Mutagenesis and mitotic recombination in Aspergillus niger, an expedition from gene to genome

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Peter J.I. van de Vondervoort

2007

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an expedition from gene to genome

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Stellingen behorend bij het proefschrift ”Mutagenesis and mitotic recombination in *Aspergillus niger*; an expedition from gene to genome”.

1. Fysieke eigenschappen van DNA zoals bepaald door purine/pyrimidine reeksen buiten coderende gebieden beïnvloeden transcriptie en mitotische recombinatie frequenties (dit proefschrift).

2. Complementatie van een auxotrofe mutatie door een heteroloog gen bewijst niet dat dit heterologe gen een ortholoog van dat gemuteerde gen is (dit proefschrift).

3. Koppelingsanalyse in *Aspergillus niger* zoals beschreven door Bos geeft slechts eenduidige resultaten voor ongekoppelde eigenschappen (Bos et al. 1988 Curr Genet 14: 437-443; dit proefschrift).

4. *Aspergillus niger* heeft waarschijnlijk een hexokinase dat nauwelijks bijdraagt aan glucose fosforylering maar toch een belangrijke functie heeft in koolstof kataboliet repressie (dit proefschrift).

5. *cspA1*, een veel gebruikte genetische eigenschap in *Aspergillus niger*, is foutief benoemd omdat het de aanwezigheid van twee mutaties aanduidt.

6. Klassiek genetische technieken zoals mitotische recombinatie zijn nog lang niet achterhaald door de thans beschikbare moleculaire technieken.

7. Taxonomische indelingen veranderen sneller dan dat schimmels evolueren. Toch zal de taxonomische indeling van schimmels nooit perfect zijn.

8. Om verwarring in genetische nomenclatuur te voorkomen is het erg belangrijk om een internet database te hebben die een overzicht geeft van alle benoemde genen en hun synoniemen.

9. Onderzoekers die aan *Phytophthora* werken zeggen soms dat ze met *Phytophthora* werken, maar ze bedoelen dat ze tegen *Phytophthora* werken en dat is wederzijds.

10. Milieuneutraal tanken van fossiele brandstof is een kortzichtige verontschuldiging voor de bijdrage aan de CO2 productie.

11. Je kunt in de ochtendspits op de snelweg alleen maar 2 seconden afstand houden op je voorganger als je stil staat in een file.

Mutagenesis and mitotic recombination in *Aspergillus niger*: an expedition from gene to genome

Peter van de Vondervoort

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Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op donderdag 25 oktober 2007

klokke 16.15 uur

door

Peter Jozef Ida van de Vondervoort

geboren te Someren

in 1963

Promotie commissie

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Cover:	Cross-section of petri dish culture of *Aspergillus niger*
Printed by:	Printpartners Ipskamp B.V. Amsterdam
ISBN/EAN:	978-90-9022318-6

Voor Therese, Bram, Maarten en mijn ouders

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Chapter 1

General introduction

Aspergillus niger is a filamentous fungus belonging to the Fungi Imperfecti. Its name is derived from the black conidia bearing structures resembling an Aspergillum, an object used for sprinkling Holy Water (Fig. 1). *A. niger* is a cosmopolitan fungus and its spores can be found in air and soil worldwide. Being a saprophyte it is particularly capable of degrading plant cell-wall material using a large variety of enzymes. The sugars that are released are used to sustain growth but can also be metabolised under particular conditions to organic acids such as citric acid, which are accumulating extracellularly. *A. niger* grows happily at low pH values and is capable of reusing most of these organic acids. Because of its ability to produce these enzymes and organic acids in large amounts, *A. niger* is an industrially important fungus. A long history of use in the food industry has provided its products with the GRAS- status, which means that these are **g**enerally **r**egarded **a**s **s**afe.

For the various production processes, the best producing strains were selected from wild isolates. Subsequently these strains have been further improved, mainly by different rounds of mutation and selection. With the appearance of molecular genetic tools, genetic modification of *A. niger* was also applied to increase the production of homologous

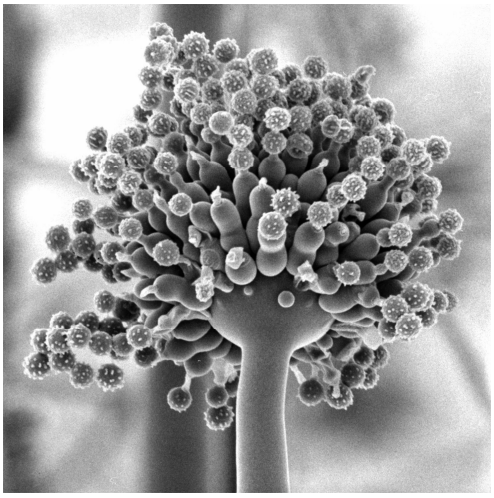

Figure 1. Electron scanning image of an *A. niger* conidiophore. This image was reproduced from Read (1991) with permission.

proteins (Verdoes et al., 1995). Encouraged by the high rates of protein production and secretion found for homologous proteins, researchers also tried to produce heterologous proteins at high levels (Punt et al., 2002). Unfortunately, several problems became apparent in these attempts, such as proteolysis of the proteins produced and genetic instability of production strains (Wiebe, 2003). Several approaches have been used to try and solve these problems, often based on the selection of better producing mutants or by genetically modifying strains, e.g. by deleting particular proteases (van den Hombergh et al., 1997a).

Optimization of various production processes has resulted in many individual *A. niger* strains with beneficial characteristics. However, further improvement by recombination of useful properties has hardly been applied. Although *A. niger* does not have a sexual cycle, recombination is possible through the parasexual cycle (Fig. 2). For *A. niger* strain breeding, the recombination of beneficial mutations could lead to a more universally optimised strain. Some of the above mentioned problems in heterologous protein production and development of tools to overcome these problems are addressed in this thesis, preceded by a general introduction to the topics experimentally approached. The topics deal with transformation and selection, strain instability, genetic recombination, carbon catabolite repression, and mutant selection in polyol metabolism.

Transformation and selection

Transformation is an important tool to study gene regulation and overexpression *in vivo*. In filamentous fungi many selectable markers are available nowadays (Ruiz-Diez, 2002), and there can be many reasons to choose for one or the other. The most frequently used selectable markers confer resistance to antibiotics or complement an auxotrophic marker. The advantage of the first category is that no special genetic background has to be engineered. Of course there are disadvantages, in particular their often toxic nature, and usually a lower transformation efficiency. The most often used auxotrophy complementing markers are those that can be selected in a bidirectional way. Mutations in *pyrA*, *niaD*, *sC* and *amdS* can be selected on fluoro-orotic acid, chlorate, selenate and fluoroacetamide, respectively while complementation leads to prototrophy or the ability to use acetamide as a nitrogen source (Tilburn et al., 1983; Ballance and Turner 1985; Buxton et al., 1989; Unkles et al., 1989).

In some cases, several consecutive transformation steps are required, for example for the disruption of several proteases or trehalases (van den Hombergh et al., 1997b; d'Enfert et al., 1999). One way to achieve this is through the use of multiple selection markers. A strain with multiple selection markers can be constructed by recombination of

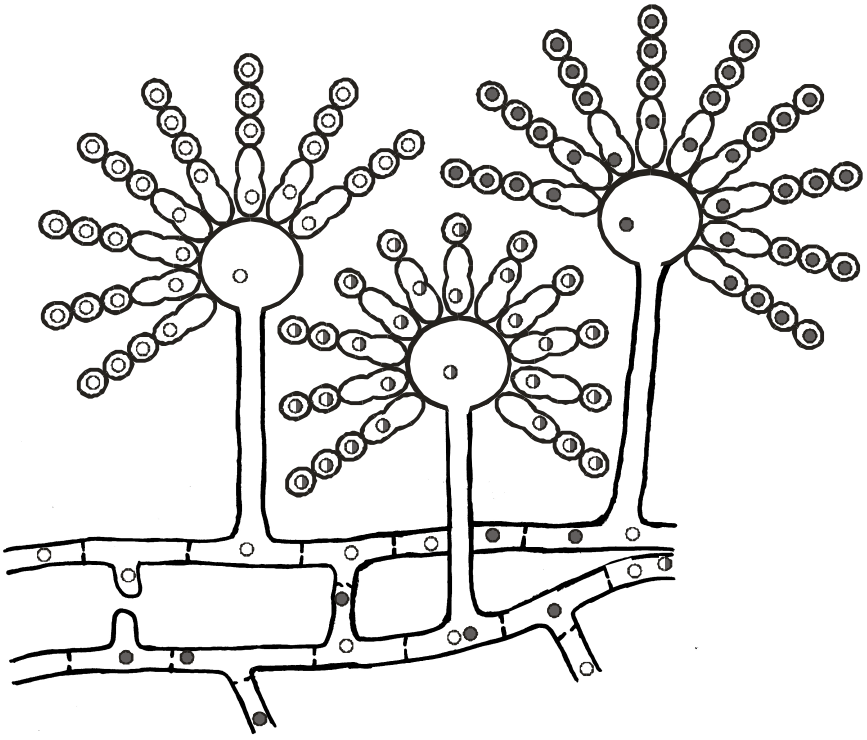

Figure 2. Schematic drawing depicting the initial phases of the parasexual cycle: hyphal fusion (anastomosis), nuclear fusion (karyogamy) and diploid spore formation. Nuclei of the two parental strains are depicted with the open and closed circles, the diploid nuclei are depicted as half open circles.

several selection markers, or by subsequent selection of transformable markers. An example of the latter is in *Aspergillus oryzae*, where a strain with quadruple transformation selection markers was developed, suitable to be complemented by the *argB*, *adeA*, *niaD* and *sC* genes (Jin et al., 2004). More recently, with several well-annotated fungal genome sequences available, the number of auxotrophic selection markers has increased tremendously (Chapters 6 and 7). Also, selection systems based on reusable markers allow subsequent transformations (d'Enfert, 1996; Krappmann and Braus, 2003).

Most fungal selection markers were shown to be complemented by orthologs of fungal origin (Buxton et al., 1985; Goosen et al., 1987; van Hartingsveldt et al., 1987). However, to increase transformation frequencies and to obtain full functionality, it usually turns out to be necessary to clone the homologous genes. A possible advantage of using a heterologous gene is a reduced frequency of integration at the homologous locus. This can

be of particular interest when trying to disrupt another gene, for example with *niaD* or *argB* in a chimeric disruption construct (Trail et al., 1994; van den Hombergh et al., 1997b).

In **Chapter 2** Lenouvel et al. (2002) examined why the *A. niger argB13* mutation is not completely complemented by the *A. nidulans argB* gene. By construction and genetic analysis of an *A. niger argB* disruption, we showed that the *argB13* mutation was not located in the *argB* gene and the mutation was renamed to *argI13*.

Strain instability

Production processes with *A. niger* are often performed as batch or fed-batch and sometimes as repeated batch fermentations. Continuous culturing may also be used to produce fungal products, but it is most often used to study production characteristics at different specific growth rates (Swift et al., 1998; Wiebe et al., 2000). A major problem in the use of continuous cultures is the rapid appearance of morphological mutants, often leading to a reduction in protein production (Mainwaring et al., 1999; Withers et al., 1998). These mutants generally have an advantage in growth rate and are affected in sporulation, branching or both (Swift et al., 1998; Wiebe et al., 1993; Withers et al., 1995). Also non-morphological mutants with altered production characteristics may occur (Mainwaring et al., 1999; Pederson et al., 2000; Wiebe et al., 1996). The high frequency of mutants appearing is troublesome, and different culturing conditions were examined to suppress strain instability (Christensen et al., 1995; Swift et al., 2000; Wiebe et al., 1996).

