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GENETIC ANALYSIS IN THE ASEXUAL FUNGUS *ASPERGILLUS NIGER**

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The genetics of *A. niger* has been developed since 1980. An overview is presented of the advances in developing methods and collecting data. Important tools have been a) the application of essentially different methods to isolate mutants, b) the adaptation to *A. niger* of *A. nidulans* methodology for analysis of the parasexual cycle, c) the choice of marker genes, and in some cases the artificial introduction of such genes, to select homozygous segregants arising from mitotic recombination. With the use of parasexual recombination, a genetic linkage map of *A. niger* has been established. In total, 110 nuclear and 1 cytoplasmic (mitochondrial) markers are available. The application of *A. niger* genetics in applied research is illustrated by examples.

Keywords: Aspergillus niger – parasexual cycle – recombination – genetic map

INTRODUCTION

Parasexual recombination in *A. niger* was first described in 1953 by Pontecorvo et al. [17]. The parasexual cycle was then recognized as an important process that allows genetic analysis in asexual fungal species (Fig. 1). Not until 1967, however, was a serious attempt undertaken to perform genetic analysis on *A. niger* [16]. Thirty-one marker genes were involved in the investigation of Lhoas, who identified 6 different linkage groups by haploidization of heterozygous somatic diploids. Whereas mitotic crossing-over occurs at a significant rate in *A. niger*, Lhoas was able to determine the linear order of several marker genes and the centromere in linkage group I. Nevertheless, his investigation suffered from low numbers of segregants that could be analyzed. Unfortunately, this work was not extended and the genetics of *A. niger* remained poor. Ten years later, Van Tuyl reported another study [21] involving the analysis of fungicide resistance in *A. niger*. Forty-two mutations were used by Van Tuyl, and again 6 linkage groups were identified. A few marker genes could not be located to these 6 linkage groups; hence, another 1 or 2 linkage groups were implicit. However, the strains used by Lhoas and Van Tuyl were derived from different wild

*Dedicated to Professor Lajos Ferenczy on the occasion of his 70th birthday.

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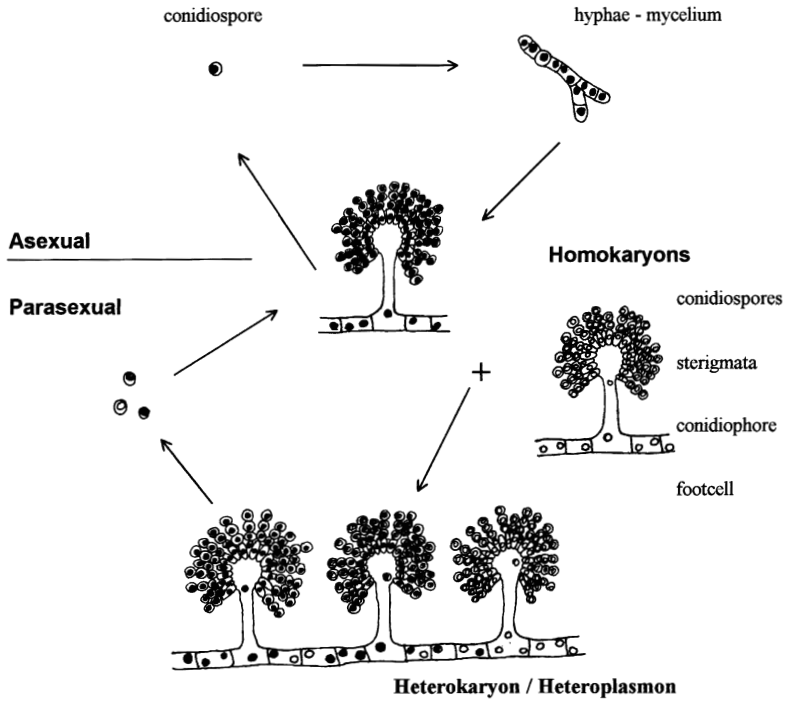

Fig. 1. The asexual and parasexual life cycles of a black *Aspergillus* (drawing by Anne van Diepeningen)

types and no attempt was undertaken to compare their results by mutual crosses. Due to poor preservation and registration, hardly any strains from the collections of Lhoas and Van Tuyl were available at the time we decided (in the early 1980s) to work out the genetics of *A. niger* in much more detail. We chose to start a new strain collection, setting out from the gluconic acid-producing wild-type strain ATCC 9029.

