns. Although the size of some of the hybridizing fragments correspond with those of the cloned trpC-lacZ hybrid gene (Figure 1) others could not be assigned. It is however obvious from digestion of the large XhoI fragment by BamHI, that the former contained both trpC, lacZ and amdS sequences. So the presumed gene replacement event at the trpC locus did not only involve the trpC-lacZ hybrid gene, but also amdS sequences.

Probing the restricted DNA from the $TrpC^+$, $LacZ^-$ transformants T1W and T13W with the labelled trpC fragment yielded a hybridization pattern undistinguishable from that obtained with WG290 DNA. At the same time hybridization with the amdS fragment showed that this pattern is also identical to the WG290 situation whereas the lacZ probe revealed the absence of bacterial β-galactosidase sequences. Thus in these transformants the wild type trpC gene has been restored and at the same time lacZ and amdS sequences have been removed. This lacZ probe showed some minor hybridization signals in all Aspergillus DNA preparations. We assume these were due to cross-hybridization between the bacterial β-galactosidase and some A. nidulans sequence.

Discussion

Cotransformation of *A. nidulans* is a very efficient process; the frequency with which it takes place can equal that of composite vectors. In this respect *A. nidulans* resembles other lower eukaryotes like the yeasts *S. cerevisiae* (Hicks et al. 1978) and *Yarrowia lipolytica* (Gaillardin et al. 1985) where cotransformation has also been reported to be efficient.

The experiments with the *amdS* and *trpC* genes indicate that the frequency of cotransformation is both determined by the absolute amount of cotransforming vector and the ratio of transforming and cotransforming vector. For best results high concentrations of both vectors should be used. The maximum cotransformation frequency to be reached depends on the nature of the cotransforming DNA: over 95% for *trpC* but only about 60% for *amdS*. This difference might reflect a relatively large number of *amdS* genes inactivated in the act of integration, since similar results are obtained with a plasmid containing both genes.

In our attempts to replace the wild type *trpC* gene by the *trpC-lacZ* hybrid gene only a small fraction of the $AmdS^+$, $LacZ^+$ cotransformants had acquired tryptophan auxotrophy, even when the hybrid gene was on a linear DNA fragment. This implies that gene replacements are rare events compared to *S. cerevisiae* (Scherer and Davis 1979) even to other *A. nidulans* transformation systems (Miller et al. 1985; Johnstone et al. 1985).

The determination of the exact frequency with which gene replacement occurs in our experiments is not straightforward. As found previously (Wernars et al. 1985) only a fraction of the conidia on a primary transformant colony actually has the transformant phenotype. For $TrpC^-$ cotransformant colonies this would mean that they will be overlooked in the stab-inoculation tests.

This problem could be overcome by pooling and replating the conidia from the primary transformants prior to testing, however the number of tryptophan auxotrophs within such a pool is no longer a measure for the frequency among the primary colonies. By using an enrichment procedure, Trp^- transformants could be isolated from seven out of eight pools, each containing conidia of about 200 colonies. This would suggest that at least 0.5% of the primary transformants contain tryptophan auxotrophic cells. It may be possible that by vegetative propagation of primary transformants, new $TrpC^-$, $LacZ^+$ colonies could have been generated by secondary events. Although such secondary rearrangements can occur in A. nidulans upon meiosis (Wernars et al. 1985; Miller et al. 1985) they, however, never have been observed upon mitosis.

The enrichment procedure for tryptophan auxotrophs showed to be both simple and effective. Especially in gene replacement studies, in which the desired transformants are rare but selectable, such an enrichment procedure may be very time saving.

Enrichment for tryptophan auxotrophs resulted in a simultaneous enrichment for $LacZ^-$, which appears to be contradictious since $TrpC^-$ conidia should be $LacZ^+$. Since the enrichment procedure is based on distinction of germinated and non-germinated conidia this is probably caused by a better germination of $LacZ^+$ conidia. It can be speculated that expression of the bacterial β-galactosidase is responsible for this phenomenon. A more likely explanation might be that the presence of one or more copies of the trpC-lacZ hybrid gene in the genome results in an increased depression of the resident trpC gene due to repressor dilution, which in turn resulted in a better germination. The possibility that the trpC-lacZ hybrid gene influences germination by specific integration on a locus involved in this process is falsified by the observation that among $TrpC^+$, $LacZ^+$ transformants analyzed no site specific

integration was found (unpublished results).

Transformation of the TrpC$^-$, LacZ$^+$ mutants to tryptophan prototrophy with pHY101 DNA results in transformation frequencies 30 times higher than obtained with the classical trpC801 mutant strain. Using a trpC801, amdS320 double mutant strain this phenomenon was not observed. A different genetic background of the strains involved may be responsible for such an increase of transformation frequency. On the other hand it may be connected with the presence of trpC-lacZ sequences in the genome.

Only from TrpC$^-$, LacZ$^+$ mutants T1 and T13 TrpC$^+$ transformants could be isolated which had simultaneously lost the LacZ$^+$ phenotype. This is characteristic for candidates having undergone a novel gene replacement. The other eight TrpC$^-$, LacZ$^+$ mutants probably contain multiple trpC-lacZ insertions, strongly decreasing the chances to abolish LacZ$^+$ expression, as it would require multiple independent replacement events.

