Genetic analysis of *Aspergillus niger*

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Fons Debets

Genetic analysis of *Aspergillus niger*

Proefschrift

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ISN= 268226

NN08201, 389

Stellingen

1. *Aspergillus niger* heeft acht chromosomen.

Dit proefschrift

2. Het genoom van *Aspergillus niger* is significant groter dan dat van *Aspergillus nidulans*.

Dit proefschrift

3. Genetische analyse van haploide segreganten van heterozygote diploiden van *Aspergillus niger* zoals door Lhoas voorgesteld, levert geen betrouwbare informatie omtrent de ligging van en de onderlinge afstanden tussen genetische markers.

P. Lhoas (1967). Genet. Res. **10**:45-61
Dit proefschrift

4. De bewering dat er geen coincidentie is tussen overkruising en haploidisatie in *Aspergillus nidulans* lijkt in strijd met de waargenomen verhoogde frequentie van mitotische overkruising in disomen.

E. Käfer (1977). Adv. Genet. **19**:33-131

5. De verklaring die Kundu & Das geven voor het ontstaan van de door hen geïsoleerde recombinanten van *Aspergillus niger* is niet de meest waarschijnlijke.

P.N. Kundu and A. Das (1985). J. Appl. Bacteriol. **59**:1-5

6. De veronderstelling van Assinder *et al.* dat het *cam*C gen van *Aspergillus nidulans* ver van het centromeer ligt op de linker arm van chromosoom I is niet correct.

S.J. Assinder, B. Giddings and A. Upshall (1986). Mol. Gen. Genet. **202**:382-387.
H. Arst (1988). Mol. Gen. Genet. **213**:545-547

7. Het gebruik van eenkernige schimmelprotoplasten in transformatie experimenten waarbij polyethyleenglycol wordt toegepast zal het ontstaan van heterokaryotische transformanten niet verhinderen.

J.R.S. Fincham (1989). Microbiol. Rev. **53**:148-170

8. De conclusie dat door mitotische overkruising in *Aspergillus nidulans* complementaire produkten ontstaan, is gebaseerd op de foutieve veronderstelling dat heteroallele recombinatie niet verschilt van recombinatie tussen niet-allele mutaties.

J.A. Roper and R.H. Pritchard (1955). Nature 175:639
M. Bandiera, D. Armaleo and G. Morpurgo (1973). Mol. Gen. Genet. 122:137-148

9. Voor eventuele regelgeving ten aanzien van inter-species recombinant DNA overdracht is met name voor asexuele schimmels de huidige taxonomische indeling niet toereikend.

10. Het belang van de parasexuele cyclus bij schimmels voor de onderzoeker is duidelijk, de functie voor de schimmel zelf verdient nader onderzoek.

11. Een stammenverzameling is voor een schimmelgeneticus als een cunet voor een stratenmaker: het is een diepte-investering die niet door iedereen op waarde wordt geschat maar het bepaalt wel het eindresultaat.

12. Naast de oproep 'vrouwen gevraagd voor mannenwerk' kan ook een stimulans aan mannen om te kiezen voor 'vrouwenwerk' bijdragen aan een verbetering van de positie van vrouwen op de arbeidsmarkt.

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13. Door de politieke omwentelingen in Oost-Europa dreigen grote omleggingen van geld- en afvalstromen te ontstaan ten nadele van de Derde wereldlanden.

14. Het gebruik van titulatuur bij het ondertekenen van ingezonden stukken in de krant aangaande 'common interest' zaken getuigt niet van veel vertrouwen in de aangevoerde argumenten.

15. Dopingjacht geeft records 'eeuwigheidswaarde'.

B. de Graaf de Volkskrant 25 augustus 1990

Stellingen behorende bij het proefschrift van Fons Debets:
'Genetic analysis of Aspergillus niger'.
Wageningen, 4 december 1990.

Aan Marita en Lynn
Aan moeder

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CHAPTER 1

Introduction

1.1 Aspergillus

Micheli (1729, cf Raper and Fennel 1965) introduced the name Aspergillus for the moulds with a characteristic pattern of conidiophores and sporeheads reminding him, as a priest, of a mop for distributing holy-water (Lat. *Aspergillium*). The fungi that belong to this form-genus are worldwide in distribution and are found on almost any type of substrate (foods, textile, leather, decaying vegetation in the fields etc.) (Raper and Fennel 1965). Because of their metabolic versatility especially the black Aspergilli are widely used in industry for the production of organic acids such as gallic acid, citric acid and gluconic acid (Lockwood 1975), for the production of industrial enzymes such as amylase, glucoamylase, cellulase, hemicellulase, pectinase, glucose oxidase and catalase (Underkofler 1976) and in food fermentations practiced in the orient (Wood 1977). Some Aspergilli can also produce mycotoxins in food and some can cause aspergillosis (Edwards and Al-Zubaidy 1977). Since De Bary (1854, cf Raper and Fennel 1965) reported the direct relationship between the ascomycetous genus Eurotium and the *Aspergillus glaucus* group, the ascosporic states have been found for many Aspergilli (Raper and Fennel 1965, Onions *et al.* 1981). They all are cleistocarpic and all but one (*A. heterothallicus*) are homothallic (Raper and Fennel 1965).

1.2 *Aspergillus niger*

The black Aspergilli are probably more common than any other group within the genus. Because of the characteristic pigmentation of the conidial heads, members of the group are commonly referred to as

'Aspergillus niger'. Indeed, within the *Aspergillus niger* group Van Tieghem's species *A. niger* is by far the most abundant member, and the degree of intragroup (morphological) variation is such that specific identifications often become quite difficult (Raper and Fennel 1965). The occurrence of this easily recognizable and selectable fungus (on the basis of specific growth on 20% tannin, Rippel 1939) is documented from all parts of the world, but in contrast to many other Aspergillus species not more than 50% of the findings are within the tropics (Domsch *et al.* 1980). The characteristic pigment production in *Aspergillus niger* is profoundly influenced by the presence or absence of minute quantities of copper in the substratum and this phenomenon has been used to estimate copper concentrations in soil (Mulder 1948). Copper dependent laccase activity has been documented to be involved in pigmentation not only in *A. niger*, but also in *Aspergillus nidulans* (Clutterbuck 1972).

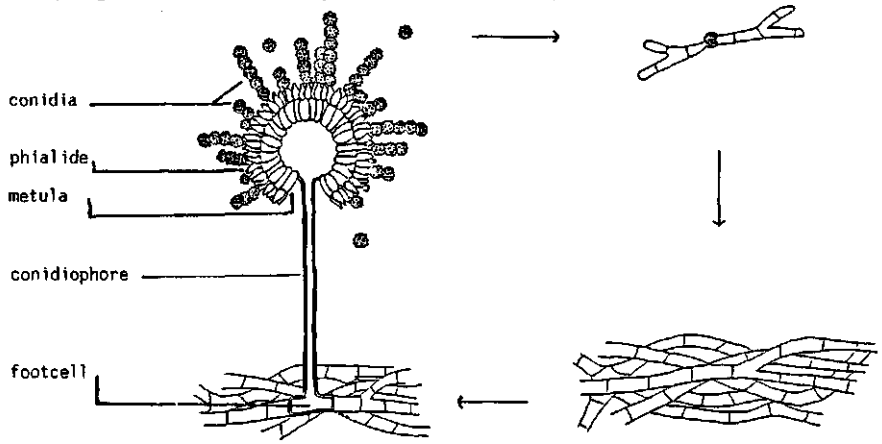

Figure 1.1. Lifecycle of A. niger.

No sexual stage is known in the life cycle of *A. niger*. Though *A. japonicus* from the *A. niger* group was reported to have a perfect state (*Saitoa japonica* Rajendram and Muthappa, Onions *et al.* 1981) this observation has not been confirmed. The growth cycle of *A. niger* therefore consists of the sequence: hyphae -- airborne conidiospores -- hyphae (Fig. 1.1). The vegetative mycelium consists of septate hyphae which are branching and partially submerged. The conidial apparatus develops from specialized, enlarged, thick-walled hyphal cells (the foot cells) as conidiophores with conidial heads. Conidia are produced by specialized conidiogenous cells called phialides that cover the surface of the vesicle,

the swollen end of the conidiophore. In some species, the phialides are borne directly on the vesicle (uniseriate), whereas in biseriate species the phialides are borne on intermediate cells or metulae which are attached to the vesicle. *A. niger* shows mixed uni- and biseriate types of arrangement sometimes even in the same conidial head (Raper and Fennel 1965). Conidial heads split during aging and at maturity the dry conidia are easily spread by air. The conidia of *A. niger* Van Tieghem are thought to be uninucleate (Yuill 1950), though also *A. niger* isolates with binucleate conidia have been reported (Baracho and Coelho 1978). In submerged cultures with agitation and temperature elevation *A. niger* may show microcycle sporulation, i.e. immediate recapitulation of sporogenesis following spore germination (Smith 1977).

1.3 Genetic analysis based on mitotic recombination

1.3.1 Parasexual recombination in fungi

Recombination without the involvement of the fungal sexual cycle was

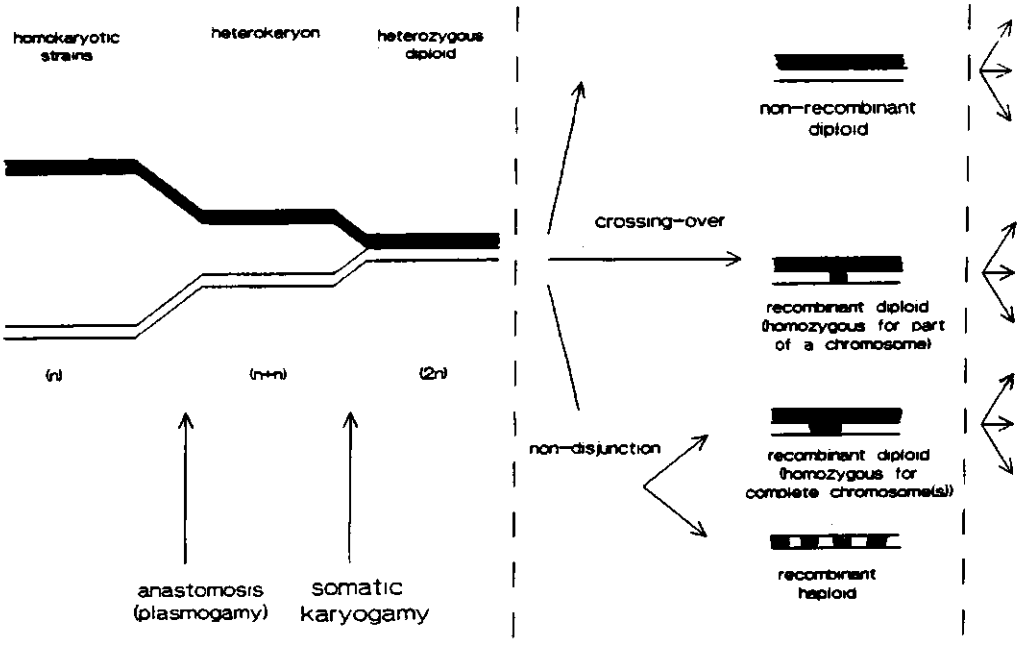

Figure 1.2. Origins of new genotypes by parasexual recombination. For genetic analysis haploid segregants and diploid crossing-over recombinants are relevant (see Figures 1.3 and 1.6).

first discovered by Pontecorvo *et al.* (1953b) in *A. nidulans* and has been termed parasexual recombination. The parasexual cycle as reviewed by Pontecorvo (1956, 1958) and Roper (1966) essentially consists of the following sequence of events (see Fig. 1.2): 1 Hyphal anastomosis of homokaryons of different genotypes resulting in heterokaryotic mycelium. 2 Fusion of two unlike haploid nuclei. 3 Occasional mitotic crossing-over during the multiplication of the diploid nuclei, resulting in recombinants homozygous for part of a chromosome, and/or 4 Occasional non-disjunction leading, via successive aneuploid states, to a haploid number of chromosomes or to recombinant diploids homozygous for one or more complete chromosomes (Fig. 1.2). In contrast to recombination at meiosis there is no precise coordination between nuclear fusion, recombination and reduction of chromosome number.

Parasexual recombination has since been demonstrated in most fungi in which it has been looked for: fungi of industrial importance e.g. *A. niger* (Pontecorvo *et al.* 1953a), *A. soyae* and *A. oryzae* (Ishitani *et al.* 1956), *Penicillium chrysogenum* (Pontecorvo and Sermonti 1954), fungal entomopathogenic fungi e.g. *Metarhizium anisopliae* (Al-Aidroos 1980) *Verticillium lecanii* (Jackson and Heale 1987), plant pathogenic fungi e.g. *Cochliobolus sativus* (Tinline 1962), *Fusarium oxysporum f. pisi* (Buxton 1956), *F. tricinctum* and *F. sporotrichioides* (Cullen *et al.* 1983), *Pyricularia oryzae* (Genovesi and Magill 1976), *Verticillium dahliae* (Hastie 1973) and fungi of medical interest e.g. *A. fumigatus* (Berg and Garber 1962) and *A. flavus* (Papa 1973), *Candida albicans* (Kakar et al 1983). *Neurospora crassa* is a well known exception in that never stages of somatic recombination (somatic diploids, somatic recombinants in heterokaryons) have been found. Nevertheless diploid mycelia have been isolated from ascospores and these produce parasexual recombinants by crossing-over and non-disjunction (Smith 1974). Evidence for parasexual recombination thus is found in members of all main classes of fungi: Ascomycetes, Phycomycetes, Basidiomycetes and Deuteromycetes. So parasexual processes seem to be widespread in fungi. The different stages of the parasexual cycle in the various fungi may vary extremely in frequency and stability. In extreme cases one of the steps may be so transient that they regularly escape detection, e.g. in *Humicola* sp. diploids arise directly in mixed cultures of auxotrophic mutants on minimal medium without detection of an intervening heterokaryon (De Bertoldi and Caten 1975), whereas in

Cephalosporium the diploid stage is transient and may remain unnoticed (Hamlyn and Ball 1979). These observations suggest that the impact of mitotic recombination in different fungal species may be different and especially for fungi that lack sexual recombination it is generally considered to be of significant importance for the contribution to natural genetic variation. So far, however, there is no valid evidence for the hypothesis that parasexuality has evolved to replace or to make up the absence of sexuality in asexual fungi (Pontecorvo 1958, Webster 1974).

Mitotic recombination can be used efficiently in genetic analysis of fungi for assigning markers to a linkage group and for chromosome mapping. For asexual fungi it is the only available mapping technique, but also in sexual fungi it may be more convenient for mapping than the conventional meiotic mapping techniques (e.g. in *Dictyostelium discoideum* (Welker and Williams 1982) and *A. nidulans* (Käfer 1977)). The efficiency of mitotic mapping in a fungal species is highly dependent upon selection and often also upon induction of stages of the parasexual cycle.

1.3.2 Haploidization analysis

The genetic analysis based on mitotic recombination in habitually haploid filamentous fungi begins with diploids heterozygous for a number of known markers. For *A. nidulans* an efficient technique of diploid selection has been described by Roper (1952). A prototrophic heterokaryon is grown (i.e. forced) on minimal medium from two auxotrophic mutant strains with different nutritional deficiencies. The conidia on the heterokaryon are uninucleate, and so all the haploid conidia will be auxotrophic whereas heterozygous diploid conidia are prototrophic and hence can be selected for on minimal medium. This method has been used for the isolation of diploids in many other uninucleate habitually haploid fungi. The method is also applicable to fungi that have multinucleate conidia which arise by successive mitoses in primary uninucleate young conidia. *A. niger* isolate ATCC 1015 has binucleate conidia, but since they are homokaryotic the diploid isolation technique can be applied successfully (Boschloo *et al.* in press). Heterokaryotic conidia that are the result of migration of several nuclei from heterokaryotic phialides into the spores have been observed in several Aspergilli e.g. *A. carbonarius* (Yuill 1950), *A. flavus* (Papa 1973),

A. parasiticus (Papa 1977) and *A. echinulatus* (Baker 1945). Heterokaryotic conidia do interfere with the diploid selection technique, but although in *A. parasiticus* 28% of the conidia of a forced heterokaryon was found to be heterokaryotic, diploid colonies could still be selected (Papa 1977)

Since there is little coincidence of mitotic crossing-over and

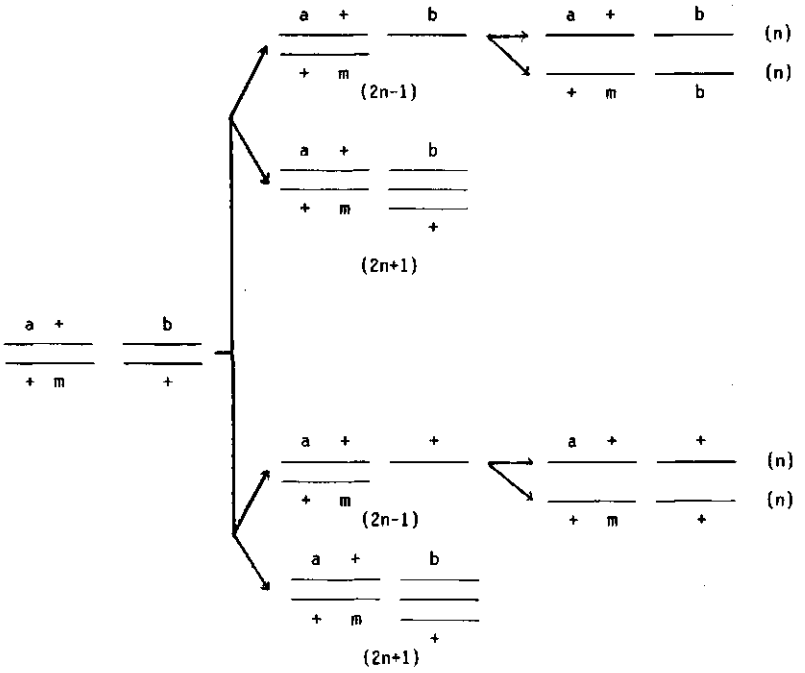

Figure 1.3. The process of haploidization in A. nidulans by subsequent non-disjunctions (only two chromosome pairs are shown). Markers linked on a chromosome (a and m) will segregate en bloc during haploidization (recombinant frequency near 0%), whereas markers on different chromosomes(a and b, m and b) segregate independently (recombinant frequency of about 50%).

haploidization in *A. nidulans*, the markers on one chromosome segregate together and independently of markers on the other non-homologous chromosomes (linkage groups) during haploidization (Pontecorvo and Käfer 1958, Fig. 1.3). Master strains have been constructed so that genes can be assigned to linkage groups for *A. nidulans* (McCully and Forbes 1965) and recently also for *A. niger* (Bos *et al.* 1988). A diploid can be constructed from these master strains with markers on each or several of the chromosomes and a strain carrying an unlocated marker. Diploid strains are rather stable, but haploids can be isolated using haploidizing agents like benomyl (Hastie 1970), p-fluorophenylalanine (Lhoas 1961) or chloralhydrate

(Singh and Sinha 1976). Upon analysis of randomly isolated haploid segregants linkage of a new marker to one of the markers of the master strain can be observed and thus, the linkage group can be assigned.

1.3.3 Mitotic recombination

1.3.3.1. Models for recombination

Mitotic crossing-over recombinants can be explained as resulting from crossing-over at the four chromatid (G2) stage of mitotis as was demonstrated by Stern (1936) for Drosophila based on the genetic analysis of twin spots that represent the reciprocal products of crossing-over. This was confirmed by Roper and Pritchard (1955) in *A. nidulans*. They analyzed prototrophic diploid segregants that were isolated from a diploid of *A. nidulans* heterozygous for a number of linked markers and carrying in a trans arrangement the two allelic mutations *ad16* and *ad8*.

In *Saccharomyces cerevisiae* Roman (1956) demonstrated in comparable experiments that heteroallelic recombination occurred predominantly by non-reciprocal recombination: gene conversion. Also in *A. nidulans* it was shown later that intragenic recombination is mainly a consequence of gene conversion (Putrament 1964; Bandiera *et al.* 1973). The possible mechanisms for mitotic recombination both reciprocal (crossing-over) and non-reciprocal (gene conversion) have been studied mainly in *S. cerevisiae* and have been reviewed recently by Orr-Weaver and Szostak (1985) and Roeder and Stewart (1988). An association of crossing-over with mitotic gene conversion has been observed for a number of genes in *S. cerevisiae* with frequencies of gene conversions resulting in crossing-over ranging from 10-55% (Esposito and Wagstaff 1981). This is supported by the observation that inducing agents stimulate both recombination events (Fogel and Hurst 1963) though not to the same extent suggesting that the two types of events can be uncoupled. Data obtained with mutants affected in recombination demonstrate also that mitotic gene conversion and mitotic crossing-over require some of the same functions, but are also separable (Orr-Weaver and Szostak 1985).

Models for recombination have been formulated to account for the observed properties of mitotic and meiotic recombination. The Meselson-Radding model and the double-strand gap repair model are summarized in Fig.

1.4 and Fig. 1.5. The Meselson-Radding model of recombination (Meselson and Radding 1975) is initiated by a single strand (or asymmetric) transfer, which may become a two-strand (or symmetric) exchange after isomerization (Fig. 1.4). The model is an extension of the model proposed by Holliday

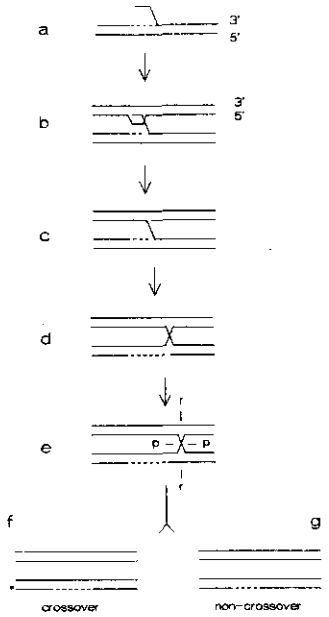

Figure 1.4. The Meselson-Radding model for recombination (Meselson and Radding 1975). (a) Initiation of recombination by a single-strand nick. The 3' end of the nicked strand acts as a primer for DNA synthesis, which displaces the strand ahead of it. The displaced single strand invades the other duplex at a homologous site, displacing a D-loop (b) and forming a small region of asymmetric heteroduplex DNA. The single-stranded D-loop is degraded, and the invading strand is ligated in place. The limited region of asymmetric heteroduplex DNA is expanded (c) by concerted DNA synthesis on the first (donor) duplex and by nucleolytic degradation on the second (recipient) duplex. Branch migration and ligation of the nicks produces a Holliday junction which can be isomerized (d). Symmetric heteroduplex DNA can be formed by branch migration of the Holliday junction (e). Resolution of the points marked p produce two molecules with the flanking arms in the parental configuration (non-crossover (g)), whereas resolution at the points marked r produce molecules with flanking arms in the recombinant configuration (crossover (f)).

(1964a) and accounts for aberrant segregation patterns observed in fungi that suggest formation of heteroduplex DNA on only one chromatid. Gene conversion, like in the Holliday model, results from enzymatic repair of mismatched basepairs in heteroduplex regions.

More recently, much additional information has been obtained about possible recombination mechanisms in fungi from transformation experiments. Especially yeast transformation has been used as a model system for the study of recombination (Orr-Weaver *et al.* 1981). Yeast cells can be transformed with plasmid DNA by integration of the plasmid into a homologous sequence on the chromosome by a single crossing-over or by substitution through gene conversion or double crossing-over (Hinnen *et al.*

1978, Orr-Weaver *et al.* 1983). Additionally, a high frequency of transformation is possible in yeast if the transforming plasmid contains chromosomal fragments by which the plasmid can be maintained extrachromosomally (Struhl *et al.* 1979). Such ARS (autonomously replicating sequences)-containing plasmids transform at high frequencies, because no integration of the plasmid into a homologous site in the genome is required. Yeast transformation has been a very useful technique for studying recombination mechanisms because the transforming DNA can be manipulated experimentally (Orr-Weaver *et al.* 1981, Orr-Weaver and Szostak 1983). It was shown that double-strand breaks in the plasmid introduced by restriction enzyme digestion are highly recombinogenic in yeast and resulted in up to 3000-fold stimulation of transformation frequencies (Orr-Weaver *et al.* 1981). It was also observed that integration occurred only at the locus homologous to the region of the plasmid containing the double-strand break. Therefore, a plasmid can be directed to integrate into a particular chromosomal locus and can be used to mutagenize chromosomal loci. Plasmids containing a double-strand gap within a yeast fragment

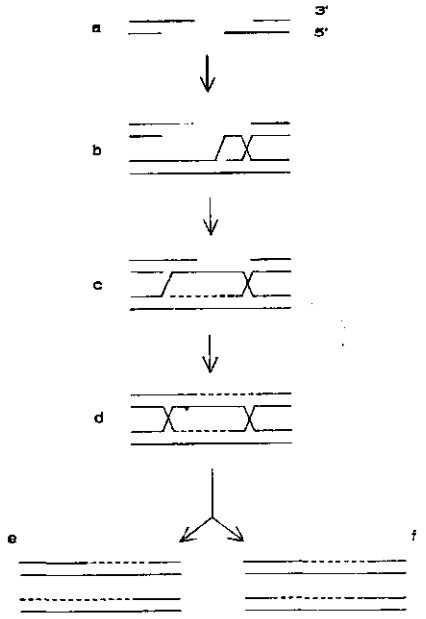

Figure 1.5. Double-strand break repair model for recombination (Szostak et al. 1983). (a) A double-strand cut is made in one duplex, and a gap flanked by 3' single-strands is formed by the action of exonucleases. (b) On 3' end invades a homologous duplex, displacing a D-loop. (c) The D-loop is enlarged by repair synthesis until the other 3' end can anneal to complementary single-stranded sequences. (d) Repair synthesis from the second 3' end completes the process of gap repair and branch migration results in the formation of two Holliday junctions. Branch migration of the two Holliday junctions will form symmetric heteroduplex. Resolution of the two junctions by cutting either inner or outer strand leads to two possible non-crossover (e) and two possible crossover (f) configurations.

transform at high frequency and are always repaired from homologous chromosomal DNA during the integration event (Orr-Weaver *et al.* 1981).

There is more evidence that double-strand breaks may play a role in the initiation of recombination. Mating-type switching in yeast is initiated by the enzymatic production of a specific double-strand break within the mating-type locus (Strathern *et al.* 1982). Mating-type switching is a conversion event at high frequency (as high as once per cell cycle) and it is resolved without crossing-over. Both radiation induced and spontaneous gene conversion require the RAD52 gene product in yeast, which is involved in the DNA repair synthesis. This gene product is also necessary for the integration and repair of linear plasmids and for double-strand break repair (Orr-Weaver *et al.* 1981). These observations prompted Szostak *et al.* (1983) to propose the double-strand gap repair model for general recombination (Fig. 1.5). The double-strand gap repair is initiated by a double-strand break in the recipient information (Fig. 1.5). In this model gene conversion can result from mismatch repair in heteroduplex DNA, but can also arise if a marker falls within a double-stranded gap; in the latter case the marker is converted by a double-strand transfer of information.

Mitotic recombination in fungi occurs at a much lower level than meiotic recombination. In mitosis homologous sequences presumably pair only by chance. This may be not the major reason for the lower frequency of recombination in mitosis compared to meiosis, since the frequency of mitotic recombination can be increased by X-rays, UV-irradiation and treatment with chemical mutagens almost up to meiotic levels (e.g. Holliday 1964b, Davies *et al.* 1975, Shanfield and Käfer 1971). This suggests a mechanistic relationship between recombination and repair. Orr-Weaver and Szostak (1985) suggest that the most likely explanation for the existence of mitotic recombination is that it is simply a consequence of DNA repair activities. Base mismatches introduced by DNA replication errors or spontaneous or induced lesions in DNA are all removed or repaired and repair may result in recombination. This phenomenon of recombination induction by mutagen treatment has been used in several fungi to facilitate isolation of mitotic recombinants.

As mentioned before, mitotic crossing-over can be explained as a G2-stage occurrence, but the initiation of both induced and spontaneous mitotic recombination can also occur in the G1-stage of the cell cycle

(Fabre 1978, Esposito 1978). Therefore, it was proposed that mitotic recombination initiates at the two-chromatid stage and that recombination intermediates are resolved during S-phase by passage of the replication fork (Esposito 1978). However, considering intergenic recombination, a possible initial heteroduplex and gene conversion will remain undetected, since it can only be observed if genetic markers are properly located (e.g. if heteroallelic recombination is studied). Thus there will be no essential difference in the outcome of recombination if initiation is by a single- or by a double-strand break in G1 or G2. Therefore in the following, crossing-over in heterozygous diploids will be modelled as a four-chromatid post replicative exchange.