From this strain, numerous morphological, auxotrophic and resistant mutants were obtained and genetic analysis was undertaken to establish a genetic map. Many procedures were developed and *A. nidulans* methods were applied and adapted to *A. niger*. This review aims to provide an overview of the progress that was made during the eighties and nineties of the previous century.

MUTANTS

Most *A. niger* wild types have relatively tall conidiophores and the conidiospores spread easily. This characteristic is undesirable in laboratory practice. Thus, the first mutant we isolated from ATCC 9029 (called N400 in our laboratory) was a morphological mutant with short conidiophores (N402, *csp*A1). All subsequent mutants are

descendents from N402. Mutants were obtained as spontaneous mutants or after low-dose UV irradiation. A low UV dose (>70% survival) will yield the highest numbers of mutants and a low risk of double mutants or genetic background damage [1]. Additionally, it is impossible to remove genetic background damage by serial back-crosses in an imperfect fungus. A low mutagen dose results in a low frequency of mutants among the survivors; hence, efficient methods must be used to recover mutants. The filtration enrichment procedure [14] was used in many experiments and yielded numerous auxotrophic mutants. In the course of these experiments, we found that aggregation of the spores hampered efficient isolation of the mutants. This drawback was overcome by using a low-pH medium ($2 < pH < 4$) during incubation. Thus, a broad variety of auxotrophic mutants were isolated, though we recognized that some auxotrophies were found very easily (arginine, leucine, lysine, methionine and adenine), whereas others were hardly found or not at all. Therefore, we searched for other methods to enrich a mutagenized spore suspension for auxotrophic mutants. A promising method using selective cell wall lysis was published in 1975 by Ferenczy et al. [13]. The principle of the method is based on the difference in susceptibility to cell wall degrading enzymes of actively growing cells with a high turnover of cell wall components versus resting cells with a tough and rigid cell wall. This method was adapted to the specific properties of growth of germinating spores of a filamentous fungus such as *A. niger* [4]. Spores were plated in selective semi-solid agar medium and were allowed to germinate. The progress of the germination could be examined by microscopy and a proper time was chosen to apply the enzymatic treatment with the commercially available Novozym 234. Microscopy was helpful again to judge the lysis of the overwhelming majority of germinating spores. Subsequently, a rich complete medium was added as a toplayer to allow germination of the resting spores, among which we expected auxotrophic mutants. This method yielded 72 new mutants comprising 29 loci, of which 50% appeared to be new ones.

Another group of useful genetic markers were isolated as resistant mutants to a variety of toxic compounds. We used chlorate, for example, to isolate mutants affected in the nitrogen assimilation pathway [9] and 5-fluoro-orotic acid to select for pyrimidine auxotrophs [3]. Additionally, several fungicide-resistant mutants were isolated [12, and Slakhorst et al. unpublished, see Table 1].

Table 1
Fungicide-resistant mutations in the linkage groups of *A. niger*

Resistance gene	Linkage group
*fpm*A, *fpm*B	II
*trf*A, *vco*A	IV
*bit*A, *fnr*A, *mtf*A	VII
*sul*A	unknown

bit: bitertanol, *fnr:* fenarimol, *fpm:* fenpropimorph, *mtf:* methylthiofanate, *sul:* sulfanilamide, *trf:* triforine, *vco:* vinchlozolin.

Each group of phenotypically similar mutants was tested for complementation in forced heterokaryons. The total number of definitely different loci was approximately 110.

In addition to the nuclear markers we also tried to isolate cytoplasmic markers. However, such markers turned out to be difficult to obtain. Among approximately 100 oligomycin-resistant mutants, only one appeared to be a mitochondrial oligomycin-resistant mutation; the others were all nuclear, as proven by heterokaryon tests. The mitochondrial oligomycin resistance marker has been used effectively in the selection of mitochondrial transfer between incompatible strains [15]. Van Diepeningen et al. [20] used this oligomycin resistance marker to monitor cytoplasmic exchange in a study of intra- and interspecies transfer of viruses in *Aspergilli*.