Southern blotting analysis of DNA from T1 and T13 revealed the absence of the wild type trpC XhoI-fragment. However, the hybridization patterns were not as expected for a simple replacement of the trpC gene by trpC-lacZ. The results suggest that it was replaced by trpC-lacZ sequences containing co-integrations with amdS vector sequences, however without abolishing LacZ$^+$ expression. Cointegrate formation has been demonstrated previously for amdS transformation (Wernars et al. 1985). However it was seldomly observed in strain WG290. The presence of cointegrated sequences in both TrpC$^-$, LacZ$^+$ transformants analyzed here could be fortuitous. On the other hand it might have been caused by the use of linear DNA fragments for transformation. The hybridization patterns obtained with DNA from T1W and T13W upon probing with trpC and amdS DNA were identical to that of WG290 and no hybridization signals to a lacZ probe could be found. The reversion of T1 and

T13 to the TrpC$^+$ phenotype and simultaneously to both the AmdS$^-$ and lacZ$^-$ phenotype implicates a close linkage of both markers. This confirms the conclusion from the Southern analysis that these strains contain a trpC-lacZ-amdS cointegrate. Considering the low reversion frequencies of T1 and T13 to the TrpC$^+$ phenotype we discard the possibility of spontaneous excision. Our results suggest that in T1W and T13W the wild type trpC situation has been restored by a novel replacement event.

In this chapter we showed that cotransformation can be used for the introduction into A. nidulans of genes for which direct selection is not possible. Cotransformation is an efficient process and the cotransformation frequency of unlinked sequences can equal that of covalently linked genes.

In our replacement study with the A. nidulans trpC gene and the trpC-lacZ hybrid gene, cotransformation proved to be a helpful tool. Although gene replacement is possible in A. nidulans the whole procedure is less straightforward than for yeast. The problems encountered, resemble those in similar studies with Neurospora crassa (Paietta and Marzluf 1985). Non-homologous integration, co-integrations and integration of more than one copy can occur and make it necessary to check transformants with the desired phenotype by Southern analysis. Nevertheless, we think that gene replacement by cotransformation in A. nidulans is a potentially powerful method for the study of gene regulation and the generation of mutants.

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CHAPTER 6

The effect of the *A. nidulans ansl* sequence on Aspergillus transformation.

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Summary

Vectors which in addition to the N. crassa pyr4 gene contain the 3.5kb A. nidulans ansl DNA fragment, show increased transformation frequencies of a uridine auxotrophic A. nidulans strain (Ballance and Turner 1985). We have assessed in detail the effect of this ansl sequence on A. nidulans transformation, using the cloned A. nidulans amdS, trpC and argB genes as well as the N. crassa pyr4 gene as selection markers. With trpC, amdS and pyr4, but not with argB, ansl increased the transformation frequency when added on a cotransforming vector. In the case where the ansl sequence was covalently linked to the amdS gene on a composite vector, location of ansl within these vectors was found to determine its influence on the frequency of transformation. Using similar vector constructs with argB the effect of ansl was marginal.

Introduction

Transformation of A. nidulans is most commonly achieved by complementation of auxotrophic mutants with the cloned wild type gene. Most of the genes available are homologous e.g. amdS (Hynes et al. 1983), trpC (Yelton et al. 1983), argB (Berse et al. 1983) prn (Durrens et al. 1986). However, Ballance et al. (1983) developed a heterologous transformation system using the cloned N. crassa pyr4 gene (Buxton and Radford 1983) to transform an A. nidulans pyrG mutant strain to uridine prototrophy. In all A. nidulans transformants obtained the vector DNA has integrated into the genome (Tilburn et al. 1983; Ballance et al. 1983; Yelton et

al. 1984; John and Peberdy 1984; Wernars et al. 1985; Durrens et al. 1986).

To increase the practical use of A. nidulans transformation, e.g. for direct selection of cloned genes in A. nidulans, attempts have been made to improve the transformation frequency. For the amdS transformation system , parameters were examined and the transforming ability of newly constructed vectors was tested (Wernars et al. 1985). However, neither increased homology between vector and genomic DNA, nor the inclusion of yeast selected A. nidulans ARS sequences (Goosen et al. 1984) resulted in an increase of the A. nidulans transformation frequency.

Similar experiments carried out by Ballance and Turner (1985) with the N. crassa pyr4 gene yielded a 3.5kb DNA fragment, designated ans1, which conferred autonomous replication to yeast vectors and was found to increase the transformation frequency of the pyr4 gene 50-100 fold. However, the A. nidulans transformants obtained invariably carried the vector sequences integrated into the genome.

In this study we have examined the effects of ans1 on transformation of A. nidulans with various selection markers, using either composite vectors containing both ans1 and the selected gene or cotransformation of two separate vectors.

Material and Methods

- A. nidulans transformation.

Strains G191 (pabaA1, pyrG89; fwA1, uaY9: Ballance and Turner 1985), FGSC237 (pabaA1, yA2; trpC801: Yelton et al. 1984), MH1277 (biA1; amdI18, amdS320; amdA7; niiA4: Hynes et al. 1983) and WG328