1.3.3.2 Determining gene order

The gene order of markers within a linkage group can be determined relative to the centromere by analysing crossing over recombinants derived from heterozygous diploid culture. Especially the partially homozygous diploid recombinants are informative (Fig. 1.6). Due to crossing-over all markers distal to the site of exchange may become homozygous whereas all proximal markers will remain heterozygous. The main problem in the mitotic mapping analysis is the fact that spontaneous crossing-over is rare so that selective systems are required to detect the products of recombination. Selection depends strongly on the availability of selective markers. For maximum information, these selective markers should be distal to and in cis position to other recessive markers on the same chromosome. Without adequate recombination, such a genetic arrangement can only be achieved by selecting successively new mutations in existing mutants. A full analysis requires a selectable distal marker on each chromosome arm. Even in the genetically well characterised fungus *A. nidulans* with a genetic map containing over 200 mapped markers (Clutterbuck 1987) it is often difficult to meet the prerequisites for efficient mitotic mapping. Therefore, strategies have been looked for that either allow easy detection and or easy analysis of spontaneous recombinants or that make use of induction of recombinants.

In *A. nidulans* there seems to be hardly any coincidence between haploidization and crossing-over. Therefore, haploids from heterozygous

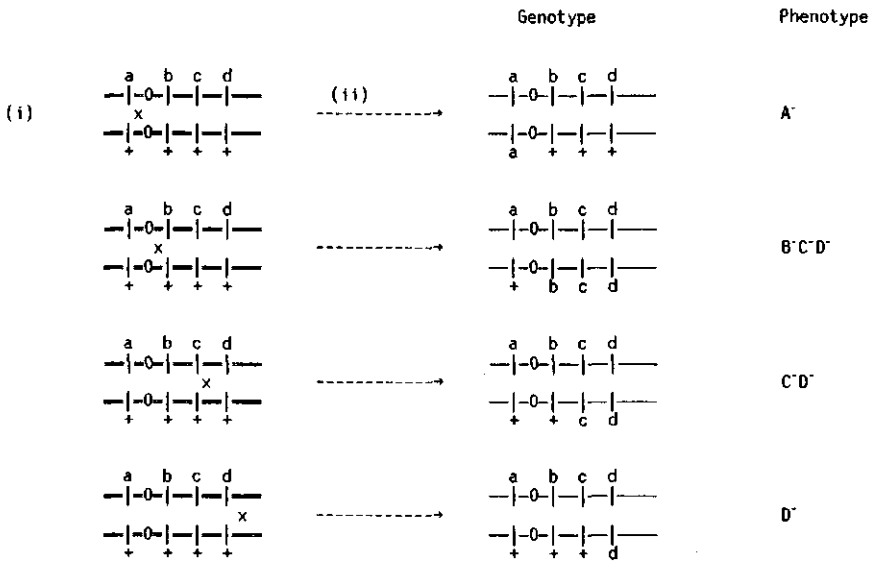

Figure 1.6. Determining gene order on the basis of mitotic crossing-over (essentially according to Pontecorvo 1958). (i) An idealized heterozygous diploid is shown carrying the recessive markers b and c in cis-position to the recessive selective markers a and d. A four chromatid stage in cell cycle is drawn. (ii) Crossing-over of chromatids 2 and 3 between the centromere and either of the markers, and segregation of chromatids (1+3) and (2+4) during mitosis gives rise to diploid recombinants. These segregants will be homozygous for all markers centromere distal to the site of crossing-over but will remain heterozygous for all proximal markers and for markers on the other chromosome-arm. The (1+3) segregants can be isolated on the basis of homozygosity of the selectable markers a and d (shown). The genotype of each of these recombinants can be concluded from the phenotype: segregants homozygous for an unselected marker will show the corresponding mutant phenotype. The site of crossing-over can be infered from the genotypes and, thus the gene order can be concluded. The relative frequency of recombinants can be used to estimate relative distances between markers and the centromere. In A. nidulans double crossing-over was shown to be no more frequent than expected from random coincidences of single exchanges (Käfer 1977) and therefore, homozygosity of all markers on both chromosome-arms is indicative of non-disjunction in this fungus.

diploids normally do not show intrachromosomal recombination. In *A. niger* however, induced haploids include a significant percentage which show recombination between linked markers (Lhoas 1967, Bos *et al.* 1988). Haploid recombinants have been used by Lhoas (1967) to determine the order of markers on a chromosome and to estimate the distances between them. In *Dictyostelium discoideum* Welker and Williams (1982) constructed a genetic map that was mainly based on mitotic homozygous recombinants, but in addition use was made of unselected recombined haploid strains that were

derived from diploids heterozygous at three or more linked loci. The use of haploids for intrachromosomal mapping in *A. niger* will be discussed later.

Disomic strains of *A. nidulans* are available that are relatively unstable and show random loss of one of the duplicated chromosomes at mitosis. Up to approximately 6% of randomly selected haploid breakdown sectors of chromosome I and III disomics were shown to result from recombination between the homologues prior to chromosome loss (Assinder and Upshall 1986, Assinder *et al.* 1986). Therefore it has been suggested to use these recombinants for mitotic mapping. It has been argued that if aneuploids are a relatively long-lasting stage in the generation of haploids on the haploidization medium, crossing-over in these aneuploids might explain for the observed coincidence of crossing-over and haploidization found in *A. niger* but not in *A. nidulans* (Käfer 1977).

In *A. nidulans* translocation strains have also been suggested for mapping markers to chromosome segments, either by analysis of homozygous translocation diploids (Ma and Käfer 1974) or by analysis of translocation disomics (Käfer 1975).

Principally heteroallelic recombination might also be used to select for associated outside marker exchange. Though such recombinants can be isolated easily (e.g. as prototrophic segregants from heteroallelic auxotrophic diploids) it has never been used efficiently for mapping.

As mentioned before spontaneous mitotic crossing-over is rare, but can be induced by treatment of the heterozygous diploid with mutagens in order to facilitate the detection of recombinants needed for mitotic mapping. In *A. nidulans* UV-induced crossing-overs appear to show the same distribution as spontaneous crossing-over and the frequency with which a marker becomes homozygous may be used as a relative measure of the mitotic distance between this marker and the centromere (Wood and Käfer 1969). Multiple exchanges were no more frequent than expected from random coincidence of single exchanges. This is also true for chemical induction of mitotic recombinants in *A. nidulans* (Shanfield and Käfer 1971). Also in *S. pombe* the mitotic mapping data based on UV-induced recombinants are consistent with the order proposed on the basis of spontaneous recombinants (Gygax and Thuriaux 1984).

As with haploidization there is considerable variation in the frequency of mitotic crossing-over in different organisms. The absolute frequency of mitotic crossing-over in higher organisms is extremely

difficult to estimate. In fungi it is feasible, but limited information is available due to the low crossing-over frequency and the clonal appearance of recombinants. For *A. nidulans* the incidence of mitotic crossing-over using colour markers has been estimated in a few experiments to be about 0.1-0.3% per chromosome arm probably correlated with arm length (Käfer 1961).

1.4 Aim and outline of this study

The main goal of the work presented was to develop and apply genetic techniques for the construction of genetic maps of the asexual fungus *A. niger* to facilitate genetic analysis. A detailed genetic (mainly meiotic) map is available for the perfect fungus *A. nidulans* (perfect state *Emericella nidulans*) (Clutterbuck 1987) which is the model organism for industrially important fungi but has no economical significance like *A. niger*. Genetic techniques for the analysis of parasexual recombination have been developed for *A. nidulans* but have been adopted only scarcely in *A. niger* (Lhoas 1967, van Tuyl 1977). Lhoas (1967) reported the assignment of 31 markers to six linkage groups and a tentative gene order of markers on one linkage group. Van Tuyl (1977) studying fungicide resistance in *A. niger* started with a different wild type isolated from hyacinth bulbs and also observed six linkage groups in *A. niger*. Since the strains of Lhoas (1967) were not available and only few of the *A. niger* strains of van Tuyl (1977) were preserved a new strain collection was started (Bos 1985) from the gluconic acid production strain ATCC 9029. Mutations were induced at low UV dose to minimize background damage (Bos 1987).

For several reasons a genetic map of *A. niger* was wanted. At first there is a fundamental reason to have more insight in the genome organization of the widespread asexual fungus *A. niger*: what is the number of chromosomes and chromosome arms, how is the linkage of markers on the chromosomes and what is the physical size of the genome. It is also of basic interest to know more about the frequency of mitotic recombination for the different markers on the chromosomes. Such information can contribute to a better understanding of the impact of parasexual recombination in storing and recombining genetic variation in imperfect fungi.

For more practical reasons it is important for breeding of the economically important fungus *A. niger* to have a linkage map of the entire genome and at least an indication of the map distances. Improvement of *A. niger* production strains is mainly based on mutation and selection (e.g. Markwell *et al.* 1989, Gunde-Cimerman *et al.* 1985). Only very limited use of genetic principles of parasexual processes has been made (Kundu and Das 1985, Ball *et al.* 1978) since formal genetics could not be adopted due to lack of marker mutations and genetic maps. Because of the ability of *A. niger* to secrete large amounts of extracellular proteins it is considered to be a good candidate for the development of expression and secretion systems of homologous as well as heterologous proteins of pharmaceutical and industrial interest. Genes of interest can be manipulated in vitro by molecular techniques and can be subsequently introduced into *A. niger* by transformation (e.g. Goosen *et al.* 1990). Formal genetics is indispensible for analysis of such transformants for it can yield information about stability of transformants and genome position of transformed sequences. Moreover, strategies for strain construction can be devised if a genetic map is available and genetic techniques can be applied so that the effect of combined mutations or transforming (heterologous) sequences originally introduced in different strains can be studied.

The genetic analysis based on mitotic mapping highly depends on efficient markers and genetic techniques. The markers present in the newly started *A. niger* strain collection were mainly auxotrophic mutations that could be used to force heterokaryon formation on minimal medium and to select heterozygous diploids. In haploidization analysis these markers can be used efficiently and master strains that can be used for the assignment of genes to six linkage groups in *A. niger* were constructed (Bos *et al.* 1988). Auxotrophic markers, however, could hardly be used for chromosome mapping since no efficient selection method was available. As also no direct selectable markers were available, at first an attempt was made to develop an efficient method for the selection of auxotrophic recombinants from originally prototrophic heterozygous diploid culture (Chapter 2). In addition, markers were introduced that enable direct selection of recombinants from heterozygous diploids or that facilitate genetic analysis of isolated homozygous diploids (Chapters 3 and 5). Furthermore, a novel mitotic mapping strategy was developed based on the introduction of the heterologous *amdS* gene of *A. nidulans* into *A. niger* by transformation

(Chapter 4). All these methods have been used in genetic analyses for the construction of a tentative genetic map of *A. niger* (Chapter 5). Karyotyping by cytological methods is extremely difficult in fungi due to the small size of the fungal chromosomes. However, the recently developed pulsed-field gel electrophoresis methodology allows the separation of large DNA molecules in the size range of chromosomes (Schwartz and Cantor 1984, Carle and Olson 1984). With this technique an electrophoretic karyotype can be made and has been used to estimate the genome size of e.g. *Neurospora crassa* (Orbach *et al.* 1988) and *A. nidulans* (Brody and Carbon 1989). The method allows the correlation of mitotic linkage groups to specific bands in the electrophoretic karyotype. Generally mitotic linkage groups are considered to be equivalent to the cytological chromosomes. In Chapter 6 the development of an electrophoretic karyotype of *A. niger* is described.

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CHAPTER 2

Mitotic mapping in linkage group V of *Aspergillus niger* based on selection of auxotrophic recombinants by Novozym enrichment

AJM Debets, K Swart, CJ Bos (1989). Can J Microbiol 35:982-988

Mitotic mapping in linkage group V of *Aspergillus niger* based on selection of auxotrophic recombinants by Novozym enrichment

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This paper describes a procedure which allows the quantitative selection of auxotrophs of the fungus *Aspergillus niger* by enzymatic killing of immobilized germinating prototrophic conidiospores. We have applied this procedure to linkage analysis on the basis of mitotic crossing-over in this fungus. Starting with a heterozygous diploid strain, we could select auxotrophic homozygous diploid recombinants quantitatively. We estimated the frequency of crossing-over after correction for clonal distribution of recombinants, and localized four auxotrophic markers as well as the centromere on chromosome V of this fungus. The Novozym enrichment procedure proved to be useful in genetic analysis and for the construction of recombinant genotypes in the case of closely linked auxotrophic markers. The determination of gene order and the estimation of distances on the basis of benomyl-induced recombinant haploid segregants may lead to incorrect conclusions. Genetic analysis on the basis of homozygous recombinants, however, can provide reliable estimates of map distances.

Key words: *Aspergillus niger*, chromosome mapping, mitotic crossing-over, Novozym enrichment, auxotrophic recombinants.

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Une méthode qui permet la sélection quantitative d'auxotrophes du champignon *Aspergillus niger* par destruction enzymatique de conidiospores prototrophes immobilisées et en cours de germination est décrite dans cet article. Cette méthode a été utilisée pour analyser les linkages résultant d'entrecroisements (crossing-over) lors des mitoses chez ce champignon. Débutant cette étude avec une souche diploïde hétérozygote, des recombinants diploïdes homozygotes auxotrophes ont pu être sélectionnés quantitativement. Après correction de la distribution clonale des recombinants, la fréquence d'entrecroisement a été évaluée. Par cette méthode, quatre marqueurs auxotrophes ont été localisés, de même que le centromère du chromosome V de ce champignon. La méthode d'enrichissement à la Novozym s'est montrée utile pour l'analyse génétique et la construction des génotypes de recombinants dans le cas de marqueurs auxotrophes voisins. La détermination de l'ordre des gènes et des distances à l'aide de recombinants haploïdes ségrégés induits par le bénomyle peut donner des conclusions erronées. L'analyse génétique basée sur des recombinants homozygotes, par contre, peut donner des estimés fiables des distances cartographiques.

Mots clés : *Aspergillus niger*, cartographie des chromosomes, entrecroisements mitotiques, enrichissement à la Novozym, recombinants auxotrophes.

[Traduit par la revue]

Introduction

Genetic analysis of asexual fungi is based on mitotic recombination in heterozygous diploid strains. Two types of recombinants can be distinguished: those resulting from chromosome segregation, and those originating from crossing-over between homologous chromosomes. The second type of recombinants can be used for mapping of linked genes. These recombinants are homozygous for all markers on a chromosome distal to the site of crossing-over and heterozygous for all proximal markers. Because the frequency of mitotic crossing-over is relatively low, it is necessary to select recombinants for genetic analysis. Usually, only markers suitable for positive selection are used to isolate recombinants homozygous for a distal marker on a given chromosome. The linear order of the proximal markers relative to the centromere can be deduced from the genotypes (Pontecorvo *et al.* 1953; Pontecorvo and Käfer 1958; Käfer 1977). The limitation of this type of analysis is that it depends on the availability of a selective marker on each chromosome arm. In *Aspergillus niger*, such markers are scarce and, therefore, linkage analysis has been restricted to chromosome I (Lhoas 1967); a few linkage groups in *A. nidulans* were also studied this way (e.g., Käfer, 1958; Arst, 1988).

During the genetic analysis of *A. nidulans*, the filtration enrichment method was used successfully for the isolation of recombinants homozygous for *pdhC* (Bos *et al.* 1981). However, the procedure proved ineffective for the isolation of auxotrophic recombinants for the genetic analysis of *A. niger*. Therefore, we were interested in another enrichment method. Many other methods are based on selective killing of prototrophs (see Fincham *et al.*, 1979). When techniques for the isolation of protoplasts became available, cell wall degrading enzymes were proposed as tools for the isolation of auxotrophic mutants (Delgado *et al.* 1979; Ferenczy *et al.* 1975; Piedra and Herrera 1976; Sipiczky and Ferenczy 1978). However, enrichment of auxotrophs by such enzymes has mainly been applied to yeasts and not to filamentous fungi.

In this paper, we describe an enrichment method based on selective killing of immobilized germinating conidiospores by the lytic enzyme preparation Novozym 234. Optimal conditions for the Novozym enrichment method were determined in simulation experiments. It will be shown that this method allows the quantitative isolation of auxotrophic recombinants. Subsequently, the method is used to make a gene map of

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chromosome V on the basis of the homozygous recombinants isolated using this method. We present evidence that frequencies of recombinants found represent real crossing-over frequencies, so that linkage maps of different chromosomes can be drawn to the same scale.

Materials and methods

Strains

The *A. niger* strains were derived from strain N402 (*cspA1*, a mutant with short conidiophores) after induction with UV and filtration enrichment (Bos *et al.* 1988). The strains used are shown in Table 1. Only low doses of mutagen were used and repeated mutagenesis was limited to avoid chromosomal abberations (Bos 1987). Strains with more markers were obtained by somatic recombination.

Media and growth conditions

Aspergillus niger was grown on complete medium (CM) and on supplemented minimal medium (SM) with a composition according to Pontecorvo *et al.* (1953). All media contained 1 mg/L of $ZnSO_4$, $FeSO_4$, $MnCl_2$, and $CuSO_4$. In platings where the amount of viable conidiospores had to be determined, 0.05% Triton X-100 was added to CM (CMT) to reduce colony size (Maleszka and Pieniazek 1981). The carbon source, mostly 0.05 M glucose, was added after autoclaving of the media. SM was prepared by adding supplements at a concentration of 200 mg/L (amino acids and nucleotides) and 2 mg/L (vitamins).

Cultures were grown on CM at 30°C for 4 days, and stored at 4°C for 1–7 days to get optimal synchronized germination of conidiospores. Conidial suspensions were made in saline–Tween (0.05% Tween 80, w/v) and vigorously shaken during 1 min with glass beads to break conidial chains. The suspensions were filtered through a cotton wool plug to remove mycelial fragments and counted using a Coulter counter.

Genetic techniques

The assignment of genes to linkage groups by haploidization on CM with benomyl has been described earlier (Bos *et al.* 1988). Benomyl was dissolved in acetone and used at a final concentration of approximately 0.25 mg/L. As mitotic crossing-over can disturb the linkage of markers on the same chromosome and can even result in the loss of a certain marker, subculturing was avoided as much as possible. One propagation step was necessary to obtain synchronized germination of conidia. Several small samples of conidiospores were taken from several diploid colonies on the original minimal medium (MM) sandwich plates and subcultured on CM propagation plates by needle plating to obtain synchronized conidia, essentially as described by Käfer (1961). Conidial suspensions were made from each of these plates and a sample of each suspension of synchronized conidia was used in an enrichment procedure.

Filtration enrichment

Conidia were subjected to filtration enrichment, essentially as described by Bos *et al.* (1981) for *A. nidulans*.

Novozym enrichment

The basis of this enrichment procedure is that the conidiospores of the original prototrophic strain are allowed to germinate and are subsequently killed enzymatically, whereas auxotrophic spores that do not germinate, survive. Novozym 234 was obtained from Novo Industries, Denmark (batch ppm 1961). Conidiospores were harvested as described and plated onto MM in small Petri dishes (10^4 conidia per plate of 5.5 cm diameter, as determined by Coulter counter and viable count) and incubated at 30°C for 10 h. The percentage of germinated conidia was estimated by light microscopy. When the conidiospores had started to germinate and when the germ tubes were about 10 times the spore diameter, 1.5 mL enzyme solution (10 mg Novozym 234/mL H_2O) was added and the plates were incubated for 1 h at 30°C. The Novozym was removed and the plates were washed twice with 1 mL H_2O. Subsequently, the plates were incubated again for 3–4 h at 30°C and examined by light microscopy. In most cases, the treatment with Novozym was then repeated. After this second incubation, the plates with Novozym were stored overnight at 4°C as it appeared to improve the method slightly. The Novozym solution was pipetted off and the plates were washed with sterile water. The nongerminating conidia that resided on the MM enrichment plates were rescued by topping the plates with a 2-mL layer of five-times concentrated CM containing only 0.8% (w/v) agar. Colonies that appeared after 2–3 days of incubation were tested by transfer onto MM and CM, respectively, using wooden toothpicks. As some ungerminated conidiospores were rinsed off in each washing of the MM + Novozym plates, all washing fluid was combined and centrifuged. The pelleted conidiospores were plated on CMT, grown to colonies, and subsequently tested for auxotrophy. Colonies failing to grow on MM were further characterized for their deficiency.

Results

Haploidization of heterozygous diploids

In principle, strains with various combinations of markers were obtained from experiments that were performed to determine the linkage group of markers by benomyl-induced haploidization of diploids. This was easily achieved for unlinked markers and, sometimes, recombinant strains could be isolated carrying linked auxotrophic markers (Bos *et al.* 1988). However, no recombinants were obtained containing the markers *nicA1* and *metB2* (or *metB10*) on chromosome V, and the position of the centromere was unknown. The haploid strain N455 (*argA1 pheA1*) was isolated as a phenylalanine mutant from strain N408 (*argA1*). The linkage group of these markers was determined in a few haploidization experiments (Table 2). Diploid strains N455//N505 and N494//N561 were haploidized using benomyl, and haploid segregants were analysed. A few (only prototrophic) recombinants were found among the haploid segregants of diploid strain N494//N561, and linkage of *argA1* as well as *pheA1* to the *nicA1* marker on chromosome V was concluded. Haploidization of diploid strain N455//N505 also showed linkage of *argA1* and *pheA1* to the *metB* gene on chromosome V. As can be seen from Table 2, again no double auxotrophic recombinants were found.

So, the construction of double auxotrophic recombinant strains by way of benomyl-induced haploidization offered no good perspectives for the relatively closely linked auxotrophic markers (*metB2, pheA1, argA1*, and *nicA1*) on linkage group V. In many other haploidization experiments, no such recombinants were ever found (data not shown). Moreover, determination of relative order and distances on the basis of the recombinant frequencies found in these experiments is not reliable, as will be discussed later.

Gene localization using homozygous diploid recombinants isolated by filtration enrichment

Strains with several markers on the same chromosome are essential for the determination of the linear order of these markers. As such strains could not be isolated by benomyl-induced haploidization, an alternative method was used. A few recombinant diploids were isolated from diploid N455//N505 by filtration enrichment (C. J. Bos. 1985. Ph.D. thesis, Agricultural University, Wageningen.) Although proper genetic analysis based on these few colonies was not possible, the haploid strain N567 (*cspA1, metB2 pheA1 argA1*) could be

TABLE 1. Strains used in this study

Strain number	Genotype
N423	*cspA1, nicA1*
N455	*cspA1, pheA1 argA1*
N494	*cspA1, nicA1, pabA1*
N505	*cspA1, fwnA1, leuD6, metB2*
N561	*cspA1, hisD4, lysA7, argA1 pheA1*
N567	*cspA1, fwnA1, metB2 pheA1 argA1*

NOTE: Various combinations of these haploid strains were used for the construction of diploids (designated, e.g., *N455//N505*). Gene symbols are as follows: *csp*, short conidiophores; *fwn*, fawn-colored conidia; deficiencies: *nic*, nicotinamide; *pab*, *p*-aminobenzoic acid; *arg*, arginine; *phe*, phenylalanine; *leu*, leucine; *his*, histidine; *lys*, lysine; *met*, methionine.

isolated from a Met$^-$ recombinant diploid upon haploidization. Subsequently, a diploid strain was constructed from strains N567 (*cspA1, metB2 pheA1 argA1*) and N423 (*cspA1, nicA1*) and used in experiments on the isolation of homozygous recombinants. In several experiments, filtration enrichment yielded only a few amino acid requiring segregants and no Nic$^-$ auxotrophic recombinants were found (data not shown). The filtration enrichment procedure is apparently not suitable for the isolation of all different types of auxotrophic recombinants. In general, the procedure has some shortcomings, which will be discussed later, that prompted us to search for another more quantitative enrichment procedure.

Enrichment of auxotrophs using Novozym 234

We developed an enrichment method for the isolation of auxotrophic recombinants based on the cell wall degrading enzymes of Novozym 234, which we had already used for the isolation of protoplasts (Debets and Bos 1986). Optimal conditions for the enrichment were determined in several simulation experiments. Novozym 234 is highly active against germinating conidia. Ungerminated conidia are not affected, and germinating conidiospores with long septated mycelium are not killed efficiently. Therefore, it is very important that the conidiospores germinate synchronously. The best results were obtained when the conidia were harvested from colonies grown at 30°C for 4 days and stored at 4°C for 1–7 days. The optimal germination time for the conidia on the enrichment plates was about 10 and 12 h at 30°C for diploid and haploid strains, respectively. Under these conditions, more than 95% of the prototrophic conidiospores germinated, whereas the conidia with the largest germ tubes were still sensitive to Novozym 234. Simulation experiments were performed in which the haploid strains N423 and N567 served as auxotrophic and prototrophic strains, respectively. The enrichment plates were supplemented for the deficiencies of strain N567. Thus, conidia of this strain could germinate, whereas conidia of strain N423 could not. A mixture of conidiospores containing about 0.1% "auxotrophic" conidia was used. The compiled results of two experiments are shown in Table 3. The first Novozym treatment resulted in a 50-fold enrichment and the second treatment showed an additional fivefold enrichment. The efficiency of the second treatment was lower, as could be observed by light microscopy, mainly because conidia that were not yet germinated when the first treatment was given did not germinate synchronously. As the enzyme treatment was not effective when germination had proceeded too far, the second incubation with Novozym had to be performed after 3–4 h, although, by then, not all prototrophs were germinating. It can be seen from Table 3, that no significant loss of auxotrophs during the enrichment procedure occurred. Therefore, this method allows quantitative isolation of auxotrophs.

Determination of linear order from homozygous diploid recombinants

In a subsequent experiment, the Novozym enrichment method was used to isolate auxotrophic recombinants from the diploid strain N423//N567. To get segregants originating from different recombination events, several experiments were performed using independent conidial suspensions from independently grown cultures. From each suspension, auxotrophs were isolated by enrichment from a sample of 10^4 conidia. Diploid as well as haploid segregants were obtained. The ploidy of the recombinants could be determined visually as haploid segregants were either fawn (*fwnA1*) or dark black (*fwnA*$^+$), whereas diploid segregants were brownish black (*fwnA1//fwnA*$^+$). In case of doubt, the ploidy was confirmed from the size of the conidia, as measured with a Coulter counter. The results of the Novozym enrichment experiments on the isolation of auxotrophs from 26 different conidial suspensions are shown in Table 4. The segregant colonies were tested for their auxotrophy and the number of diploid recombinants of each type is shown. Among the haploid auxotrophs, equal amounts of Nic$^-$ and Met$^-$Phe$^-$Arg$^-$ segregants were found.

As one of the markers was in repulsion to the others, the genotype of the diploid recombinants was then determined by haploidization in order to distinguish between homozygosity and heterozygosity of a marker originally in repulsion to the selective marker. This was rather laborious. When the order of *metB2* and *pheA1* relative to the centromere became obvious, it was decided that if more than one Met$^-$ or Met$^-$Phe$^-$ segregant was isolated from a suspension only one of these would be analysed by haploidization as they might result from the same recombination event. Differences in viability were not observed upon haploidization of the homozygous diploids. The genotypes of the segregants and the most likely genetic explanation for each homozygous recombinant are given in Table 5. Nearly all recombinants can be explained by a single crossing-over event. Two recombinants remain that could be explained only by either nondisjunction or double crossing-over. The deduced map order and the position of the centromere are shown in Table 5.