MULTIPLE MARKED STRAINS AND RECOGNITION OF LINKAGE GROUPS

Forced heterokaryons could easily be selected when differently marked strains were co-cultivated according to the *A. nidulans* protocols [2, 17]. Also similarly as in such protocols, heterozygous diploids were selected in minimal medium. Haploidization was performed on complete medium with benomyl, and haploid colonies were tested for marker genes. Thus, colonies were collected which contained a combination of markers of the parental strains. These experiments revealed whether markers recombined randomly or must be linked. In the course of these experiments, we recognized important features of the parasexual cycle in *A. niger*. The frequency of heterozygous diploids among spores from a heterokaryon is 10^{-5}–10^{-6}. In heterozygous diploids of *A. niger,* a significant degree of mitotic recombination takes place, giving rise to recombinant sectors in a diploid colony. Therefore, propagation of diploids should be limited to avoid a genetic linkage bias due to such clonal segregation. We also introduced a method that allowed the recognition of recombinant sectors, which could hence be excluded from linkage analysis: several independent samples of spores were taken from different parts of a diploid colony and were each separately subjected to haploidization and phenotypic testing. If one of the samples showed results that did not agree with those of the other samples, the first sample was probably taken from a recombinant sector and was excluded from further analysis. A large number of such experiments finally led to numerous multiple marked strains and to the conclusion that *A. niger* had a total of 8 linkage groups [2, 3, 9]. Several 8 chromosome marked strains were constructed which can be applied as master strains in genetic analysis. Many mutants and different master strains are available at the Fungal Genetics Stock Center (http://www.fgsc.net/)

GENE ORDER IN LINKAGE GROUPS

Lhoas [16] carried out several experiments to establish the linear order of a number of markers on linkage group I. His analysis relied on the phenotype of haploid segregants that were obtained from heterozygous diploids. Relatively few haploid segregants are derived from mitotic crossovers and, if so, they may be of clonal origin, as explained in the previous section. Hence, this type of analysis does not provide quantitative data on genetic distances. In our collection of mutants, we had several phenotypes which in principle could be used to positively select homozygous diploids. Especially the chlorate-resistant mutants proved very useful to isolate mitotic crossover segregants (Fig. 2) [9]. Unfortunately, only a few chlorate-resistance markers appeared to be sufficiently distal to allow genetic mapping in the major part of a chromosome arm. To obtain more tools for interchromosomal mapping, we again took advantage of the selective killing of prototrophic germinating spores by cell wall degrading enzymes, as used by Ferenczy et al. [13] to select for auxotrophic mutants. A spore suspension of a heterozygous diploid was plated on partially supplemented minimal medium. The prototrophic spores germinated and were then killed by the addition of Novozym 234. Auxotrophic (presumably homozygous for an auxotrophic marker) spores were not attacked and were recovered after the Novozym treatment by the addition of complete medium [8]. Phenotypic characterization of the selected segregants allowed quantitative mapping.

In the meantime, a series of *amd*S transformants of *A. niger* were generated in our laboratory [11]. The *A. nidulans amd*S gene (encoding acetamidase, enabling growth

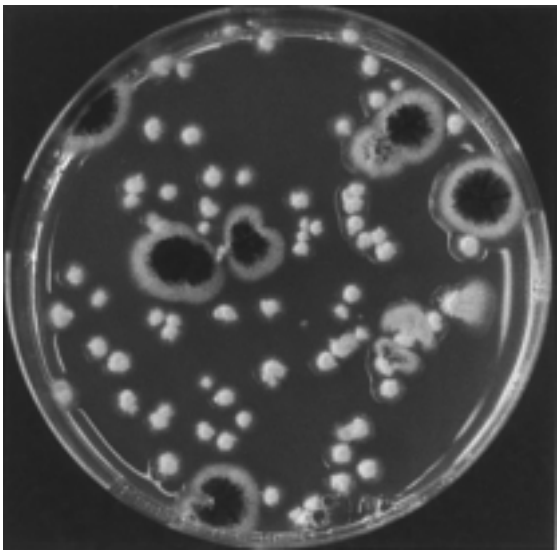

Fig. 2. Colonies of an *A. niger* diploid heterozygous for *cnx*AE8 (recessive chlorate-resistant) on complete medium with chlorate (0.2 M $KClO_3$). Heterozygous colonies are small, yellowish and have no spores. Homozygous segregants emerge as well-growing colonies with normal sporulation

on acetamide as sole C and N source; an equivalent is lacking in *A. niger*) integrates at ectopic sites into the host genome. Thus, several strains were obtained that now contained a selectable marker (the ability to grow on acetamide). Even more important in genetic mapping was the feature that this marker gene could be used for counter-selection: loss of this gene (due to mitotic crossing-over, for instance) confers on the segregant the ability to grow on the otherwise toxic compound fluoroacetamide. In the course of these experiments, the *amd*S sequence appeared to be integrated in each independent transformant at a rather distal site in a chromosome arm, thereby enabling efficient mapping in a significant area of a chromosome.