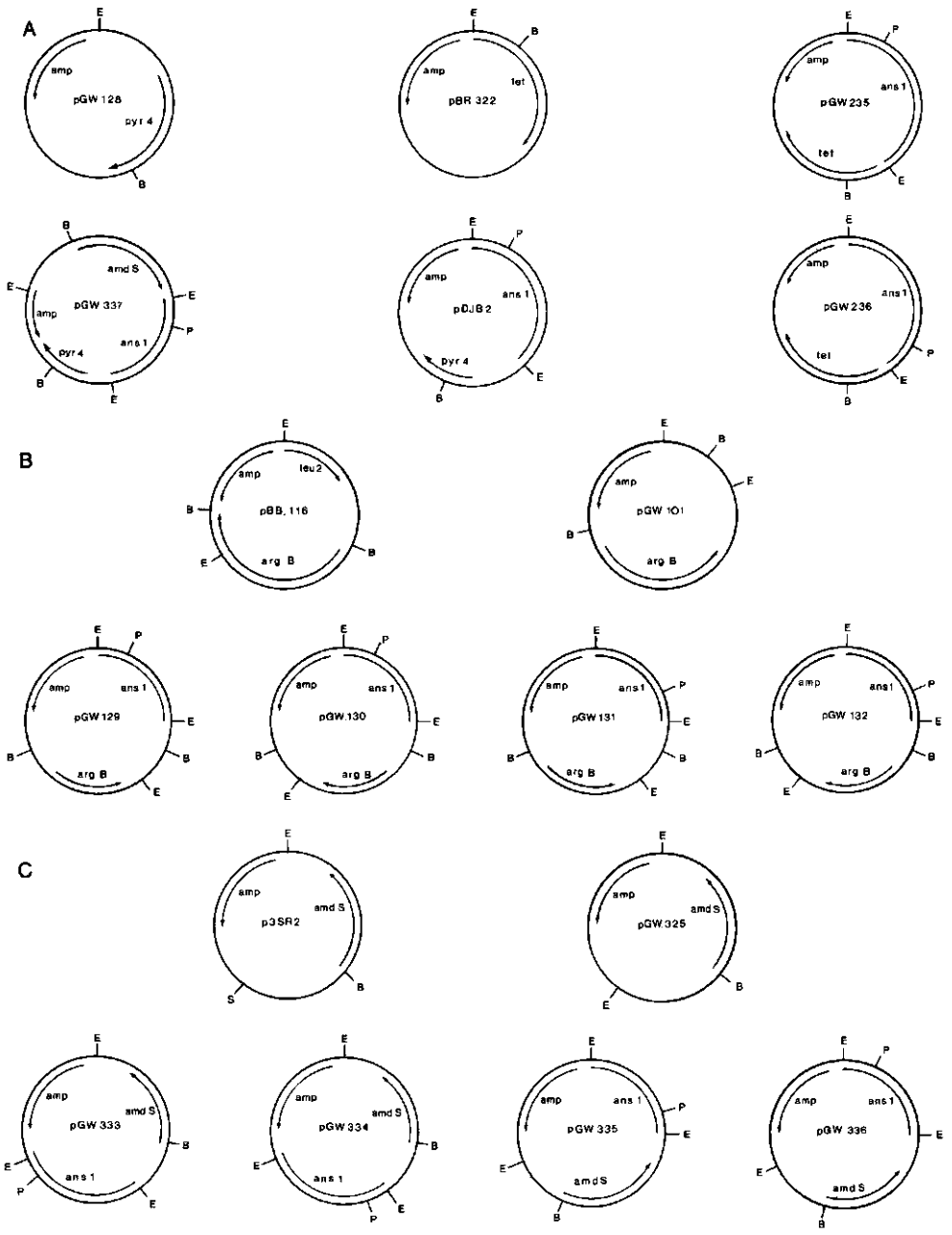
(biA1; methH2, argB2: constructed in our laboratory by dr. C.J. Bos) were used for transformation. Media have been described previously (Wernars et al. 1985). For growth of auxotrophs the appropriate supplements were added: tryptophan (1mg/ml), arginine (0.5 mg/ml), uridine (3 mg/ml), D(+)-biotin (10 µg/ml), para-aminobenzoic acid (0.2 µg/ml) and methionine (20 mg/ml).

Transformation was carried out with conidial protoplasts (Bos and Slakhorst 1981) using the following procedure: A maximum of 10 µlDNA solution in sterile bidest was mixed with 2.10^7 protoplasts in 0.2 ml sorbitol solution (1.0 M sorbitol, 50 mM $CaCl_2$). After addition of 50 µl PEG-buffer (25% w/v polyethylene glycol 6000, 50 mM $CaCl_2$, 10 mM Tris pH 7.5), the mixture was incubated on ice for 20 minutes. Then 2 ml PEG buffer was added and after a 5 minutes interval 4 ml sorbitol solution, both at room temperature. Aliquots were plated on MMS using a toplayer of 4 ml MMS containing 1.3% agar, held liquid at 47 °C. To test A. nidulans transformants for the expression of bacterial β-galactosidase activity, these were screened for their ability to give blue coloured colonies on solid M9 medium (pH 7.5), containing 2% glucose and 40 µg/ml X-gal (van Gorcom et al. 1985).

- Construction of plasmids.

Plasmids p3SR2 (Hynes et al. 1983; Wernars et al. 1985), pDJB2 (Ballance and Turner 1985), pAN92-20 (chapter 5) and pBB116 (Berse et al. 1983) have been described. The constructions of the other plasmids used in this study are schematically shown in figure 1.

Figure 1: Construction of vectors



A) Plasmid pGW128 was constructed by elimination of the ansl containing EcoRI fragment from pDJB2. Plasmid pGW128 is equivalent to pDJB1 (Ballance and Turner 1985). Plasmids pGW235 and pGW236 contain the EcoRI ansl fragment inserted in pBR322 in opposite orientations. Plasmid pGW337 was constructed by insertion of the EcoRI amdS fragment from pGW325 (panel C) into one of the EcoRI sites of pDJB2.

B) Plasmid pGW101 was constructed by insertion of the argB containing BamHI fragment from pBB116 into pBR322. Insertion of this BamHI fragment into pGW235 and pGW236 (panel A) yielded pGW129, pGW130, pGW131 and pGW132.

C) Plasmid pGW325 was obtained from p3SR2 by converting the SalI site into an EcoRI site, using Klenow DNA polymerase and a synthetic linker. Insertion of the EcoRI fragment containing the ansl sequence into one of the EcoRI sites of pGW325 yielded plasmids pGW333, pGW334, pGW335 and pGW336.

Only relevant restriction sites are indicated.

E = EcoRI; B = BamHI; P = PvuII; S = SalI.Results

Results

- ansl and the N. crassa pyr4 selection marker.

Since our transformation procedure is slightly different from the one used by Ballance and Turner (1985), the effect of ansl was reassessed (table 1). Indeed the ansl containing vector pDJB2 gave rise to an increased number of Pyr^+ transformants of both type I (stable) and type II (abortive) (Wernars et al. 1985) as compared with vector pGW128.

Since it is known from our previous studies (chapter 5) that A.