As the Novozym procedure allowed quantitative isolation of nonleaky auxotrophs in simulation experiments, an estimation of absolute recombination frequencies can be made. When mitotic crossing-over occurs in a diploid colony, segregation of recombinant nuclei may occur. So, it is essential to take into account the possible clonal origin of recombinants. To get many crossing-over products originating from different recombinational events, parallel experiments were performed using several conidial suspensions from independently grown cultures of a diploid strain; relatively small quantities of conidia (10^4) were used from each suspension. The absolute cross-over frequency for each region was calculated after correction for clonal distribution of segregants (Luria and Delbrück 1943). These frequencies are included in Table 5. The Nic$^-$ diploid, explained by a crossing-over in region III, was found at the same frequency as the reciprocal Arg$^-$ recombinant. Isolation of reciprocal pairs from the same suspension was observed in a number of cases, indicating simultaneous production, although our data are insufficient to allow a clear-cut conclusion. New recombinant strains, N667 (*cspA1,*

TABLE 2. Genetic analysis of induced haploid segregants from heterozygous diploids N494//N561 and N455//N505

Diploid strain	Linked markers		Segregant phenotypes				Percentage of recombinants
	a	b	ab	ab⁺	a⁺b	a⁺b⁺	
N494//N561	*nicA1*	*argA1*	0	81	52	6	4.3
	argA1	*pheA1*	52	0	0	87	0.0
	pheA1	*nicA1*	0	52	81	6	4.3
N455//N505	*argA1*	*pheA1*	98	2	1	212	1.0
	argA1	*metB2*	0	100	211	2	0.6
	pheA1	*metB2*	0	99	211	3	1.0

NOTE: Only linkage data of markers on chromosome V are shown. The number of segregant phenotypes for each combination of linked markers is given as well as the percentage of recombinants. Diploid strains used:
N494 *cspA1*, + + + + *nicA1*, *pabA1*
N561 *cspA1*, *hisD4*, *lysA7*, *argA1 pheA1* + +

N455 *cspA1*, + + + *argA1 pheA1*
N505 *cspA1*, *fwnA1*, *leuD6*, *metB2* + +

TABLE 3. Simulation of enrichment of auxotrophs by Novozym 234

Novozym treatments	Colonies recovered		Auxotrophs (%)	Enrichment factor[a]
	N567	N423		
0 (start)	6×10^4	66	0.11	—
1	1.2×10^3	70	5.5	50
2	196	73	27.1	246

NOTE: Haploid strain N567 (*cspA1*, *metB2 argA1 pheA1*) was used as "prototrophic" strain and N423 (*cspA1*, *nicA1*) as "auxotrophic" mutant. The results of two experiments are compiled. The effects of one or two Novozym treatments are shown.
[a]The enrichment factor was calculated from the frequency of auxotrophs.

fwnA1, *argA1 nicA1*) and N668 (*cspA1*, *fwnA1*, *metB2 pheA1 nicA1*), containing new combinations of these linked markers were isolated by haploidization of the recombinant diploids. The frequency of spontaneous haploidization was calculated from the same experiment on the basis of the average number of haploids (Table 4). After correction for clonal distribution according to Luria and Delbrück (1943), the haploidization frequency was found to be 5.5×10^{-4}.

Discussion

The Novozym enrichment procedure described in this paper and used to isolate homozygous recombinants proved to be useful in the genetic analysis of *A. niger*. The method showed several advantages over filtration enrichment. A first limitation of the latter is that not all types of recombinants could be isolated. Upon application of this method for the isolation of auxotrophic mutants, we had already observed that mutants requiring vitamins or nucleic acid bases were especially scarce. Similar observations were described by Woodward *et al.* (1954) with *Neurospora crassa*. The most likely explanation is cross-feeding of minute traces of growth factors (e.g., vitamins) in the liquid medium by germinating conidia.

Another important limitation of the filtration enrichment method is the fact that many ungerminated conidia are discarded together with the germinated ones. Firstly, part of the ungerminated conidia become trapped into the mycelial network upon filtration. Secondly, conidia of *A. niger* show aggregation during incubation in liquid medium. So, quantitative data cannot be obtained using this method.

The Novozym enrichment procedure is based on selective killing of immobilized germinating conidia so that aggregation does not occur and cross-feeding will be limited. We imitated the enrichment of auxotrophs in simulation experiments. We found a 250-fold enrichment factor and no significant loss of auxotrophs. Therefore, we concluded that auxotrophs can be isolated quantitatively provided nonleaky mutants are used.

Linkage analysis, as used by Lhoas (1967), was mainly based on recombinants found in haploidization experiments. This method was ineffective for the determination of the linear order of auxotrophic markers on linkage group V of *A. niger*. We found low frequencies of recombinants in haploidization analyses. Most of these recombinants were prototrophic and no double auxotrophic recombinants could be isolated.

Lhoas (1967), who found relatively high rates of haploid recombinants for the markers he studied, also observed a predominance of prototrophic ones. He explained this by reduced viability of the auxotrophic recombinants on the *p*-fluorophenylalanine-containing haploidization medium. In our experiments, however, we supplemented the benomyl-containing haploidization medium with the essential growth factors and observed no significant difference in viability of haploid segregants carrying one or more of the auxotrophic markers used. It is therefore unlikely that the absence of haploid auxotrophic recombinants is caused by reduced viability during haploidization.

A more likely explanation for the predominance of prototrophic recombinants is the occurrence in the heterozygous diploid of a recombinant homozygous for the wild-type alleles. Such a recombinant might result from crossing-over during outgrowth of the heterozygous diploid. Segregation of a prototrophic diploid recombinant is likely on MM, whereas a reciprocal auxotrophic recombinant would not be able to segregate on this medium.

Finally, recombination frequencies are not reproducible in haploidization experiments. The concentration of benomyl in the medium is critical. If the concentration is rather low, relatively high numbers of diploids and aneuploids are observed. When a slightly higher concentration is used, almost only haploid segregants are found. The high variance of recombinant frequencies between markers in different experiments

TABLE 4. Number and classification of auxotrophic segregants isolated from different conidial suspension of diploid strain N423//N567 by Novozym enrichment (see also Table 5)

Conidial suspension[a]	Auxotrophs					
	2n					
	Met⁻	Met⁻Phe⁻	Arg⁻	Nic⁻	Arg⁻Met⁻Phe⁻	n[b]
1	1		2	2		5
2	2					5
3	2	2	1			53
4	3	3	1			43
5	1	1	1			14
6	1					8
7			1			4
8	1					2
9						1
10	2					15
11		2	2			4
12		3				10
13	1					9
14	2		1	1		0
15	1					5
16	1			1		6
17	1					6
18						0
19		1				7
20	1		2	2		5
21				1		2
22	3	1	2			7
23	2		1	1	1	4
24			1			1
25	1	1	2			25
26	1			1		33
Total	27	14	18	9	1	274

NOTE: Diploid strain: N423 *cspA1*, + + + *nicA1* +
N567 *cspA1*, *fwnA1*, *metB2 pheA1* + *argA1*

[a] From each suspension, 10^4 conidiospores were used in the enrichment procedure.

[b] Among the haploid auxotrophs, equal amounts of *nicA1* and *metB2 pheA1 argA1* haploid segregants were found. Haploids were distinguished from diploids visually or by Coulter counter determination.

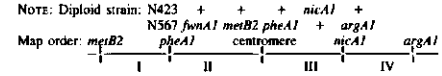
may be due to this phenomenon. For disomics of *A. nidulans*, it has been shown that the frequency of randomly selected haploid breakdown sectors that are recombinant can be up to 6% (e.g., Assinder *et al.* 1986; Assinder and Upshall, 1986). These studies also demonstrated that the occurrence of crossovers in the centromeric region was more pronounced in disomics than in diploid nuclei. It has been argued that if aneuploids are a relatively long-lasting stage in the generation of haploids on the haploidization medium, crossing-over in these aneuploids might explain the observed coincidence of crossing-over and haploidization in *A. niger* (Käfer 1977).

For the construction of the genetic map of chromosome V, we isolated and analysed homozygous diploid recombinants. From the diploid strain N423//N567, diploid recombinants homozygous for either of the auxotrophic markers were found, using the Novozym method. The reciprocal recombinants of crossing-over in region III (Nic⁻ and Arg⁻, respectively) were found at the same frequency. To get proper estimates of recombination frequencies, we isolated recombinants from many small samples of independent suspensions and, thus, correction for clonal segregation could be performed (Luria and Delbrück 1943). A map order for the auxotrophic markers on linkage group V could be determined (Table 5). The total recombination frequency resulting from a crossing-over event in each of the regions is probably twice the value based on the auxotrophic recombinants, assuming that recombination giving rise to the reciprocal homozygous prototrophic recombinants occurs at the same rate. Therefore, the frequencies of recombination leading to homozygous diploid segregants in a heterozygous diploid culture varied from 3.8×10^{-5} for the region between the centromere and *nicA1* up to 8.6×10^{-5} for that between *metB2* and *pheA1*. The frequency of haploidization was estimated to be 5.5×10^{-4} and, therefore, the frequency of nondisjunction leading to homozygosity is probably below this figure. Only two recombinants were found that could be explained by either nondisjunction or double crossing-over. Taking into account the frequency of mitotic crossing-over (in between 10^{-4} and 10^{-5} for each region), these two recombinants most likely arose by nondisjunction. Even in disomics of *A. nidulans* that show high rates of crossing-over in the centromeric region, most recombinants could be explained by a single crossing-over (e.g., Assinder and Upshall 1986; Käfer 1977). The conclusion of Assinder *et al.* (1986) that most of the recombinants

TABLE 5. Genetic analysis of homozygous auxotrophic segregants of diploid strain N423//N567 isolated by Novozym enrichment (see also Table 4)

No. segregants				
Total[a]	Independent[b]	Phenotype	Genotype[c]	Explanation
27	18	Met⁻	*metB2pheA1* + *argA1* *metB2* + *nicA1* +	Crossing-over I
14	8	Met⁻Phe⁻	*metB2pheA1* + *argA1* *metB2pheA1nicA1* +	Crossing-over II
11	7	Arg⁻	*metB2pheA1* + *argA1* + + *nicA1argA1*	Crossing-over IV
7	6	Arg⁻	*metB2pheA1* + *argA1* + + + *argA1*	Crossing-over III
8	6	Nic⁻	*metB2pheA1nicA1* + + + *nicA1* +	Crossing-over III
1	1	Nic⁻	+ + *nicA1* + + + *nicA1* +	Nondisjunction or crossing-over II and III
1	1	Met⁻Phe⁻Arg⁻	*metB2pheA1* + *argA1* *metB2pheA1* + *argA1*	Nondisjunction or crossing-over II and III

NOTE: Diploid strain: N423 + + + *nicA1* +
N567 *fwnA1 metB2 pheA1* + *argA1*



The recombination frequencies based on the average number of recombinants from 26 suspensions according to the method of Luria and Delbrück (1943) were: I, 4.3×10^{-5}; II, 2.7×10^{-5}; III, 1.9×10^{-5}; and IV, 2.3×10^{-5}.

[a] The total number of homozygous diploids isolated from 2.6×10^{5} conidiospores obtained from 26 different conidial suspensions.

[b] The number of different conidial suspensions from which the auxotrophs were isolated.

[c] The genotype was based on haploidization.

of chromosome I disomics of *A. nidulans* resulted from double exchange events was shown to be erroneous, as most of the recombinants could be explained by a single cross-over event in the centromeric region (Arst 1988).

Though the method is rather laborious if the recessive markers are in repulsion, it is useful if no direct selective marker located distally on the chromosome is available. The most likely map order and recombination frequencies of the auxotropic markers on linkage group V and the position of the centromere were deduced from genetic analysis of the homozygous segregants isolated by Novozym enrichment. In addition to the determination of the map order of markers on chromosome V, we were able to isolate recombinant haploid genotypes containing new combinations of closely linked auxotrophic markers on this chromosome.

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CHAPTER 3

Genetic analysis of *Aspergillus niger*: Isolation of chlorate resitance mutants, their use in mitotic mapping and evidence for an eighth linkage group

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Genetic analysis of *Aspergillus niger*: Isolation of chlorate resistance mutants, their use in mitotic mapping and evidence for an eighth linkage group

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Summary. This paper describes the use of chlorate resistant mutants in genetic analysis of *Aspergillus niger*. The isolated mutants could be divided into three phenotypic classes on the basis of nitrogen utilization. These were designated *nia*, *nir* and *cnx* as for *Aspergillus nidulans*. All mutations were recessive to their wild-type allele in heterokaryons as well as in heterozygous diploids. The mutations belong to nine different complementation groups. In addition a complex overlapping complementation group was found. Evidence for the existence of eight linkage groups was obtained. Two linked chlorate resistance mutations and two tryptophan auxotrophic markers, which were unlinked to any of the known markers (Goosen et al. 1989), form linkage group VIII. We used the chlorate resistance mutations as genetic markers for the improvement of the mitotic linkage map of *A. niger*. We determined the linear order of three markers in linkage group VI as well as the position of the centromere by means of direct selection of homozygous *cnxA1* recombinants. In heterozygous diploid cultures diploid chlorate resistant segregants appeared among conidiospores with a frequency of 3.9×10^{-5} (*cnxG13* in linkage group I) to 2.1×10^{-2} (*cnxD6* in linkage group III). The mean frequency of haploid chlorate resistant segregants was 1.3×10^{-3}. The *niaD1* and *niaD2* mutations were also complemented by transformation with the *A. niger niaD*$^+$ gene cloned by Unkles et al. (1989). Mitotic stability of ten Nia$^+$ transformants was determined. Two distinct stability classes were found, showing revertant frequencies of 5.0×10^{-3} and 2.0×10^{-5} respectively.

Key words: Chlorate resistance *Aspergillus niger* Linkage groups Transformation Recombinant frequency

Introduction

Aspergillus niger is an economically important fungus, which has been the subject of somatic hybridization and molecular genetic manipulations. For the analysis of the results of such manipulations and for breeding, a linkage map of the entire genome is a prerequisite. For the imperfect fungus *A. niger* such a map can be constructed only on the basis of analysis of mitotic recombinants isolated from originally heterozygous diploid colonies. Because mitotic crossing over is a relatively rare event, the homozygous diploid segregants have to be selected. Then the order of markers on a chromosome arm proximal to a selective marker can be determined. So it would be useful if such a selective marker were available on each chromosome arm. For several reasons we expected a priori that chlorate resistance mutations would be very useful in genetic analysis:

1. Mutations in many *Aspergillus nidulans* genes can result in chlorate resistance (for a review see Cove 1979), and these genes are located in five different linkage groups (Clutterbuck 1987). Each of these genes is required for nitrate utilization (Cove 1979). Similar mutations leading to chlorate resistance and nitrate non-utilization have been found in other filamentous fungi (for a review see Unkles 1989) such as *Neurospora crassa* (Marzluf 1981), industrial strains of *Penicillium chrysogenum* (Birkett and Rowlands 1981) and *Fusarium* spp. (Correl et al. 1987; Klittich and Leslie 1988).
2. The selection and characterization of chlorate resistant mutants in these fungi is relatively easy.
3. The phenotypic expression of the mutant alleles is recessive or semidominant in *A. nidulans* heterozygous diploids (Cove 1976b; Tomsett and Cove 1979). Complementation of the mutant alleles is thus possible.
4. Finally, two way selection of chlorate resistance and nitrate utilization is possible, which can be a great benefit in genetic analyses and also in transformation studies.

Here we describe the isolation and the characterization of chlorate resistant mutants of *A. niger*, as well as the use of these mutations in genetic analyses of *A. niger*.

Materials and methods

Strains. A. niger strains used are descended from N402, a strain with short conidiophores (*cspA1*) (Bos et al. 1988). The genotypes are shown in Table 1. Strains N423, N500 and N502 were used to isolate chlorate resistant mutants. The *trpC* mutant was available as transformant N593-T1 [*cspA1*, *trpC*::(*trpC-lacZ*)] and was obtained by gene replacement in a cotransformation experiment of strain N593 (*cspA1*, *pyrA5*) using plasmids carrying *pyrA* and *trpC*::*lacZ* respectively (Goosen et al. 1989).

Media and growth conditions. The media and growth conditions have been described before (Bos et al. 1988; Debets et al. 1989). The abbreviations CM, MM and SM are used for complete, minimal and supplemented minimal medium, respectively. To test chlorate resistance, 150 mM $KClO_3$

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Table 1. Genotypes of *Aspergillus niger* strains used

Strain	Linkage group							
	I	II	III	IV	V	VI	VII	?[a]
N423					*nicA1*			
N470	*olvA1*							*trpB2*
N500					*metB10*			
N502	*olvA1*		*bioB2*					
N591						*pyrB4*		
N619	*fwnA1*	*hisD4*	*bioA1 lysA7*	*leuA1*	*nicA1*	*pabA1*		
N662		*trpA1*	*argB2*	*leuA1*	*nicA1*	*pabA1*		
N717					*nicA1*		*adeF12*	
N738	*fwnA1*	*hisD4*	*bioA1 lysA7*	*leuA1*	*metB1*	*pdxA2*		

Gene symbols are as described before (Debets et al. 1989). All strains carry *cspA1* in addition to the markers shown

[a] Unlinked to markers in linkage groups I–VII

and 10 mM urea was added to CM (CMC) or MM (MMC). For testing of growth on different nitrogen sources the final concentrations were: 1 mM nitrite, 735 µM hypoxanthine, 20 mM nitrate, 20 mM glutamate or 595 µM uric acid as sole sources of nitrogen. Resistance to 5-fluoro-orotic acid (5-FOA) was tested on SM + 0.8 mg/ml 5-FOA and 10 mM uridine.

Isolation of mutants. Conidiospores were irradiated with ultraviolet light (UV) at a dose of 120 J/m^2 (for the black strains N423 and N500) or 60 J/m^2 (for the olive-green strain N502). This resulted in at least 80% survival (Bos 1987), and 5×10^6 conidia were then plated onto CM containing 150 mM $KClO_3$ and either urea, glutamate or uric acid (essentially according to Cove 1976a). Up to a few hundred small chlorate resistant colonies per plate were found. Resistant colonies were subcultured on fresh selective plates and pure colonies were isolated from these by subculturing single conidiospore colonies.

Classification of chlorate resistant mutants. Purified mutant colonies were phenotypically characterized on the basis of their ability to use various sole nitrogen sources, according to the criteria developed by Cove (1976a) for *A. nidulans*. Phenotypically indistinguishable mutants were further characterized by complementation analysis. For this purpose heterokaryons were constructed using the auxotrophic markers of the original strains (N423, N500 and N502).

Assignment of mutant genes to linkage groups. The isolation and haploidization of diploids were done essentially as described before (Bos et al. 1988). We used N619, N662 and N738 as master strains for the analysis of chlorate resistant mutants derived from N500, N502 and N423, respectively (see Table 1). Special precautions were taken so that segregation of recombinants during outgrowth of the diploids could be recognized: conidiospore suspensions were made from six conidial heads from different (parts of the) diploids; a sample of each suspension was plated onto CM containing 0.25 µg/ml benomyl, and haploid segregants from each suspension were isolated, purified, and tested separately.

Linkage analysis in linkage group VI. For the mapping of markers in linkage group VI we made use of the chlorate resistance mutation *cnxA1* and of *pyrB4* in a heterozygous diploid. Diploids were isolated by plating concentrated conidial suspensions from heterokaryon culture on MM covered by a thick MM overlay (sandwich plate). Conidiospores from single conidial heads from different diploid colonies were point inoculated on CMC. We selected 100 chlorate resistant diploid segregants. The ploidy of the colonies was determined by the colony colour, which is lighter in heterozygotes for *fwnA1* than in *fwnA*$^+$ haploids, and in case of doubt the size of the conidiospores was measured with a Coulter counter. In addition we selected homozygous PyrB$^-$ segregants on the basis of resistance to 5-FOA [the *pyrB4* mutant was isolated as a mutant resistant to 5-FOA (Goosen et al. 1987)]. The *pyrB4* mutation is recessive and therefore, segregants homozygous for *pyrB4* could be selected on SM + 5-FOA. The genotype of the segregants (resistant to either chlorate or 5-FOA) was further analysed as follows: Heterozygosity for the *pyrB4* marker was concluded from the appearance of 5-FOA resistant segregants on SM + 5-FOA (homozygous *cnxA1* segregants); the genotype for the *cnxA* gene was similarly tested on CMC (homozygous *pyrB4* segregants). For the chlorate resistant segregants the genotype with respect to the recessive marker *pabA1* could be deduced directly from the phenotype (Pab$^+$ or Pab$^-$ indicating respectively heterozygosity or homozygosity for the *pabA1* mutation).

Quantitative isolation of diploid recombinants. To achieve reproducible quantitative isolation of chlorate resistant recombinants, the following procedure was used. Single conidial heads from primary diploid colonies on the MM-sandwich plate were isolated and suspended in 0.5 ml saline-Tween (0.8% NaCl, 0.05% Tween 80). From these suspensions 0.1 ml (containing about 400 conidiospores) was plated onto CM and CMC. After 2 days of incubation at 30° C the total number of colonies on the CMC plates could be determined. The chlorate sensitive colonies appeared on CMC plates as small compact aconidial colonies with yellowish mycelium, whereas chlorate resistant colonies showed normal growth and sporulation. The latter could be counted on the 3rd day. If no chlorate resistant colonies appeared on the CMC plates, the corresponding inoculum was considered also to be free of chlorate resistant conidia. Then, the conidia of the colonies on the corresponding CM

plates were harvested, and dilutions were plated on CMC. Thus, chlorate resistant conidiospores would result from recombination events during outgrowth of the diploid on the CM plate, and disturbance by clonal effects would be limited. The frequency of chlorate resistant recombinants was determined after 3 days incubation at 30° C.

Transformation. Putative nitrate reductase minus mutants were studied in transformation experiments using the plasmid pSTA10 containing the *A. niger* wild-type *niaD* gene (Unkles et al. 1989; Campbell et al. 1989). Transformation was performed essentially as described by Goosen et al. (1987). Transformants were selected on SM containing 20% of the nutritional supplements of the strains used (avoiding nitrogen supplementation) and 20 mM nitrate as the only nitrogen source.

Results and discussion

Isolation and identification of chlorate resistant mutants

From the parental strains N423, N500 and N502 150 different chlorate resistant mutants were isolated and classified (Table 2). The phenotypic classes are designated by the same gene symbols as comparable mutants of *A. nidulans* (Cove 1976a). In experiments with *A. nidulans* Cove (1976a) observed that the proportions of different mutant classes were strongly influenced by the nitrogen source in the selection medium. This was also observed for *Fusarium moniliforme* (Klittich and Leslie 1988). Therefore, we used either urea, glutamate or uric acid as nitrogen source in the chlorate-containing selection medium. As can be seen in Table 2, *nia* and *cnx* mutants were isolated predominantly when glutamate served as a nitrogen source, whereas the highest proportion of *nir* mutants was isolated on medium with uric acid. With urea as nitrogen source all classes were found. So far no chlorate resistant nitrate-utilizing mutants have been isolated. All mutants appeared to be recessive in heterokaryons as well as in heterozygous diploids. This concerned not only nitrate utilization, but also chlorate resistance. Heterokaryons between phenotypically indistinguishable mutants derived from different strains could be isolated on MM + urea. Small pieces of the established heterokaryon were transferred to MMC and MM. Complementation of most *cnx* mutants was straightforward, e.g. combinations of non-complementing *cnx* mutants showed growth on MMC, but not on MM with nitrate as the sole nitrogen source, and heterokaryons from complementing *cnx* mutants were chlorate sensitive and nitrate utilizing. The chlorate resistant mutants of *A. niger* fall into nine defined complementation groups (Table 2). The *cnx* mutants belong to seven complementation groups and an additional overlapping complementation group. This latter class of *cnx* mutants (designated *cnxAE*) complemented neither *cnxA* nor *cnxE*. A similar overlapping complementation pattern has been found in *A. nidulans*, with *cnxB* failing to complement *cnxA* and *cnxC*, whereas *cnxA* and *cnxC* complement each other (Cove 1979). In *A. nidulans* eight complementation groups have been described including the overlapping *cnxB* complementation group (Clutterbuck 1987). Comparable results have been obtained for *P. chrysogenum* (Birkett and Rowlands 1981) and *N. crassa* (Marzluf 1981).

In *A. nidulans nirA* mutants have similar growth characteristics to *niiA niaD* double mutants (Cove 1976a). The double mutants comprised about 1% of all spontaneous *niaD* mutants, but remarkably after UV mutagenesis at low survival level the percentage of double mutants was much lower. The double mutants of *A. nidulans* have been shown to have deletions covering the closely linked *niiA* and *niaD* genes (Cove 1976a; Tomsett and Cove 1979). In our experiments with *A. niger* we found that all mutants that had lost the capacity for nitrate as well as nitrite utilization did not complement each other, but did complement *niaD* mutants. Therefore, we assume that they are not *niaD* or *niaD niiA* mutants. Furthermore, these mutants are not *niiA* mutants as they show no nitrite excretion whereas *A. nidulans niiA* mutants did (Cove 1976a). Thus we conclude that these mutants are equivalent to the *A. nidulans nirA* regulatory mutants.

Table 2. Chlorate resistant mutants of *Aspergillus niger* classified on the basis of nitrogen utilization according to the criteria described by Cove (1976a)

Phenotypic class	Nitrogen source[a]			Number of complementation groups involved	Designation
	urea	glutamate	uric acid		
Nia	32	47	6	1	*niaD*
Nir	14	1	17	1	*nirA*
Cnx	14	18	1	7[b]	*cnxA* to *F*

[a] Chlorate resistant mutants were isolated on chlorate-containing medium using different nitrogen sources

[b] In addition to these seven complementation groups a class of *cnx* mutants was found showing overlapping complementation with *cnxA* and *cnxE*. These mutants were designated *cnxAE*

Complementation of Nia⁻ mutants by transformation

Recently Unkles et al. (1989) isolated the wild-type *A. niger niaD* gene and it was subsequently cloned in a pUC based plasmid (designated pSTA10). We found that our putative nitrate reductase mutants could be complemented by this *niaD*$^+$ gene by transformation with this plasmid. From strains N693 (*metB10, niaD1*) and N694 (*olvA1, bioB2, niaD2*) 100–150 transformants/μg DNA were obtained using unlinearized DNA.

The revertant frequency of ten Nia$^+$ transformants was determined by selection for chlorate resistant segregants. It appeared that they could be divided into two distinct classes. Three of these transformants formed a class of very stable transformants and gave rise to chlorate resistant segregants at a frequency of about 2.0×10^{-5}. The other seven transformants were rather unstable: about 0.5% of the conidia were chlorate resistant. Southern hybridization of three transformants suggested that one stable transformant was a gene replacement (no detectable bacterial sequence and a hybridization pattern similar to the wild type, generally designated a type III transformant after Hinnen et al. 1978), whereas two rather unstable transformants were single copy type I (insertion) transformants. The stability of transformants probably depends on the recombination event by which they have arisen. Possibly the stability of transformants can be used as indication of the type of integration. Spontaneous chlorate resistant mutants were found at a frequency of about 2×10^{-6}.

Table 3. Assignment of chlorate resistance markers to linkage groups

Diploid[a]	Chlorate resistance marker	Linked[b] marker	Segregants				Recombinants (%)	Linkage group
			ab	a+	+b	++		
N706//N619	*cnxG13*	*fwnA1*	0	53	35	0	0.0	I
N819//N738	*cnxF12*	*fwnA1*	0	98	19	0	0.0	I
N702//N619	*cnxD6*	*bioA1*	0	64	38	1	1.0	III
	cnxD6	*lysA7*	7	55	32	10	16.4	III
N699//N662	*cnxB3*	*leuA1*	0	21	73	1	1.1	IV
N697//N619	*cnxA1*	*pabA1*	13[c]	40	56	3	14.3	VI
N703//N619	*cnxE7*	*pabA1*	0	59	31	0	0.0	VI
N760//N717	*cnxC5*	*adeF12*	0	9	71	2	2.4	VII
N774//N470	*niaD3*	*trpB2*	0	55	41	0	0.0	VIII
N716//N593-T1	*nirA2*	*trpC*	0	16	51	1	1.5	VIII
N751//N696	*niaD3*	*nirA2*		105		0		VIII

[a] The chlorate resistant strains were derived from N423 (N819); N500 (N697, N702, N703, N706) and N502 (N696, N699). N716 (*bioA1, leuA1, nicA1, pabA1, oliC2, nirA1*), N751 (*fwnA1; hisD4; nicA1; niaD3*), N760 (*fwnA1; hisD4; vcoA1, leuA1; cnxC5*) and N774 (*metB10; fnrA1; niaD3*) are derivatives of the original mutants obtained by recombination. N760 and N774 carry markers conferring resistance to a fungicide (*vcoA*, vinchlozolin; *fnrA*, fenarimol; S.M. Slakhorst et al., personal communication)

[b] Only the linked markers are shown

[c] N711, *cspA1, metB10, pabA1 cnxA1*, was isolated

Assignment of chlorate resistance mutations to linkage groups

Clear linkage was found for six of the chlorate resistance markers to a marker of the tester strain containing markers in six linkage groups. These chlorate resistance mutations mapped in four different linkage groups (Table 3). Three other chlorate resistance mutations, *niaD, nirA* and *cnxC*, were unlinked to any of the markers of the linkage groups I to VI (data not shown). Subsequently, the *cnxC* gene was shown in our laboratory to be linked to some other genes (e.g. *adeF*), that were also unlinked to the markers of the tester strains (see Table 3) and formed linkage group VII (Bos et al. 1989). Both *niaD* and *nirA* were unlinked to these genes, but were concluded to be mutually linked as only chlorate resistant haploid segregants were found in the haploidization experiment (Table 3). About 25% chlorate sensitive segregants would be expected if the mutations were on different chromosomes. A transformant (N593-T1) with a disrupted *trpC* gene and a *trpB* mutant were also included in these analyses, as these mutations formerly could not be assigned to one of the six linkage groups described (Goosen et al. 1989). *TrpB2* showed close linkage to *niaD3* and *trpC* was linked to the *nirA* gene (Table 3). They were both unlinked to genes in linkage group VII. Therefore, we concluded that these four genes form an eighth linkage group in *A. niger*.