At present, we have selective marker genes in our strain collection in at least every chromosome of *A. niger*. A summary is given in Table 2.

Details of the linkage map of *A. niger* have been published by Bos et al. [5] and Debets et al. [10].

Table 2
Direct selectable recessive markers available in the linkage groups of *A. niger*

Selectable markers	Linkage group
*cnx*F, *cnx*G, *olv*A, *fwn*A, *brn*A	I
*fpm*A, *fpm*B	II
*cnx*D, *pyr*A	III
*cnx*B, *trf*A	IV
	V
*cnx*A/E, *pyr*B	VI
*cnx*C, *fnr*A	VII
*nia*D, *nir*A	VIII

*cnx*A-G, *nia*D, and *nir*A mutations result in chlorate resistance; *olv*A (olive green), *fwn*A (fawn), and *brn*A (brown) are colour markers; *fnr, fpm, trf,* are fungicide resistance genes, see Table 1 for explanation; *pyr*A and *pyr*B mutations confer resistance to 5-fluoro-orotic acid.

PHYSICAL KARYOTYPE

CHEF gel electrophoresis provided a tool to separate chromosomes according to their length. In *A. niger* (our laboratory strain), 4 distinct bands were obtained and, by using already allocated genes as probes, we could conclude that 8 chromosomes were present [7]. Extension of this work using strains with introduced chromosomal size variation showed that all 8 chromosomes could be distinguished and that separated chromosomes could be used to assign cloned genes to a linkage group [22].

We also applied CHEF gel electrophoresis to examine chromosome length polymorphism (CLP) in a series of different culture collection strains of the *A. niger* aggregate [19]. A broad range of variation in banding pattern and also in hybridization of the rDNA sequence was detected among these strains. Analysis of numerous natural isolates again showed extensive CLP (unpublished).

APPLICATIONS

Strain improvement in applied research and industrial applications is traditionally achieved by repeated mutagenesis. We used the available genetics of *A. niger* in a number of investigations to construct new strains harboring a combination of already characterized mutations. Swart et al. [18] localized the mutant genes of 7 glucose oxidase-overproducing mutants and in addition utilized auxotrophic genetic markers to construct heterozygous diploids and to select haploid segregants, which now contained 2, 3, and 4 mutations combined. In this way, it is possible to analyze each single mutation thoroughly (e.g. which metabolic route/step is affected) in order to make the best choice to combine specific mutations. The final effect is more or less predictable and much less uncertain than the result of random mutagenesis. Verdoes et al. [23] used the same strategy to combine glucoamylase genes which were introduced by transformation at different sites in different strains. Genetic markers and genetic maps are indispensable for this type of strain constructions.

Resistance to economically important fungicides is often difficult to explore in phytopathogens. Studies in model fungi can be helpful to unravel resistance development and to find genes involved in the resistance. Engels et al. [12] performed genetic analysis of resistance to fenpropimorph in *A. niger*. One of the goals of the study was to determine whether resistance to this compound is mono- or polygenic. Genetic analysis of different mutants revealed that at least 2 genes can mutate to resistance.

The behavior of an autonomously replicating plasmid (pAB4-ARp1), originally isolated from *A. nidulans*, was studied in the parasexual cycle of *A. niger* [6]. Genetic analysis revealed that the *Pyr*$^{+}$ marker gene on the plasmid was not linked to any of the 8 linkage groups. It was also shown, by using differently marked nuclei in a heterokaryon, that the plasmid could 'jump' from one nucleus to another. Thus, a possible route for horizontal transfer of DNA elements is indicated. The same plasmid, which confers a relatively high transformation frequency, was shown to provide an efficient gene-cloning tool when co-transformation with a cosmid library was applied [24].

CONCLUSIONS

The genetics of *A. niger* was successfully developed. Genetic tools were adapted, if necessary, for application in *A. niger*. Methods and master strains are available for the assignment of mutated genes to linkage groups, and even non-mutated, but cloned genes can be assigned by using the physical karyotype of this fungus. Applied research can benefit from utilizing the genetics of *A. niger*.

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