Table 1: Effects of the ansl sequence on the frequency of transformation using the pyr4 gene

vectors	Pyr^+ transf./µg DNA	
	type I	type II
pGW128(pyr4)	$2.6x10^2$	$1.3x10^3$
pDJB2(pyr4, ansl)	$3.0x10^3$	$3.0x10^3$
pGW128(pyr4)+pAN92-20(trpC-lacZ)	$1.5x10^2$	$7.0x10^2$
pDJB2(pyr4, ansl)+pAN92-20(trpC-lacZ)	$1.5x10^3$	$3.0x10^3$
pGW128(pyr4)+pGW236(ansl)	$2.4x10^3$	$3.0x10^3$

Strain G191 was transformed with 5 µg of each vector. Pyr^+ transformants were classified according to their morphology (Wernars et al. 1985): type I (well growing) and type II (abortive). Transformation frequencies are per µg pyr4 containing vector.

nidulans can be cotransformed very efficiently, we investigated the effect of ansl on the frequency of cotransformation. Strain G191 was transformed with pGW128, or pDJB2, in the presence of an equal amount of vector pAN92-20. From both experiments 100 type I colonies were tested for bacterial β-galactosidase expression. Among the pGW128 transformants 57% expressed the hybrid trpC-lacZ gene, whereas of the pDJB2 transformants this was only 15%. Although pyr4 transformation is stimulated, the frequency of trpC-lacZ cotransformation is decreased.

The enhancing effect on transformation is not dependent on covalent linkage of the ansl and pyr4 sequences. Cotransformation

of strain G191 with a mixture of pyr4 containing (pGW128) and ansl containing (pGW236) vectors is almost as efficient as transformation with the composite vector pDJB2 (Table 1).

- ansl and the A. nidulans trpC selection marker.

The effect of ansl on transformation with the A. nidulans trpC gene (Yelton et al. 1984) was examined by cotransforming ansl containing vectors with the trpC vector pHY101. The results are summarized in Table 2.

Transformation of the tryptophan auxotrophic strain FGSC237 with vector pHY101 yielded about 500 type I (stable) $TrpC^+$ transformants per µg of DNA. Cotransformation of pHY101 with the ansl containing vectors pGW236 or pDJB2 resulted in a five fold in-

Table 2: Effects of the ansl sequence on the frequency of transformation using the trpC gene

vectors	$TrpC^+$ transf./µg DNA	
	type I	type II
pHY101(trpC)	$5.0x10^2$	$>5x10^3$
pHY101(trpC)+pDJB2(pyr4, ansl)	$2.5x10^3$	$>5x10^3$
pHY101(trpC)+pGW236(ansl)	$2.5x10^3$	$>5x10^3$

Strain FGSC237 was transformed with 5 µg of each vector. $TrpC^+$ transformants were classified according to their morphology (Wernars et al 1985): type I (stable) and type II (abortive). Transformation frequencies are per µg of trpC containing vector.

crease of this frequency. In all experiments, type II (abortive) TrpC$^+$ transformants were observed in such high frequencies that an increase by ansl would have escaped notice.

These results suggest that ansl can also stimulate the frequency of trpC transformation in trans. Furthermore, the ansl sequence does not require the N. crassa pyr4 gene to exert its stimulating effect.

- ansl and the A. nidulans amdS selection marker.

The effect of ansl on transformation with the A. nidulans amdS gene was studied using a variety of vectors, each containing both ansl and amdS sequences but with different relative orientations (see Fig. 1, C) and the results are presented in table 3.

With pGW333 and pGW334 the frequency of type I (stable) AmdS$^+$ transformants obtained was 20 to 30 times higher than with the ansl-less vector pGW325, whereas the number of type II (abortive) AmdS$^+$ transformants about doubled. These two composite vectors contain ansl inserted upstream of the amdS gene. However, plasmids pGW335 and pGW336, having ansl inserted downstream of the amdS gene, showed no increase. Apparently in the case of amdS the position of the ansl sequence with respect to the EcoRI amdS containing DNA fragment is decisive for the increase of the transformation frequency.

Also for amdS, cotransformation with pGW236 (ansl) or pDJB2 (pyr4, ansl) resulted in a 4 to 5 fold increase of the number of type 1 transformants, again showing the ability of ansl to act in trans.

Surprisingly when pGW236 (ansl) was cotransformed with vectors pGW333, pGW334, pGW335 or pGW336, no increased transformation frequencies were observed (Table 3). With pGW333 and pGW334 the stimulating effect of the ansl sequence which is present on the

Table 3: Effects of the ansl sequence on the frequency of transformation using the amdS gene

vectors	AmdS$^+$ transf./µg DNA	
	type I	type II
pGW325(amdS)	$2.0x10^2$	$1.0x10^3$
pGW333(amdS, ansl)	$3.6x10^3$	$2.0x10^3$
pGW334(amdS, ansl)	$6.0x10^3$	$2.0x10^3$
pGW335(amdS, ansl)	$2.0x10^2$	$1.0x10^3$
pGW336(amdS, ansl)	$2.0x10^2$	$1.0x10^3$
pGW325(amdS)+pGW236(ansl)	$9.0x10^2$	$1.0x10^3$
pGW325(amdS)+pDJB2(pyr4, ansl)	$9.0x10^2$	$1.0x10^3$
pGW236(ansl)+pGW333(amdS, ansl)	$3.0x10^3$	$1.5x10^3$
pGW236(ansl)+pGW334(amdS, ansl)	$4.8x10^3$	$1.5x10^3$
pGW236(ansl)+pGW335(amdS, ansl)	$2.0x10^2$	$1.0x10^3$
pGW236(ansl)+pGW336(amdS, ansl)	$2.0x10^2$	$1.0x10^3$

Strain MH1277 was transformed with 5 µg of each vector. AmdS$^+$ transformants were classified according to their morphology: type I (stable) and type II (abortive). Transformation frequencies are per µg of amdS containing vector.