An example of mitotic recombination mapping with chlorate resistance marker genes

Linkage group VI to which the *cnxA* gene belongs also contains the *pyrB* gene (Bos et al. 1989). Both are very suitable as selective markers as described in Materials and methods and were used to isolate homozygous mitotic recombinants. Table 4 shows the results of the analysis of the diploid chlorate or 5-FOA resistant segregants of strain N711// N591, together with the simplest genetic explanation for each recombinant type. All homozygous *pyrB4* segregants were still heterozygous for *cnxA1* and vice versa (Table 4). We conclude therefore that the position of the centromere is in between *pyrB4* and *cnxA1*. Some of the segregants homozygous for *cnxA1* were also homozygous for *pabA1* while others were still heterozygous; thus it was concluded that these two markers are on the same chromosome arm and that *cnxA1* is centromere distal to *pabA1*. The relative distances of the markers on this chromosome arm are also shown in Table 4. Most genotypes can be explained by a single crossover event. One diploid may have arisen as a double crossover, and another one by either a double crossing over or non-disjunction. We prefer the former explanation because partially homozygous diploids arise very rarely by non-disjunction as will be discussed below.

Estimation of recombination frequencies

In fungi the determination of distances between markers and the centromeres by means of mitotic recombination mostly concerns relative frequencies. Comparison of recombination frequencies on different chromosomes or chromosome arms is usually not possible. Often recombinant diploids cannot be isolated quantitatively and in addition they appear as clones.

For the estimation of the absolute recombination frequencies between the chlorate resistance mutations and the centromeres, we took advantage of the fact that these mutations allow the quantitative selection of chlorate resistant mitotic recombinants from heterozygous diploids. We constructed a series of diploids which were each heterozygous at one of the chlorate resistance loci, and (except N738// N819) also at two closely linked colour loci *olvA1* and *fwnA1*. These colour markers facilitated the distinction between haploids (fawn or olive) and diploids (black). In diploid N738//N819 the closely linked auxotrophic markers *metB1* and *nicA1* were in repulsion. This allowed the detection of haploids as these are either Nic^- or Met^-, whereas

Table 4. Mitotic mapping in linkage group VI

Diploid: N711 + *pabA1 cnxA1*
N591 *pyrB4* + +

Selected phenotypes	Genotype of segregants[a]	No. of occurrences	Recombination event[b]
Chlorate resistant	+ *pabA1 cnxA1* *pyrB4* + *cnxA1*	90	Crossover III
	+ *pabA1 cnxA1* *pyrB4 pabA1 cnxA1*	8	Crossover II
	+ *pabA1 cnxA1*	1	Crossover I and II
	+ *pabA1 cnxA1*		(or non-disjunction)
	+ *pabA1 cnxA1* + + *cnxA1*	1	Crossover I and III
5-FOA resistant	*pyrB4* + + *pyrB4 pabA1 cnxA1*	36	Crossover I

Conclusion: *pyrB4* — c — *pabA1* — *cnxA1*

Map interval: I | II | III

Relative recombination frequencies: 8(9) | 91

N761, *cspA1; fwnA1, pyrB4 pabA1 cnxA1*, was isolated. 5-FOA, 5-fluoro-orotic acid
[a] Only the relevant genotypes of the diploids are shown (see text)
[b] For map intervals see concluded map positions

Table 5. Estimation of recombination frequencies in diploids heterozygous for a chlorate resistance marker

Marker	Diploid	Inocula analysed[a]	Recombinant frequency[b] ($\times 10^3$)	Standard deviation ($\times 10^3$)
cnxF12	N738//N819	9/20	2.4	0.84
cnxG13	N764//N523	0/40	0.039	0.022
cnxD6	N702//N502	15/40	21	12
cnxB3	N699//N523	0/40	2.4	0.25
cnxA2	N698//N523	36/90	20	5.2
cnxE8	N704//N523	34/80	19	2.5
cnxC5	N701//N523	1/17	1.6	0.86
niaD2	N694//N523	3/20	3.5	2.9
nirA2	N696//N523	3/20	11	8.7

Diploids heterozygous for the chlorate resistance marker were constructed containing the closely linked colour markers *olvA1* and *fwnA1* in repulsion to allow detection of haploid recombinants. For diploid N738//N819 the closely linked markers *nicA1* and *metB1* that are in *trans* position on chromosome V were used for this purpose. The mean frequency of chlorate resistant haploids in all these suspensions was 1.3×10^{-3} (SD 1.4×10^{-3})
[a] The fraction of conidial suspensions containing chlorate resistant conidia is shown. Suspensions that were free of such recombinants were used to inoculate CM propagation plates
[b] The mean frequency of diploid chlorate resistant recombinants of at least ten conidial suspensions

the diploid was prototrophic for these markers. For each of these diploid strains we determined the fraction of diploid chlorate resistant conidia by plating conidiospore suspensions on CMC. The results are shown in Table 5. For some markers (*cnxD6*, *cnxA2* and *cnxE8*) a relatively high number of the conidial heads of the original diploid already contained chlorate resistant conidia (Table 5). In few cases conidial heads were isolated that were completely chlorate resistant. In one suspension no chlorate resistant conidia could be isolated from the CM propagation plate indicating the loss of the chlorate resistance marker allele in the original conidial head. These observations indicate a high recombination frequency, and show the necessity to test the inocula used for the propagation of the heterozygous diploid. For the other chlorate resistance markers only occasionally conidial heads were found that already contained chlorate resistant conidia. The calculated recombinant frequencies varied from 3.9×10^{-5} for *cnxG1* up to 2.1×10^{-2} for *cnxD6*.

In these experiments it was not possible to distinguish between diploids homozygous through mitotic crossing over and diploids homozygous through non-disjunction. However, from the results in Table 4 it is clear that only 1 out of 100 diploids homozygous for *cnxA1* could have resulted from non-disjunction. In the case of markers on chromosome V only 2 out of 69 homozygous diploids analysed (recombinant frequency of 7.7×10^{-6}) possibly originated from non-disjunction (Debets et al. 1989). This indicates that non-disjunction is rather infrequent. Assuming that the frequency of non-disjunction for each of the chromosomes is similar, non-disjunction would hardly contribute to the frequency of homozygous diploids found for most of the markers. If the reciprocal recombinants occur at the same rate as chlorate resistant colonies, the total recombinant frequency would be twice the value indicated in Table 5. The recombination frequencies between other markers and the centromere can be estimated from the relative proportion of the recombinants. For instance, the recombination frequency between *pabA1* and the centromere can be esti-

mated to be about 10% of that between *cnxA1* and the centromere (Table 4).

In conclusion, the chlorate resistant mutants of *A. niger* are very similar to those in *A. nidulans* and other filamentous fungi. The chlorate resistance genes are distributed over six linkage groups and they revealed an eighth linkage group in *A. niger*. They can be used efficiently for the quantitative isolation of mitotic recombinants, and are very useful for mitotic mapping. Finally, two-way selection of mutants, recombinants and transformants is possible and this is a useful tool in genetic analyses.

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CHAPTER 4

Genetic analysis of *amdS* transformants of *Aspergillus niger* and their use in chromosome mapping

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Genetic analysis of *amd*S transformants of *Aspergillus niger* and their use in chromosome mapping

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Summary. The *Aspergillus nidulans* gene coding for acetamidase (*amd*S) was introduced into *A. niger* by transformation. Twelve Amd$^+$ transformants were analysed genetically. The *amd*S inserts were located in seven different linkage groups. In each transformant the plasmid was integrated in only a single chromosome. Our (non-transformed) *A. niger* strains do not grow on acetamide and are more resistant to fluoroacetamide than the transformants. Diploids hemizygous for the *amd*S insert have the Amd$^+$ phenotype. We exploited the opportunity for two-way selection in *A. niger*: transformants can be isolated based on the Amd$^+$ phenotype, whereas counter-selection can be performed using resistance to fluoroacetamide. On this basis we studied the phenotypic stability of the heterologous *amd*S gene in *A. niger* transformants as well as in diploids. Furthermore, we mapped the plasmid insert of transformant AT1 to the right arm of chromosome VI between *pab*A1 and *cnx*A1, providing evidence for a single transformational insert. The results also show that the *amd*S transformants of *A. niger* can be used to localize non-selectable recessive markers and that the method meets the prerequisites for efficient mitotic mapping. We suggest the use of *amd*S transformants for mitotic gene mapping in other fungi.

Key words: *Amd*S transformants – *Aspergillus niger* – Mitotic mapping – Recombination – Mitotic stability

Introduction

Several efficient transformation systems for *Aspergilli* have been developed during the last few years, and these can be applied to the study of homologous or heterologous gene expression (for review see Fincham 1989; Goosen et al. 1990). Hybridization analysis of *Aspergillus nidulans* transformants revealed firstly that integration of a homologous transforming gene into the genome may occur by homologous as well as non-homologous recombination and, secondly, that the number of integrated copies could vary widely (Tilburn et al. 1983; Yelton et al. 1984; Wernars et al. 1985; Ballance and Turner 1986; De Graaf et al. 1988; Upshall 1986). If no or very low homology exists between the transforming plasmid and the fungal genome, transformation seems to result from integration into different sites in the genome (Ballance and Turner 1985). Therefore, we used the *amd*S transformation system in order to study heterologous transformation in *A. niger* which does not contain an equivalent gene, but in which the *amd*S gene is subject to regulation if introduced by transformation (Kelly and Hynes 1985). *Amd*S transformants can be selected in this fungus on the basis of acetamide utilisation. On the other hand *amd*S mutants of *A. nidulans* can be isolated by selecting for resistance to fluoroacetamide (Hynes and Pateman 1970b).

Genetic analysis of Amd$^+$ transformants of *A. nidulans* has shown that non-homologous integration can take place in various chromosomes (Wernars et al. 1986). Also in *A. niger*, Southern hybridization experiments suggested that plasmid DNA carrying the heterologous *argB* gene of *A. nidulans*, could integrate into different sites in the genome (Buxton et al. 1985). The genetic techniques and strains of *A. niger* developed in our group allow genetic analysis of transformants (Bos et al. 1988; Debets et al. 1989). Master strains with markers in six linkage groups are available and recently two additional linkage groups have been identified (Bos et al. 1989; Debets et al. 1990).

In this paper we describe the genetic analysis of heterologous *amd*S transformants of *A. niger*. We quantified the reversion of Amd$^+$ transformants to the Amd$^-$ phenotype, and also the loss of the Amd$^+$ phenotype by mitotic recombination in diploids hemizygous[1] for the *amd*S insert. Furthermore, we show that the heterologous *amd*S gene can be used very efficiently as a counter-selection marker in chromosome mapping of non-selectable recessive markers in *A. niger*.

Materials and methods

Strains and plasmids. The *A. niger* strains used are listed in Table 1. The plasmids p3SR2 (Hynes et al. 1983) and pGW325 (p3SR2 in which the *Sal*I site has been changed to an *Eco*RI site using a synthetic linker), both containing

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[1] The term hemizygous is used to indicate that the plasmid insert is present only on one of the two homologous chromosomes in the diploid

Table 1. Genotype of *Aspergillus niger* strains used

Strains	Linkage group							
	I	II	III	IV	V	VI	VII	VIII
N423 (*cspA1*)					*nic*A1			
N593 (*cspA1*)			*pyr*A6					
N695 (*cspA1*)					*met*B1			*nir*A1
N722 (*cspA1*)	*fwn*A1	*his*D4	*bio*A1 *lys*A7	*leu*A1		*pab*A1	*cnx*C5	
N761 (*cspA1*)	*fwn*A1					*pyr*B4 *pab*A1 *cnx*A1		

Gene symbols are as described previously (Bos et al. 1989; Debets et al. 1989; 1990)

the *A. nidulans* wild-type *amd*S gene, were used for transformation.

Media. The media used have been described before (Bos et al. 1988; Debets et al. 1989). Chlorate resistant recombinant colonies were selected on complete medium (CM) containing 150 mM potassium chlorate and 10 mM urea (CMC). For viable counts 0.05% (w/v) Triton-X100 was added to CM (CMT) to reduce colony size. If necessary 10 mM uridine was added to the media (to allow growth of PyrB$^-$ segregants). PyrB$^-$ mitotic recombinants were isolated on supplemented minimal medium (SM) containing 0.8 mg/ml 5-fluoroorotic acid (SM + 5FOA). Selection of fluoroacetamide resistant segregants was performed on SM supplemented with 5 mM urea, 1 mg/ml fluoroacetamide (FA) and 100 mM acetate as carbon source (SM + FA), essentially according to Hynes and Pateman (1970b). Growth tests for acetamide and acrylamide utilization were carried out on SM containing 20% of the standard concentration of the required auxotrophic supplements and either 20 mM acetamide as a sole carbon and nitrogen source or 10 mM acrylamide as the sole nitrogen source.

Transformation. The transformation procedure was performed essentially according to Wernars et al. (1987), except that mycelial protoplasts were used. The transformants analysed in this paper are listed in Table 2.

Haploidization analysis. Diploids between transformants and master strains N722 and N695 (for genotype see Table 1) were isolated as described previously (Bos et al. 1988).

For haploidization we prepared conidial suspensions from different heterozygous diploid colonies by taking single conidial heads from the original MM isolation plate. These conidia were subjected to haploidization by plating on CM with 0.25 µg/ml benomyl as described earlier (Bos et al. 1988; Debets et al. 1990).

Isolation of Amd$^-$ segregants. The quantitative selection of Amd$^-$ segregants (revertants) from the haploid transformants was performed on SM + FA. Conidia of purified Amd$^+$ transformants were grown on CM for 3–4 days at 30° C; the inocula were also tested by plating on SM + FA. If no resistant colonies appeared on the SM + FA plates, conidia of the CM plates were harvested and plated onto SM + FA and CMT. Plates were incubated for 3–4 days at 30° C, and the frequency of FA resistant colonies was determined. FA resistant colonies were tested for their inability to utilize acetamide as the sole nitrogen source. The quantitative selection of mitotic segregants from diploid strain N761//AT1 was performed with similar precautions against clonal segregation: suspensions of single conidial heads from primary diploid cultures were screened for mitotic recombinants, i.e. Amd$^-$ segregants (on SM + FA) and chlorate resistant CnxA$^-$ segregants (on CMC). Parallel cultures from the same suspensions were grown on CM to propagate the diploid. If no segregants resistant to FA or chlorate were found, conidia harvested from the corresponding CM plate were tested for resistant segregants.

Results

Linkage analyses of the amd*S gene(s) in transformants*

The results of the haploidization analyses of the transformants are compiled in Table 2. Clear linkage of the Amd$^+$ phenotype to a single chromosomal marker of tester strain N722 was observed in 11 *amd*S transformants. For AT7 no linkage to any of the markers of the tester strain was found but, in a haploidization experiment using N695 as a test strain, we found linkage of the plasmid insert to the *nir*A1 marker on linkage group VIII (Table 2). For AT18 a rather high percentage of recombinants was found with *his*D4 (16.8%). In these experiments six spore suspensions, each derived from a single conidial head, were subjected to haploidization and one suspension showed over 50% *amd*S-*his*D recombinants and accounted for about 50% of the total number of recombinants. Probably, these segregants have a common origin in a crossing-over event in the diploid colony. Omitting the results from this conidial head, the linkage between *his*D4 and the *amd*S insertion is closer (about 10% recombinants). The Amd$^+$ and Amd$^-$ segregants were equally represented in all haploidization experiments (not shown).

Determination of the frequency of reversion of Amd$^+$ transformants

In *A. nidulans*, Amd$^-$ mutants could be isolated as fluoroacetamide resistant mutants (Hynes and Pateman 1970b). On testing our *A. niger* strains we observed that these are considerably more resistant to FA than transformants carrying the *amd*S gene. Loss of the Amd$^+$ phenotype will thus result in resistance to FA. The mitotic phenotypic stability of the Amd$^+$ transformants was estimated by selection of FA resistant segregants after growth of the transfor-

Table 2. Linkage data of *A. niger amdS* transformants in haploidization analysis

Transformant	Parental strain	Plasmid	Linked markers[a]	Number of segregants analysed	Recombinant frequency	Linkage group
AT20	N593	pGW325	*fwn*A1–*amdS*$^+$	61	0.0	I
AT8	N423	pGW325	*his*D4–*amdS*$^+$	109	1.8	II
AT16	N423	pGW325	*his*D4–*amdS*$^+$	123	4.1	II
AT18	N423	pGW325	*his*D4–*amdS*$^+$	125	16.8[b]	II
AT19	N423	pGW325	*lys*A7–*amdS*$^+$	104	1.9	III
			*lys*A7–*bio*A1		10.6	
			*bio*A1–*amdS*$^+$		8.7	
AT6	N423	p3SR2	*leu*A1–*amdS*$^+$	105	1.9	IV
AT17	N423	pGW325	*nic*A1–*amdS*$^+$	97	2.1	V
AT2	N423	pGW325	*nic*A1–*amdS*$^+$	79	3.8	V
AT3	N423	p3SR2	*nic*A1–*amdS*$^+$	78	5.1	V
AT4	N423	p3SR2	*nic*A1–*amdS*$^+$	115	2.6	V
AT1	N423	pGW325	*pab*A1–*amdS*$^+$	98	11.2	VI
AT7	N423	p3SR2	*nir*A1–*amdS*$^+$	86	0.0	VIII

[a] Only the markers of the test strain linked to the Amd$^+$ character are shown
[b] About 50% of the recombinant haploids were derived from one conidial head

Table 3. Mitotic stability of *amdS* transformants

Amd$^+$ strain (transformant)	Frequency of FA resistant segregants[a]	Standard deviation	Copy number[b]	Growth on acrylamide[c]
AT1	1.8×10^{-4}	1.4×10^{-4}	3–4	
AT2	2.3×10^{-6}	6.5×10^{-7}	>6	c
AT3	9.5×10^{-5}	4.7×10^{-5}	>8	
AT4	1.2×10^{-5}	9.7×10^{-6}	>4	c
AT6	1.5×10^{-4}	1.3×10^{-4}	≥6	c
AT7	9.5×10^{-5}	1.5×10^{-5}	>5	
AT8	1.3×10^{-5}		>4	c
AT16	2.1×10^{-4}	2.0×10^{-5}	2–3	
AT17	3.0×10^{-5}	3.2×10^{-5}	1–2	
AT18	2.6×10^{-3}	3.0×10^{-3}	≥7	
AT19	6.4×10^{-6}	7.5×10^{-5}	2	
AT20	3.1×10^{-5}	2.7×10^{-5}	>10	c

[a] The results shown are the mean frequencies among conidiospores in three experiments, except for AT8 which has been analysed once. FA, fluoroacetamide
[b] The plasmid copy number was estimated on the basis of Southern analysis by comparing hybridization signals obtained from equal amounts of chromosomal DNA of each transformant using wild type *A. nidulans* as standard (results not shown)
[c] c, growth on medium containing 10 mM acrylamide as the sole nitrogen source, indicating constitutive expression of the *amdS* gene(s) (Kelly and Hynes 1985)

mants on non-selective medium (CM). In order to obtain reproducible quantitative data the inocula used were tested for the frequency of revertants as described in Materials and methods. The results of these experiments are shown in Table 3.

One transformant (AT8) was only slightly more sensitive to FA than wild type and in this case proper estimation of the frequency of FA resistant segregants was laborious, as all segregants on the SM + FA plates had to be retested. Therefore, the frequency of revertants of AT8 was only determined once, whereas that of the other transformants has been analysed in three experiments. All FA resistant segregants had lost the Amd$^+$ phenotype, as judged by testing growth on acetamide. The frequency of mitotic recombination leading to loss of the Amd$^+$ phenotype ranged from 2.6×10^{-3}–2.3×10^{-6} for the various transformants. The plasmid copy number in each of the transformants was estimated by Southern analysis and varied from 1 to more than 10 (Table 3). In Table 3 the transformants that showed growth on acrylamide are also indicated. In *A. nidulans* the capacity to utilize acrylamide as nitrogen source is an indication that the *amdS* gene is constitutively expressed (Hynes and Pateman 1970a). Kelly and Hynes (1985) showed that the *A. nidulans* amdS gene is also subjected to regulation in *A. niger* transformed with *amdS*.

Mitotic mapping of the integration site

The transformant AT1 with an *amdS* insert in chromosome VI, was very suitable for mitotic mapping analysis. The linear order of *pyr*B4, *pab*A1 and *cnx*A1 relative to the centromere (between *pyr*B4 and *pab*A1) on chromoso-

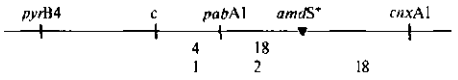
Table 4. Determination of the linear order of markers on linkage group VI relative to the *amdS* insertion

Diploid strain:

N761	*fwn*A1	+	*pyr*B4	*pab*A1	*	*cnx*A1[a]
AT1	+	*nic*A1	+	+	*amdS*$^+$	+

Segregants isolated						Number of occurrences
Phenotype selected	Genotype[b]					
FA resistant	*pyr*B4	*pab*A1	*	*cnx*A1		18
	+	+	*	*cnx*A1		
	*pyr*B4	*pab*A1	*	*cnx*A1		4
	+	*pab*A1	*	*cnx*A1		
Chlorate resistant	*pyr*B4	*pab*A1	*	*cnx*A1		18
	+	+	*amdS*$^+$	*cnx*A1		
	*pyr*B4	*pab*A1	*	*cnx*A1		2
	+	+	*	*cnx*A1		
	*pyr*B4	*pab*A1	*	*cnx*A1		1
	+	*pab*A1	*	*cnx*A1		
5-FOA-resistant[c]	*pyr*B4	*pab*A1	*	*cnx*A1		32
	*pyr*B4	+	*amdS*$^+$	+		

Linear order:



[a] The absence of the *amdS* insert is marked by *, i.e. the site on chromosome VI of N761 corresponding to the position of the *amdS* insert on the homologous chromosome of AT1

[b] The relevant genotype was deduced from the phenotype on the various test plates: homozygosity of the recessive markers *pyr*B4, *pab*A1 and *cnx*A1 that were in *cis* in the original diploid is indicated by Pyr$^-$, Pab$^-$ and chlorate resistance, respectively. Segregants hemizygous for *amdS* are Amd$^+$ and FA^s and loss of the *amdS*$^+$ gene(s) resulted in FA^r and Amd$^-$ phenotype

[c] 5-FOA, 5-fluoroorotic acid

me VI was established recently and a strain (N761) containing these markers was then isolated (Debets et al. 1990). The diploid N761//AT1 (see Table 4 for the genotype) is heterozygous for *pyr*B4, *pab*A1 and *cnx*A1 and hemizygous for *amdS*$^+$. Segregants homozygous for *pyr*B4 on the left chromosome arm can be selected as colonies resistant to 5-FOA, whereas homozygosity of the *cnx*A1 marker allele on the right arm results in chlorate resistance. Furthermore, diploid N761//AT1, hemizygous for the *amdS* insert, was sensitive to FA and loss of the *amdS* insert resulted in FA resistance. Different types of mitotic recombinants were isolated from this diploid and were subsequently analysed (Table 4). The FA resistant segregants were all homozygous for *cnx*A1, but still heterozygous for *pyr*B4. Four such recombinants were Pab , indicating homozygosity of the *pab*A1 marker. The recombinants homozygous for *cnx*A1 were all heterozygous for *pyr*B4, whereas some were FA resistant. One of the latter was Pab , the others were heterozygous for *pab*A1. The Pyr segregants were all heterozygous for the other markers, and hemizygous for *amdS*. From these results we conclude that the position of the *amdS* insert is between *pab*A1 and *cnx*A1 (Table 4).

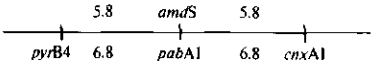
Table 5. Analysis by haploidization of diploid strain N761//AT1

Diploid:

N761	*fwn*A1	+	*pab*A1	*pyr*B4	*cnx*A1	*
AT1	+	*nic*A1	+	+	+	*amdS*$^+$

Linked markers		Segregants				Recombinant frequency
a	b	ab	a+	+b	++	(%)
*pyr*B4	*pab*A1	45	7	1	67	6.7
*pyr*B4	*amdS*$^+$	46	6	1	67	5.8
*pyr*B4	*cnx*A1	46	6	6	62	10.0
*pab*A1	*amdS*$^+$	46	0	1	73	0.8
*pab*A1	*cnx*A1	45	1	7	67	6.7
amdS$^+$	*cnx*A1	46	1	6	67	5.8

Linear order:



A total of 120 haploid recombinant colonies was tested. *, no *amdS* insert present

Table 6. Estimation of mitotic recombination frequencies in diploid culture using *cnx*A1 and the *amdS* insert as selectable and counter-selectable marker respectively

Conidial suspension	Frequency of resistant spores[a]	
	Chl^r	FA^r
1	4.5×10^{-3}	8.0×10^{-4}
2	6.0×10^{-3}	1.3×10^{-3}
3	2.6×10^{-3}	1.2×10^{-3}
4	7.1×10^{-3}	2.0×10^{-3}
5	1.2×10^{-2}	2.6×10^{-3}
6	2.5×10^{-3}	3.2×10^{-3}
7	5.5×10^{-3}	2.7×10^{-3}
8	4.0×10^{-2}	1.2×10^{-3}
9	3.2×10^{-3}	4.1×10^{-3}
10	2.0×10^{-2}	8.6×10^{-3}
Mean	1.3×10^{-2}	2.8×10^{-3}
Standard deviation	1.3×10^{-2}	2.3×10^{-3}

[a] The frequency of fluoroacetamide resistant (FA^r) and chlorate resistant (Chl^r) diploid recombinants in ten different conidial suspensions of diploid strain N761//AT1 (for genotype see Table 4) was determined

In addition, diploid N761//AT1 was haploidized to study the segregation of the transformational insert of AT1. A rather close linkage of the *amdS* insert and *pab*A1 was observed (Table 5).

Estimation of the mapping distance of the amdS *insert to the centromere*

In a recent study (Debets et al. 1990) chlorate resistance markers were used for gene mapping. If the same precautions are taken, a similar procedure (see below) can also be used to locate the position of the *amdS* insert. We used AT1 with an *amdS* insert on chromosome VI and the *cnxA* gene as reference to show that both markers provide comparable data. From diploid N761//AT1 the frequencies of

chlorate resistant and FA resistant segregants were determined. Small suspensions of conidiospores obtained from the original diploids that were used as inocula for propagation of the heterozygous diploids were tested for the presence of mitotic segregants on SM + FA and CMC. In this way 10 out of 20 inocula were found to be free of both types of recombinants and spores from the corresponding CM plates were harvested. The FA resistant and chlorate resistant recombinants isolated from these spores originate from recombination events during colony development. In Table 6 the frequencies of mitotic segregants from these ten conidial suspensions are shown. On the CMC plates well-growing and sporulating chlorate resistant plus the very small aconidial chlorate sensitive colonies were enumerated for viable count as previously described (Debets et al. 1990). Nearly all the chlorate resistant colonies were black, only a few fawn haploid colonies were observed. The data presented were corrected for haploids assuming that half of the haploid segregants would be fawn (the *fwn*A1 marker is unlinked to *cnx*A1). The segregants on FA plates were aconidial and were not further analysed for ploidy. Haploid and non-disjunctional diploid segregants would not be able to grow on these plates, as no uridine was added to the FA-containing medium in this experiment. Therefore, the data for FA resistant segregants represent only recombination events due to crossing-over in diploids. The distances of the *cnx*A1 marker and the *amd*S insert of AT1 from the centromere are 1.3×10^{-2} and 2.8×10^{-3}, respectively (Table 6).