Amongst these strain variants which appear, some were found to have improved production characteristics. Swift et al. (1998) isolated a white aconidial strain with higher glucoamylase production than the parental strain. Such changes could originate from alterations in physiology, or from reduced autolysis as some “fluffy” genes were demonstrated to be involved in the regulation of this process (Emri et al., 2005). Van de Vondervoort et al. (2004; **Chapter 3**) showed that the parasexual cycle can be used to genetically characterise an aconidial strain, and conidia from an heterokaryon can be used as an inoculum for liquid cultures. Possibly, an aconidial strain could have advantages as a host for homologous and heterologous protein production.

Carbon catabolite repression

Saprophytic filamentous fungi such as *Hypocrea jecorina*, *A. nidulans* and *A. niger* can use a wide range of substrates. They are able to degrade complex substrates, in particular plant cell walls, through secretion of a large variety of hydrolysing enzymes (Bauer et al., 2006;

de Vries et al., 2002). Sugars thus released are taken up and used for growth and maintenance. To be able to compete with other microorganisms there are two important control mechanisms that ensure efficient use of resources. Specific induction of hydrolytic enzymes enables the use of a wide variety of available substrates (Aro et al., 2005), and a mechanism called carbon catabolite repression (CCR) ensures that energetically favourable carbon sources are used first (Ruijter and Visser, 1997).

There have been studies to elucidate the CCR signaling pathway from the perception of a repressing carbon source to the exertion of CCR on the targeted genes. Genetic screens in *A. nidulans* for the isolation of mutants with reduced CCR have identified mutations in *creA*, *creB* and *creC*, and a suppressor of the latter two, designated *creD*. Of these mutants only *creA* mutants show a general derepressed phenotype affecting a variety of inducible systems. The other *cre* mutations were described to be pleiotropic (Hynes and Kelly, 1977). More recently, their gene products were shown to be involved in (de)ubiquitination (Boase and Kelly, 2004; Lockington and Kelly, 2002).

CreA is a DNA-binding transcriptional repressor that exerts CCR on its target genes. On the mode of action of CreA a lot of detailed knowledge has been obtained, for example in studies of the inducible ethanol utilization pathway which involves the DNA-binding activator AlcR and CreA (Felenbok et al., 2001). It was found that CreA activity is regulated at both transcriptional and the post-transcriptional level and there are indications that sugar phosphorylation is required to obtain CCR (Strauss et al., 1999).

In *Saccharomyces cerevisiae*, many components of the glucose signaling pathway leading to glucose repression have been identified (reviewed by Santangelo, 2006). Hexose phosphorylating enzymes not only have a catalytic function in glycolysis but are also involved in carbon catabolite repression (Gancedo, 1998). Although a possible correlation between residual phosphorylating activity of Hxk2 mutant alleles and carbon catabolite repression has been rejected (Moreno and Herrero, 2002), glucose phosphorylation is required for the glucose signaling via Ras2-GTP (Colombo et al., 2004). Besides hexose phosphorylation, Hxk2 is believed to play another important role in CCR, and the protein is found in the nuclei of glucose grown cells (Santangelo, 2006 and Fig. 3). The gene encoding the main mediator of D-glucose repression in *S. cerevisiae*, *mig1*, is an ortholog of *creA* encoding the main CCR effector in *A. niger* (Ruijter et al., 1997) and appears to mediate the nuclear localisation of Hxk2 (Moreno et al., 2005).

As glucose phosphorylation is a very important step in CCR signaling, Flipphi et al. (2003; **Chapter 4**) studied its role in *A. nidulans*. They showed only one glucokinase and one hexokinase to be responsible for glucose phosphorylation, and none of the two enzymes appeared to have the unique importance that Hxk2 has in *S. cerevisiae*.

However, as genome sequencing of *A. nidulans* and *A. niger* revealed other putative hexokinase genes to be present, one of their products could play a role in CCR,

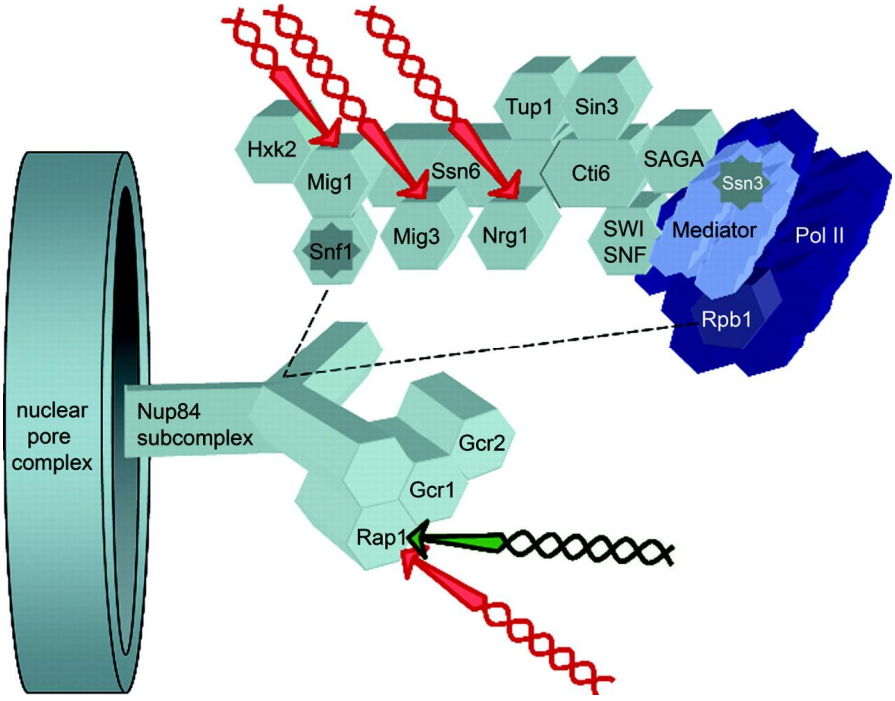

Figure 3. A model for glucose regulation at the nuclear periphery of *S. cerevisiae*. Numerous regulators involved in the transcriptomic response to glucose are components of a large nuclear assemblage that is tethered to nuclear pores (dashed lines) via multiple interactions with the Nup84 subcomplex. Arrows indicate repressed or activated target genes. This picture is copied from Santangelo with permission (2006).

comparable to yeast Hxk2. In view of the observation that glucose phosphorylating activity appeared not to be correlated to the CCR signaling function of hxk2 of yeast, lack of their involvement in glucose phosphorylation (Flipphi et al., 2003, Panneman, personal communication) does not rule this out. Besides glucose, other carbon sources also exert CCR. A rank-order can be defined of carbon sources that exert CCR to all of its "weaker" carbon sources. In general, a carbon source that support good growth is a stronger carbon catabolite repressor than a carbon source that supports less growth. From these observations, it is unclear whether the CCR signaling starts with a sugar receptor, uptake, carbon flux, intermediates or anabolic charge.

Polyol metabolism and mutant selection

Several *Aspergillus* species have been shown to accumulate polyols in mycelium and in conidiospores. In mycelium the polyols trehalose, D-mannitol, L-arabitol, xylitol, D-erythritol and glycerol can be found, while in conidiospores mostly mannitol and erythritol are found. The polyol pool levels in mycelium are influenced by several factors. The polyol predominantly formed is often the one closest in structure to the carbon source consumed in the metabolic network (Fig. 4). An exception is trehalose, which is found at low levels under gluconeogenetic conditions. Dijkema et al. (1985) found that the nature of the carbon source and aeration are of great importance, indicating a role of polyols in carbon storage and cofactor balance in *A. nidulans*. Witteveen and Visser (1995) found osmotic stress and the age of the mycelium to be important determinants for the polyol pool composition in *A. niger*. Glycerol was found to be important for osmoregulation. In *A. nidulans*, trehalose is needed to overcome heat and oxidative stress (Fillinger et al., 2001). In *A. niger*, xylitol is an intermediate of both the D-xylose and L-arabinose catabolism, and it is produced in low amounts during growth on these pentoses (de Groot et al., 2005; Prathumpai et al., 2003; Witteveen et al., 1989). Another pentitol, L-arabitol, is found upon growth on L-arabinose, whilst D-arabitol is produced by several aspergilli during growth on hexoses (Dijkema et al., 1985; Kelavkar and Chhatpar, 1993; Ramos et al., 1999; Ruijter et al., 2004; Witteveen and Visser, 1995).

Secretion of polyols is also observed particularly under oxygen limitation and carbon excess. It is not a big problem in enzyme production in *A. niger*, especially under carbon limited fermentation conditions where polyols produced and secreted in an early stage of fermentation are taken up again and metabolized. However, in the production of fuel ethanol from lignocellulose by recombinant yeast strains xylitol formation is a problem, causing a reduced yield. Wild type yeast is not able to degrade hemicelluloses and its main monomeric components D-xylose and L-arabinose, but genetically modified yeast, expressing heterologous microbial enzymes, can. Despite several strategies to reduce xylitol byproduct formation, this remains a problem (reviewed by Jeffries and Jin, 2004). In thesefermentations, xylitol is formed as a result of cofactor imbalance, because in yeast D-xylose reductase can use both NADPH and NADH, whereas for the oxidation of xylitol only NAD+ is used. More recently Karhumaa et al. (2006) described a genetically modified yeast strain with an independent D-xylose and L-arabinose catabolic pathway, resulting in reduced xylitol and arabitol production.

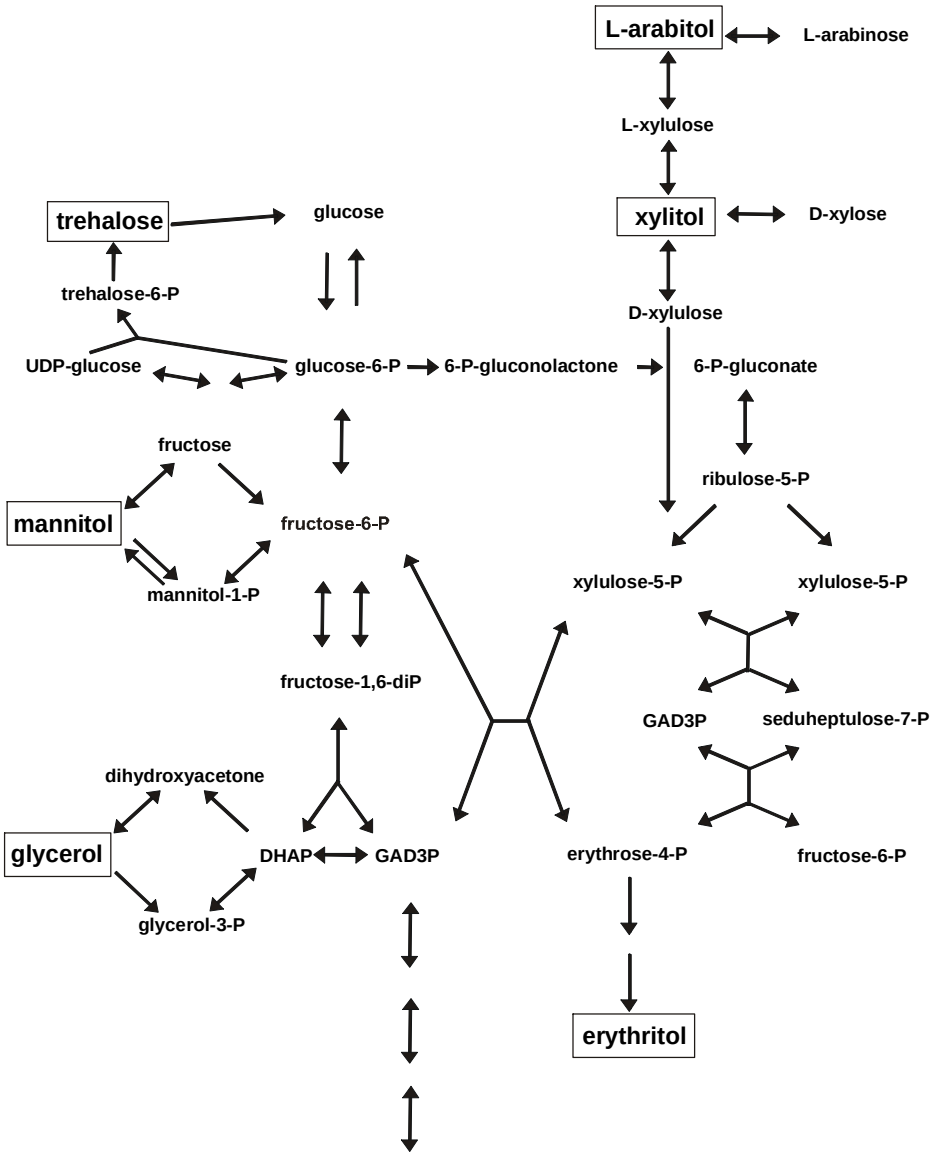

Figure 4. Carbon catabolite pathways leading to polyols commonly found in *Aspergillus* species. Abbreviations: GAD3P = glyceraldehyde-3-phosphate, DHAP = dihydroxyacetone phosphate