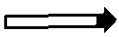
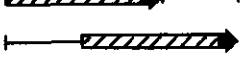
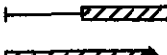
vector was not further reinforced by supplying ansl simultaneously in trans. In vectors pGW335 and pGW336 apparently the interaction of ansl and amdS is such that even the stimulating action of ansl, supplied on a cotransforming plasmid, is abolished.

Moreover, as demonstrated with vector pGW337 (amdS, ansl, pyr4) (Fig. 1,A), placing ansl downstream of the amdS fragment, also

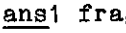
resulted in the loss of stimulation of pyr4 transformation.

This plasmid showed neither increased transformation frequencies in amdS nor in pyrG A. nidulans strains (Table 4).

Table 4: Influence of the amdS containing DNA fragment on the ansl effect

vectors	position and orientation of vector components	relative transformation frequency Pyr$^+$	relative transformation frequency AmdS$^+$
pGW128		low	-
pDJB2		high	-
pGW337		low	low
pGW336		-	low
pGW334		-	high
pGW325		-	low

Strains MH1277 (AmdS$^-$) and G191(Pyr$^-$) were transformed using 5 µg of vector DNA and the type I (stable) transformants were scored. Transformation frequencies are related to those of pGW128 (low) and pDJB2 (high) for pyr4 and to those of pGW325 (low) and pGW334 (high) for amdS.

 : pyr4 fragment,  :ans1 fragment,  amdS fragment.

The transcriptional direction of the amdS and pyr4 genes is shown (➧).

- ansl and the A. nidulans argB selection marker.

Analogous to amdS a set of four plasmids was constructed carrying the A. nidulans argB gene (Berse et al. 1983) in combination with the ansl sequence Ffig. 1,B). These vectors, designated pGW129, pGW130, pGW131 and pGW132, were used to transform strain WG328 to arginine prototrophy. As indicated in Table 5 none of these constructs yielded an increased number of type I (stable) transformants compared to the ansl-less vector pGW101. The plasmids pGW131 and pGW132 however, showed some increase of type II (abortive) transformants. The transformation frequency of pGW101

Table 5: Effects of the ansl sequence on the frequency of transformation using the argB gene

vectors	$ArgB^+$ transf./µg DNA	
	type I	type II
pGW101(argB)	$1.0x10^2$	$5.0x10^3$
pGW129(argB, ansl)	$1.0x10^2$	$5.0x10^3$
pGW130(argB, ansl)	$1.0x10^2$	$5.0x10^3$
pGW131(argB, ansl)	$1.0x10^2$	$2.4x10^4$
pGW132(argB, ansl)	$1.0x10^2$	$2.4x10^4$
pGW101(argB)+pGW236(ansl)	$1.0x10^2$	$5.0x10^3$
pGW101(argB)+pDJB2(pyr4, ansl)	$1.0x10^2$	$5.0x10^3$

Strain WG328 was transformed with 5 µg of each vector. $ArgB^+$ transformants were classified according to their morphology: type I (stable) and type II (abortive). Transformation frequencies are per µg of argB containing vector.

was not affected by cotransforming ansl containing vectors.

Thus the effect of ansl on argB transformation is very small, may be even negligable, independent on relative orientations of the two sequences.

Discussion

The results presented here show that the increasing effect of the ansl sequence isolated by Ballance and Turner (1985) on A. nidulans transformation is not limited to the heterologous pyr4 gene, but is also found for other (homologous) selection markers. Moreover, for its stimulating effect ansl does not require the N. crassa pyr4 sequences. It is obvious however that the effect of ansl on transformation with various selection markers is not consistent.

Ballance and Turner (1985) reported a 50 to 100 fold increase oi the number of type I (stable) Pyr^+ transformants upon inclusion of the ansl sequence into the transforming vector and a simultaneous decrease of the number of type II (abortive) transformants. In our experiments only a 12 fold increase of the number of type I Pyr^+ transformants was observed, whereas we also found an increase in the number of type II colonies. These differences may be due to experimental circumstances.

Inclusion of the ansl sequence in argB vectors had a marginal effect on the frequency of transformation. Only two of the composite vectors constructed gave a slight increase in the number of type II $ArgB^+$ transformants.

With all selection markers examined (pyr4, trpC, argB and amdS), but with the exception of argB, cotransformation with a

separate vector containing ansl gave rise to increased transformation frequencies, indicating that for the ansl dependent stimulation, covalent linkage of ansl and the selection marker is not a prerequisite.

One might argue that the formation of cointegrates by the two cotransforming vectors, resulting in covalent linkage of ansl and the selection marker, preceeds the integration into the genome (Wernars et al. 1985). If such a model should be the basis of ansl dependent stimulation, then ansl should increase the formation of cointegrates and thus increase the overall cotransformation frequency. However, the decrease in frequency of trpC-lacZ cotransformants (Table 1) argues against this model.

For the amdS selection marker inclusion of ansl in the transforming vector did increase the transformation frequency but exclusively if the ansl sequence was inserted upstream of the amdS gene. Insertion of ansl downstream of amdS not only showed no increase of transformation frequency but also abolished the trans-acting stimulating effect of cotransforming ansl vectors.

Even more striking is the effect on Pyr^+ transformation with vector pGW337, which was constructed by insertion of a DNA fragment containing the amdS gene into the high-frequency transforming vector pDJB2. Vector pGW337, again with the ansl sequence downstream of the amdS gene, had lost the stimulating effect of ansl on the transformation frequency for both the amdS and the pyr4 selection marker.

The principle by which the ansl sequence increases the transformation frequency is not clear yet. However, our results are suggestive at some points. The observation that ansl can exert its stimulation in trans makes it likely that an ansl gene product is involved. Since ansl does not enhance the transformation frequency of all selection markers apparently also a more or less specific

site is required for this gene product to act upon. One could speculate that limited autonomous replication of the vector is the cause of the stimulation of ansl on transformation. Such replication would of course need a site to be initiated and one or more gene products to do so properly.