Discussion

In this paper we describe the genetic analysis of 12 transformants of *A. niger* with an *amd*S insert. In each individual transformant, the Amd^+ character could be assigned to one single linkage group. The inserts mapped in seven of the eight linkage groups known in *A. niger*. Comparable results were found for Amd^+ transformants of *A. nidulans* (Wernars et al. 1986) where the *amd*S sequences of six transformants mapped on five different chromosomes. It is likely that the multiple copy transformants contain a compound integrate at one position, and that complex hybridization patterns of our multiple copy *amd*S transformants (not shown), like those observed by Kelly and Hynes (1985), represent a single rearranged plasmid insert, that probably resulted from recombination between plasmids before or during integration into the genome. Multiple integrates at different sites in the genome, which could also explain the results of Southern analysis in these transformants, are not likely as the *amd*S insert behaved as a single genetic marker. A similar conclusion was recently drawn by Mohr et al. (1989) from the fact that from a multicopy *amd*S transformant of *A. niger* only one fusion fragment between the plasmid insert and the chromosome could be isolated.

Constitutive expression of the *amd*S gene(s), as indicated by growth on acrylamide, was found for some but not all of the multicopy transformants. Whether this reflects the number of active *amd*S genes in the integrate or the position on the chromosome is not clear.

In general little information is available about the phenotypic stability of transformants. Most reports on mitotic stability are based on tests of several transformants grown under non-selective conditions (e.g. Tilburn et al. 1983; Yelton et al. 1984; Wernars et al. 1986). Dunne and Oakley (1988) studied the frequency of benomyl resistant revertants from single copy transformants integrated at the homologous *ben*A locus of *A. nidulans*. They found a revertant frequency (referred to by the authors as recombination frequency) of 4.6×10^{-4} and showed that more than half of the revertants resulted from gene conversion and the others from crossing-over. In *A. niger* the stability of *nia*D transformants can be determined easily by selection for chlorate resistance, and it has been suggested that the frequency of reversion of such transformants might be helpful in distinguishing allelic integrants (type I, according to Hinnen et al. 1978) from gene replacement transformants (type III). The two types show reversion frequencies of 5.0×10^{-3} and 2.0×10^{-5}, respectively (Debets et al. 1990). Significant loss of the transforming sequence during the parasexual cycle as reported for *A. nidulans pyr*G transformants (Ballance and Turner 1985) and some Amd^+ transformants (Wernars et al. 1985) was not observed in the Amd^+ transformants of *A. niger*. As can be seen from Table 5, Amd^+ segregants did not predominate and no aberrant segregation of *amd*S was observed. In order to determine the stability of the different ectopic (type II) heterologous *amd*S transformants of *A. niger*, Amd^- revertants were isolated using selection for resistance to fluoroacetamide. All FA resistant segregants tested could not utilize acetamide, thus indicating loss of the *amd*S^+ sequence. The frequency of such revertants varied from 2.6×10^{-3} for AT18 to 2.3×10^{-6} for AT2. Both AT18 and AT2 are multicopy transformants but the reversion frequencies differ significantly. For multicopy transformants it is likely that phenotypic reversion occurs either by non-homologous recombination resulting in excision of all or most of the transforming sequence, or by recombination between homologous regions in the rearranged integrate resulting in excision of the active *amd*S genes. If the likelihood of both events in multicopy transformants is different (e.g. because of structure or chromosomal position of the integrate), this might explain the observed differences in stability. The low (one or two) copy number transformants AT17 and AT19 show rather high mitotic stability (revertant frequencies of 3.0×10^{-5} and 6.4×10^{-6}, respectively). Assuming that single copy heterologous *amd*S transformants (type II) result from rare non-homologous recombination, one might expect that such transformants would be stable, since they can only revert by either mutation or (rare) non-homologous recombination. Heterologous *amd*S transformants were found at a frequency of about 2/μg plasmid DNA (using 10^6 protoplasts) whereas, for example, homologous types I plus III *pyr*A transformants had been found at a frequency of 30–50/μg plasmid DNA (10^6 protoplasts) (Goosen et al. 1987). Both phenomena, the high stability observed for the low copy number transformants and some of the multicopy *amd*S transformants and the low transformation frequency, may have a common basis in a low frequency of non-homologous recombination in *A. niger*. However, lack of phenotypic expression of primary transformants may also contribute to the low transformation frequency observed for the *amd*S transformation system. As long as the mechanism of non-homologous integration is unknown, and as long as no quantitative data about other type II transformants (using either heterologous or homologous DNA) are available, the question of whether single copy type II transformants in general are stable must remain open. Such data may,

however, be of great practical importance, for example in the improvement of production strains by transformation techniques.

The *amd*S insert in transformant AT1 was shown to be in linkage group VI. The linear order of three markers in linkage group VI relative to the position of the centromere has been determined recently (Debets et al. 1990). Strain N761, containing these three markers, was used to determine the position of the transforming sequence of AT1. In this way the *amd*S insert was mapped between the markers *pab*A1 and *cnx*A1 (Table 4). The map order *pyr*B4, centromere, *pab*A1 and *cnx*A1 as concluded from these data is in full agreement with the results described before (Debets et al. 1990). The data show that mitotic mapping based on *amd*S transformants meets the prerequisite for efficient chromosome mapping in providing a selectable marker (Amd^-, FA^r) in *cis* to all recessive markers in the test strains. The different *amd*S transformants described in this paper provide such a marker on various chromosomes, mostly centromere-distal to all known markers (our unpublished results), thus extending the genetic map. The application of *amd*S inserts for mitotic mapping of markers on *A. niger* chromosomes may be of great value especially when no other suitable selectable markers are available on a particular chromosome arm.

Selection for homozygous FA resistant recombinants can also be used to quantify mitotic recombination. This is shown for diploid N761//AT1 (Table 6). The frequency of chlorate resistant (1.3×10^{-2}) and of FA resistant diploid recombinants (2.8×10^{-3}) determined from ten different conidial suspensions indicate that the *amd*S insert is closer to the centromere than is *cnx*A1. This is in good agreement with the relative map distances found (Table 4).

Recombination between homologous chromosomes is apparently the main mechanism for loss of the Amd^+ phenotype of AT1 in such hemizygous diploids, as all FA resistant diploid segregants were found to be cross-overs. Thus, the recombinant frequency reflects the distance from the integrate to the centromere. Reversion events like those found in haploid transformants (e.g. revertant frequency of 1.2×10^{-4} for AT1) probably also occur in diploids, but there is no indication that such events are more frequent in diploid than in haploid cultures (compare revertant and recombinant frequencies in Tables 3 and 6, respectively).

The results of the analysis of diploid N761//AT1 by haploidization (Table 5) are consistent with the map derived from mitotic recombination (Table 4), but do not reveal the exact position of the *amd*S insert: the recombinant frequencies for *pyr*B4, *pab*A1 and *amd*S might suggest that the *amd*S insert is between *pyr*B4 and *pab*A1, whereas recombinant frequencies between *cnx*A1, *pab*A1 and *amd*S suggest a position of the *amd*S insert between *cnx*A1 and *pab*A1. In general, recombinant frequencies found in haploidization experiments are not suitable for mapping (Debets et al. 1989).

In conclusion, the *amd*S inserts are shown to be very useful for genetic mapping in *A. niger*. The method may be applicable to other filamentous fungi which are Amd^- and can be transformed to the Amd^+ phenotype (e.g. *Penicillium chrysogenum*, Beri and Turner 1987; Kolar et al. 1988; *Trichoderma reesei*, Pentillä et al. 1987; for review see Goosen et al. 1990). The *amd*S transformants can also be used in electrophoretic karyotyping of *A. niger*. Experiments are in progress in which the cloned *amd*S gene is used as a molecular probe for seven of the eight chromosomes described so far.

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CHAPTER 5

Genetic maps of eight linkage groups of *Aspergillus niger* based on mitotic mapping

(To be published)

Summary

In this paper a genetic map of *Aspergillus niger* is presented. The order of 60 loci in eight linkage groups relative to the centromere is based on mitotic crossing-over. In this study various methods for selection and analysis of homozygous recombinants were applied, using colour, auxotrophic and resistance markers. In addition, transformants carrying the heterologous *Aspergillus nidulans* gene coding for acetamidase (*amd*S) were used for mitotic mapping of markers in several linkage groups. The most likely chromosomal position of the *amd*S insert of nine transformants was determined and in most of the transformants the *amd*S insert appeared to be centromere distal to all known genetic markers, thus extending the genetic map. These *amd*S transformants could be used efficiently in mitotic mapping by using the *amd*S insert as counter selectable marker for the isolation of mitotic crossing-over recombinants. Genetic markers were found on both arms of the chromosomes, except for chromosomes II and IV, which may be acrocentric.

Key words: *Aspergillus niger* - genetic maps - mitotic mapping - AmdS transformants

Introduction

The industrially important asexual fungus *Aspergillus niger* has been subjected to somatic hybridization, transformation and molecular studies. Genetic analysis is indispensable in these studies for the analysis of recombinants and transformants and for strain construction. A genetic map is very helpful in devising strategies for such experiments and can greatly contribute to understanding the genetics of the fungus. For strain improvement of *A. niger* production strains, so far mainly subsequent mutagenesis and selection cycles are used, due to lack of fundamental genetic knowledge of the fungus.

Genetic analysis in *A. niger*, as in other asexual fungi, is based on genetic recombination in somatic cells. This so called parasexual cycle consists of heterokaryon formation between haploid strains, nuclear fusion and mitotic recombination by crossing-over and non-disjunction (Pontecorvo *et al.* 1953). The frequency of each of these events is low. However, heterozygous diploids can be selected and subcultured and haploidization can be induced. Haploid recombinants can be used to establish linkage groups. Previously, six linkage groups were described in *A. niger* (Lhoas 1967), but the strains were no longer accessible and this work could not be extended. Starting with another *A. niger* wild type (ATCC 9029) we found at first also six linkage groups and we constructed master strains with markers for these linkage groups (Bos *et al.* 1988). If special care is taken to avoid segregation of crossovers prior to haploidization, markers can be assigned explicitly to a linkage group. Recently we obtained evidence for the existence of two additional linkage groups (Bos *et al.* 1989, Debets *et al.* 1990a).

Mitotic crossing-over can be used to elucidate the linear order of markers on a chromosome. For efficient mitotic mapping, a distal selectable marker is required to isolate the homozygous recombinants from originally heterozygous diploids. The analysis of such segregants is strongly facilitated if recessive markers are in cis-position to the selectable marker on the chromosome (Käfer 1977). Recombinant strains carrying several linked recessive markers as well as the selectable marker must be constructed by recombination. Some methods for the selection of homozygous

recombinants were applied and the gene order of some markers in linkage groups V (Debets *et al.* 1989) and VI (Debets *et al.* 1990a) were determined based on the analysis of recombinants respectively selected as auxotrophic segregants, using Novozym enrichment, and as recombinants resistant to chlorate or 5-fluoroorotic acid. Recently, we showed the use of an *amd*S transformant in mitotic mapping of *A. niger* (Debets *et al.* 1990b). A major advantage of the method is that recessive markers in mutant strains *a priori* are cis to the selectable marker (fluoroacetamide resistance), thus facilitating the analysis.

In this paper we present a first tentative genetic map of eight linkage groups of *A. niger* based on mitotic crossing-over. In addition to standing methods for selection and analysis of homozygous recombinants, the general applicability in mitotic mapping of transformants carrying the heterologous *amd*S gene is studied and the position of the insert in nine transformants was determined.

Materials and methods

Strains.

The *A. niger* strains used in this study are listed in Table 1 and descend from wild-type strain N400 (CBS120-49 = ATCC9029).

Media and growth conditions.

The media (complete, minimal and supplemented minimal medium; CM, MM and SM respectively) and the growth conditions have been described before (Bos *et al.* 1988, Debets *et al.* 1989). Chlorate resistance was tested on complete medium containing 150 mM $KClO_3$ and 10 mM urea (CMC). Segregants resistant to 5-fluoroorotic acid (FOA) were isolated on medium containing 0.8 mg FOA/ml and 10 mM uridine. The fungicides acriflavine, carbendazim and fenarimol were added to CM to a final concentration of 50 µg/ml, 1 µg/ml and 0.8 mg/ml respectively. Benomyl was added to CM to a final concentration of 0.25 µg/ml. Fluoroacetamide (FA) resistant segregants were isolated on SM + FA (2 mg/ml) + 5 mM urea and 100 mM acetate as carbon source (Debets *et al.* 1990b).

Table 1. Genotype of A.niger strains used.

Strains	Linkage groups							
	I	II	III	IV	V	VI	VII	VIII
N423					nicA1			
N428				lysC5				
N431		hisA1						
N436	fwnA1		lysA7					
N440		leuC5						
N441	metC3							
N442	fwnA1			leuD6				
N446		hisE5						
N457	fwnA2			leuB3				
N464	fwnA1			leuD6		argE5		
N478	olyA1			adeA1				
N479	olyA1					argG11		
N491		argH12			nicA1			
N495			adeB2		nicA1			
N499		adeC3						
N502	olyA1		bioB2					
N512	fwnA1					lysD18		
N518	fwnA1			adeD5				
N523	fwnA1 metD12							
N531	fwnA3	pabB2 trpA1						
N567	fwnA1				metB2 pheA1 argA1			
N571						lysB24		
N639	brnA2				metB2			
N656	fwnA1		lysA7 bioA1		metB2		oliC2	
N665	acrA1				nicA1			
N676		hisD4	bioA1 pyrA5		nicA1			
N680	fwnA1		proC3 lysA7 bioA1	leuA1				
N687	fwnA1		proC3 lysA7 cysA2					
N694	olyA1		bioB2					niaD2
N696	olyA1		bioB2					nirA2
N699	olyA1		bioB2	cnxB3				
N701	olyA1		bioB2				cnxC5	
N702			cnxD6		metB2			
N704	olyA1		bioB2			cnxE8		
N721			lysA7 cnxD6		metB10	pabA1		
N722	fwnA1	hisD4	bioA1 lysA7	leuA1		pabA1	cnxC5	
N724	acrA1 brnA2				nicA1			
N725	cnxG13 fwnA1 metD12					pabA1		
N726	cnxG13		bioA1 lysA7		nicA1	pabA1		
N728					metB10			crbA1
N729				vcoA1	metB10			
N731					metB10		mtfA1	
N732					metB10		fnrA1	
N738	fwnA1	hisD4	bioA1 lysA7	leuA1	metB1	pdxA1		
N740			bioA1 lysA7			proA4 pabA1	cnxC5	
N742					metB10		bitA1	
N743			proC3 pyrA5 bioA1					
N745			argL2 pyrA5 lysA7					
N749			thiA1		metB10			
N753				argF8	nicA1			
N759			bioA1 proB5		metB10	pabA1		
N763	olyA1			lysC5 cnxB3				
N764	olyA1			argF8 cnxB3				
N768	olyA1		bioB2	leuD8				
N796		hisD4		leuD8	metB10	pabA1	cnxC5 nicB5	
N819	cnxF12				nicA1			
AT1					nicA1	amdS+		
AT2,3,4,17					nicA1 amdS+			
AT8,16,18		amdS+			nicA1			
AT6				amdS+	nicA1			
AT19			amdS+		nicA1			
recAT19			amdS+ cnxD6		metB2			

Genetic techniques.

Selection of heterozygous diploids. Diploids were isolated from heterokaryon cultures as described before (Bos *et al.* 1988).

Selection and analysis of haploid segregants. Haploidization analysis was performed by plating conidiospores of a heterozygous diploid on CM containing benomyl as described before (Bos *et al.* 1988, Debets *et al.* 1990a).

Selection of diploid mitotic segregants. The following techniques were used: **A.** Colour segregants were isolated from diploids originally heterozygous for a recessive colour marker. Single conidial heads of the primary diploid were inoculated on CM and incubated for 4-5 days at 30 °C. From each colony if possible, one colour segregant was isolated, purified and tested to establish the genotype. **B.** Auxotrophic segregants were isolated using Novozym enrichment. Samples of 10^4 and 10^5 conidiospores derived from different diploid cultures heterozygous for a recessive deficiency marker were plated on MM in small Petri dishes (6 cm diameter) and incubated at 30 °C for 10 h. Germinating conidia were subsequently subjected to Novozym enrichment as described earlier (Debets *et al.* 1989). Non-germinating conidia that survived the enzyme treatments, were rescued by adding a 2 ml toplayer of a five times concentrated CM containing 0.8% ($^{w}/_{v}$) agar. The colonies that appeared after 2-3 days were tested for auxotrophy. **C.** Drug resistant segregants. From diploids heterozygous for a chlorate resistance mutation, single conidial heads were inoculated on CMC. Well growing chlorate resistant (Chl^r) segregants that appeared after 2-4 days were purified and tested (Debets *et al.* 1990a). Similarly the recessive mutations conferring resistance to FOA (*pyr*B4 and *pyr*A6), carbendazim (*crb*A1) and fenarimol (*fnr*A1) were used to isolate mitotic recombinants. FA-resistant (FA^r) diploid segregants were isolated from diploids hemizygous for the heterologous *amd*S sequence (i.e. the plasmid insert is present only on one of the two homologous chromosomes in the diploid). When the recessive markers were in repulsion to the selected marker, recombinant diploids were haploidized to determine the genotype. The distinction between haploid and diploid mitotic recombinants could be made on the basis of closely linked markers in repulsion in the diploid (e.g. *olv*A1 and *fwn*A1 in linkage group (LG) I, or *met*B2 and *nic*A1 in LG V), or by measuring spore size with a Coulter counter.

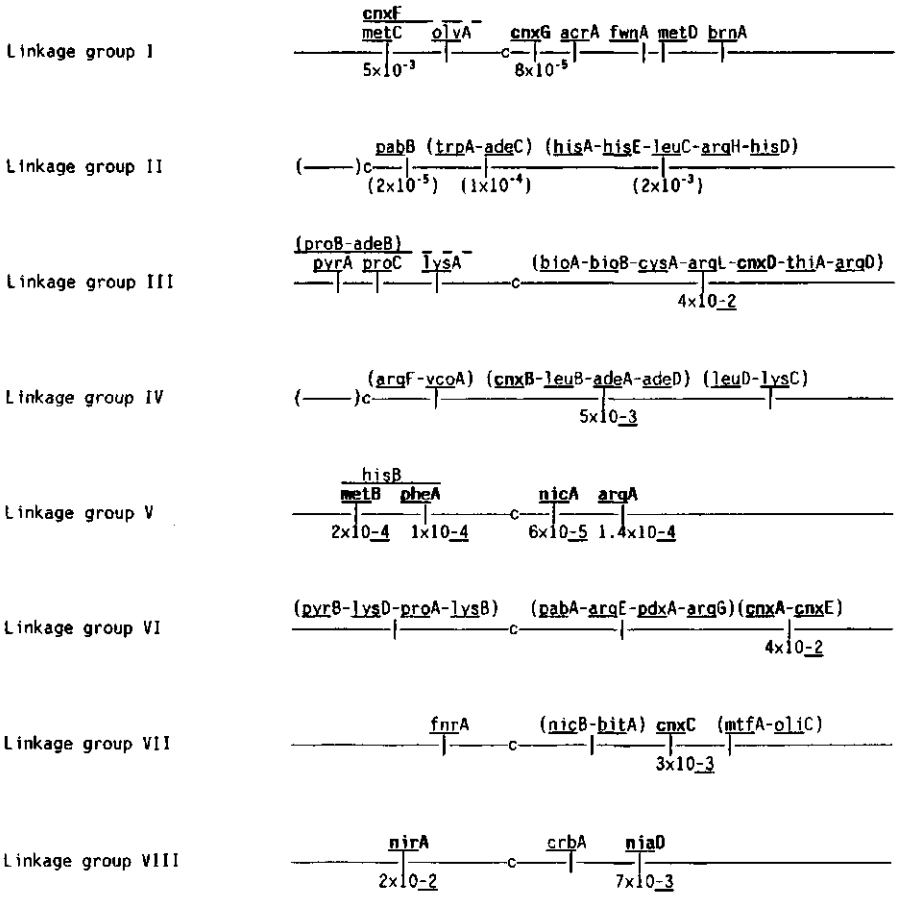
Induction of mitotic recombination. In some cases the frequency of

mitotic recombination was increased by UV irradiation. Heterozygous diploid conidia were germinated on CM plates for 16 h at 30 °C, irradiated with UV to a final dose of 10 J/m^2 and were further incubated.

Results

Haploidization analyses showed clear linkage of the markers in the different linkage groups of *A. niger* (cf Bos *et al.* 1989, Debets *et al.* 1990a). In many experiments, only occasionally recombinants were found that contained linked markers from both original strains. For closely linked markers such recombinants were not found. The efficiency of mitotic mapping depends, however, highly on the possibility to construct heterozygous diploids that carry a distal selectable marker in cis-position to other recessive markers. Only then sufficient independent homozygous recombinants can be isolated and analyzed. Therefore, haploid strains with several genetic markers in the same linkage group were constructed by successive rounds of isolation and haploidization of homozygous diploid segregants. In the course of these experiments we obtained also information about the tentative map order. This was useful for planning the experiments in which the linear order was confirmed. The mitotic recombinants in this study were isolated in different ways. Colour segregants were isolated from diploids originally heterozygous for either of the recessive colour markers *olv*A1, *fwn*A1 and *brn*A2 that were all located on chromosome I. The Novozym enrichment, based on the selective killing of prototrophic germinating conidia, has been used to isolate auxotrophic diploids, homozygous for a recessive deficiency marker. Such markers are available on most of the chromosome arms. Chlorate resistant (Chl^r) segregants were isolated from diploids that were heterozygous for either of the recessive chlorate resistance mutations (*cnx*A-G, *nia*D and *nir*A) located on six different chromosomes. The other recessive drug-resistance mutations (*pyr*B4, *pyr*A6, *fnr*A1 and *crb*A1, located on different chromosomes) were used in similar experiments. Finally, FA resistant (FA^r Amd$^-$) segregants were isolated from diploids hemizygous for the dominant heterologous *amd*S gene (from *A. nidulans*). The *amd*S inserts were introduced in haploid strains by transformation and were shown to be located at different loci in various transformants (Debets *et al.* 1990b). The transformants analyzed provided

Table 2. Genetic map of A.niger



Note: The map is not drawn to scale. Markers of which the gene order is unknown are shown in parentheses and/or above each other. The map distances indicate absolute recombinant frequencies as estimated before (Debets et al. 1989, 1990a) and represent the distances of the markers in bold type to the centromere. For LG II tentative map distances are shown in parentheses. These distances are based on the relative frequency of recombinants isolated using transformants AT8 and AT16. For further details see text.
c = centromere

the counter selectable *amd*S marker in either of six chromosomes. A tentative genetic map of *A. niger* is shown in Table 2. Details and evidence are presented in Tables 3 - 14 and are described below. Previously published data are included in the Table.

Linkage group I. The linear order of seven markers in LG I has been determined. Except the dominant *acr*A1 mutation all markers were recessive and have been used to select mitotic recombinants. Most diploid mitotic

segregant types were rather scarce and predominantly haploid segregants were isolated, indicating low recombination frequencies for these markers. Therefore, mitotic recombination has been induced in some experiments by UV irradiation of the heterozygous diploid. For strain construction we have used only spontaneous diploid recombinants.

Table 3. Mitotic mapping in LG I.

Diploid[1]: N724 + acrA1 + + brnA2
N725 cnxG13 + fwnA1 metD12 +

Segregants							Interpretation
Genotype[1]					Selected phenotype		
					Brown		
+	acrA1	+	+	brnA2			
+	acrA1	+	+	brnA2	2		
+	acrA1	+	+	brnA2			
cnxG13	acrA1	+	+	brnA2	3		
+	acrA1	+	+	brnA2			
cnxG13	+	+	+	brnA2	3		
+	acrA1	+	+	brnA2			cnxG acrA fwnA metD brnA
cnxG13	+	fwnA1	+	brnA2	4		—c— 2 3 3 4 9
+	acrA1	+	+	brnA2			
cnxG13	+	fwnA1	metD12	brnA2	9		
					Met⁻	Fawn	
cnxG13	+	fwnA1	metD12	(+)			
cnxG13	+	fwnA1	metD12	(+)	2	2	
+	+	fwnA1	metD12	(+)			
cnxG13	+	fwnA1	metD12	(+)	8	4	
+	acrA1	fwnA1	metD12	(+)			cnxG acrA fwnA metD brnA
cnxG13	+	fwnA1	metD12	(+)	4	2	—c— 2 8 4 2
+	acrA1	+	metD12	(+)			2 4 2
cnxG13	+	fwnA1	metD12	(+)	2		

[1] Only the relevant genotype of the diploid strain and segregants is shown. For complete genotypes of strains N724 and N725 see Table 1.
Note: Recombinant strains N724 and N725 carrying several linked markers were isolated by haploidization of homozygous diploid colour segregants derived from diploids N665//N639 and N523//N726 respectively. The diploid constructed from these strains was subsequently used in mitotic mapping. Recombinants were isolated as brown or fawn coloured or as methionine (Met⁻) requiring segregants as described in Materials and methods. The number of independent mitotic segregants is shown. The genotype of the brown recombinants was concluded upon haploidization; the fwnA1 mutation appeared to be epistatic to brnA2. The genotype of segregants isolated as methionine deficient or as fawn coloured recombinants could be concluded directly from their phenotype on the various test media. The likely (but not further analyzed) genotype for the brnA locus is shown in parentheses. The concluded gene order and relative frequency of crossing-over between the genes is given.

In Table 3 the establishment of the gene order on the right arm of LG I is shown. In order to obtain a suitable diploid, haploid strains N724 carrying the markers *acr*A1 and *brn*A2 in LG I and strain N725 carrying *cnx*G13, *fwn*A1 and *met*D12 in this linkage group were constructed by somatic recombination. From diploid N724//N725 brown and fawn coloured segregants

as well as Met⁻ segregants were isolated. The independent diploid segregants were used to determine the gene order. For most markers the genotype could be concluded from the phenotype. The *fwn*A1 mutation is epistatic to *brn*A2, and therefore, the *fwn*A genotype of the brown diploid segregants could only be concluded after haploidization.

As diploid recombinants are homozygous for all markers distal to the point of crossing-over, the concluded gene order on the left arm of chromosome I is (*met*C-*cnx*F) - *olv*A - centromere. From several experiments we concluded that *met*C and *olv*A are on the other side of the centromere than are *cnx*G, *acr*A, *fwn*A, *met*D and *brn*A. Some of these experiments are

Table 4. Mitotic mapping in LG I.

Diploid[1]	Segregants		Interpretation
	Genotype	Selected phenotype	
		Fawn Met⁻	
a. N441 metC3 N436 fwnA1	metC3 fwnA1 + fwnA1	6	metC3 fwnA1
	metC3 + metC3 fwnA1	5	
		Olive[2] Chl^{r} [2]	
b. N478 olvA1 N726 cnxG13	olvA1 cnxG13 olvA1 +	5	
	olvA1 cnxG13 + cnxG13	5	olvA1 cnxG13
	+ cnxG13 + cnxG13	6	non-disjunction or d.c.o.
		Met⁻ [2] Olive[2]	
c. N478 olvA1 N441 metC3	metC3 olvA1 metC3 +	3	
	metC3 + metC3 +	8	metC3 olvA1 3 8
	+ olvA1 + olvA1	2	

[1] Only the relevant genotypes of the diploid strains and segregants are shown. For complete genotypes of strains see Table 1.
[2] Recombinants isolated after UV-induction (see Materials and methods).