A comparable situation exists in *A. niger*. However, only NADP-dependent xylose reductase activity is found in *A. niger* (de Groot et al., 2005; Witteveen et al., 1989). Despite the importance of xylitol, only little information is available with regard to transport of xylitol or regulation of metabolism by xylitol. Van de Vondervoort et al. (2006; **Chapter 5**) investigated whether the mutant selection system used by de Groot et al. (2003) could be applied to identify functions involved in xylitol metabolism. We show that this selection system was capable of selecting a new mutant affected in xylitol-mediated repression, and detailed investigation of a xylitol-derepressed mutant shows it to be severely hampered in xylitol transport.

Genetic recombination

Several production processes using Aspergillus have been improved by strain breeding (Punt et al., 2002). *A. niger* production strains can be improved by subsequent rounds of mutation and selection. For specific problems such as those caused by the presence of proteases or the need for specific foldases, separate mutations or genetic modifications have been obtained (Lombrana et al., 2004; Mattern et al., 1992; van den Hombergh et al., 1997c). In plant breeding programs useful traits of an individual can easily be crossed into another genetic background with a few sexual crosses. In contrast to the situation in *A. nidulans*, genetic recombination is more troublesome in *A. niger* because it lacks a sexual cycle. An alternative is recombination using the parasexual cycle, which first was described for *A. nidulans* (Pontecorvo et al., 1953). The parasexual cycle starts with a heterokaryon, which can be obtained via anastomosis or protoplast fusion. In a heterokaryon, two different nuclei can fuse to form a diploid nucleus. This event occurs al low frequency and is called karyogamy (Fig 2). This heterozygous diploid can be selected from conidia. Through chemically induced loss of chromosomes, this diploid is reduced to an unstable aneuploid, finally giving rise to stable haploids. Essentially in this way complete chromosomes are exchanged between the starting strains. Markers with less than 25% recombination are believed to be located on the same chromosome. At low frequencies, recombination of linked markers is achieved by mitotic crossing-over of homologous chromosomes. In *A. nidulans*, these recombination events most commonly occur near the centromeres (Käfer, 1977). As presently physical maps of filamentous fungi become available, it would be interesting to know where these recombinations actually occur. This could contribute to the evaluation of the use of mitotic recombination in strain breeding strategies. Recently, the physical and genetic map of *A. niger* were linked (Debets et al., 1993; Pel et al., 2007; **Chapter 6**) and this advantage was used to study mitotic crossing-over in the case of chromosome III of *A. niger* (**Chapter 7**).

Aim and outline of this thesis

The aim of this thesis is to investigate several aspects of strain improvement for biotechnological applications using *A. niger*, particularly on how mutant selection and mitotic recombination can be used as tools. The aspects investigated are of interest in heterologous protein production in *A. niger*, but can be interesting for other applications as well. **Chapter 2** addresses a problem that occurred with the transformation selection marker *argB*. When using the auxotrophic selection marker *argB13* in combination with the heterologous *argB* gene from *A. nidulans*, only partial complementation was found. In this study we concluded that the mutation *argB13* was not located in the corresponding ortholog of the *A. nidulans argB* gene. **Chapter 3** addresses the problem of strain instability, often encountered in continuous cultures. In chemostat cultures of an *A. niger* strain aconidial mutants appeared, a morphologic instability which has been described also to occur in other strains. A genetic characterisation of an aconidial (fluffy) mutant in *A. niger* is described, showing that such a mutation can be handled very well using the parasexual cycle. In **Chapter 4** the role of hexose phosphorylation in glucose mediated CCR is investigated, concluding that the catalytic enzymes HxkA and GlkA do not play a unique role in CCR, as HxkII in yeast does. In **Chapter 5** we used pentose mediated carbon catabolite repression to isolate mutants with altered pentose metabolism. One of the mutants showed reduced growth on xylitol and analysis of this mutant surprisingly shows that both xylitol uptake and secretion are affected. Such a mutation might be of interest in ethanol bio fuel production by yeast, where xylitol accumulation was described to be a problem. The last two chapters look at *A. niger* genetics from a more general perspective. **Chapter 6** describes how we linked the genetic and the physical map of *A. niger*, part of which is included in the publication describing the *A. niger* genome sequence (Pel et al., 2007). Some unpublished details on the possible correlation between auxotrophic markers and annotated genes is included in **Chapter 6**. This research is extended in **Chapter 7** where we focused on chromosome III to investigate mitotic crossing-over frequencies and identified a mitotic recombination hotspot.

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Chapter 2

Disruption of the *Aspergillus niger argB* gene: a tool for transformation

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Curr Genet (2002) 41: 425–431
DOI 10.1007/s00294-002-0320-0

Abstract

We disrupted the *Aspergillus niger* gene *argB*, encoding ornithine transcarbamylase. Full characterisation of the *argB* deletion was performed by Southern blot analysis, growth tests and by means of mitotic recombination, complementation and transformation. The *argB* locus was found to be physically removed, thus creating an auxotrophic mutation. The latter can be supplemented by addition of arginine into the culture medium. The *argB* gene and its disruption do not correlate to the *argI13* (formerly *argB13*) allele described. The Δ*argB* is on chromosome I whereas *argI13* is on V. In addition, the *argI13* mutation can only be complemented by the *A. nidulans argB* gene, whereas the new *argB* deletion can be complemented by both the *A. niger* and *A. nidulans argB* genes. The Δ*argB* strain has been used to generate several strains in a breeding programme and to study the expression of important genes, such as *areA* and *kexB*.

Introduction

Transformation of micro-organisms is an important goal for the study of gene expression. The selectable markers that are available are not always the most suitable. In filamentous fungi, homologous complementation of an auxotrophic marker is preferred. For instance, the *pyrA* gene from *Aspergillus niger* is often used (Goosen et al., 1987). Because of its relatedness, the *argB* gene from *A. nidulans* is also considered as a good marker (Berse et al., 1983; Goosen et al., 1987). *A. niger* is an industrially important fungus able to secrete a variety of commercially interesting extracellular enzymes, including pectinases, hemicellulases, amylases, lipases and several oxidases in large amounts. Moreover, there is an increasing interest in *A. niger* and related Aspergillus species as expression systems for heterologous proteins (reviewed for example by Devchand and Gwynne 1991; Davies

1994; Archer and Peberdy 1997). A selection marker frequently used by us to manipulate *A. niger* is the *pyrA6* allele combined with homologous complementation with a *pyrA*-carrying plasmid (Goosen et al., 1987). Another is the *argI13* (formerly *argB13*) allele, which can be complemented by the *A. nidulans argB* gene (Swart et al., 1992). The latter system has been used to eliminate three different protease-encoding genes in *A. niger* (van den Hombergh et al., 1996). The *argB* system was originally described by Buxton et al., (1987), who isolated the *argB52* allele in *A. niger*, which could be complemented with both the $argB_{niger}$ and $argB_{nidulans}$ genes. We decided to construct an *argB* deletion strain for two reasons. The first was the observation that the *A. niger argI13* and *argI15* mutants are not fully complemented by the $argB_{nidulans}$ gene. The second was to avoid targeting to the *A. niger argB* locus when using the $argB_{niger}$ gene as a selection marker (Buxton et al., 1987). Thus, in this paper we report the construction of such an *argB* disrupted strain. We also show that the original *argI13* and *argI15* mutations (Swart et al., 1992) are not allelic to the disrupted gene.

Materials and methods

Strains

The gene symbols are as according to Martinelli (1994). The *A. niger* strains used in this study are all descendants of N402, a mutant of N400 (CBS 120.49) which carries the *cspA1* mutation conferring the short conidiophore phenotype. Strains N408 (*argA1*), N409 (*argL2*), N430 (*argD6*), N479 (*argG11 olvA1*), N491 (*argH12 nicA1*), N492 (*argI13 nicA1*), N647 (*fwnA6 nicA1 argI13*), N748 (*argK16 nicA1*) and N902 (*fwnA1 pyrA*5 *argI15 metB10*) are different arginine auxotrophs (Swart et al., 1992). N912 (*fwnA1 trpA1 bioA1 lysE28 pdxA2 crnB12*) was used as a tester strain. Strain NW219 (*pyrA*6 *nicA1 leuA1*) was used to make an *argB* disruption. We also used *A. nidulans* WG164 (*yA2 wA3 argB*2) and WG096 (*yA1 pabaA1*). Further, the following strains were constructed: NW245 (Δ$argB_{niger}$::*pyrA pyrA*6 *leuA1 nicA1*) and NW250 (Δ*argB*::*pyrA goxC17 bioA1 prtF28*); 1012.9 (Δ$argB_{niger}$::*pyrA goxC17*).

Media and growth conditions

A. niger was grown on complete medium and supplemented minimal medium Pontecorvo et al., (1953) containing 0.02% of a trace metal solution as described by Vishniac and Santer (1957). In the plate assays, 5 mM of arginine, citrulline, ornithine or nitrate were used as the sole nitrogen source. The plates were incubated for 3 days at 30°C.

Nucleic acid manipulation

Plasmid DNA manipulation was done according to standard protocols (Sambrook et al., 1989). The *argB* gene from *A. niger* was cloned by homologous hybridisation, using a 1.1-kb PCR fragment probe that was generated with the following primers: ARGBNIG-1 (5'-CCTCTACTCCTCCACCAG-3') and ARGBNIG-2 (5'-CACAAGCTATATACTAGC-3'). A 3.5-kb insert was recovered from a

N400 genomic phage library (Harmsen et al., 1990) and cloned into pUC19, leading to pIM2100. Partial sequencing confirmed the nature of the $argB_{niger}$ gene. Deleting a 1.0-kb SphI 3' fragment, creating pIM2101, generated a shorter functional fragment. From this, the $argB_{niger}$ disruption construct was made as described in Fig. 1.

Transformation

The procedure to transform protoplasts using the $pyrA_{niger}$ (Goosen et al., 1987) and the $argB_{niger}$ (this study) and $argB_{nidulans}$ (Berse et al., 1983; Buxton et al., 1985) genes was essentially as described by Kusters-van Someren et al., (1991).