On the other hand one could imagine that stimulation of recombination might be the basis of ansl action. The situation however becomes much more complicated if the negative effect of amdS has to be accounted for. Also this inhibition seems to work in trans, as if some repressor for ansl function is involved. However, repression is dependent on the orientation of the amdS fragment rather then of the ansl fragment, suggesting that such repressing activity is located on the amdS fragment. In view of the size of the fragment and the approximate location of the amdS structural gene (Hynes, pers. comm.) this would certainly be possible.

Our results indicate that the ansl sequence might not be universally applicable to increase transformation frequency in A. nidulans. Moreover the observation that the amdS containing DNA fragment can completely abolish the stimulating effect of ansl implies a warning for the use of high frequency transforming ansl vectors to construct gene libraries in A. nidulans. Cloned DNA fragments exhibiting a similar effect will be underrepresented or even absent.

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CHAPTER 7

Summary and general conclusion

Although transformation of S. cerevisiae and N. crassa already could be achieved at the end of the seventies, positive results for A. nidulans had to await the isolation of useful selection markers. As soon as cloned fungal genes of homologous (amdS, trpC and argB from A. nidulans) and heterologous (pyr4 from N. crassa) origin became available transformation procedures for A. nidulans were developed (Ballance et al. 1983; Tilburn et al. 1983; Yelton et al. 1984; John and Pederby 1984). They all are based on the ability of these selection markers to complement auxotrophic A. nidulans mutants.

A disadvantage of these transformation markers is the need for an auxotrophic recipient strain. With dominant selection markers even wild type strains should be good recipients for transformation. However, dominant selection markers like bacterial drug resistance genes, could not be developed due to the insensitivity of A. nidulans for most antibiotics (chapter 2). As found later in our studies in some conditions the amdS gene may serve as a dominant selection marker. All A. nidulans transformation protocols originate from that or S. cerevisiae, being based on the incubation of protoplasts with DNA in the presence of $CaCl_2$ and polyethylene glycol (PEG).

In our study on A. nidulans transformation we initially focussed on the amdS marker (chapter 2, 3 and 4). Transformation of $AmdS^-$ strains with vectors containing the wild type amdS gene gives rise to two types of transformant colonies, viz. well growing, sporulating ones (type I) and tiny non-sporulating ones, with stagnating growth (type II). This latter type is not specific

for the amdS marker, since with variable frequencies these have also been observed with other transformation markers (Yelton et al. 1984; John and Peberdy 1984; Ballance and Turner 1985; chapter 6). In general, these colonies have been indicated as "abortives". This, however is not correct since at least 50% of the type II AmdS$^+$ transformant colonies can be converted into type I (chapter 2).

All type I AmdS$^+$ transformants, obtained with amdS containing vectors have integrated the transforming vector DNA sequences into the fungal genome DNA, as could be shown by Southern blotting analysis (chapter 2) and confirmed by genetic analysis (chapter 3). The integration of the transforming vector DNA into the genome is a common feature of the amdS gene and other cloned genes (pyr4, trpC, argB). However, between the various selection markers, differences exist with respect to mode of vector DNA integration and transformation frequencies obtained (Ballance et al. 1983; Yelton et al. 1984; John and Peberdy 1984). The mode of amdS integration depends on the recipient A. nidulans AmdS$^-$ strain. Whereas strain WG290 usually integrates one single vector copy at the homologous, partially deleted amdS locus, virtually all AmdS$^+$ transformants of strain MH1277 contain multiple vector copies, integrated in tandemly repeated fashion. Integration is not preferentially at the homologous locus, nor at another specific site in the genome (chapter 2, chapter 3). Although integration of multiple vector copies into the A. nidulans genome has been observed using other selection markers, such a strain dependency has not been reported before. A model to explain the tandem type of integration in strain MH1277 (chapter 2) assumes the presence of a cryptic mutation in this acceptor. Such a locus has not been identified by genetic analysis. However, in diploid combinations of MH1277 derived AmdS$^+$ transformants and a master strain, unusu-

ally high levels of mitotic recombination are found (chapter 3). It is suggested that this is the basis of the peculiar mode of vector integration in MH1277.

Genetic analysis of MH1277 derived AmdS$^+$ transformants confirms the conclusion derived from the biochemical analysis (chapter 2), that the transformant property is genome-linked; in six transformants analyzed the AmdS$^+$ property resides on five different chromomes. One of the transformants contains a translocation between two chromosomes of which at least one carries the AmdS$^+$ property. Translocation and vector integration in this strain may have occurred as two unrelated events. On the other hand it can be speculated that the former is a result of the latter.

In chapter 4 a study is presented concerning the isolation of transforming vector sequences from the DNA of MH1277-derived AmdS$^+$ transformants *via E. coli*. Digestion of the *A. nidulans* DNA with *Eco*RI, followed by ligation prior to *E. coli* transformation, yields plasmids even from a strain carrying only one single integrated vector copy. Following this procedure with AmdS$^+$ transformants containing multiple copy vector inserts, plasmid molecules can be recloned at higher frequencies. The length polymorphism found among these plasmids probably reflects the sequence rearrangements within the tandem inserts (chapter 2) and the recloning frequency shows a correlation with the number of vector copies integrated in each *A. nidulans* transformant.

Similar vector plasmids could also be reisolated from undigested AmdS$^+$ transformant DNA. CsCl/EtBr centrifugations clearly demonstrate the presence of free covalently closed circular plasmid molecules within these *A. nidulans* DNA preparation. Our opinion is that these plasmids arise *in vivo* from recombination events between the individual copies within then tandem vector inserts, which are present in the genomic DNA of MH1277-derived

AmdS$^+$ transformants. Also for A. nidulans transformants, obtained with other selection markers indications have been found for the presence of free vector molecules. Although some favour the idea of autonomous vector replication (Barnes and McDonald 1986) we consider this possibility unlikely.