Note: Recombinants were isolated as fawn (Fawn) and olive-green (Olive) coloured, chlorate-resistant (Chl^{r}) or methionine deficient (Met⁻) segregants as described in Materials and methods. See also note of Table 3.

shown in Table 4. The analyses with the first two diploids (Table 4a,b) lead to the conclusion that *met*C and *olv*A are on the left arm and the third diploid (Table 4c) shows that *met*C is centromere distal to *olv*A. In the

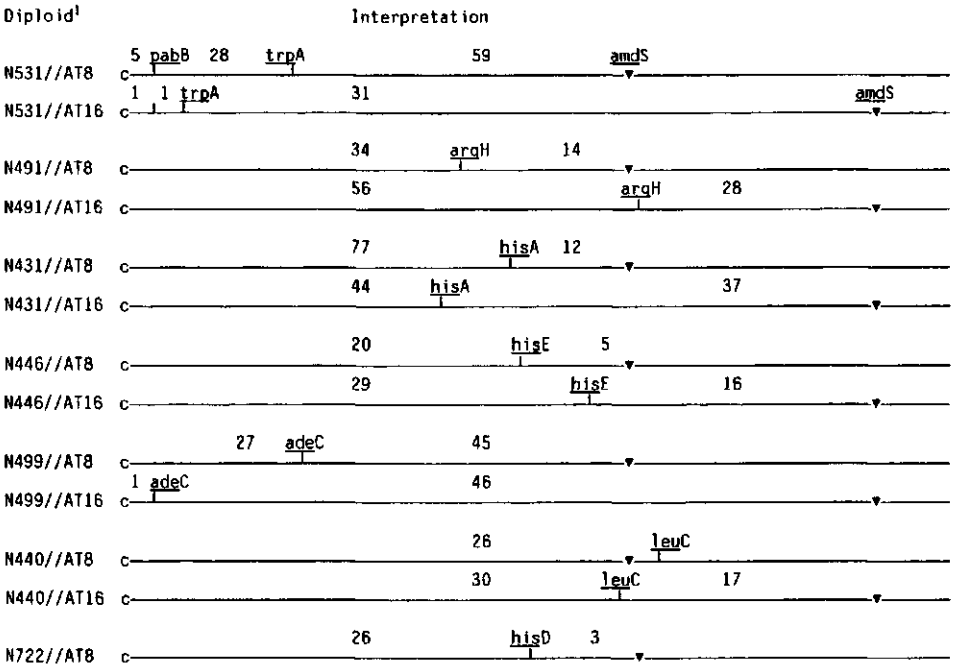
experiment with diploid N478//N726 (Table 4c) about half of the Chl^r diploid segregants probably resulted from non-disjunction. This is in agreement with the low frequency of crossing-over found earlier for this marker (Debets *et al.* 1990a). From another diploid (N819//N738, data not shown) carrying *cnx*F12 and *fwn*A1 in repulsion, Chl^r segregants were isolated that were all heterozygous for *fwn*A1 and *vice versa*, indicating that *cnx*F is to the left of the centromere.

Linkage group II. The genetic markers in LG II were all recessive auxotrophic mutations, and no selectable marker was available. Tight linkage of these markers was observed in haploidization experiments, and again no recombinant haploids were obtained in this way (data not shown). Only strain N531 carried two such markers (*pab*B2 *trp*A1), because this strain was isolated from a Trp$^-$ strain after mutagenesis and enrichment. However, two suitable transformants with the *amd*S insert in LG II were available. Diploids were constructed from these transformants (AT8 and AT16), and various mutant strains carrying an auxotrophic marker in this linkage group. These diploids were hemizygous for the *amd*S insert, and segregants were isolated on the basis of FA-resistance. The frequency of the diploid FA^r recombinants for AT8 and AT16 was 1.7×10^{-4} and 1.6×10^{-3} respectively. This is about ten times higher than the frequency of revertants from each of the haploid transformants (see Table 14). Transformant AT18 was also studied in this way, but in this case the revertant frequency is almost as high as the recombinant frequency in diploid culture (2.6×10^{-3} and 4.0×10^{-3} respectively, Table 14). So the exact position of this insert in LG II could not be concluded, but crossing-over recombinants could be isolated from the diploid with this transformant strain also.

For all eight markers in this linkage group, loss of the Amd$^+$ phenotype of AT8 or AT16 was associated with homozygosity of the auxotrophic mutation. The relative frequency of such FA^r auxotrophic recombinants among the total number of FA^r diploids was different for the various markers depending on the distance of each of the auxotrophic markers to the centromere (Table 5). So, the tentative gene order in LG II is: centromere - *pab*B - (*ade*C - *trp*A) - (*leu*C - *arg*H - *his*A - *his*D - *his*E) (see Table 2). This is based on the different relative recombinant frequencies, assuming that FA^r segregants result from crossing-over of homologous chromosomes between the centromere and the *amd*S insert (see

Table 5. Mitotic mapping in LG II using the amdS$^+$ inserts of transformants AT8 and AT16.

Diploid[1]		FA^r Segregants		
		Phenotype	AT8	AT16
a. N531 AT8,16	pabB2 trpA1 * / + + amdS$^+$	Pab$^+$ Trp$^+$	59	31
		Pab$^+$ Trp$^-$	28	1
		Pab$^-$ Trp$^-$	5	1



Note: From many diploids, like N531//AT8 and N531//AT16, fluoro-acetamide resistant (FA^r) segregants were isolated and cosegregation of the other markers was tested. Heterozygosity of an auxotrophic marker (i.e. prototrophy) is interpreted as a crossing-over between that marker and the amdS$^+$ insert. Auxotrophic recombinants isolated as FA-resistant segregants, are interpreted as resulting from crossovers centromere proximal to the auxotrophic marker. The number of independent auxotrophic and prototrophic recombinants for each of the markers is shown. For further explanation see text.
c = tentative position of the centromere at the leftside of the chromosome.
▼ = The position of the amdS sequences in AT8 and AT16. As the frequency of FA^r recombinants for AT16 is higher than for AT8, the position of the heterologous amdS insert of AT16 is drawn distal to that of AT8.
* = No amdS insert present.
[1] Only the relevant genotype of the diploids is given, for complete genotypes see Table 1.

Discussion). Tentative mapping distances can be calculated from these data in combination with the absolute recombinant frequencies (see Table 14) for the *amd*S insert of AT8 (for *pab*B and (*trp*A-*ade*C)) or AT16 (for *arg*H-*his*A-

*his*D-*his*E-*leu*C) (Table 14). For these estimates it is assumed that reversion in haploid and diploid culture is the same, and that the recombinant frequencies of AT8 and AT16, corrected for the reversion frequencies, represent crossing-over frequencies between the *amd*S inserts and the centromere. It is also assumed that the FA^r recombinants are equally frequent as the reciprocal recombinants. Thus, the tentative mitotic map distances of the *amd*S insert of AT8 and AT16 are 3.1×10^{-4} and 2.8×10^{-3} respectively.

Linkage group III. Rather high recombinant frequencies were found for the markers in LG III in several haploidization experiments (up to 10% for e.g. *bio*A1 and *lys*A7 in several experiments), and also some recombinant haploid strains with two auxotrophic markers could be obtained in this way. Several markers suitable for positive selection were available in this linkage group. Recombinants were isolated on the basis of resistance to FOA (using *pyr*A5), chlorate (using *cnx*D6) or FA (using *amd*S transformant AT19). Furthermore, auxotrophic diploid segregants were isolated using Novozym enrichment.

In the analysis of diploid N676//N687 (Table 6a) the frequency of crossing-over for the markers in this linkage group was rather high and double crossovers were observed relatively frequent. One recombinant must even have resulted from multiple crossing-over. The other recombinants isolated from this diploid lead to the concluded gene order: *pyr*A - *pro*C - *lys*A - centromere - (*bio*A - *cys*A). The position of *lys*A7 relative to *pro*C3 could also be concluded from an experiment with diploid N687//N502 (Table 6b). From these data we also conclude that the *bio*B gene is located on the right arm close to the *cys*A locus. Likewise it was inferred that the *cnx*D gene is also on the right arm (Table 6c).

The position of some genetic markers relative to the $amdS^+$ insert in AT19 was determined in several experiments. The frequency of FA^r diploid segregants isolated from a diploid hemizygous for AT19 is about 100 times higher than the frequency of haploid revertants of transformant strain AT19 (Table 14). Again a rather high incidence of double crossing-over was observed, which complicated the analysis. Nonetheless, a genetic map could be drawn on the basis of the results of the analyses of six diploids as shown in Table 7. In a diploid heterozygous for *cnx*D6 and hemizygous for *amd*S (Table 7a.) two different selection procedures could be performed. Of 40 chl^r diploid segregants analyzed 39 were still hemizygous for the *amd*S

Table 6. Mitotic mapping in LG III.

a. Diploid[1]: N676 pyrA5 + + bioA1 +
N687 + proC3 lysA7 + cysA2

Segregants: Genotype	Selected phenotype: FOA^r	Auxotroph	Interpretation
pyrA5 + + + cysA2 + proC3 lysA7 + cysA2		9	
pyrA5 + + bioA1 + + proC3 lysA7 bioA1 +		11	
+ proC3 + bioA1 + + proC3 lysA7 + cysA2		3	
+ proC3 + bioA1 + + proC3 lysA7 bioA1 +		1	d.c.o.
pyrA5 + + bioA1 + pyrA5 + + + cysA2	4		
pyrA5 + + bioA1 + pyrA5 + lysA7 + cysA2	2		pyrA proC lysA (bioA cysA)
pyrA5 + + bioA1 + pyrA5 proC3 lysA7 + cysA2	1		
pyrA5 + + bioA1 + pyrA5 proC3 + + cysA2	1		multiple exchanges
pyrA5 + + bioA1 + pyrA5 + lysA7 bioA1 +	1		d.c.o.

b. Diploid[1]: N687 proC3 lysA7 cysA2 +
N502 + + + bioB2

Segregants: Genotype	Selected phenotype: Auxotroph	Interpretation
proC3 lysA7 + cysA2 proC3 + bioB2 +	1	
proC3 lysA7 + cysA2 + + + cysA2	6	proC lysA (bioB cysA)
proC3 lysA7 bioB2 + + + bioB2 +	1	

c. Diploid[1]: N680 proC3 bioA1 +
N721 + + cnxD6

Segregants: Genotype	Selected phenotype: Chl^r	Auxotroph	Interpretation
proC3 bioA1 + + bioA1 +		4	proC (bioA cnxD)
proC3 + cnxD6 + + cnxD6	5		

Note: Recombinants were selected on the basis of resistance to 5-fluoroorotic acid (FOA^r), chlorate (Chl^r) or as auxotrophs by Novozym selection (as described in the Materials and methods section). The number of independent segregants is indicated. The genotypes of segregants were determined upon haploidization (see also note of Table 3) and the most likely explanation for each of the recombinants is given.

[1] For complete genotypes of diploid strains see Table 1.

Table 7. Mitotic mapping in LG III using the amdS$^+$ insert of AT19.

Diploid[1]	Segregants			Interpretation
	Genotype	Selected phenotype		
		FA^r	Chl^r	
a. N702 cnxD6 AT19 amdS$^+$	* cnxD6 * +		4	
	* cnxD6 * cnxD6	1	1	amdS — cnxD
	* cnxD6 amdS$^+$ cnxD6		39	
b. N743 pyrA5 proC3 bioA1 AT19 amdS$^+$	* pyrA5 proC3 bioA1 * + + +	33		
	* pyrA5 proC3 bioA1 * pyrA5 + +	6		amdS pyrA proC bioA 33 6 1
	* pyrA5 proC3 bioA1 * pyrA5 proC3 +	1		
c. N745 pyrA5 lysA7 argL2 AT19 amdS$^+$	* pyrA5 lysA7 argL2 * + + +	9		
	* pyrA5 lysA7 argL2 * pyrA5 + +	5		amdS pyrA lysA argL 9 5 1
	* pyrA5 lysA7 argL2 * pyrA5 lysA7 +	1		
d. N759 proB5 bioA1 AT19 amdS$^+$	* proB5 bioA1 * + +	34		
	* proB5 bioA1 * proB5 +	6		amdS proB bioA 34 6
	* proB5 bioA1 * proB5 bioA1	1		d.c.o.
e. N749 thiA1 recAT19 amdS$^+$ cnxD6	* thiA1 + * + cnxD6	45		amdS (thiA-cnxD)
	* thiA1 + * thiA1 +	1		d.c.o.
f. N495 adeB2 recAT19 amdS$^+$	* adeB2 + * + cnxD6	11		
	* adeB2 + * adeB2 cnxD6	2		amdS adeB cnxD 11 2

Note: Segregants resistant to fluoro-acetamide (FA^r) or chlorate (Chl^r) were isolated and analyzed as described in Materials and methods. * = No amdS gene present. See also notes of Tables 3 and 5 for general remarks.
1 For complete genotypes of diploid strains see Table 1.

insert. One segregant has probably arisen by double crossing-over. Four out of five FA^r segregants were still heterozygous for *cnx*D6. So the centromere is between the insert and *cnx*D. From the analysis of diploids N743//AT19 and N745//AT19 (Tables 7b,c) we conclude that *pyr*A and *pro*C are located between the *amd*S insert and the centromere on the left arm. We also

conclude that *arg*L is to the right of the centromere which is in agreement with the results of other experiments (personal data). From recombinants of diploid N759//AT19 (Table 7d) we conclude that *pro*B5 is on the left arm. The FA^{r}Pro$^-$Bio$^+$ and the six FA^{r}Pro$^-$Bio$^-$ recombinants might also be explained by double crossing-overs with a position of *pro*B5 distal to *bio*A1 on the right arm. However, in a haploidization experiment we found four double auxotrophic recombinants carrying *bio*A1 (on the right arm) and *pro*B5 whereas in the same experiment no such double auxotrophic recombinants were found for *lys*A7 (on the left arm) and *pro*B5. So, we assume single crossovers.

Also in other experiments it was sometimes not clear whether recombinants resulted from single or double crossing-over as the frequency of the latter for markers in this linkage group could be rather high. From diploid N749//AT19 one Thi$^-$Amd$^-$ recombinant and 24 Thi$^+$Amd$^-$ segregants were found, and from diploid N495//AT19 one Ade$^-$Amd$^-$ and 16 Ade$^+$Amd$^-$ recombinants. If the rare recombinants resulted from a single crossing-over, *thi*A and *ade*B would be near to the centromere on the same chromosome arm as the *amd*S insert. If the markers are on the other chromosome arm these recombinants could only have arisen by double crossing-over. Because the position of the auxotrophic marker could not be concluded from these data, diploids were constructed with a recombinant of AT19 (isolated from the diploid in Table 7a) carrying the additional marker *cnx*D6 on the right arm (Table 7e,f). As the *amd*S insert and the *cnx*D are on different chromosome arms, analysis of these diploids led us to conclude that *ade*B is centromere proximal to the *amd*S insert of AT19 and that *thi*A is at the other side of the centromere. The single *thi*A1//*thi*A1 recombinant (Table 7e) thus most likely resulted from double crossing-over.

Linkage group IV. In general low frequencies of recombinants were found in haploidization analyses for the markers in LG IV. In the first series of experiments (Table 8) mitotic recombinants were isolated on the basis of resistance to chlorate (using *cnx*B3), and by Novozym enrichment (using *lys*C5 and *leu*D6). Only few diploid recombinants were isolated in these experiments as either selection or analysis of the recombinant diploids was time consuming (markers in repulsion in the diploids). No Arg$^-$ recombinants could be isolated from diploid N699//N753 despite recurrent Novozym enrichment experiments in which haploid auxotrophs were isolated. So *arg*F is probably close to the centromere. Recombinant strains from these

Table 8. Mitotic mapping in LG IV.

Diploid[1]	Segregants		Interpretation
	Genotype	Selected phenotype	
		Chl^r Auxotroph	
a. N699 cnxB3 N729 vcoA1	+ cnxB3 vcoA1 cnxB3	2	
	+ cnxB3 + cnxB3	2	(——)c vcoA cnxB 2 2
b. N699 cnxB3 N442 leuD6	cnxB3 + cnxB3 +	5	
	cnxB3 leuD6 + leuD6	3	(——)c cnxB leuD 3 2
	+ leuD6 + leuD6	2	
c. N699 cnxB3 N428 lysC5	cnxB3 + cnxB3 +	3	
	cnxB3 lysC5 + lysC5	3[a]	(——)c cnxB lysC 3 3
	+ lysC5 + lysC5	3	
d. N699 cnxB3 N753 argF8	cnxB3 + cnxB3 argF8	4[b]	(——)c argF cnxB or argF cnxB ——c——

Note: Segregants were isolated as chlorate resistant (Chl^r, cnxB3//cnxB3) or as auxotrophs (lysC5//lysC5 or leuD6//leuD6; no Arg$^-$ recombinants were found). For general remarks see note of Table 3 and text.
[a], [b] Recombinant strains N763 (cnxB3 lysC5) and N764 (cnxB3 argF8) were isolated after haploidization.
[1] For complete genotypes of diploid strains see Table 1.

experiments were used in mitotic mapping experiments with transformant AT6, which carries an *amd*S insert on chromosome IV. The frequency of FA^r segregants could not be determined as it was very difficult to quantify this due to the leaky growth of the hemizygous diploid. Nonetheless, the position of several markers relative to the insert could be concluded. The results of the mapping experiments with AT6 are shown in Table 9. In conclusion the gene order in LG IV is: centromere - (*arg*F - *vco*A) - (*leu*B - *cnx*B -*ade*A - *ade*D) - (*leu*D - *lys*C). *arg*F and *vco*A are centromere proximal to *cnx*B as can be concluded from analysis of diploids N699//N729 and N764//AT6 (Tables 8a and 9a respectively). The results indicate that all markers are on the same chromosome arm. The position of *leu*D and *lys*C centromere distal to *cnx*B is determined from the data in Table 8 b,c. On the basis of the relative recombinant frequencies of FA^r segregants (Table 9), we assume that *leu*B, *ade*A and *ade*D are between (*arg*F - *vco*A) and (*leu*D - *lys*C), and that the *amd*S insert of AT6 is distal to all these

Table 9. Mitotic mapping in linkage group IV using amdS$^+$ transformant AT6.

Diploid[1]	Segregants			Interpretation
	Genotype	Selected phenotype		
		Chl^r	FA^r	
a. N764 argF8 cnxB3 AT6 amdS$^+$	argF8 cnxB3 * + + *		10	
	argF8 cnxB3 * + cnxB3 *	18	9	
	argF8 cnxB3 * argF8 cnxB3 *	4	1	(———)c—argF—cnxB—amdS▼— 1 9 10
b. N763 cnxB3 lysC5 AT6 amdS$^+$	cnxB3 lysC5 * + + *		2	
	cnxB3 lysC5 * + lysC5 *		10	
	cnxB3 lysC5 * cnxB3 lysC5 *	11	15	(———)c—cnxB—lysC—amdS▼— 15 10 2

Diploid[1]	
c. N518//AT6	(———)c— 9 —adeD— 4 —amdS▼— (AT6)
d. N768//AT6	(———)c— 34 —leuD— 6 —▼—
e. N478//AT6	(———)c— 19 —adeA— 12 —▼—
f. N457//AT6	(———)c— 8 —leuB— 7 —▼—

Note: Analysis of recombinants isolated as chlorate resistant (Chl^r) or fluoro-acetamide resistant (FA^r) segregants. The position of the amdS insert (▼) relative to the other markers is concluded from the phenotype (reflecting genotype) of the various recombinants derived from different diploids. See also notes of Tables 3 and 5.
[1] For complete genotypes of the diploid strains see Table 1.

markers.

Linkage group V. Part of the analysis of LG V was published before (*met*B - *phe*A - c - *nic*A - *arg*A, Debets *et al.* 1989). These results were the basis for some experiments using the *amd*S transformants in order to confirm the gene order (Table 10a). The marker *nic*A1, although present in the diploid, was omitted in Table 10 because no conclusion about this marker could be drawn without haploidization of the diploid segregants. The results show that *met*B and *phe*A are on the other arm than *arg*A and the *amd*S insert, and that the position of the *amd*S insert in the transformants AT2, AT3, AT4 and AT17 are centromere distal to *arg*A. The frequency of FA^r recombinants for each of the transformants was different, and we assume

Table 10. Mitotic mapping in linkage group V using amdS$^+$ transformants AT2, AT3, AT4 and AT17.

Diploid[1]	FA^r segregants					Interpretation
	Phenotype	AT2	AT3	AT4	AT17	
a. N567 metB2 pheA1 argA1 AT amdS$^+$	Met$^+$Phe$^+$Arg$^-$	23	17	24	16	metB pheA — c — argA AT2,3,4,17
	Met$^+$Phe$^+$Arg$^+$	8	17	34	15	
b. N423 hisB2 AT amdS$^+$	His$^-$	0	0	0	0	hisB — c — AT2,3,4,17
	His$^+$	17	14	16	28	

Note: The most likely position of the amdS inserts of transformant strains AT2, AT3, AT4 and AT17 was determined by isolation and analysis of fluoroacetamide resistant (FA^r) recombinants (see note of Table 5). The order of the auxotrophic markers metB, pheA and argA was determined before (Debets et al. 1989). For general remarks see notes of Tables 3 and 5.
[1] Only the relevant genotype of the diploid strains is shown. For complete genotypes see Table 1.

different insertion sites (Table 14). The analysis of FA^r recombinants from the various diploids N423//AT (Table 10b) suggest a left arm position of *his*B.

Table 11. Summary of mitotic mapping in linkage group VI using the amdS$^+$ insert of transformant AT1.

Diploid[1]	Interpretation
a. N571//AT1	lysB — 29 — amdS (AT1)
b. N512//AT1	lysD — 36
c. N740//AT1	proA — 11 pabA 8
d. N738//AT1	14 pdxA 14
e. N464//AT1	15 argE 14
f. N479//AT1	11 argG 8
g. N704//AT1	8 — 32 — cnxE

Note: The position of the amdS insert of strain AT1 (▾) as concluded from analysis of recombinants isolated on the basis of resistance to fluoro-acetamide or chlorate. The number of independent recombinants as explained by a crossing-over in either of the regions is shown. If no auxotrophic recombinants were found, this marker is drawn to the left arm. For further explanation see note of Table 5 and text.
[1] For genotypes of the diploid strains see Table 1.

Linkage group VI. In haploidization experiments rather low frequencies of mitotic crossovers were observed. For *pab*A1 and *arg*E5, *arg*K11 and *cnx*A1, and for *cnx*E8 and *pdx*A1 this was less than 3%, and all recombinants were prototrophic. Rather high recombinant frequencies (up to

20%, including auxotrophic recombinants) were, however, found for *pab*A1 and either of the markers *pro*A4, *lys*B24 and *lys*D18. Such high recombinant frequencies may indicate a position on different chromosome arms. The map order *pyr*B - centromere - *pab*A - *cnx*A was already determined before (Debets et al. 1990a). The position of the other markers is determined by mitotic mapping using transformant strain AT1. The results are summarized in Table 11.

Table 12. Mitotic mapping in linkage group VII.

Diploid[1]	Segregants		Interpretation
	Genotype	Selected phenotype	
		Chl^r Fnr^r	
a. N701 cnxC5 N732 fnrA1	cnxC5 + cnxC5 fnrA1	32	
	cnxC5 fnrA1 + fnrA1	32	fnrA cnxC c
b. N701 cnxC5 N731 mtfA1	cnxC5 + cnxC5 +	91	cnxC mtfA c
c. N701 cnxC5 N742 bitA1	+ cnxC5 bitA1 cnxC5	27	
	+ cnxC5 + cnxC5	8	bitA cnxC c 8 27
d. N796 nicB5 cnxC5 N656 oliC2	nicB5 cnxC5 + + cnxC5 +	44	
	nicB5 cnxC5 + nicB5 cnxC5 +	13	nicB cnxC oliC c 13 44

Note: Segregants were isolated as recombinants resistant to chlorate (Chl^r) or fenarimol (Fnr^r). For general remarks see Table 3 and text.
[1] For complete genotypes of diploid strains see Table 1.

As can be seen from Table 11 (c,d,e,f), many of the FA^r diploids isolated from diploids heterozygous for *pab*A, *pdx*A, *arg*E or *arg*G are homozygous for either of these markers. Therefore, it was concluded that these markers are centromere proximal to the *amd*S insert. The relative order of these markers can not be concluded from these data. Again the recombinant frequency of diploid FA^r segregants in diploids exceeds the frequency in the haploid transformant AT1 (Table 14). None of the FA^r recombinants found was homozygous for *pro*A4, *lys*B24 or *lys*D18 (Table 11a,b,c). So, we conclude that the most likely map position of these markers is to the left of the centromere. A left arm position of *lys*D was confirmed by other data (not shown). All 18 FA^r segregants isolated from diploid N704//AT1 (Table 11g) were Chl^r whereas eight of 40 Chl^r segregants

isolated were FA^r, indicating that the *amd*S insert is nearer to the centromere. The most likely gene order in LG VI is: (*pyr*B - *lys*D - *lys*B - *pro*A) - centromere - (*pdx*A - *arg*E - *arg*G - *pab*A) - (*cnx*A - *cnx*E).

Table 13. Mitotic mapping in LG VIII.

Diploid[1]	Segregants			Interpretation
	Genotype	Selected phenotype		
		Chl^r	Crb^r	
a. N728 crbA1 N694 niaD2	niaD2 crbA1 niaD2 +	25		
	niaD2 crbA1 niaD2 crbA1	10	27	crbA niaD c 10 25
		Chl^r	Crb^r	
b. N728 crbA1 N696 nirA2	nirA2 crbA1 nirA2 +	38		
	nirA2 crbA1 nirA2 crbA1		14	non-disjunction
	nirA2 crbA1 + crbA1		21	nirA crbA c

Note: The number of chlorate resistant (Chl^r) and carbendazim resistant (Crb^r) segregants is shown. From these results the gene order: nirA - centromere - crbA - niaD was concluded. For general remarks see Table 3 and text.
[1] For complete genotypes of diploid strains see Table 1.

Table 14. Frequency of fluoroacetamide (FA) resistant segregants from diploid (recombinant frequency) and haploid (revertant frequency) culture carrying the heterologous amdS gene.

Linkage group	Strain[1]	Recombinant %	Revertant %
II	AT8	1.7×10^{-4}	1.3×10^{-5}
II	AT16	1.6×10^{-3}	2.1×10^{-4}
II	AT18	4.0×10^{-3}	2.6×10^{-3}
III	AT19	7.0×10^{-4}	6.4×10^{-6}
IV	AT6	N.D.	1.5×10^{-4}
V	AT2	5.0×10^{-4}	2.3×10^{-6}
V	AT3	6.0×10^{-3}	9.5×10^{-5}
V	AT4	3.6×10^{-3}	1.2×10^{-5}
V	AT17	1.0×10^{-2}	3.0×10^{-5}
VI	AT1	2.8×10^{-3}	1.8×10^{-4}

[1] Transformant strain used as haploid to estimate reversion frequency and as part of diploid to estimate the recombination frequency in diploids hemizygous for the amdS insert.
N.D. = Not done, the rather leaky growth on FA containing medium did not allow quantification.

Linkage group VII. Most markers in this linkage group are resistance mutations. The linear order of six markers in LG VII has been determined. For the selection of mitotic recombinants the recessive resistance mutations *cnx*C5 and *fnr*A1 were used. The results and interpretations of these experiments are listed in Table 12. The concluded gene order is

*fnr*A - centromere - *bit*A - *cnx*C - *mtf*A.

Linkage group VIII. In LG VIII only few markers are available. Diploid mitotic segregants were isolated on the basis of resistance to chlorate (NiaD$^-$ or NirA$^-$) or carbendazim (CrbA$^-$). A rather high frequency of non-disjunctional mitotic segregants was found among carbendazim resistant diploids (Table 13a,b). This is not surprising as carbendazim is the active compound of benomyl, an inducer of non-disjunction. From the data in Table 13, we conclude that the most likely gene order in LG VIII is: *nir*A - centromere - *crb*A - *nia*D.

Discussion

A tentative genetic map of *A. niger* with 60 genetic markers in eight linkage groups based on somatic recombination is presented. The loci have been located in the various linkage groups by mitotic mapping using different methods for selection and analysis of homozygous diploid crossing-over recombinants.

All genetic markers of *A. niger* have been assigned to a specific linkage group by haploidization analyses. Generally clear linkage of markers to one of the eight linkage groups was observed. In most experiments special care was taken to minimize the effect of clonal outgrowth of mitotic segregants prior to benomyl induced haploidization (Bos *et al.* 1988; Debets *et al.* 1990a). In the haploidization experiments circumstantial information about linkage of markers was obtained, but haploid recombinants arisen by crossing-over cannot be used for determining gene order and estimating distances. We discussed earlier that genetic maps on the basis of such data as used by Lhoas (1967) may lead to incorrect conclusions (Debets *et al.* 1989). Generally recombinants carrying linked markers that were in repulsion in the original diploid are only found if they are on different chromosome arms. So, if such recombinants are found (e.g. for some markers in LGs III and VI) this may indicate a position on opposite chromosome arms. On the other hand attempts to construct multiply marked strains with closely linked markers on the same chromosome arm in this way are unsuccessful. Therefore, we optimized and used mitotic mapping methods based on the isolation and analysis of diploid crossover recombinants.