Southern blotting

Fungal genomic DNA was extracted as described by de Graaff et al., (1988). Southern blot analysis was performed as indicated by Sambrook et al., (1989). Probes were prepared by the random priming method (Sambrook et al., 1989). Membranes (Hybond N, Amersham) were hybridised overnight at 65°C, washed once with 2·SSC/0.1% SDS (w/v) for 20 min. at 42 °C and autoradiographed on Agfa films at –80 °C with intensifying screens. The following DNA fragments were used as probes: a 868-bp AvrII-SphI $argB_{niger}$ fragment from plasmid pIM2101 (contains the 3' end of the $argB_{niger}$ gene) and a 2.2-kb XhoI-XhoI $pyrA_{niger}$ fragment from plasmid pGW635 (Goosen et al., 1987).

Genetic analysis

Linkage group assignment was done using heterozygous diploid obtained from tester strain N912 and NW245. After haploidization using benomyl, the recombinants were analysed for linkage of markers (Bos et al., 1988). The complementation assay was performed by growing a forced heterokaryon on minimal medium in the presence and absence of arginine (Swart et al., 1992).

Results and discussion

Disruption of the *argB* locus in A. *niger*

The need for several reliable and easy to use selection markers for transformation purposes in *A. niger* led us to design a disruption strategy for the *argB* locus in *A. niger*. The *A. niger argB* gene was cloned from a N400 genomic library (Harmsen et al., 1990). We confirmed the nature of the clone by DNA sequencing and sequence comparison. Multiple protein alignment shows that our *argB* clone does encode ornithine transcarbamylase (OTCase; Fig. 2). Recently, the complete genomic sequence of a natural isolate of *A. niger* NRRL3122 was determined by DSM N.V., revealing only one OTCase encoding gene (van Peij, personal communication). The *argB* marker was used as a selectable marker in many filamentous fungi to complement OTCase-defective mutants (Ventura et al., 1992). The disruption strategy we employed is depicted in Fig. 1. The first step consisted of cloning an $argB_{niger}$-containing fragment to be used in preparing the disruption

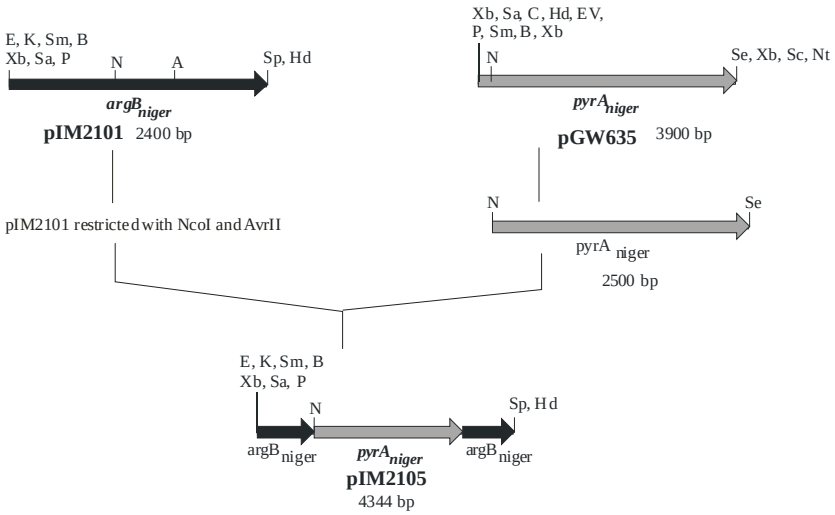

Fig. 2. Plasmids used to generate the *argB*$_{niger}$ disruption construct. pIM2105 was made using both pIM2101 (see Materials and methods) and pGW635 (Goosen et al., 1987). pIM2101 was digested with NcoI and AvrII, eliminating a 557-bp internal *argBniger* fragment. pGW635 was digested with NcoI and SpeI to liberate a 2.5-kb *pyrA*$_{niger}$ fragment. Both the linearised pIM2101 and the insert were gel-purified prior to ligation, generating pIM2105. The latter was subsequently linearised with XbaI, gel-purified and utilised to transform *A. niger* N902. Note that AvrII and SpeI sites are compatible but none is recovered after the ligation. Restriction enzyme sites are indicated as follows: E EcoRI, K KpnI, Sm SmaI, B BamHI, Xb XbaI, Sa SalI, P PstI, N NcoI, A Av rII, Hd HindIII, Sp SphI, Se SpeI, C ClaI, EV EcoRV, Sc SacI, Nt NotI.

construct (see Methods and methods). Briefly, an internal 0.5-kb NcoI-AvrII *argB*$_{niger}$ insert was replaced by the functional 2.5-kb NcoI-SpeI fragment containing the *pyrA*$_{niger}$ gene (Goosen et al., 1987), leading to pIM2105. Transformation was carried out with pIM2105 linearised by XbaI in the strain NW219 carrying a *pyrA*6 point mutation (Goosen et al., 1987). *pyrA*+ transformants were selected, purified and replica-plated to identify arginine auxotrophs. One transformant out of 60 presented the expected phenotype. Southern blot analysis was consistent with a disruption of the *argB* gene, as seen in Fig. 3. The expected 1.8-kb and ±8.5-kb EcoRI wild-type fragments were replaced by a 3.6-kb (Fig. 3a, left panel) and 3.6-kb (Fig. 3a, right panel) EcoRI fragment, respectively, in the transformant, thus demonstrating the integration of the *pyrA*$_{niger}$ gene at the *argB* locus. The single PstI and double EcoRI/NcoI restrictions confirmed that the *argB*$_{niger}$ gene was disrupted.

The PstI and the EcoRI-NcoI wild-type fragments are ±3.8 kb and 1.7 kb,

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A.oryzae   MTCGLKLAAARYGNHTLRQKIPLNAVRRYTSHTATSTTPPTSPFAPRHFLSIADLTSTEFATLV
A.terreus  -------MIPTARCGALRQKIPVQAVRQYS----SSTTLKTSPFAPRHLLSIADLTPTEFTTLV
A.nidulans -------------MASLRSVLKSQSLRHTVRSYSSQTMPPASPFAPRHFLSIADLSPSEFATLV
A.niger    ---MPSPLRTAPQPPLRAFHNPPALRRLYSSTSHSAATPATSPFAPRHLLSIADLTPTEFATLV
M.grisea   -------------------MRPSTLRAINRALAGNEARTYSSSASPRHLMSIADLTPTELTTLV
N.crassa   ---------------MMSRATTRTIKSAVGQIQARSVSNSAASSTPRHLLSISQLSPAEFSKLV
                                                .   ::. :***::**::*:.:*::.**

A.oryzae   RNASSHKRTIKSGS------IPQNLLGSMTGQTVAMLFSKRSTRTRISTEGAVVRLGGHPMFLG
A.terreus  RNASSHKHSIKSGS------IPTNLQGSLAGKTVAMMFSKRSTRTRISTEGATVQLGGHPMFLG
A.nidulans RNASSHKRAIKSGS------MPQNLQGSLLGKTVAMIFSKRSTRTRVSTEGAVVQMGGHPMFLG
A.niger    RNASSHKRAIKSGS------IPQSLHGALSGKTVAMMFSKRSTRTRISTEGAVVQMGGHPMFLG
M.grisea   RSAATHKHAVKSGAG-----APLHLAQSLTGKTVAMMFSKRSTRTRVSTEAAVAMMGGHPMFLG
N.crassa   LNASAYKQATKAAFAAGPGQVPRTLDGKLKGRTVAMMFSKRTTRTRVSTEAAVASWRGHPMFLG
            .*:::*:: *:.       *  *   : *:****:****:****:***.*..   *******

A.oryzae   KDDIQLGVNESLYDSAVVISSMVSCIVARVGKHAEVADLAKHSTVPVINALCDSFHPLQAIADF
A.terreus  KDDIQLGVNESLYDTAVVVSSMVSAIVARVGKHAEVADLAKHSTVPVINALCDSFHPLQAIADF
A.nidulans KDDIQLGVNESLYDTSVVISSMVSCIVARVGKHAEVADLAKHSSVPVINALCDSFHPLQAVADF
A.niger    KDDIQLGVNESLYDTAVVVSSMVECIVARVGKHADVADLAKHSTKPVINALCDSYHPLQAIADF
M.grisea   KDDIQLGVNESLLDTSTVISSMTSCMVARVGPHSDVTGLAKHSSVPVINALSDDFHPLQTIADF
N.crassa   KDDIQLGVNESLYDTSKVISSMTSCMVARVGPHSDVADLARDSSVPVINALSDDFHPLQAIADF
           *********** *:: *:***...:***** *::*:.**:.*: ******.*.:****::***

A.oryzae   QTIYETFTPKAHR-SDSLGLEGLKIAWVGDANNVLFDMAIAATKMGIDIAVATPKGYEIPAPML
A.terreus  QTIYETFTPKAHH-LSSLGLEGLKIAWVGDANNVLFDMAISAAKMGVDLAVATPKGYEIPASMR
A.nidulans QTIYEAFTPKAHH-LSSLGLEGLKIAWVGDANNVLFDMAIAATKMGVDIAVATPKGYEIPPHML
A.niger    QTISEHFAASGKGKLEGLGLNGLKIAWVGDANNVLFDMAISARKMGVDVAVATPKGYEIPKEML
M.grisea   LTIHSHHPSSTPG---SLGLEGLKIAWIGDSNNVLFDLALGAAKLGCHVAVASPTGYGIPENMR
N.crassa   QTIHEAFAPSPPTRPASDSRHEGRLGRR--SNNVLFDMATAASCWASTLPSPRPPATDSRPHEA
            ** . ....      . . .  ::.    :******:* .*   .  :. . * .

A.oryzae   ELIKQASNGVSKPGKIIETNVPEEAVKGADILVTDTWVSMGQEAESIKRVKDFEG-FQITSELA
A.terreus  ELIQEAGKGVANPGKLIQTNVPEEAVKKADILVTDTWVSMGQEEESLKRMKAFEG-FQITSELA
A.nidulans ELIKSAGEGVSKPGKLLQTNIPEEAVKDADILVTDTWVSMGQEEEKAQRLKEFDG-FQITAELA
A.niger    EIIEKAGEGVKSPGKLVQTNVPEEAVKGADVLVTDTWVSMGQEEEAAKRLRDFAG-FQITSELA
M.grisea   TLIQSASKASGSGGSLSETTVPEEAVKDADILVTDTWVSMGQEAEAKKRLAAFAG-FQITNDLA
N.crassa   DHPQRR-RGLAKPGKLIETTVPEEAVKDADILVTDTWVSMGQEAETQRRLKDCLPASKITNELA
              :   ..  . *.: :*.:****** **:************ *  :*:       :** :**

A.oryzae   KRGGANEGWKFMHCLPRHPEEVSDEVFYSPRSLVFPEAENRLWAAISAMEGFVVNKGRIE
A.terreus  KRGGANENWKFMHCLPRHPEEVSDEVFYSNRSLVFPEAENRLWAAISALEGFVVNKGKIA
A.nidulans KRGGAKEGWKFMHCLPRHPEEVSDEVFYSNRSLVFPEAENRLWAAISALEGFVVNKGKIE
A.niger    KRGGAKEGWRFMHCLPRHPEEVADEVFYGHRSLVFPEAENRLWAAISALEGFVVNKGKIE
M.grisea   KRGGAKKDWKFMHCLPRHPEEVHDEVFYSPRSLVFEEAENRLWAAVAALEAFVVNKGKI-
N.crassa   KRGGAKPGWKFMHCLPRHPEEVDDEVFYGPQSLVFPEAENRLWAAVSALEAFVVNNGRIL
           *****: .*:************ *****. :**** *********::*:*.****:*:*
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Fig. 2. Amino-acid (aa) comparison of *Aspergillus niger* ornithine transcarbamylase (OTCase) with other OTCases. The aa sequences were aligned using the program Clustal W (Jeanmougin et al., 1998). The aligned sequences are as follows: *A. oryzae* (372 aa; Nagashima et al., 1998), *A. terreus* (361 aa; Ventura et al., 1992), *A. nidulans* (359 aa; Berse et al., 1983), *A. niger* (370 aa),; *Magnaporthe grisea* (351 aa; Hamer et al., 2001), *Neurospora crassa* (362 aa; Flint and Wilkening 1986). A dash indicates a gap in the sequence. Fully conserved residues are indicated by a star.