Chapter 5 deals with the phenomenon of cotransformation. When amdS mutants of A. nidulans are transformed with a mixture of an amdS containing vector and another, unlinked DNA sequence, a large fraction of the AmdS$^+$ transformants also contains this second, unselected sequence (chapter 5). The cotransformation frequency is demonstrated to depend both on the molar ratio of the two vectors and the concentration of the cotransforming vector. Although there may be some variation in the extent of cotransformation, it is in general such an efficient process in A. nidulans that the DNA of the unselected sequence can be found in almost every transformed cell.

Cotransformation has been applied to induce gene replacement events in the A. nidulans genome (chapter 5). The amdS mutant WG290 was transformed with an amdS vector in the presence of a DNA fragment, containing an A. nidulans trpC - E. coli lacZ (TrpC$^-$, LacZ$^+$) hybrid gene and among the AmdS$^+$ transformants we have screened for TrpC$^-$, LacZ$^+$ colonies. Since tryptophan auxotrophs arise very infrequently, an enrichment procedure for TrpC$^-$ conidia has been applied to demonstrate the presence of the TrpC$^-$ transformants. We used ten such AmdS$^+$, TrpC$^-$ transformants, which were all lacZ$^+$, to study gene replacement. They were each transformed to TrpC$^+$ phenotype with a DNA fragment containing the wild type A. nidulans trpC gene. Only 2 strains yielded at a low frequency, transformants which had simultaneously lost their LacZ$^+$ phenotype. These TrpC$^+$, lacZ$^-$ colonies had the AmdS$^-$ phenotype. Southern blotting analysis of the two AmdS$^+$, TrpC$^-$, LacZ$^+$ mutants showed

replacement of their wild type trpC gene by a trpC, lacZ, amdS-cointegrate. These results show that gene replacement by cotransformation is possible in A. nidulans, although less straight forward than directly selectable gene replacements (Miller et al. 1985). Due to the integrative behaviour of DNA sequences in A. nidulans, gene replacement procedures are more complex than in S. cerevisiae; in the latter case homologous recombination in the dominant mode of stable integration.

In chapter 6 experiments are described in which the effect of the A. nidulans ansl DNA fragment (Ballance and Turner 1985) on the frequency of Aspergillus transformation is examined, using the N. crassa pyr4 gene and the A. nidulans amdS, argB and trpC genes as selection markers. We find that ansl can increase transformation frequencies when added on a cotransforming vector with trpC, amdS and pyr4, but not with argB. When ansl is inserted into the vector, again with argB no stimulation is found. In amdS vectors, the position of ansl with respect to the amdS gene determined its influence on transformation: ansl upstream of amdS increased the frequency, whereas ansl downstream of amdS has no effect. Moreover the transformation frequency of the latter type of vector can not be stimulated by addition of ansl on a cotransforming vector. We suggest that ansl dependent stimulation involves an ansl gene product which, due to its inconsistency in effect may need a specific site for its action. The abolishing effect of DNA sequences like amdS may complicate the general applicability of this sequence in transformation.

Transformation of A. nidulans has now evolved to a stage in which many problems can be tackled at a molecular level: cloning of genes in A. nidulans, introduction and expression of cloned genes, either from A. nidulans itself or from other organisms, study of the regulation of gene expression in A. nidulans using

gene replacements, site directed mutagenesis etc. Moreover, the experience obtained with A. nidulans transformation can now be applied to other, biotechnologically important species like A. niger (see chapter 1).

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Samenvatting

In de chemische, farmaceutische en voedingsmiddelenindustrie spelen schimmels een belangrijke rol bij tal van omzettingen, fermentatieprocessen, productie van antibiotica en enzymen. Een aantal van deze industrieel belangrijke schimmelsoorten behoort tot de groep der Aspergilli. Tot voor kort waren er uitsluitend klassiek genetische methoden beschikbaar om de eigenschappen van deze schimmels te veranderen maar de snelle ontwikkelingen op het gebied van de recombinant DNA technologie hebben daarin verandering gebracht. Dit proefschrift behandelt aspecten van de DNA-afhankelijke genetische transformatie van de schimmel Aspergillus nidulans, daarbij gebruikmakend van zowel moderne moleculair biologische technieken als genetische analyse.

Hoofdstuk 1 geeft een beschrijving van de biologie van A. nidulans. Dit organisme bezit zowel een vegetatieve (asexuele) als een generatieve (sexuele) voortplantingscyclus. Bovendien kent A. nidulans afwisseling van een haploide en diploide fase zonder dat daarbij meiose optreedt (parasexuele cyclus). Genetisch onderzoek aan deze schimmel heeft veel informatie opgeleverd over de chromosomale organisatie, recombinatie processen, genregulatie e.d. In dit hoofdstuk wordt ook een overzicht gegeven van de ontwikkeling van de genetische transformatie. Om de resultaten betreffende de transformatie van A. nidulans binnen een bredere context te kunnen plaatsen, worden enkele resultaten beschreven van de genetische transformatie bij dierlijke cellen, plantencellen en enkele schimmels.

Hoofdstuk 2 behandelt de genetische transformatie van de protoplasten van een A. nidulans amdS mutant (MH1277) met een vector

die het gekloneerde wild type amdS gen bevat. De verkregen AmdS$^+$ transformanten konden worden ingedeeld in twee categorieën: goed groeiende kolonies (type I) en kleine compacte kolonies, die niet zonder meer verder uitgroeien (type II). De transformatie-omstandigheden zijn geoptimaliseerd, terwijl de invloeden van vectorveranderingen nader zijn geanalyseerd.

Analyse van DNA uit AmdS$^+$ transformanten van stam MH1277 toont aan dat integratie van vector DNA sequenties in het genoom is opgetreden. Bijna alle transformanten bevatten vele vector copieën, gerangschikt in lange, niet perfecte tandems en deze bevinden zich meestal niet op het homologe, gedeeltelijk gedeleteerde amdS locus. Een andere amdS mutant (WG290) vertoont dit opvallende integratie fenomeen niet, wat suggereert dat de genetische achtergrond van de recipient van invloed is op het integratie gedrag van de transformerende amdS vector. Er wordt een model gepresenteerd dat een verklaring geeft voor de tandem integratie in stam MH1277 en de afwezigheid ervan in WG290.