Isolation of recombinants. Markers suitable for positive selection were available on six of the eight chromosomes. As most of the genetic markers are auxotrophic markers, Novozym enrichment can be used for the selection of auxotrophic recombinants (Debets *et al.* 1989). Although laborious, the general applicability of the method makes it a useful tool in genetic analysis. If suitable selectable markers are available, homozygous diploids can be isolated more easily and recombinant frequencies of up to 4 % for *cnx*D6 (LG III) are found (Debets *et al.* 1990a). For some of the markers in LG I recombinant frequencies were very low (e.g. $8x10^{-5}$ for *cnx*G, Debets *et al.* 1990a) and after selection mainly haploid segregants were found (haploid frequency of $3x10^{-3}$, Debets *et al.* 1990a). In some experiments a very low dose of UV irradiation was used to increase the mitotic crossing-over frequency. In preliminary experiments such UV treatment resulted in an increase of diploid mitotic segregants of up to 100x, whereas the haploid frequency was only slightly higher, indicating an increase in mitotic crossing-over rather than non-disjunction. An increase of the frequency of mitotic crossing-over by UV irradiation has been observed in many fungi (see Fincham *et al.* 1979). UV irradiation has been used for the induction of mitotic segregation in several yeasts like *Saccharomyces cerevisiae* (Kunz and Haynes 1981), *Candida albicans* (Whelan and Magee 1981) and *C. maltosa* (Klinner *et al.* 1984). In *Schizosaccharomyces pombe* mitotic mapping data based on UV-induced recombination were consistent with the order proposed on the basis of spontaneous recombinants (Gygax and Thuriaux 1984). In these experiments the yeast cells were irradiated to a survival level of 50% (150 J/m^2). Also in *A. nidulans* UV-induced crossing-overs appeared to show the same distribution as spontaneous crossing-overs (Wood and Käfer 1969). We assume that in our experiments the UV dose given to germinating conidia (10 J/m^2) was sublethal, as the viable count of *A. niger* mycelial protoplasts was not significantly influenced by such UV treatment (personal data). However, as mutagenic effects cannot be ruled out in these experiments, UV-induced recombinants were not used in the development of the strain collection. The order of the markers on the left arm of chromosome I is based on UV-induced recombinants.

Analysis of recombinants. The genotypes of the isolated recombinants can be concluded directly from their phenotypes if recessive markers are in cis-position to the selected marker in the original diploid. Otherwise

genotypes must be determined by haploidization. To avoid the latter time consuming procedure, recombinant strains were constructed in several rounds of construction of diploids, isolation of mitotic crossing-over recombinants and haploidization.

An alternative mitotic mapping strategy was found in the use of the heterologous *amd*S transformants (ATs) (Debets *et al.* 1990b). For transformant strain AT1 it was shown that Amd$^-$ segregants, isolated from diploids hemizygous for *amd*S, (mainly) resulted from crossing-over between homologous chromosomes and not by excision of the transformational insert (Debets *et al.* 1990b). Strains in which the heterologous *amd*S gene was introduced 'randomly' by transformation at a unique position in the genome, therefore, might be used in mitotic mapping. The major advantages of the method are that the dominant *amd*S gene(s) can be used as a counter-selectable marker and that the *amd*S insert is in repulsion to the non-selectable recessive markers if diploids are constructed from a transformant and a mutant strain. Selection and analysis of crossing-over recombinants from such diploids is straight forward: Amd$^-$ segregants can be isolated on the basis of resistance to fluoroacetamide and from their phenotype (auxotrophy or prototrophy) the genotype concerning the linked recessive markers can be concluded directly. The Amd$^-$ segregants that were still heterozygous for the other markers can have arisen by mitotic crossing-over, but may in principle also result from excision of (part of) the plasmid insert (reversion). Furthermore, those homozygous for all other marker(s) on the chromosome formally can be explained by non-disjunction. Diploid non-disjunction segregants are rather infrequent in *A. niger* so the latter explanation seems unlikely as for each transformant rather high recombinant frequencies were found (Table 14). As can be seen from Table 14, for all transformants the frequency of FA^r segregants in diploids is considerably higher than the revertant frequency in the original haploid. Assuming that the reversion frequency in haploid and diploid cultures is the same, we conclude that most of the FA^r segregants in the hemizygous diploids result from crossing-over. The positions of the *amd*S inserts of AT19, AT6 and AT1 (carrying the *amd*S insert on chromosomes III, IV and VI respectively) concluded on basis of mitotic crossing-over are in good agreement with results of recombinants isolated on the basis of homozygosity of other selectable markers. LG II does not contain a selectable marker and, therefore, the tentative map of this chromosome is

solely based on mitotic mapping using transformant strains AT8 and AT16. All recessive auxotrophic markers mapped on one chromosome arm, suggesting an acrocentric chromosome. For AT8 the frequency of FA^r segregants in hemizygous diploid culture is about ten times higher than that in the haploid transformant strains. Therefore, if the relative frequency of prototrophic FA^r recombinants is low (e.g. for *his*D, *leu*C, *his*A and *his*E) such recombinants may all result from excision and the exact position of the *amd*S insert cannot be concluded. In the case of AT16 both recombination and excision frequencies are about ten times higher than with AT8. The relative frequency of prototrophic FA^r recombinants using AT16 is too high to be explained by excision. This indicates that the *amd*S insert of AT16 is distal to all markers on the chromosome. Results obtained with transformant strains AT8 and AT16 are in agreement with each other. No effort was made to order all genes, only major differences in relative recombinant frequencies were assumed to be significant and were used to make a tentative gene order and to estimate map distances using the tentative map distances for the *amd*S inserts of AT8 and AT16 (Table 2). The other map distances are based on recombinant frequencies for markers as estimated before (Debets *et al.* 1989, 1990a). The stability of the transformants can be a limitation to the use of the heterologous *amd*S gene in mitotic mapping. If the reversion frequency of the transforming sequence is relatively high the majority of the Amd^- segregants from hemizygous diploids probably results from excision of the plasmid insert rather than from crossing-over. In such a case (e.g. for AT18) the relative position of the *amd*S insert cannot be determined by selecting FA^r segregants.

Several of the *amd*S inserts introduced by transformation seem to expand the genetic map of the chromosomes (e.g. in chromosomes II, III, IV and V *amd*S inserts were found to be located distal to all genetic markers known). In *S. cerevisiae* it was shown that introduction of a new marker by transformation on chromosome I substantially increased the (meiotic) genetic map length (Kaback *et al.* 1989). Analysis of transformants with randomly introduced heterologous genes may contribute to the understanding of the genetic organization in the genome. The frequency of FA^r diploid recombinants probably indicates (at least to some extent) the map distances of the *amd*S genes to the centromere in each transformant. Together with the map distances obtained with markers introduced by mutation (Table 2) they may serve as reference points on the chromosomes.

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CHAPTER 6

An electrophoretic karyotype of *Aspergillus niger*

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Mol Gen Genet, in press

Summary

An electrophoretic karyotype of *Aspergillus niger* has been obtained using contour-clamped homogeneous electric field (CHEF) gel electrophoresis. Chromosome-sized DNA was separated into four bands. Seven of the eight linkage groups could be correlated with specific chromosomal bands. For this purpose DNA preparations from seven transformant strains of *A. niger* each carrying the heterologous *amd*S gene of *Aspergillus nidulans* on a different chromosome were analyzed. Some of the assignments were confirmed with linkage group specific *A. niger* probes. The estimated size of the *A. niger* chromosomes range from 3.5 Mb to 6.6 Mb, as based on gel migration relative to the chromosomes of *Schizosaccharomyces pombe* strains, *Saccharomyces cerevisiae* and *A. nidulans*. The total genome size of *A. niger* significantly exceeds that of *A. nidulans* and is estimated to be about 35.5 Mb - 38.5 Mb. Electrophoretic karyotyping was used to allocate non-mutant rRNA genes and to estimate the number of plasmids integrated in a high copy number transformant.

Introduction

Pulsed field gel electrophoresis methodology allows the separation of large DNA molecules. For lower eukaryotes this can be in the size range

of complete chromosomes (Schwartz and Cantor 1984, Carle and Olson 1984). This technique has been applied to study aspects of genome organization in several yeasts and fungi including *Saccharomyces cerevisiae* (Carle and Olson 1985, Schwartz and Cantor 1984), *Schizosaccharomyces pombe* (Vollrath and Davis 1987, Smith et al. 1987), *Neurospora crassa* (Orbach et al. 1988) and *Aspergillus nidulans* (Brody and Carbon 1989). Using the contour-clamped homogeneous electric field (CHEF) gel system (Vollrath and Davis 1987) the upper limit of resolution has been extended to about 10 megabases (Orbach et al. 1988).

Aspergillus niger is an asexual filamentous fungus with eight linkage groups as determined genetically (Bos et al. 1989, Debets et al. 1990a). Karyotyping by cytological methods is extremely difficult in fungi, but using the better morphology of chromosomes in meiotic cells and in post-meiotic mitosis it is feasible (e.g. Raju 1980). In asexual fungi however, this approach is impossible and only electrophoresis can be used for physical mapping. So far the genome size of *A. niger* has not been estimated. Generally the genome size is assumed to be similar to that of *A. nidulans* which has recently been estimated to be 31 Mb by pulsed field gel electrophoresis (Brody and Carbon 1989).

In this paper we demonstrate the use of the CHEF gel technique for electrophoretic karyotyping of *A. niger* using the chromosomes of *A. nidulans*, *S. cerevisiae* and *S. pombe* as size standards. In this way the genome size of *A. niger* (CBS120-49) was estimated. For the recognition of seven of the eight linkage groups in the resolved DNA bands of the karyotype we have used *amdS* transformants of *A. niger* each carrying the heterologous marker on a different single chromosome (Debets et al. 1990b). Thus, the *A. nidulans amdS* gene can be used as a linkage group specific probe in each transformant. Furthermore we show that the electrophoretic karyotype can be used for the assignment of genes that are not easily identified by the phenotype(s) of mutations.

Materials and methods

Strains and plasmids. All *A. niger* strains used originate from N402 a mutant strain with short conidiophores derived from strain N400 (CBS120-49). Intact chromosomal DNA molecules of *A. niger* were prepared from wild-

type *A. niger* and from *amd*S transformants AT20, AT16, AT19, AT6, AT17, AT1 and AT7 which carry the *amd*S insert in linkage group (LG) I, II, III, IV, V, VI and VIII respectively (Debets et al. 1990b). *Schizosaccharomyces pombe* strains CBS356 (Centraal Bureau Schimmelcultures Baarn) and 972 (h^-) (Bio-Rad), *Saccharomyces cerevisiae* strain YNN295 (Bio-Rad) and *A. nidulans* strain WG132 (*wA3,acrA1,pyroA4*) derived from Glasgow wild-type strain FGSC4 were used as sources of chromosomal DNA for size markers. The following plasmids were used as linkage group specific probes for chromosomal DNA blots: p3SR2 (carrying the *A. nidulans amd*S gene, Hynes et al. 1983), pRRR2 (carrying a fragment of the *A. nidulans* rDNA repeat unit, T. Goosen, unpublished results) and pAB82 (R. van Gorcom et al. in prep.) carrying the *A. niger bph*A gene which is located on chromosome I (Boschloo et al. in press).

Preparation of intact chromosomal DNA. Agarose plugs containing intact chromosomal DNA from *S. pombe* (972) and *S. cerevisiae* were purchased from Bio-Rad. For the isolation of chromosomal DNA from *A. niger*, *A. nidulans* and *S. pombe* strain CBS356, protoplasts were prepared using Novozym 234 (NOVO BioLabs). *A. nidulans* and *A. niger* mycelial protoplasts were isolated using standard procedures (Debets and Bos 1986). Similarly, *S. pombe* protoplasts were prepared from cells grown in 1% yeast extract, 1% malt extract, 2% glucose for 18 h at 30° C. Yeast cells were washed in saline (0.8% NaCl) prior to protoplast formation. Protoplasts were washed once with 1.2 M sorbitol, 50 mM EDTA and mixed with 1% low-melting point agarose at 50° C in 1M sorbitol, 500mM EDTA containing 2 mg/ml proteinase K. The final agarose concentration was 0.6% and the final concentration of protoplasts was about $2x10^8$/ml. The mixture was pipetted into a mold (2x10x45 mm) on ice. The agarose plugs were cut to size and incubated in 1% N-lauroylsarcosine containing 500 mM EDTA and 1 mg/ml proteinase K at 50° C for 48 h. Subsequently the plugs were washed in 50 mM EDTA and stored at 4° C. Extended storage of the plugs (over 1 year) did not interfere with the final resolution.

Electrophoresis conditions. CHEF gel electrophoresis was performed using Bio-Rad's CHEF DRII apparatus. DNA-agarose plugs were loaded as follows: The plugs were placed to a comb in a mold (21x13 cm) and fixed with agarose. A 100 ml gel containing either 0.8% DNA-grade agarose (Bio-Rad) or

0.5% chromosomal-grade agarose (Bio-Rad) was then gently poured, thus embedding the plugs. After cooling the comb was removed and the holes were filled with agarose. Gels were electrophoresed at 9° C in circulating 0.5xTAE buffer (Maniatis et al. 1982) at 45 V with pulse intervals of 55, 47 and 40 min. The duration of each pulse interval for the 0.5% agarose gel was 48 h, whereas the duration of the pulse intervals for the 0.8% agarose gels was 72, 48 and 48 h, respectively. Gels were stained in ethidium bromide (0.5 μg/ml) for 1 h and then destained in water for 1 h and photographed under UV illumination (at 302 nm).

Hybridization conditions. Procedures for hybridization using Hybond N^+ (Amersham) nucleic acid transfer membranes and probe labeling using the random hexamer priming method (Boehringer, Mannheim) were according to the manufacturers recommendations.

Results and discussion

Size estimation of A. niger chromosomes. A. niger chromosomal DNA as subjected to CHEF electrophoresis as indicated in Materials and methods. Chromosomal DNA of both wild-type and transformant strains was efficiently released from mycelial protoplasts embedded and t eated in agarose plugs; degradation of DNA was very limited. Using 0.8% agarose (DNA grade) and pulse intervals of 55 min, 47 min and 40 min for a total period of 168 h, the *A. niger* genome was resolved into four distinct bands (Fig. 1A). Under the same electrophoresis conditions the *A. nidulans* genome was separated into six bands which is in good agreement with the results of Brody and Carbon (1989). The lower three bands of the *A. niger* karyotype are estimated to be 5.0, 4.1 and 3.5 Mb respectively, using the size estimates for the *A. nidulans* chromosomes and the size standards *S. cerevisiae* (YNN295) and *S. pombe* (972) (Mortimer and Schild 1985, Fang et al. 1989). Clearly, the eight *A. niger* chromosomes fall in to four distinct size classes. The three lower bands are in the region of resolution of the *A. nidulans* chromosomes that could be separated although they differed in size by only several 100 kb. Using the electrophoresis conditions shown in Fig.1A no clear separation in the higher molecular weight range was observed. Therefore, gel electrophoresis was performed using 0.5%

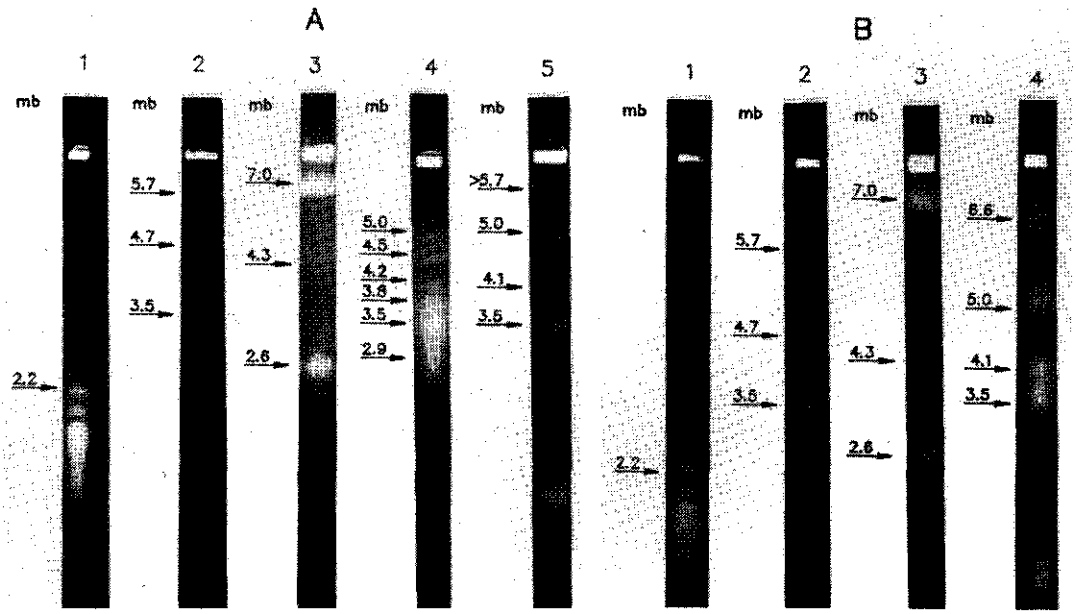

Figure 1A and B. Separation of Aspergillus niger chromosomal DNA on a contour-clamped homogeneous electric field (CHEF) gel relative to Saccharomyces cerevisiae, Schizosaccharomyces pombe and Aspergillus nidulans at pulse intervals of 55, 47 and 40 min. A. The agarose gel was electrophoresed for 168 hr (pulse durations 72, 48 and 48 h respectively) using 0.8% agarose (DNA grade). Lane 1, S. cerevisiae (YNN295); lane 2, S. pombe (972); lane 3, S. pombe (CBS356); lane 4, A. nidulans; lane 5, A. niger. B. Chromosomal DNAs electrophoresed for 144 h (pulse durations of 48 h each) using 0.5% agarose (chromosomal grade). Lane 1, S. cerevisiae, lane 2, S. pombe (972); lane 3, S. pombe (CBS356); lane 4, A. niger. The estimated sizes of the chromosomes in the electrophoretic bands are indicated.

chromosomal grade agarose (Bio-Rad) and 48 h duration of the pulse intervals (Fig.1B). Using this type of agarose (with a very low electro-endo-osmosis) at a low concentration, DNA mobility is considerably increased and the molecular weight range for maximum resolution is shifted upwards. As can be seen from Fig.1B further separation between the upper bands of the *S. pombe* strains and of *A. niger* was obtained. The upper band of *A. niger* chromosomal DNA showed a lower mobility than *S. pombe* 972 chromosome I, but a higher mobility than the largest chromosome of *S. pombe* CBS356. As reliable estimation of chromosomal size can only be made within the range covered by *S. pombe* strain 972 for which chromosomal sizes have been determined by physical mapping (Fang et al. 1989), the upper size limit of the chromosome(s) in the upper band remains unclear. Assuming that the total genome size of *S. pombe* CBS356 is the same as that of strain 972 the chromosomes can be estimated to be about 7.0 Mb, 4.3 Mb and 2.6 Mb respectively. If this assumption is correct, *S. pombe* strain CBS356 gives

an extension of the size range that can be estimated on the basis of relative mobility. Using the 7.0 Mb chromosome as a reference the upper band of *A. niger* represents a DNA size of about 6.6 Mb. The tentative DNA sizes of the bands of the *A. niger* karyotype are indicated in Fig.1B.

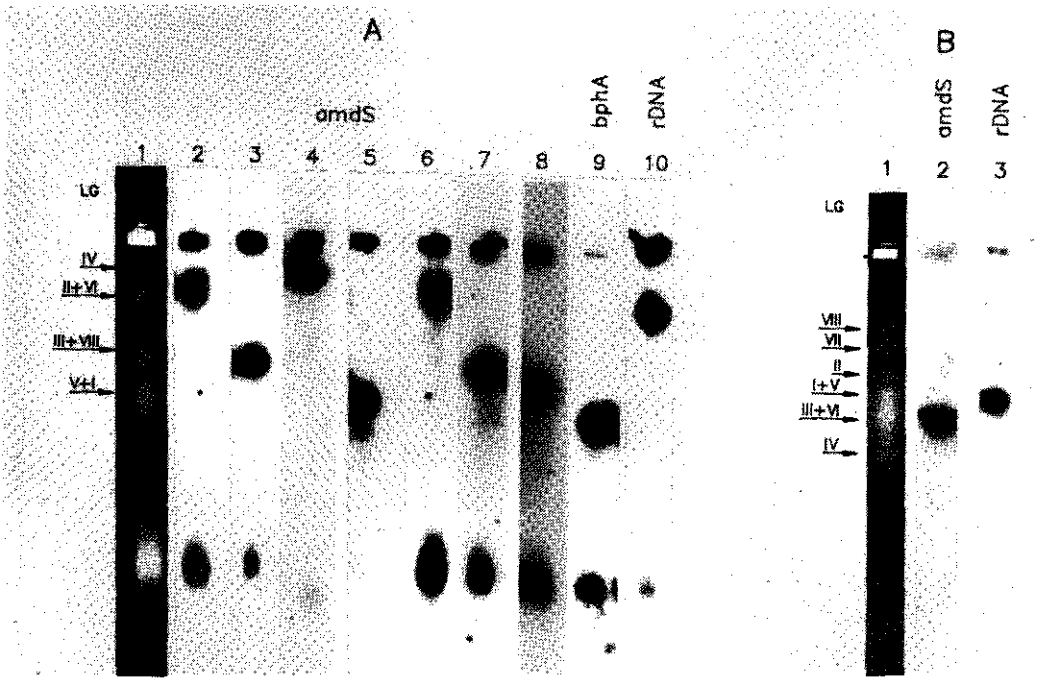

Figure 2A and B. Linkage group (LG) identification by hybridization analysis. Electrophoresis conditions were as in Fig 1A. A. Separated Aspergillus niger chromosomes. Lane 1, ethidium bromide stained chromosomal DNA of wild-type A. niger; lanes 2-8, hybridization of separated chromosomes of amdS transformants with labelled amdS probe. Lane 2, AT16 (LG II); lane 3, AT19 (LG III); lane 4, AT6 (LG IV); lane 5, AT17 (LG V); lane 6, AT1 (LG VI); lane 7, AT7 (LG VIII); lane 8, AT20 (LG I). Lanes 9 and 10, hybridization of wild-type A. niger chromosomes with labeled bphA (LG I) and rDNA (LG unknown) probes. B. Separated Aspergillus nidulans chromosomes. Lane 1, ethidium bromide stained chromosomal DNA of A. nidulans ; lanes 2 and 3, hybridization of separated A. nidulans chromosomes to amdS (LG III) and rDNA (LG unknown) probes, respectively.

Recognition of linkage groups. Although several *A. niger* genes have been cloned, the genetic linkage group is known for only a few. Only the following can be used as specific molecular markers for LGs in the electrophoretic karyotype of *A. niger*: *bph*A (LG I, Boschloo et al. in press), *pyr*A (LG III, Bos et al. 1989), *arg*B (LG V, unpublished results), *nia*D and *trp*C (both LG VIII, Debets et al. 1990a). Well-characterized translocation strains as described for *A. nidulans* and *Neurospora crassa* for example are not available for *A. niger*. Therefore, we used *amd*S transformants of *A. niger* to identify the position of the linkage groups in the karyotype. *A. niger* wild-type strains do not contain a gene equivalent

to the *A. nidulans amd*S gene, but can be transformed to the Amd$^+$ phenotype (Hynes et al. 1983). Recently we have subjected several *amd*S transformants of *A. niger* to genetic analysis (Debets et al. 1990b). In this study the *amd*S insert in each transformant could be assigned to a single chromosome and most probably to a single site on the chromosome. Seven of these transformants with the plasmid insert on chromosomes I-VI and VIII were protoplasted, embedded in agarose and electrophoresed. After transfer of the DNA to a Hybond membrane and hybridization with the labelled *amd*S probe, seven linkage groups could be detected (Fig. 2A). As can be seen in this figure, hybridization was restricted to a single band in the karyotype, which is in agreement with the unique chromosomal position of the integrated *amd*S DNA in the transformants found by genetic analysis. Thus, the position of the linkage groups in the electrophoretic karyotype of *A. niger* was concluded to be: LG IV (6.6 Mb band), LGs II and VI (5.0 Mb band), LGs III and VIII (4.1 Mb band) and LGs V and I (3.5 Mb band). Some of the results were confirmed using available LG-specific probes (LG VIII *trp*C, *nia*D; LG V *arg*B; LG III *pyr*A; data not shown). The position of linkage group VII could not be inferred as no appropriate transformant was found among a total of 20 transformants analyzed genetically (unpublished results). Since no LG VII-specific cloned marker is available either, the position of LG VII remains unclear. The relative intensity of ultraviolet fluorescence of the bands after ethidium bromide staining suggests that the upper band contains only chromosome IV, so the other bands probably represent two doublets and a triplet. In all lanes of the *A. niger* strains an additional low molecular weight band was observed. The relative intensity of this smear is different for each embedding. The DNA in this band hybridized to all LG-specific probes so we conclude that the band contains products of chromosome degradation.

Copy number estimation. Strain AT20 was shown to be a high copy number transformant (more than ten copies of the 9 kb pGW325 plasmid, as estimated by Southern analysis, Debets et al. 1990b). In ethidium bromide stained gels a broader lower band than that of the wild type was observed. On Southern hybridization LG I of AT20 shows a significant shift in migration position compared with the position of the hybridization signal for the LG I-specific probe *bph*A in the wild-type strain (Fig 2A). The shift of LG I was confirmed by probing AT20 with *bph*A. The multiple copy

insert of AT20 apparently resulted in a significant increase in molecular weight of the relatively small chromosome I. Using the relative mobility of LG I in AT20 and in wild type, we estimated the increase in chromosomal length to be about 300 kb. Thus, the plasmid copy number of the transformant strain is about 30. Although other transformants carry multiple copies of the *amd*S plasmid, the increase in chromosomal size was not as significant and did not interfere with linkage group identification. The presence in transformant AT6 of six or more copies of the plasmid in the largest linkage group II (6.6 Mb), did not result in a noticeable change in mobility. Transformant strain AT17 with an insert in the small LG V (3.5 Mb) on the other hand has one or two plasmid copies (Debets et al. 1990b). Occasional increases in chromosomal size due to integration of plasmids in high copy number, thus, may limite the use of transformant strains for initial linkage group identification in electrophoretic karyotypes.

Allocation of non-mutant genes. As mentioned above most of the cloned genes of *A. niger* are never used in genetic analysis either because no mutants of the gene exhibit a usable phenotype (e.g. *pel* (pectate lyase), *g6pd* (glucose-6-phosphate-dehydrogenase), rDNA), or because mutations were induced in *A. niger* isolates which are incompatible with the available test strains (unpublished results). Therefore, we were interested in testing the possibility of allocating genes to a linkage group by hybridization analysis of electrophoretic karyotypes. In *A. niger* and *A. nidulans* the chromosomal locus of the rRNA genes has never been determined because no mutants with a detectable phenotype are available for formal genetic analysis. The rRNA genes have been located by cytological studies of chromosomal attachment to the nucleolus in e.g. *N. crassa* (Barry and Perkins 1969) and by genetic analysis of meiosis using a restriction fragment length polymorfism (RFLP) marker in *S. cerevisiae* (Petes 1979). We used the *A. nidulans* rDNA repeat unit (pRRR2) and found specific hybridization to the 5.0 Mb band (LG's II and VI, possibly also VII) of *A. niger* (Fig.2A). Although we did not check the karyotype of *A. nidulans* by gene hybridization, we assume that we have a similar *A. nidulans* karyotype to that described by Brody and Carbon (1989) and by May et al. (1989): the *amd*S gene showed hybridization to the 3.5 Mb doublet which corresponds to its position on chromosome III (Hynes and Pateman 1970). Using the rDNA

probes of *A. nidulans* we observed clear hybridization to the upper doublet (3.8 Mb band) of *A. nidulans* (LG's I and V) (Fig.2B). With this probe we also found hybridization to the lower band of *S. pombe* 972 (in agreement with a position on chromosome III as described by Toda et al. 1984) and to the upper band of *S. cerevisiae* YNN295 (chromosome XII, Petes 1979) (data not shown). Remarkably, in strain CBS356 of *S. pombe* the rDNA probe clearly hybridized to the middle band, suggesting that the different electrophoretic karyotypes of the two *S. pombe* strains are due to complex chromosomal rearrangements.

Genome size of A.niger. The total genome size of *A. niger* has never been estimated, but has generally been considered to be similar to that of the *A. nidulans* genome. As can be seen from Fig. 1, the total genome size of *A. niger* is actually considerably larger than that of *A. nidulans* which has been estimated to be about 31 Mb by electrophoretic karyotyping (Brody and Carbon 1989) and 26 Mb by DNA-DNA reassociation analysis (Timberlake 1978). Based on the assumptions detailed earlier in this paper concerning size estimations, the total genome size of *A. niger* is between 35.5 Mb and 38.5 Mb depending on the size of LG VII. However, it must be noted that the size estimates of the chromosomes of *A. nidulans* of May et al. (1989) using electrophoretic karyotyping are significantly lower than those of Brody and Carbon (1989). On the basis of co-electrophoresis of *S. cerevisiae* and *S. pombe* they estimated a chromosome size range from 2.3 Mb for the smallest to 5.0 Mb for the largest chromosome and a total genome size of 21.4 Mb. Our results for *A. nidulans* are in full agreement with the findings of Carbon and Brody (1989), so we think that our analysis gives a good estimate of the genome size of *A. niger*, nonetheless size estimations must be considered as tentative.