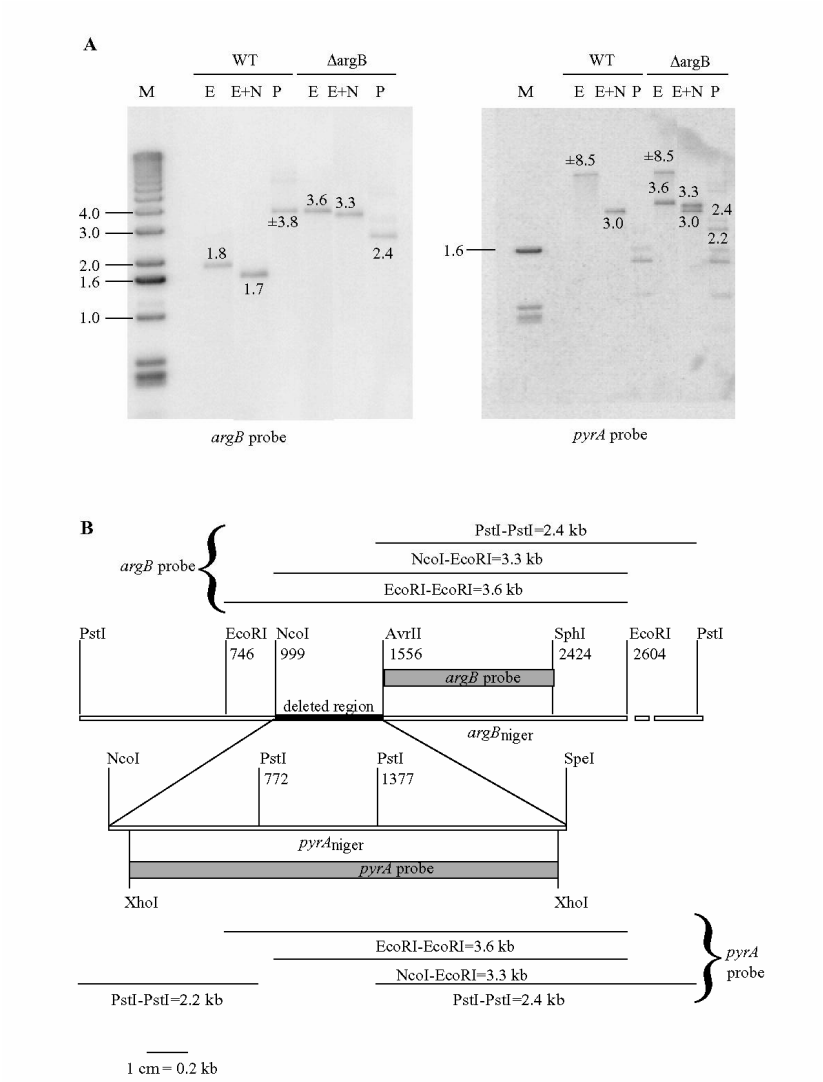

Fig. 3a, b. Disruption of the *argB*$_{niger}$ gene. Gene disruption was detected by Southern blot analysis. Genomic DNA from the *A. niger* wild-type strain (N402) and a transformant (NW245) was digested by EcoRI (E), EcoRI+NcoI (E+N) and PstI (P). a The 868-bp AvrII-SphI fragment of the *argB*$_{niger}$ gene and the 2.2-kb fragment of the *pyrA*$_{niger}$ gene were used as probes. WT wild type, M marker. b The relevant restriction sites are indicated. In Δ*argB*, the deleted region is replaced by the NcoI-SpeI fragment containing the *A. niger pyrA* gene. The expected fragments with the *argB* and *pyrA* probes in the Δ*argB* are indicated. The grey boxes represent the probes used (*argB*, *pyrA*).

respectively, whereas in the disruptant fragments of 3.3 kb and 2.4 kb appear (Fig. 3a, left panel). The faint band observed at 3.3 kb (Fig. 3a, left panel, lane P for Δ*argB*) is the result of a partial cut by PstI, thus revealing the upstream PstI (Fig. 3b, position 772) site in the *pyrA* gene. The *pyrA* probe confirmed these results (Fig. 3a, right panel). The EcoRI-NcoI cut generates a 3.3-kb fragment in the Δ*argB* strain. The PstI restriction shows several internal *pyrA* bands in the wild type, since the *pyrA*6 allele is a point mutation (Goosen et al., 1987). In the Δ*argB* transformant, two fragments are detected, at 2.2 kb and 2.4 kb (Fig. 3b). Taken together, these data are in agreement with a disruption of the $argB_{niger}$ locus. In order to assign the disruption to a linkage group, a somatic diploid was obtained with a tester strain N912 and NW245. Surprisingly, the $argB_{niger}$ disruption (Δ*argB*) was found to belong to linkage group I, whereas the *argI13* mutation was previously localised onto linkage group V (Swart et al., 1992). It appears that in CBS 120.49, *eglA*, *argB*, *rhgB*, *pelA*, *pepA* and *aguA* are located on the same chromosome as in NRRL 3122, where these genes were found in the order mentioned (van Peij, personal communication). The location of *pepA* is known from both pulsed-field electrophoresis–Southern blot experiments (Verdoes et al., 1994) and from parasexual analysis of the *pepA* disruption (van den Hombergh et al., 1997). The other genes were allocated by the method of Verdoes et al., (1994; van de Vondervoort, unpublished data). As the *argI13*/*argI15* and the $\Delta argB_{niger}$ alleles apparently

Table 1. Complementation tests of *Aspergillus niger* arginine requiring mutants.

Mutant	Genotype	Linkage group	Complementation	
			$argB_{nidulans}$	$argB_{niger}$
N408	*argA1*	V	+	-
N492	*argI13*	V	+	-
N902	*arg115*	V	+	-
N430	*argD6*	III	-	-
N479	*argG11*	VI	-	-
N491	*argH12*	II	-	-
N748	*argK16*	VI	-	-
N409	*argL2*	III	-	-
NW245/NW250	Δ*argB::pyrA*	I	+	+
WG164	*argB2*	III	+	+

The various strains were transformed with both the $argB_{nige}$ and $argB_{nidulans}$ genes. *A. nidulans* WG164 strain was used as a control. All strains and linkage groups were established by Swart et al., (1992), except NW245 and NW250 carrying the Δ*argB* allele (this study). + Prototrophic transformants for arginine obtained, – no transformants obtained.

do not belong to the same linkage group, they should complement in a heterokaryon. Strains N902, carrying the *argI15* allele, and NW245, carrying the Δ*argB* one, were used in a complementation test. The heterokaryon obtained grew perfectly well in the absence of arginine in a plate assay. This result agrees with the linkage group analysis and indicates that the *argI13*/*argI15* and Δ*argB* mutations are not allelic. As the *argB* gene was already defined in 1983 (Berse et al., 1983), we propose to rename the *argB13* and *argB15* alleles as *argI13* and *argI15*, to prevent confusion with the Δ*argB* allele we have constructed.

Non-specific complementation of different arg– mutations

The method used by Swart et al. (1992) to identify the mutation that corresponds to the $argB_{niger}$ gene apparently led to the wrong conclusion. Based on growth tests, they chose a limited number of mutations to be tested for complementation by transformation with the $argB_{nidulans}$ gene. In order to study the extent of non-specific complementation, we decided to transform all the mutations with the $argB_{niger}$ and $argB_{nidulans}$ genes. Data are presented in Table 1. As expected the $argB_{niger}$ and $argB_{nidulans}$ genes fully complemented the $argB_{niger}$ disruption strains (NW245/NW250) and the control *argB*2 strain from *A. nidulans* (WG164). Interestingly, the $argB_{nidulans}$ gene was found able to partially complement the *argA1* (N408), *argI13* (N492) and *argI15* (N902) alleles, whereas the $argB_{niger}$ gene did not. Transformants with a partially restored phenotype grew more slowly than the wild type on arginine-free media and the same as the wild type on arginine-supplemented media.

To further analyse the set of arginine-requiring mutants, we performed growth tests on plates as described by Swart et al., (1992), including the *argB* disruption strain (NW245). This did not lead to the expected phenotype, as only arginine was able to supplement the mutants properly. Normally an *argB* mutant is rescued by the addition of arginine or citrulline in the plate but not by the addition of ornithine. In our original tests, we used NW245 (Δ$argB_{niger}$::*pyrA pyrA*6 *leuA1 nicA1*) and therefore added leucine in the test medium. It appeared however, that the presence of other amino acids in the plate, such as leucine, methionine or lysine, inhibits the uptake of citrulline and ornithine, but less that of arginine. The competition for uptake between ornithine and basic and neutral amino acids had already been reported in *A. nidulans* (Pontecorvo et al., 1953; Piotrowska et al., 1976) and *Saccharomyces cerevisiae* (Soetens et al., 1998). Also, the absence of a nitrogen source was found to induce basic and neutral amino acid uptake (Piotrowska et al., 1976). Therefore, the tests were redone using strains with only arginine-requiring markers and not having other amino acid requirements. Arginine, citrulline and ornithine were employed both as nitrogen source and as supplement. Under these conditions, the plate tests are much more reliable. Results of the plate tests are presented in Table 2. As expected, both arginine and citrulline supplemented the *argB* disruption strain (1012.9) but ornithine did not (Table 2). These results are fully comparable with the situation observed in *A. nidulans* (Table 2,

strain WG164). The other mutants showed the same behaviour, as described by Swart et al., (1992), with the exception of the *argA1* strain N408, which in our tests could also be supplemented by citrulline. Our results present the construction of a well defined loss of function $argB_{niger}$ locus, using a disruption strategy in *A. niger*. The transformation efficiency was tested using both the $argB_{nidulans}$ and $argB_{niger}$ genes, leading to 1,000 and 400 transformants/mg plasmid, respectively. In addition, we found that the *argI13* and argA1 alleles did not concern the *argB* locus, although the $argB_{nidulans}$ gene could still partially complement the defect. The nature of these alleles remains to be determined. Nevertheless, a hypothesis can be made based on the arginine anabolism pathway, which has been investigated in detail in *A. nidulans*, *Neurospora crassa* and *S. cerevisiae* (Cybis et al., 1972; Cybis and Davis 1975; Karlin et al., 1976; Crabeel et al., 1995). Since the OTCase itself must be functional in the *argI13* and *argA1* strains, these mutations affect a process that is circumvented by the use of the $argB_{nidulans}$ gene and by supplementation with citrulline. A difference between the *A. niger* and *A. nidulans* OTCase is its targeting to the mitochondrion. Primary sequence analysis showed that the *A. niger* protein has a 74% prediction to be in the mitochondrion, whereas the *A. nidulans* one has only a 52% prediction (Nakai and Kanehisa 1992). If the *A. niger* OTCase is directed to the mitochondrion, mutations affecting the transport of ornithine into the mitochondrion and of

Table 2. Growth experiments.