In hoofdstuk 3 is geprobeerd een verband te leggen tussen de genetische achtergrond van stam MH1277 en het vector integratiegedrag, beschreven in hoofdstuk 2. Daarbij bleek dat bij uitwisseling van chromosomen van MH1277 tegen die van een A. nidulans testerstam het integratie gedrag van MH1277 niet veranderd kon worden. Daarnaast bevat dit hoofdstuk een genetische analyse van enkele AmdS$^+$ transformanten van stam MH1277. Uit deze analyse blijkt dat de AmdS$^+$ eigenschap genoom-gebonden is en dat integratie van de amdS vector in verschillende chromosomen kan plaatsvinden. In een van de AmdS$^+$ transformanten bleek een chromosoom translocatie te zijn opgetreden.

Hoofdstuk 4 beschrijft de isolatie van transformerende DNA sequenties uit het DNA van A. nidulans AmdS$^+$ transformanten. Dit is gedaan door digestie van chromosomaal DNA met EcoRI gevolgd door zelf-ligatie en transformatie naar E. coli. Dit levert ampicilline resistente kolonies op die plasmiden bevatten, welke sterk lijken op of identiek zijn aan de orginele vector. Dergelijke plasmiden kunnen echter ook gereïsoleerd worden uit onbehandeld chromosomaal DNA. Er is bewijs aangedragen dat deze A. nidulans transformanten een zeer klein aantal vrije vector moleculen bevatten.

Hoofdstuk 5 gaat in op de mogelijkheden van cotransformatie bij A. nidulans. Als tijdens de transformatie van amdS mutanten met een amdS vector gelijktijdig een andere vector wordt toegevoegd, dan kan deze worden aangetroffen in het merendeel van de AmdS$^+$ transformanten. Cotransformanten van een amdS, trpC A. nidulans dubbel mutant laat zien dat de cotransformatie frequentie afhangt van zowel de molaire verhouding van de cotransformerende vectoren als van de concentratie van de cotransformerende vector. Daarnaast behandelt dit hoofdstuk het gebruik van cotransformatie als hulpmiddel bij het tot stand brengen van gen vervangingen in het A. nidulans genoom. Daartoe is amdS mutant WG290 getransformeerd met een lineair DNA fragment waarop een A. nidulans trpC - E. coli lacZ (TrpC$^-$, LacZ$^+$) hybride gen ligt. Tien transformanten met een AmdS$^+$, LacZ$^+$, Trp$^-$ phenotype zijn geselecteerd. Allen geven na transformatie met een functioneel A. nidulans trpC gen, TrpC$^+$ transformanten, doch bij slechts 2 zijn TrpC$^+$ transformanten met een LacZ$^-$ fenotype gevonden. Analyse van het DNA uit deze 2 AmdS$^+$, LacZ$^+$, TrpC$^-$ transformanten bevestigde de inactivering van het wild-type trpC gen, doch liet tegelijkertijd zien dat er amdS sequenties betrokken waren in de vervangingsgebeurtenissen.

Hoofdstuk 6 beschrijft een aantal experimenten waarin de invloed van de A. nidulans ansl DNA sequentie op de Aspergillus transformatie wordt vastgesteld. Van vectoren die, behalve het N. crassa pyr4 gen, deze ansl sequentie bevatten was bekend dat zij met sterk verhoogde frequenties een uridine behoeftige A. nidulans mutant konden transformeren. Nagegaan is of dit verschijnsel universeel is. Gebruikmakend van de A. nidulans genen trpC, argB en amdS als selectiemarkers voor transformatie, zijn geheel verschillende effecten van ansl gevonden. Als ansl aanwezig is op een cotransformerende vector, dan heeft dit een positieve invloed op de transformatie frequentie met pyr4, trpC en amdS. In het geval dat ansl aanwezig is in de amdS vector dan is de locatie van ansl ten opzichte van het amdS gen bepalend voor het effect.

Hoofdstuk 7 tenslotte bevat een concluderende samenvatting.

Curriculum vitae

De auteur van dit proefschrift, Karel Wernars, werd op 22 januari 1956 te Hilversum geboren. Na een opleiding aan het Gemeentelijk Gymnasium aldaar, die in 1975 werd afgesloten met het behalen van het Gymnasium-B diploma, ving hij aan met een studie aan de Landbouwhogeschool te Wageningen. Na een natuurwetenschappelijk georiënteerd propaedeuse jaar, werd de studierichting Moleculaire Wetenschappen, biologische oriëntatie als afstudeerrichting gekozen. Na het afronden van de kandidaatsfase in september 1979 vond verdere specialisatie plaats middels de keuze van een 6-maands hoofdvak Organische Chemie (prof.dr. Ae. de Groot) en een 9-maands verzwaard hoofdvak Moleculaire Biologie (prof.dr. A. van Kammen en dr. P. Zabel). In het kader van de doctoraalstudie werd een stageperiode van ruim een half jaar doorgebracht op de afdeling Bacteriologie van het Rijksinstituut voor de Volksgezondheid te Bilthoven waar gewerkt werd onder leiding van dr. J. van Embden. Het doctoraal-diploma werd behaald in februari 1982. In maart 1982 volgde aanstelling als assistent-onderzoeker bij de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO) en werd op het Laboratorium voor Erfelijkheidsleer aan de Landbouwhogeschool te Wageningen gestart met het in dit proefschrift beschreven onderzoek, waarvan de dagelijkse (bege)leiding in handen was van dr.ir. H.W.J. van den Broek. In oktober 1986 zal hij als moleculair bioloog in dienst treden bij het Rijksinstituut voor Volksgezondheid en Milieuhygiene te Bilthoven.