In conclusion, an electrophoretic karyotype for *A. niger* has been obtained using CHEF electrophoresis. Four chromosomal bands were separated and seven of the eight linkage groups were assigned to a distinct band using seven *amd*S transformants each carrying the insert on one specific chromosome. Chromosomal size estimation was based on the relative mobility of the chromosomes of the size standards *S. pombe* and *S. cerevisiae*, and of *A. nidulans*. As the upper band of the *A. niger* karyotype is larger than the largest chromosomes of these size standards, we estimated the size of this

chromosomal DNA using another *S. pombe* strain (CBS356). The tentative size estimates of the chromosomes obtained in this way are: IV (6.6 Mb), II+VI (5.0 Mb), III+VIII (4.1 Mb), V+I (3.5 Mb) and VII (unknown). Thus the total genome size of *A. niger* (CBS120-49) is 35.5 - 38.5 Mb. We also tested the method for assignment of non-mutant genes to a chromosomal band in the electrophoretic karyotype by hybridization. In this way we found that the rRNA genes are probably located on either chromosome II or VI. Furthermore, we used band displacement in the electrophoretic karyotype to estimate the number of plasmids integrated in a high copy number *amd*S transformant.

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CHAPTER 7

Summary and general discussion

As was outlined in section 1.4, the main goal of this study was the development and application of genetic techniques for the construction of a genetic map of *Aspergillus niger* to allow genetic analysis in this economically important asexual fungus. The establishment of a strain collection of *A. niger* was started from N400, the gluconic acid production strain ATCC 9029 (= CBS 120-49). The first mutants were mainly auxotrophic. We performed various haploidization analyses to assign these markers to a linkage group. Crossing-over recombinants were rather frequent in these experiments, but in general they provided no reliable information about map order (see Chapter 2). Therefore, diploid mitotic crossing-over recombinants were used to elucidate the linear order of markers on the chromosomes and to construct recombinant strains. Because such recombinants arise relatively infrequent, methods for selection are needed. Since most auxotrophic mutants were isolated by filtration enrichment, initially such a method was tried for the selection of auxotrophic homozygotes to enable mitotic mapping.

A shortcoming of the classical filtration enrichment method was observed during the many mutant isolation experiments. The method was not successful for the isolation of all types of auxotrophic mutations. Most of the mutants isolated were amino acid deficient, but especially mutants requiring vitamins were scarce. The most likely explanation for this is cross-feeding by minute traces of growth factors excreted in the liquid medium by germinating conidia. An additional limitation of the method became apparent at the isolation of auxotrophic recombinants for mitotic mapping in *A. niger*. Upon filtration of the germinated conidia ungerminated conidia are lost as well by aggregation and trapping of the conidia into the mycelial network. Therefore, the method cannot be used for the quantitative isolation of auxotrophs. In Chapter 2 an enrichment procedure

is described that was developed to circumvent these problems. The method is based on selective killing of immobilized germinating conidia by the lytic enzyme preparation Novozym 234 and is termed Novozym enrichment. In this procedure crossfeeding is limited and vitamin deficient recombinants can be isolated as efficiently as those requiring amino acids. A high enrichment factor can be achieved without significant loss of auxotrophs as was shown in simulation experiments. Thus, auxotrophs can be isolated quantitatively, provided non-leaky mutants are used. The procedure has been applied to linkage analysis on the basis of mitotic crossing-over (Chapters 2 and 5). Though the method is rather laborious, it is useful if no selective marker properly located on the chromosome is available. The enzyme preparation Novozym 234 has been successfully used for the isolation of protoplasts in many filamentous fungi. Therefore, the Novozym enrichment may be applicable to many other fungi. Upon minor, but essential, modifications we have used the method successfully for the isolation of induced mutants (to be published). In addition to the development of the Novozym enrichment method that allows the use of the auxotrophic markers in mitotic mapping, we were interested in selective markers.

For several reasons we expected *a priori* that chlorate resistance mutations would be very useful in the genetic analysis of *A. niger*. In *Aspergillus nidulans* and some other fungi it is known that mutations in many genes can lead to chlorate resistance and that the isolation and characterization of such mutants is rather easy. Furthermore, the phenotypic expression of the mutant alleles in *A. nidulans* is recessive. In Chapter 3 the isolation, characterization and use of chlorate resistance mutations for direct selection of recombinants is described. The isolated mutants could be classified in three phenotypic classes on the basis of nitrogen utilization. A total of nine different complementation groups was found. All chlorate resistance mutations were recessive and they were distributed over six different linkage groups. Three of these chlorate resistance markers (*nia*D, *nir*A and *cnx*C) were unlinked to any of the markers of the six linkage groups described so far for *A. niger*. With these and some other markers in our laboratory we obtained evidence for the existence of eight linkage groups in *A. niger* (Chapter 3, Bos *et al*. 1989). Experiments have been set up to determine absolute frequencies of recombinants from heterozygous diploids. Recently we have isolated also chlorate resistant mutants that are nitrate utilizing and are probably

uptake mutants comparable to the *crn* mutants of *A. nidulans* (unpublished results). So far these markers have not been used in genetic analysis, but since these mutations are also recessive, they may be used as selectable markers.

Table 7.1. Direct selectable markers available in the linkage groups of A. niger.

Selectable markers	Linkage group
cnxF, cnxG, olvA, fwnA, brnA	I
	II
cnxD, pyrA	III
cnxB	IV
	V
cnxA/E, pyrB	VI
cnxC, fnrA	VII
niaD, nirA, crbA	VIII

cnxA-G, niaD and nirA mutations result in chlorate resistance; olvA (olive-green), fwnA (fawn) and brnA (brown) are colour markers; crbA and fnrA are carbendazim and fenarimol resistance genes respectively; pyrA and pyrB mutations confer resistance to 5-fluoroorotic acid.

The available selective markers that have been used for mitotic mapping sofar are shown in Table 7.1. These markers were induced by mutation. In Chapter 4 it is shown that selectable markers can also be introduced by transformation. Transformation in *Aspergillus* results from integration of the transforming DNA sequences in the genome of the recipient. Recombination of homologous transforming DNA may be at the homologous site either by single crossing-over or by double crossing-over or gene conversion. Ectopic integration apparently occurs by heterologous recombination with different integration events for one transformant to another (for reviews see Fincham 1989, Goosen *et al.* 1990). If there is no significant homology between the transforming DNA and the recipient genome as in the case of transformation of *A. niger* with the *A. nidulans amd*S gene (our *A. niger* strains seems to have no equivalent gene) it might be expected that transformation would yield ectopic transformants. Transformation of *A. niger* with the *amd*S gene was already reported (Kelly and Hynes 1986) and Southern analysis indicated differences between the transformants, but genetic analysis of the transformants had not been done. In *A. nidulans*, AmdS$^-$ mutants can be isolated as fluoroacetamide (FA) resistant mutants (Hynes and Pateman 1970). When testing our *A. niger* strains we observed that these are considerably more resistant to FA than *A. niger* transformants carrying the *A. nidulans amd*S gene. This prompted us to exploit the opportunity for two-way selection in genetic analysis: Amd$^+$ transformants can be isolated on the basis of growth on acetamide, whereas

counter selection can be performed using resistance to FA. The isolation and linkage group analysis on the basis of *amd*S transformants is described in Chapter 4. The *amd*S inserts of the isolated transformants were shown to be located in seven different linkage groups. Though most transformants carried several copies of the plasmid, in each transformant one insert was located on a single chromosome.

The application of the two-way selection using *amd*S transformants in genetic analysis is shown in chapters 4 and 5. The *amd*S insert of the different transformants could be localized rather easily and in fact provided an efficient mitotic mapping strategy. As mentioned before, a distal selective marker in cis-position to the recessive markers on the chromosome facilitates the mitotic mapping analysis. In our *A. niger* strain collection most of the linked recessive markers are in different strains as a result of our mutagenesis strategy. So strain construction is generally necessary. Especially when markers are tightly linked it is difficult and time consuming to obtain the desired recombinants. Moreover, the number of properly located selective markers is limited as can be seen from Table 7.1. Linkage groups II and V do not carry a selective marker thus necessitating the use of the rather laborious Novozym enrichment for linkage group II as was performed previously for linkage group V. The available *amd*S transformants provided however, an efficient alternative method for gene mapping. The main advantages of the method are: 1. The *amd*S gene may be introduced at different loci in the genome. Assuming random heterologous integration, each transformant would carry the dominant *amd*S$^+$ insert at a unique position. 2. Diploids constructed from such transformant strains and a strain carrying linked recessive markers are Amd$^+$ and thus sensitive to FA. From such diploids that are essentially hemizygous for the recessive FA resistance, homozygous crossing-over recombinants can be isolated on the basis of resistance to FA. 3. The selective FA resistance 'marker' is in the desired cis-position to all recessive markers of the non-transformed strains. The method has been used to localize many recessive non-selective markers relative to the *amd*S insert of the different transformant strains (Chapters 4 and 5). The tentative gene order of markers on chromosome II is based solely on this strategy. The *amd*S inserts of the analysed transformant strains seem to expand the genetic map (Chapter 5). The method may be applicable to all other fungi that can be transformed with the *amd*S gene and are resistant to FA itself.

Knowledge of the fate of homologous or heterologous genes of economical importance that are introduced by transformation is very important. In members of the Ascomycetes meiotic instability has been observed. Selker *et al.* (1987) discovered that when a normally single-copy sequence was duplicated by transformation in *Neurospora crassa* and the duplication strain was crossed with an other strain, a proportion of the meiotic products had both duplicated copies extensively changed. The corresponding single-copy sequence from the normal parent was unaffected. The changes occurred specifically during the period between fertilization and nuclear fusion, and were evidenced by the appearance and disappearance of restriction sites. The phenomenon was called the RIP effect (originally: rearrangements induced premeiotically, later: repeat induced pointmutations). The mechanism and function of this phenomenon is still unknown. It is clear that both heavy methylation and nucleotide sequence changes of the duplicated sequences are involved. About mitotic stability of transforming sequences little information is available. Especially two-way selectable markers that can be used in transformation are useful for the analysis of mitotic stability. For example *pyrA*$^-$ mutations confer resistance to 5-fluoroorotic acid, whereas counter selection for the Pyr$^+$ phenotype can be performed on the basis of pyrimidine prototrophy. Another two-way selectable gene that can be used in transformation is *nia*D. *Nia*D$^-$ mutants can be selected for on the basis of chlorate resistance, and complementation by transformation with the *nia*D$^+$ gene will restore the ability to utilize nitrate. Subsequently, loss of the transforming sequence can be selected for on chlorate containing medium and the phenotypic stability can be determined quantitatively. In this way, the mitotic stability of ten Nia$^+$ transformants has been determined (Chapter 3). Two distinct stability classes were found. Southern analysis of three transformants suggested that the rather unstable transformants (revertant frequency 5×10^{-3}) resulted from integration at the homologous site, whereas the stable transformants (revertant frequency about 2×10^{-5}) were gene replacements. Unkles *et al.* (1989) studying Nia$^+$ transformants of another *A. niger* strain (ATCC 10864) did not observe gene conversion transformants and could not make any correlation between the mitotic stability and the location of the incoming *nia*D. Whether the occurence of the different transformant types is strain dependent is unclear. Dunne and Oakley (1988) studied the frequency of benomyl resistant revertants from single copy

transformants (carrying the *pyrA*$^+$ gene in between a mutant and the resident wild type *ben*A locus) of *A. nidulans* that were Pyr$^+$ and intermediate resistant for benomyl. In this experiment benomyl resistant revertants (revertant frequency 4.6×10^{-4}) could be studied for loss of the Pyr$^+$ phenotype. They concluded that more than 50% of the revertants resulted from gene conversion and the others from single crossing-over. In Chapter 4 the analysis of ectopic transformants in *A. niger* has been performed using the *amd*S two-way selection system. The genetic analysis of twelve *amd*S transformants is described. The frequency of revertants of the transformants varied from 2.6×10^{-3} to 2.3×10^{-6}. As yet, no clear correlation between chromosomal position (Chapter 5), copy number and mitotic stability (Chapter 4) can be made.

Application of the described genetic tools and strains provided basic information about the genetics of *A. niger*. In addition to the six linkage groups of *A. niger* described earlier (Lhoas 1967, Bos *et al.* 1988) we found two additional linkage groups. A modification of the haploidization analysis proved to be helpful for conclusive assignment of markers to a linkage group (Chapters 3 and 4). A tentative gene order of 60 loci in the eight linkage groups relative to the centromere is proposed (Chapter 5). Genetic markers were found on both arms of the chromosomes, except for chromosomes II and IV, which may be acrocentric. Also information about the absolute frequency of mitotic recombination in heterozygous diploids was obtained. The frequency of homozygous diploid recombinants through non-disjunction in *A. niger* is less than 10^{-5}, whereas the frequency of haploid segregants is about 10^{-3} (Chapters 2 and 3). The frequency of homozygosity of a marker resulting from crossing-over depends on the position relative to the centromere and varies from 6×10^{-5} (*nic*A1) up to 4×10^{-2} (*cnx*D6) (Chapters 2 and 3). The recombinant frequencies for the *amd*S insert of the different transformants varied from about 10^{-4} up to 10^{-2} (Chapter 5). Though loss of the Amd$^+$ phenotype by reversion may interfere, the recombinant frequencies (corrected for the reversion frequencies of the original haploid transformants) probably reflect the distance of the inserts to the centromere. Together with the map distances obtained for other markers, these may tentatively serve as reference points on the chromosomes. So far, we have estimated the mitotic crossing-over frequency for markers on ten of the fourteen known chromosome-arms. In addition, a tentative estimate of map distances for markers on LG II has

been made. If we add up all these estimates a total map length of over $1.2x10^{-1}$ is obtained. This is of course an underestimate of the total incidence of mitotic crossing-over in *A. niger*: For three chromosome-arms no estimate of genetic length is available, and for at least three other chromosome-arms the length is based on a marker that is not the most distal one. Furthermore, the *amd*S inserts in the transformants analysed, seem to expand the genetic map of several chromosome-arms.

In Chapter 6 the analysis of the *A. niger* physical karyotype by pulsed field gel electrophoresis is described. For the identification of mitotic linkage groups in the electrophoretic karyotype of *A. niger*, linkage group specific molecular markers are needed, but only few were available. Therefore, we made use of the *amd*S marker that was introduced by transformation in several transformants. Chapter 6 shows the analysis of DNA preparations in pulsed field gel electrophoresis from seven transformant strains each carrying the *amd*S gene on a different chromosome. Thus, assigment of seven of the eight linkage groups to a specific electrophoretic band could be done using one single molecular probe (*amd*S). Chromosome sizes of *A. niger* were estimated using the available yeast size standards as well as the recently published electrophoretic karyotype of *A. nidulans* (Brody and Carbon 1989). As the upper band of the *A. niger* karyotype contains DNA of lower mobility than the largest chromosome of these size standards, the size of this chromosomal DNA was estimated using a *Schizosaccharomyces pombe* strain with a deviating karyotype assuming that the total genome size of this strain is similar to that of the size standard strain. The size of the *A. niger* chromosomes range from 3.5 Mb to 6.6 Mb. The total genome size of *A. niger* (35.5 Mb - 38.5 Mb) was found to be significantly higher than that of *A. nidulans* (31 Mb, Brody and Carbon 1989). So far we have not found a clear correlation between the physical size, the genetic length and the number of genetic markers on the chromosomes.

Electrophoretic karyotyping has several other applications. For example, we have used the method to estimate the number of plasmids integrated in a high copy number transformant (Chapter 6). The method may also be used for the assignment of genes to linkage groups. Molecular techniques, e.g. cloning and sequencing of genes, allow the isolation and analysis of fungal genes (for review see Goosen *et al.* 1990). Detailed information at the molecular level of many cloned genes is available, while

mutants defective in the expression of such a gene have not been isolated and thus no information about genome position is available. Sometimes mutants can be isolated by transformational substitution of the wild-type gene by an *in vitro* mutated cloned gene. In this way e.g. *trp*C mutants of *A. nidulans* (Wernars *et al.* 1987) and *A. niger* (Goosen *et al.* 1989) have been isolated. The thus isolated *trp*C mutation of *A. niger* (*trp*C::*lacZ*$^+$) could be assigned to linkage group VIII by formal genetics (Chapter 3). In this particular case genetic analysis could be performed either using the Trp$^-$ phenotype or the Lac$^+$ phenotype since these were linked (personal results). So, even if there is no suitable phenotype of a mutant gene, a cotransformed marker may be used for gene mapping of the cloned gene if site-directed transformation can be performed. This approach of introducing a cotransforming marker at or adjacent to a locus of interest has been used for both meiotic and mitotic mapping of the *A. nidulans* gene *alc*C coding for a third alcohol dehydrogenase (ADHIII) of which no physiological role is known (Jones and Sealy-Lewis 1989, 1990). This approach may be used also for other cloned genes of which no mutants are available or can be obtained. However, another more straightforward approach would be the assignment of non-mutant genes to a genetic linkage group through hybridization to a specific electrophoretic band. This has been done for rRNA genes in *A. niger* (Chapter 6). So far, the electrophoretic karyotype of *A. niger* cannot be used for the exact recognition of a single linkage group, but if more high copy number transformants or translocation strains become available it may be possible to separate also the chromosomes that are very similar in size and thus are comigrating. Pulsed field gel electrophoresis may also be applied for other purposes, for example construction of chromosome libraries and analysis of chromosome length polymorphisms.

Prospects for application. The genetic data and the mutant strain collection of the *A. niger* isolate studied may be of significant importance for determining strategies in applied projects. Kundu and Das (1985) tried to analyse calcium gluconate production of diploid strains heterozygous for some auxotrophic markers that were introduced in the original production strain. The genome position of the markers was unknown and the balance of diploid and haploid nuclei in the fermentor culture could not be controlled and monitored. For example, tightly linked auxotrophic markers spanning the

centromere (e.g. *pheA* and *nicA* on chromosome V) may be used to limit segregation of undesired haploid nuclei whereas directly selectable markers (see Table 7.1) can be used to enable detection of segregation of recombinants. If the genome position of an important trait is unknown and no mutants with an suitable phenotype are available it may be localized by either 'reversed genetics' or by hybridization to a specific band in the electrophoretic karyotype. Strain improvement can subsequently be performed. The possibility to determine the position and stability of transformational inserts has also an important impact. Another application is the construction of strains carrying transforming sequences that were originally introduced and analysed in different transformants. Thus an increase of the gene dosage of the transforming sequences should be possible without the necessity of subsequent transformations. Also the effect (physiological and genetical) of either the same or different transforming sequences within one strain can be studied.

The now available genetic data may be important also for fundamental knowledge of this asexual fungus. They permit a better estimation of the mitotic recombination index as proposed by Pontecorvo (1958) and tentatively estimated by Lhoas (1967). An omission in this index however, is the fact that the heterokaryon frequency is not included. A proper estimation of the impact of mitotic recombination in *A. niger* in nature still awaits a population genetical approach. It has been recognized that taxonomical classification of members of the *'Aspergillus niger'* group solely based on morphological characters is not accurate. The genetic techniques and strains obtained in our laboratory may help to provide a genetic basis for species definition in the group of the black Aspergilli.

So far, we know that our *A. niger* strain collection that is derived from the gluconic acid production strain ATCC 9029 is compatible with the glucoamylase production strains that are being investigated by Bonatelli *et al.* (personal communication). Furthermore, it is isogenic to strains that are used for glucose oxidase production (Markwell *et al.* 1989, Witteveen *et al.* in press) thus allowing genetic analysis of specific *gox* mutations (Swart *et al.* submitted). *A. niger* strains derived from another wild type (ATCC 1015) that are used for studying the hydroxylation of benzoate for industrial application are also compatible with our strains, and therefore, application of our strain collection and genetic techniques was possible (Boschloo *et al.* in press). Genetic compatibility with other *A. niger*

strains will be studied in the near future.

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SAMENVATTING

Dit proefschrift handelt over genetische analyse van de voor de biotechnologie belangrijke schimmel *Aspergillus niger*. *A. niger* is een imperfecte schimmel, met andere woorden *A. niger* heeft geen geslachtelijk stadium, en mist daardoor meiotische recombinatie. Toch is genetisch onderzoek aan imperfecte schimmels mogelijk en wel op basis van af en toe optredende mitotische recombinatie in heterozygote diploiden. Twee typen recombinanten zijn hierbij van belang. Ten eerste kunnen door opeenvolgende non-disjunctie gebeurtenissen haploiden ontstaan die recombinant zijn als gevolg van hergroepering van chromosomen. Dergelijke recombinanten geven informatie omtrent de koppelingsgroep ('chromosoom') waartoe een bepaald gen behoort. Ten tweede kunnen door overkruisingen tussen homologe chromosomen diploide recombinanten ontstaan. Deze recombinanten kunnen worden gebruikt voor het in kaart brengen van genen die tot dezelfde koppelingsgroep behoren. De belangrijkste vereisten voor de genetische analyse van *A. niger* zijn: (1) De aanwezigheid van genetische markers. (2) Mogelijkheid tot selectie van de (zeldzame) recombinanten. (3) De mogelijkheid tot karakterisering van de recombinanten. Het primaire doel van het hier beschreven onderzoek was het ontwikkelen en toepassen van genetische technieken voor de constructie van een genetische kaart van *A. niger*. De genetische kaart kan vervolgens worden gebruikt voor genetisch onderzoek en bij de veredeling van stammen. De gevolgde strategie was tweeledig. Allereerst zijn methoden (verder) ontwikkeld waarmee genetische analyse op grond van de reeds aanwezige markergenen mogelijk is. Daarnaast zijn nieuwe markers geintroduceerd door mutatie en transformatie.

In hoofdstuk 2 wordt een procedure beschreven voor de verrijking van auxotrofe recombinanten. De methode is gebaseerd op selectieve afdoding van geimmobiliseerde kiemende conidiosporen door celwand afbrekende enzymen (Novozym 234). De optimale condities voor deze 'Novozym-verrijking' zijn in simulatie experimenten bepaald. De methode is gebruikt voor het in kaart brengen van auxotrofe markers ten opzichte van het centromeer op chromosoom

V.

Hoofdstuk 3 beschrijft de isolatie en karakterisatie van chloraat-resistente mutanten alsmede het gebruik ervan voor genetische analyse van *A. niger*. Deze resistentie mutaties behoren tot negen verschillende complementatiegroepen (plus één overlappende). De mutanten zijn onderverdeeld in drie klassen op grond van groei op verschillende stikstofbronnen. De chloraatresistentie genen liggen verspreid over zes koppelingsgroepen. Drie van deze markers bleken ongekoppeld met markers van de zes tot dan toe bekende koppelingsgroepen en leverden bewijs voor het bestaan van nog twee chromosomen in *A. niger* (n=8). De recessieve resistentie markers bleken zeer geschikt voor directe selectie van mitotische recombinanten. Ze zijn gebruikt voor het schatten van recombinatie-frequenties. Tevens is de lineaire volgorde van enkele markers op chromosoom VI bepaald aan de hand van direct selecteerbare overkruisingsprodukten. Enkele nitraatreductase mutaties zijn door transformatie met het *A. niger* *nia*D$^+$ gen gecomplementeerd. Van tien transformanten is de mitotische stabiliteit bepaald, en enkele zijn moleculair nader geanalyseerd.

Hoofdstuk 4 beschrijft de genetische analyse van *amd*S transformanten van *A. niger*. Het *A. nidulans amd*S gen (coderend voor een aceetamidase) is door transformatie geintroduceerd in *A. niger*. De transformerende sequentie bleek in elk van de twaalf geanalyseerde transformanten in één koppelingsgroep geïntegreerd te zijn, waarschijnlijk in elke transformant op een unieke plaats. In totaal is op zeven van de acht chromosomen een *amd*S insertie gevonden. Onze (niet getransformeerde) *A. niger* stammen groeien niet op aceetamide en zijn minder gevoelig voor fluor-aceetamide dan de transformanten. Hierdoor is het mogelijk om naar twee kanten te selecteren: transformanten kunnen worden geselecteerd als groeiers op aceetamide, terwijl verlies van het AmdS$^+$ fenotype leidt tot fluoro-aceetamide resistentie. Diploiden die ontstaan zijn uit een transformant en een niet-transformant zijn hemizygoot voor de *amd*S insertie en zijn fenotypisch Amd$^+$. De mitotische stabiliteit van het Amd$^+$ fenotype van transformanten en hemizygote diploiden is gekwantificeerd. De positie van het geïntegreerde plasmide ten opzichte van andere markers op het chromosoom bleek eenvoudig te bepalen.

In hoofdstuk 5 is een genetische kaart van *A. niger* beschreven. Voor het bepalen van de ligging van 60 markers ten opzichte van het centromeer op de acht chromosomen is voornamelijk gebruik gemaakt van de genetische markers en technieken die in de hoofdstukken 2,3 en 4 beschreven zijn. Tevens is voor de genetische analyses gebruik gemaakt van *amd*S transformanten. Daartoe is de vermoedelijke positie van de *amd*S insertie van negen transformanten bepaald. In de meeste gevallen bleek de *amd*S marker distaal van de andere markers te liggen. Daardoor zijn de *amd*S transformanten zeer geschikt voor het in kaart brengen van (niet selecteerbare) recessieve markers omdat ze aan de volgende eisen voldoen:
(1) *Amd*S transformanten zijn betrekkelijk eenvoudig te isoleren.
(2) In hemizygote diploiden kan geselecteerd worden op recombinanten die de *amd*S marker kwijt zijn geraakt. De frequentie waarmee dergelijke recombinanten door overkruising ontstaan is afhankelijk van de relatieve afstand van de *amd*S marker tot het centromeer.
(3) De analyse van recombinanten is eenvoudig: het genotype kan direct van het fenotype worden afgeleid.
(4) De *amd*S marker kan op vele plaatsen in het genoom worden geïntegreerd, zodat in principe voor elke chromosoomarm een transformant met een distaal gelegen *amd*S marker kan worden verkregen.

In hoofdstuk 6 is een electroforetisch karyotype van *A. niger* beschreven. Met behulp van 'pulsed-field' gel electroforese zijn de chromosomen van *A. niger* in vier banden gescheiden. Zeven van de acht koppelingsgroepen konden worden toegekend aan een specifieke chromosomale band. Hiervoor zijn zeven transformanten gebruikt die elk op een ander chromosoom de *amd*S marker dragen. De grootte van de chromosomen is geschat en varieert van 3,5 tot 6,6 megabasen. Electroforetische karyotypering is toegepast voor het localiseren van rRNA genen en voor het schatten van de grootte van de insertie bij een transformant met vele copien van het *amd*S plasmide.

Hoofdstuk 7 tenslotte bevat een samenvatting en een algemene discussie.

Nawoord

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CURRICULUM VITAE

Fons Debets werd op 1 juni 1958 te Twello geboren. Na het behalen van het einddiploma atheneum B in 1976 aan het Veluws College te Apeldoorn, studeerde hij van 1976 tot 1982 aan de Landbouwhogeschool te Wageningen in de studierichting planteziektenkunde. De doctoraalvakken waren toxicologie (bijvak), genetica (hoofdvak) en virologie (hoofdvak). De stageperiode voor het hoofdvak virologie werd doorgebracht aan het landbouwproefstation van het ministerie van LVV te Paramaribo, Suriname. Tevens werd de eerstegraads onderwijsbevoegdheid voor het vak biologie behaald. In september 1982 trad hij in dienst bij de vakgroep Erfelijkheidsleer van de Landbouwhogeschool, aanvankelijk als studentassistent later als toegevoegd docent/onderzoeker op verscheidene projecten. Daarnaast is hij tijdelijk werkzaam geweest als docent in de periode van januari-juli 1983 (biologie, MAVO-Zuid Apeldoorn) en van augustus 1985 tot jan 1987 (microbiële genetica, RHAS Stova Wageningen). Sinds augustus 1986 verrichtte hij bij de sectie microbiële genetica o.l.v. C.J. Bos genetisch onderzoek aan *Aspergillus niger*, zoals in dit proefschrift is beschreven. Daarnaast heeft hij in deze periode onderwijstaken voor de vakgroep verricht. Sinds maart 1990 is hij werkzaam als universitair docent bij de vakgroep Erfelijkheidsleer van de Landbouwuniversiteit.