Mutant	Relevant genotype	NO_3^-	Arg	Cit	Orn
N402		+	+	+	+
N408	*argA1*	-	+	+	-
N647	*argI13*	-	+	+	-
N430	*argD6*	-	+	-	-
N479	*argG11*	-	+	-	-
N491	*argH12*	-	+	-	-
N748	*argK16*	-	+	-	-
1012.9	Δ*argB::pyrA*	-	+	-	-
WG096		+	+	+	+
WG164	*argB2*	-	+	+	-

Plate assays were done by replica plating a set of strains on minimal medium containing either arginine, citrulline, ornithine or sodium nitrate at 5 mM. Glucose 1% (w/v) was used as carbon source and pH was set to 6.0. Growth was scored as: – (no growth) or + (good growth) after 3 days incubation at 30°C. All strains originate from Swart et al. (1992), except for 1012.9, which was constructed during the course of this work. NO_3^- Sodium nitrate, Arg arginine, Cit citrulline, Orn ornithine.

citrulline from the mitochondrion to the cytosol would also result in arginine requirement. The *A. nidulans* OTCase would however be able to complement this deficiency by its activity in the cytosol, thus circumventing the need for transport of citrulline or ornithine over the mitochondrial membrane. We therefore suggest that *argI* and *argA* could encode proteins needed for these transport functions. The Δ*argB* strain prepared here is particularly suitable for the elimination of other genes by disruption, since targeting of the selection marker to the *argB* locus itself is prevented. Complementation of this mutation with its endogenous gene results in well defined transformants, very suitable for comparing new disruptions to wild-type control strains. For instance, two important genes, the wide domain regulator *areA* (controlling nitrogen metabolite repression; Lenouvel et al., 2001) and *kexB* (a kexin-like encoding gene; Jalving et al., 2000), were disrupted and characterised.

Acknowledgements

The European Community (grant ERBBIO4CT975046) financially supported F.L. Part of this work was financed by grants from the Technology Foundation STW (grant WBI4100) and the European Community in the Biotech programme CT93-0174 to J.V. F.L. is grateful to Dr Mary Callanan for correcting the English. The authors thank Yvonne Muller for technical assistance, Dr Noël van Peij from DSM Food Specialities (Delft, The Netherlands) for information about the *A. niger* genomic sequence and Dr. Klaas Swart for helpful discussions.

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Chapter 3

Isolation of a fluffy mutant of *Aspergillus niger* from chemostat culture and its potential use as a morphologically stable host for protein production

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Biotech Bioeng 86: 301-307
DOI: 10.1002/bit.20046

Abstract

Chemostat cultivation of *Aspergillus niger* and other filamentous fungi is often hindered by the spontaneous appearance of morphologic mutants. Using the Variomixing bioreactor and applying different chemostat conditions we tried to optimise morphologic stability in both ammonium and glucose-limited cultures. In most cultivations mutants with fluffy (aconidial) morphology became dominant. From an ammonium-limited culture, a fluffy mutant was isolated and genetically characterized using the parasexual cycle. The mutant contained a single morphological mutation, causing an increased colony radial growth rate. The fluffy mutant was subjected to transformation and finally conidiospores from a forced heterokaryon were shown to be a proper inoculum for fluffy strain cultivation.

Introduction

Aspergillus niger is an industrially important fungus, often used for the production of organic acids and proteins. Production processes using filamentous fungi are often designed as batch or fed-batch cultivations, but some of them seem to be suitable for continuous culturing (Swift et al., 1998; Wiebe et al., 2000). Chemostat cultivation is mainly used for determining process variables and for characterizing strains at different specific growth rates. A major problem in the use of continuous cultures is the rapid appearance of morphological mutants, often leading to a reduction in protein production and altered growth characteristics (Christensen et al., 1995; Mainwaring et al., 1999; Wiebe et al., 1994; Withers et al., 1998). The mutants were affected in sporulation, branching or both (Swift et al., 1998; Wiebe et al., 1993; Withers et al., 1995) and were often demonstrated to have a selective advantage over the parental strain. Also non-morphological mutations with altered production characteristics may occur (Mainwaring et al., 1999; Pederson et al.,

2000; Wiebe et al., 1996). The high frequency of mutants appearing is a symptom of genetic instability, which is even more problematic when genetically engineered strains are used. Usually these strains carry multiple copies of the gene of interest. Losing some of these copies is advantageous for the organism, but reduces the desired protein production (Christensen et al., 1995; Mainwaring et al., 1999; Swift et al., 1998).

Wiebe et al. (1994) have isolated stable "variants" of *Fusarium graminearum* from a chemostat culture. These variants had a selective advantage over their parent and were morphologically more stable 1n chemostat cultures. Similarly, from a glucoamylase overproducing *A. niger* strain, mutants with increased selective advantage were isolated, but unfortunately, in most cases their productivity appeared to be reduced (Swift et al., 2000; Withers et al., 1998). However, Withers et al. (1998) isolated a mutant with less densely, brown spores, which sustained production for 735 hours, and Swift et al. (1998) isolated a white aconidial strain with higher glucoamylase production than the parent strain. However, an aconidial strain is difficult to maintain and use as an inoculum. In *A. niger* chemostat cultures, lowering pH from pH 5.4 to 4.0 increased morphological and genetic stability (Swift et al., 1998), but in other cases mutations still occurred (Mainwaring et al., 1999; Withers et al., 1998). Another method to increase morphologic stability was used in *Fusarium graminearum*. There, Wiebe et al. (1996) frequently changed growth conditions, limiting the selective advantage of a mutant to a short period of the cultivation. Although these approaches increased morphologic and genetic stability in individual cases, they have not led to the development of a morphologically stable generally applicable host, suitable for the production of different proteins in *A. niger*.

According to the theory of Monod (1942) spontaneous mutants with increased maximum specific growth rate will always dominate a continuous culture at high dilution rates and spontaneous mutants with increased affinity for the limiting substrate will always dominate a continuous culture at low dilution rates (Tempest, 1970). However, Swift et al. (1998) found that a mutant without a significant higher maximum specific growth rate than that of the original strain (0.27 ± 0.03 h^{-1}) constituted around 90% of a continuous culture at a dilution rate of 0.26 h^{-1} within 100 hours after appearance of the first mutant. This shows that the mutation rate and/or other factors also are of importance. If such a fast exchange would originate from a single mutant and be caused by growth competition the mutant should have a very high maximum specific growth rate of around 0.35 h^{-1}. Theoretically, a combination of genetic instability and selection pressure in the culture seems to define the problem of morphologic instability in using continuous cultures. In order to increase genetic stability in *A. niger* chemostats, one could try to manipulate genetic factors controlling it. We focussed initially on the selection pressure, which we tried to reduce by optimising growth conditions. We used a bioreactor specially designed to avoid wall growth with filamentous fungi, the Variomixing (Larsen et al., 2003), and monitored the

appearance of morphological mutants. Finally, the isolation of a fluffy mutant proved to be a further solution to the problem of morphologic instability. We show that such an aconidial strain is well accessible for modern genetic techniques. Using classical methods, conidiospores of an aconidial strain can be produced in a forced heterokaryon, which can serve as a proper inoculum for a liquid culture.

Materials and methods

Strains and media

Strain NW131 is derived from N400 (CBS 120.49) and carries the *cspA1* mutation affecting conidiophore height and density (Bos et al., 1988) and the *goxC17* mutation which is a glucose oxidase loss-of-function mutation (Swart et al., 1990). Fluffy mutant 982.1 was isolated from NW131 in this study and its mutation was designated *fluA1*. Masterstrain 982.2 was constructed as described by Bos et al. (1988) using mutations *cspA1, leuA1, nicA1* (Bos et al., 1988), *pyrA6* (Goosen et al., 1987), *goxC17* and Δ*argB* (Lenouvel et al., 2002) and by adding an UV induced colour mutation *fwnA22*. Strains 982.7 (*goxC17; cspA1 fluA1; niaD22*) and 982.11 (*ΔargB fwnA27; goxC17; cspA1 fluA1; leuA1*) were constructed in this study as described in Genetic manipulations. Strains 1012.9 (Δ*argB; goxC17; cspA1*, Lenouvel et al., 2002) and N402 (*cspA1*, Bos et al., 1988) were used as control strains in the colony radial growth rate measurement. In plates, synthetic minimal medium containing 1 % (w/v) glucose (MM) or complete medium (CM) was used, both described by Pontecorvo et al. (1953). To CM 5 mM uridine and 2 mM arginine was added. The MM of the ammonium-limited continuous cultures contained 50 g glucose per litre and 1.13 g L^{-1} NH_4Cl, while the glucose-limited continuous cultures contained 7.5 g glucose per litre and 4.5 g L^{-1} NH_4Cl. After germination 0.15 mL polypropylene glycol (P 2000, Fluka chemicals) per litre was added as antifoam.

Chemostat conditions

Chemostat cultures were performed in a 5 L Variomixing bioreactor especially designed for growth of filamentous fungi with minimal wall growth (Larsen et al., 2003). This was achieved by computer controlled rotation of the baffles at a similar speed and direction as the impeller for 5 s every 5 min, which resulted in a temporary cancellation of the effect of the baffles; a deep vortex and high peripheral liquid flow rates at the reactor wall then developed. When baffles were rotating slowly (5-10 rpm for 5 min) the highly turbulent flow regime characteristic of conventional bioreactors with high mixing and mass transfer capacities was developed. Temperature was controlled at $30^oC \pm 0.1$ and pH was controlled at 3.0 ± 0.05. Spores were harvested with saline water containing 9 g L^{-1} NaCl and 0.5 g L^{-1} Tween 80. After filtering through glass wool and washing in distilled water the spores were inoculated directly into the Variomixing bioreactor to a density of 10^6 spores mL^{-1}, in a working volume of 4.3 L. For proper germination 0.03 g L^{-1} yeast extract was added. During the initial germination period of 5-7 hours aeration was through the headspace at 1 L min^{-1} and stirrer rate was 450 rpm. After germination the aeration was performed through spargers at 1 L min^{-1}, stirrer speed was increased to 750 rpm, computer controlled intervals of turbulent (5 min) and laminar (5 sec) flow, characteristic of the Variomixing bioreactor, was activated and antifoam added. Before the final cell

density-limiting substrate, ammonium or glucose, was exhausted from the germination batch culture i.e. late in the exponential phase, the bioreactor was emptied, flushed once with medium and re-inoculated in 4.2 L medium by addition of 100 mL of the culture broth just sampled and kept under axenic conditions. Late in the exponential phase of this second batch the continuous medium flow was started at dilution rates between 0.05 and 0.27 h^{-1}. Ammonium and glucose were determined enzymatically essentially as described by Bergmeyer and Beutler (1985) and Bergmeyer et al. (1974), respectively.

Colony radial growth rate measurements

Colony radial growth rate measurements were performed essentially as described by Withers et al. (1995). Per strain six Petri dishes with a 9 cm diameter, containing 20 ml MM + 50 mM glucose supplemented with 20 mg/L leucine and 20 mg/L arginine were used. Fluffy strain 982.11 was inoculated using a round 2 mm diameter mycelial plug from a plate with the same medium. The *fluA+* control strains 1012.9 and N402 were stab inoculated.

Genetic manipulations

Selection of chlorate-resistant mutants was done as described by Debets et al. (1990). UV irradiated spores of 982.1 were plated on medium containing 0.2 M chlorate and 10 mM urea, resulting in the selection of nitrate non-utilizing mutant 982.7. Heterokaryosis of this mutant with masterstrain 982.2 was induced using protoplast fusion as described by van Diepeningen et al. (1998) with the modification that the enzymatic treatment of mycelium was done in 1.3 M L-sorbitol, 50 mM $CaCl_2$, 10 mM TrisHCl pH7.5 (STC, Kusters-van Someren et al., 1991). For the selection of heterokaryons on MM with nitrate, we used p. a. chemicals and no. 1 agar from Oxoid, to avoid contamination of a nitrogen source other than nitrate. This was important because the chlorate resistant mutant was dependent on the other strain only for supply of nitrate reductase. The masterstrain was dependent on the chlorate resistant mutant for the biosynthesis of leucine, nicotinamide, uridine and L-arginine, and was therefore much less likely to disturb the balance between the two different types of nuclei in the heterokaryon. Heterozygous diploids were isolated as intense black sectors of heterokaryotic colonies and haploidised by plating 100 cfu of the diploid spores on CM plates containing 66 ppm L, D-benomyl. Sporulating and fluffy colonies were transferred to masterplates using tweezers and replica plated on MM-based test medium using thick needles. The procedure to transform protoplasts was essentially as described before (Kusters-van Someren et al., 1991), using $5 * 10^6$ protoplasts and 1 µg of the *argB* gene of *A. niger* (Buxton et al., 1985).

Heterokaryon inoculation

Heterokaryons were grown on MM plates, using p.a. chemicals and no. 1 agar from Oxoid. After 5 days incubation at 30 °C spores were harvested, filtered and washed as described above. A 250 mL Erlenmeyer flask containing 100 mL MM with 1.13 g L^{-1} NH_4Cl instead of $NaNO_3$, 50 g L^{-1} glucose and 0.5 g L^{-1} yeast extract was inoculated with 10^8 spores. The proportion of both parents and of the diploid in the culture was estimated after 19 and 26 h, using two different approaches: 1) by plating dilutions of culture samples on supplemented medium (SM) and 2) by generating protoplasts by Novozyme 234 (Novo Nordisk A/S, Copenhagen Denmark) treatment of mycelium samples in STC, filtering out the protoplasts on a glass wool plug, and plating out dilutions on sucrose-stabilised SM plates. After incubation for 3 days at 30°C the colonies appearing were identified by their spore colour.

Results and discussion

Optimising growth conditions to increase morphologic stability

Genetic instability is a problem in studies using continuous cultures, disturbing the steady state (Swift et al., 1998). Also morphologic heterogeneity of the culture caused by wall growth can disturb this steady state (Topiwala, 1971). The Variomixing bioreactor design reduces the problem of wall growth, therefore, we used this bioreactor to study the problem of genetic instability in NW131 using different growth regimes. We monitored morphologic stability by taking samples at regular intervals and plating them directly on CM. After incubation the plates were checked for morphological mutants (see Table 1). The cultures were performed at pH 3 because low pH increases strain stability in *A. niger* (Mainwaring et al., 1999; Wallis et al., 1999). We used both ammonium-limited and glucose-limited cultures.

The ammonium-limited culture Ammonium-One-Dilution-rate was grown at one steady-state at a dilution rate of 0.16 h^{-1}, which resulted in a very fast appearance of the first morphological (aconidial) mutant after 110 h. This aconidial mutant resembles *A. nidulans* fluffy mutations (Dorn, 1970). Ammonium-Wash-out/Batch was performed as an alternating wash-out and batch culture. During the wash-out (Jannasch, 1969) the dilution rate exceeds the specific growth rate, avoiding nutritional limitation. The batch growth periods allow the restoration of biomass, after which we switched back to wash-out conditions, exposing the fungus to ammonium limitation for only very short periods.

The glucose-limited cultures were grown at different dilution rates. In Glucose-Chemostat-Batch-Chemostat an intermediate batch experiment was performed after 140 hours. The rationale behind both alternating growth regimes is to counteract selection of mutations only advantageous during a part of the cultivation. Both the wash-out and batch growth conditions result in selection pressure for increased maximum specific growth rate whereas steady-state growth conditions result in selection pressure for increased maximum specific growth rate and/or increased affinity for the limiting substrate (Tempest, 1970).

Morphologic mutations appeared earlier in the ammonium-limited cultures than in the glucose-limited ones, although the number of generations was almost the same in cultivation Ammonium-Wash-out/Batch compared to Glucose-Low-Medium-Low and Glucose-Low-to-High. The use of alternating growth regimes in cultivation Ammonium-Wash-out/Batch and Glucose-Chemostat-Batch-Chemostat delayed the appearance of the first morphologic mutant, especially when considering the number of generations. The condition of the chemostat phase of Ammonium-One-Dilution-Rate was a constant ammonium limitation, whereas the Ammonium-Wash-out/Batch cultivation was alternating between ammonium-limiting and non-limiting conditions. The conditions for cultivation Glucose-Chemostat-Batch-Chemostat were more alternating than for the other two glucose-

limiting cultures because the interruption by a batch phase resulted in non-limiting conditions, whereas during all phases of the other two glucose-limiting cultures the limitation remained.

In all cultures, the fluffy mutants were the main morphological mutant type found. No densely sporulating mutants or yellow pigment producing mutants were found and only a few brown mutants were observed.

Isolation of a stable fluffy mutant

A rapid appearance of a fluffy mutant was observed in cultivation Ammonium-One-Dilution-rate (Table 1). Ten generations (43 hours) after the appearance of the first fluffy mutant, it constituted approximately 99% of the culture, at which time it was isolated and purified by repeated cultivation on CM plates. This fluffy morphology appeared to be stable for the rest of the culture (600 h). A similar acondial "white" *A. niger* mutant was isolated by Swift et al. (1998, see figure 3 of their report) and was reported to have an increased colony radial growth rate. Also our fluffy or "white" mutant had a higher colony radial growth rate compared to wild type (202 ± 17 µm h^{-1} versus 152 ± 15 µm h^{-1}). Assuming the increase of the fluffy morphology from first appearance (approximately 0.1%) to approximately 99% in 43 hours is caused by an increased maximum specific growth rate only, the maximum specific growth rate of the mutant should be almost four times that of the parent. This is very unlikely. Another explanation for the rapid increase of the fluffy morphology could be an increased affinity for the limiting substrate, ammonium. However, the fluffy strains would have to have a hundredfold lower Ks, which is also very unlikely. Therefore we suggest that mutations resulting in fluffy phenotype have occurred in several cells independently during the 43 hours. The fluffy mutation in the different fluffy mutants might not be the same. We have isolated only one of these mutants and denoted its fluffy mutation as *fluA1*.

The fluffy mutant did not sporulate at 30°C, but slight sporulation was observed at the centre of a colony after a rather long incubation of 7 days at 37°C on CM. In a colony with a diameter of 8 cm, the sporulating centre had a diameter of 2.5 cm, carrying $2.5\ 10^7$ spores, which is only 1/100 of the amount required to inoculate a 2.5 L bioreactor.

In previous studies with *A. niger* chemostats grown at pH 5.4, the morphologic mutants that appeared were mainly brown conidia bearing mutants, and those were scarcely observed in cultures grown at pH 4.0 (Mainwaring et al., 1999; Swift et al., 1998 and 2000; Wallis et al., 2001; Withers et al., 1998). In these studies, Swift et al. (1998) and Withers et al. (1998) reported the appearance of white, non-sporulating mutants, but only as a small proportion of the total population and only at pH 5.4 or 5.5. It is therefore striking that we found mainly non-sporulating mutants, at pH 3.0. The difference in the morphologic mutants appearing could be caused by several factors. We used different culture conditions

with a different pH, we used a different reactor design and we used an *A. niger* strain with different genetic markers. The *A. niger* strains used in above mentioned studies as well as our strain are all descendants of N402 carrying the *cspA1* mutation conferring low conidiospores. The strain used by Mainwaring et al. (1999) was constructed from N402 via AB4.1 (van Hartingsveldt et al., 1987), a *pyrG* mutant transformed with a fusion gene of *glaA* and a cDNA encoding the mature hen egg white lysozyme. The strains used by Swift et al. (1998 and 2000), Wallis et al. (2001) and Withers et al. (1998) were constructed directly from N402 using acetamidase selection with four copies of *glaA* on the same cosmid vector. Our strain, NW131, is not transgenic and carries only one additional mutation, a glucose oxidase null mutation *goxC17* selected at a low UV dose (Swart et al., 1990).

For understanding the selective advantage of fluffy mutations it is useful to look at the situation in *A. nidulans* where sporulation has been studied quite well (Adams et al., 1998). Mutants in all stages of sporulation have been isolated, ranging from the total aconidial *fluffy* mutations (Dorn, 1970), to mutants having aberrant conidial structures such as the *bristle*, *wet* and *abacus* mutants (Timberlake, 1980). In liquid cultures, asexual sporulation is induced by nitrogen or carbon limitation (Lee & Adams, 1995; Skromne et al., 1995). The regulatory cascade leading to sporulation in *A. nidulans* starts with *fluG*, and is directed via a number of *flb* genes, towards modulation of genes at the end of the central regulatory pathway, *brlA*, *abaA* and *wetA* (Adams et al., 1998). By modulation of *fluG* Yu et al. (1996) showed that the onset of sporulation directly antagonises filamentous growth itself, which explains why most of the *A. nidulans* fluffy mutants have an increased maximum specific growth rate. Similarly, an *A. niger* fluffy mutant might also have an increased maximum specific growth rate, giving it a selective advantage at high dilution rates.

Schrickx et al. (1993) have shown that at dilution rates lower than 0.12 h^{-1} N402 starts to differentiate. In *A. nidulans*, sporulation was shown to involve the expression of many genes (Sanchez et al., 1998; Timberlake, 1980), and it is likely that this differentiation uses a considerable part of the fungal resources. Therefore, a fluffy mutant disabled in an early part of the sporulation signalling pathway is also expected to have a selective advantage at lower dilution rates. With the fluffy mutation located in an early part of the sporulation pathway, no other mutations in this pathway can arise, which probably makes the fluffy phenotype morphologically more stable than its parent.

Genetic analysis and manipulation of the *fluA1* mutant

The *fluA1* containing mutant isolated from cultivation Ammonium-One-Dilution-rate was designated 982.1. Genetic analysis of *A. niger* strains can only be done by mitotic recombination, which requires at least one auxotrophic mutation to force a heterokaryon

with a complementary auxotrophic master strain (Pontecorvo et al., 1953). For this purpose a *niaD* mutant of 982.1 was isolated, using 10^7 spores on a selection plate containing chlorate. Of the resulting mutants 21 were rescreened by inoculating pieces of mycelium on plates with different nitrogen sources. A strain fitting the phenotype of a *niaD* mutant (Debets et al., 1990) was designated 982.7 (*goxC17; cspA1 fluA1; niaD22*). Protoplast fusion of 982.7 with master strain 982.2 (*ΔargB fwnA27; goxC17; cspA1 pyrA6; leuA1; nicA1*) resulted in heterokaryotic colonies, bearing both black and fawn coloured spores. The black conidiospores originated from 982.7 cells. Therefore, the *fluA1* mutation appeared to be recessive and was complemented in a heterokaryon.

Although *A. niger* heterokaryons rarely form spontaneous diploid sectors, the heterokaryon of 982.7//982.2 produced them at high frequency. The diploids sporulated normally, indicating that the *fluA1* mutation is completely recessive in a diploid. We inoculated one representative diploid on plates with CM + benomyl and analysed 66 of

Table 1. Culture time and number of generations[a] before the appearance of the first morphological (fluffy) mutant in cultures limited in nitrogen (ammonium) or carbon source (glucose).

Culture[b]	Time (h)	Generations[a]
Ammonium-One-Dilution-rate[c]	110	27
Ammonium-Wash-out/Batch[d]	130	37
Glucose-Low-Medium-Low[e]	210	44
Glucose-Low-to-High[f]	240	40
Glucose-Chemostat-Batch-Chemostat[g]	>240	>60

[a]Total no of generations since spore germination. Duration of batch periods before start of medium flow was 8 to 12 generations (28 to 39 h, including 6 hour germination) in all cultures.

[b]30°C, pH 3.0, 750 rpm, 1 L air min^{-1}

[c]Ammonium-limited chemostat culture with a dilution rate of 0.16 h^{-1}.

[d]Alternating wash-out and batch culture with ammonium as final cell density-limiting substrate. The duration of the batch and wash-out phases were 14 and 25 h, respectively, and the dilution rate during wash-out was 0.27 h^{-1}.

[e]Glucose-limited chemostat culture with increase followed by decrease in dilution rates: 0.08, 0.12, 0.16 and 0.08 h^{-1} with a duration of 61, 33, 26 and 54 h, respectively.

[f]Glucose-limited chemostat culture with increase in dilution rates: 0.05, 0.08, 0.16, 0.20 and 0.23 h^{-1}, with a duration of 94, 55, 27, 22 and 11 h, respectively

[g]Glucose-limited chemostat culture interrupted by a batch and with the following dilution rates: 0.16, 0.05, 0, 0.08, 0.18 and 0.21 h^{-1} with a duration of 36, 70, 17, 8, 48, and 25 h, respectively.

the resulting haploids, with approximately an equal number of fluffy recombinants and normal ones. The *fluA1* mutation had a recombination frequency of 17% with *pyrA6*, locating *fluA1* on linkage group III. As expected a linkage between *ΔargB* and *fwnA27* was found, and no linkage between any of the other markers.

For a fluffy mutant to serve as a suitable host for protein production, it should be easily transformable. In order to see if the fluffy mutant indeed could be transformed using a common procedure, an arginine requiring recombinant, 982.11 (*ΔargB fwnA27; goxC17; cspA1 fluA1; leuA1*), was used in a standard transformation protocol (Kusters-van Someren et al., 1991). From 10^7 protoplasts and 1 µg pIM2101, containing $argB_{niger}$ (Lenouvel et al., 2002) 91 transformants were obtained, which is at least tenfold lower than usual. However, this transformation frequency is quite acceptable for routine transformations.

The use of conidiospores from a heterokaryon as an inoculum for a fluffy strain

Conidiospores are the preferred inoculum to obtain a proper morphology in submerged cultures. As shown above, a sufficient number of spores of the fluffy mutant is difficult to obtain because of the reduced sporulation. As described in the previous section, spores of the recessive aconidial mutant (982.7) are fairly easily obtained from a heterokaryon. Because conidiospores from *Aspergillus* are mononucleate, the spore suspension from heterokaryon 982.2//982.7 contains spores from both 982.2 and 982.7, as well as spores from the heterozygous diploid.

We wanted to investigate if such a mixture of spores could be used as an inoculum for the fluffy strain 982.7. Spores from the heterokaryon were harvested as described. To determine the proportion of the three different strains present in the inoculum and in the culture samples, samples were spread on plates. The different strains could easily be distinguished phenotypically, 982.7 as non-sporulating, 982.2 having fawn coloured spores and the diploid carrying black spores.

In the inoculum the proportion of the three different strains could be determined accurately, from the mycelium samples that was more difficult. Plating dilutions of the mycelium samples could give an overestimation of the sporulating strains because a pellet containing at least one of the sporulating strains was scored as black (diploid) or fawn (982.2). Assuming Novozyme 234 is equally active on the cell wall of all three strains, an estimation of the proportion based on plating out protoplasts would be more accurate. Surprisingly, using both methods we did not find much difference in the estimation of proportions of the different strains in the culture (data not shown). Strain 982.2 was hardly able to proliferate, which is not surprising as it lacks a proper amount of its auxotrophic growth requirements, L-arginine, uridine, L-leucine and nicotinamide (Fig 1). The diploid as prototroph had no obvious disadvantage compared to 982.7, but still decreased in proportion, indicating a lower maximum specific growth rate. This could be due to the

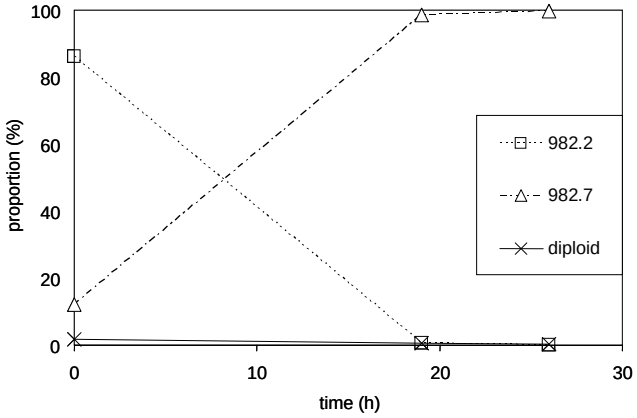

Fig. 1. Proportion of 982.2 (*fluA*+) 982.7 (*fluA1*) and diploid 982.2//982.7 in a batch culture on minimal medium containing ammonium as a nitrogen source. Estimation of proportion was done using spores at 0 h and protoplasts at 19 and 26 h.

auxotrophic mutations present in the heterozygous diploid, which, although assumed to be recessive, could also have a slightly intermediate effect. It is more likely, however, that the diploid has a lower maximum specific growth rate compared to the fluffy mutation because the beneficial effect of the recessive fluffy mutation is complemented, and therefore absent in the heterozygous diploid. The fluffy mutation appeared to be beneficial in this culture, allowing the heterokaryon to be a proper source for 982.7 inoculum.

Despite the difficulty to manipulate fluffy mutants, their morphologic stability in continuous cultures may be a great advantage. A fluffy mutant would be a suitable strain to test if increased morphological stability results in more stable production and thus would be an improved host for protein production.

Conclusions

We showed a fluffy mutant to be manageable in commonly used laboratory procedures. Using the very few conidiospores it produces we were able to select additional chlorate resistance markers and use the resulting nitrate auxotroph in a mitotic recombination. Thus adding selectable transformation markers to its genotype, we successfully transformed a fluffy strain using a regular transformation protocol. Although we expect the presence of multiple copy integrations of a gene of interest to be at least as stable as in the wild type, this still has to be investigated. Also the enzyme production and secretion properties may be different in a fluffy mutant, although Swift et al. (1998) showed a similar "white" mutant to produce even more glucoamylase compared to its parent in a batch culture, despite it

contained a lower number of glucoamylase genes. For large-scale inoculations an inoculum obtained from a forced heterokaryon was shown to be appropriate.

Acknowledgements
The Danish Research Agency financially supported BRP and the Novo Nordic Foundation financially supported TS. We thank Raymond P. Cox, University of Southern Denmark for performing competition calculations.

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Chapter 4

Onset of carbon catabolite repression in *Aspergillus nidulans*; parallel involvement of hexokinase and glucokinase in sugar signalling

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JBC (2003) 278: 11849-11857
DOI 10.1074/jbc.M209443200

Abstract

The role of hexose phosphorylating enzymes in the signaling of carbon catabolite repression was investigated in the filamentous fungus *Aspergillus nidulans*. A D-fructose non-utilizing, hexokinase-deficient (*hxkA1*, formerly designated *frA1*) strain was utilized to obtain new mutants lacking either glucokinase (*glkA4*) or both hexose kinases (*hxkA1*/*glkA4*). D-Glucose and D-fructose phosphorylation is completely abolished in the double mutant, which consequently cannot grow on either sugar. The glucokinase single mutant exhibits no nutritional deficiencies. Three repressible diagnostic systems, ethanol utilization (*alcA* and *alcR* genes), xylan degradation (*xlnA*), and acetate catabolism (*facA*), were analyzed in these hexose kinase mutants at the transcript level. Transcriptional repression by D-glucose is fully retained in the two single kinase mutants, whereas the hexokinase mutant is partially derepressed for D-fructose. Thus, hexokinase A and glucokinase A compensate each other for carbon catabolite repression by D-glucose in the single mutants. In contrast, both D-glucose and D-fructose repression are severely impaired for all three diagnostic systems in the double mutant. Unlike the situation in *Saccharomyces cerevisiae*, the hexose phosphorylating enzymes play parallel roles in glucose repression in *A. nidulans*.

Introduction

To survive among competing microorganisms in an environment with limited resources, saprophytic filamentous fungi such as *Aspergillus nidulans* and *Aspergillus niger* adapt rapidly to changing nutrient conditions. Two major control circuits, specific induction and general carbon catabolite repression (CCR), enable considerable versatility in utilizing a wide range of carbon sources while preferentially consuming readily available substrates of

high nutritional value before less accessible ones. In *A. nidulans*, CCR is ultimately mediated by the DNA-binding transcriptional repressor CreA, which prevents activation of the catabolism of less preferred carbon sources if a more favorable growth substrate is available (Arst and Cove 1973) (reviewed in Arst and Bailey 1977; Felenbok and Kelly 1996; Felenbok et al., 2001). CreA function somewhat resembles that of Mig1p, the main mediator of D-glucose repression in *Saccharomyces cerevisiae* (Nehlin and Ronne 1990) (reviewed in Gancedo 1998). The presence of a high concentration of a repressing growth substrate, not restricted to D-glucose, is necessary to trigger the CCR response.

Much is known about the targeting of transcriptional control of nutrient utilization in *A. nidulans*, the molecular means by which induction and repression are imposed on the promoter regions of genes subject to catabolic regulation. In the inducible ethanol utilization pathway, the functional cis-acting elements conferring induction and repression, the target sequences of the DNA-binding activator AlcR and the general CCR-repressor, have been identified in the regulatory *alcR* gene encoding the pathway-specific activator and the structural genes for alcohol dehydrogenase I and aldehyde dehydrogenase, *alcA* and aldA, respectively (reviewed in Felenbok et al., 2001). In this model system, various mechanisms by which induction and repression of induction are mediated were evidenced, and a subtle interplay between the two regulatory circuits was shown to fine-tune the expression of each of these three genes in distinct ways (Fillinger et al., 1995; Flipphi et al., 2001; Mathieu and Felenbok 1994; Mathieu et al., 2000; Panozzo et al., 1997; Panozzo et al., 1998). Functional regulatory target sites for CreA have also been identified in two other catabolic systems, the xylanase gene *xlnA* and the proline permease gene *prnB* (Arst and MacDonald 1975; Cubero and Scazzocchio 1994; Cubero et al., 2000; Orejas et al., 1999).

Far less is known about the means by which the CreA repressor becomes functional in response to repressing carbon sources and how the repressional regulatory circuit adapts to changing nutrient conditions, e.g. upon exhaustion of a preferable carbon source. It has been shown that transcription of the *creA* gene itself is negatively autoregulated in response to repressing carbon sources, leading to a reduced steady-state *creA* transcript level (Strauss et al., 1999). However, the CCR-repressor function appears to be mainly controlled at the post-transcriptional or post-translational level (Cziferszky et al., 2002; Strauss et al., 1999; Vautard-Mey et al., 1999a; Vautard-Mey and Fèvre 2000). In *A. nidulans*, strains mutant in two additional genes, *creB* and *creC*, exhibit some derepressed characteristics similar to those observed in loss-of function *creA* mutants but also show a number of phenotypes not related to CCR (Arst 1981; Hynes and Kelly 1977). These two genes have been characterized recently and, interestingly, *S. cerevisiae* does not appear to harbour any close homologues (Lockington and Kelly 2001; Todd et al., 2000).

Still less is known about the sensing of repressing compounds and the means by which such compounds trigger the CCR response in filamentous fungi. In *S. cerevisiae*, an

important role in the glucose-sensing process has been ascribed to hexokinase Hxk2p, an enzyme catalyzing the first step in glycolysis and glucose fermentation, phosphorylation of D-glucose at C6 (reviewed in Gancedo 1998). Baker's yeast actually specifies three enzymes capable of this phosphorylation, hexokinases (Hxk: EC 2.7.1.1) Hxk1p and Hxk2p, and glucokinase (Glk: EC 2.7.1.2) Glk1p (Lobo and Maitra 1977). Any one suffices for growth on glucose, but Hxk2p is the main activity for phosphorylating glucose because it is predominantly expressed during fermentation (Gancedo et al., 1977; Rodriguez et al., 2001). Concomitantly, only this isozyme is essential for repression of catabolism of alternative carbon sources such as sucrose and maltose; loss-of-function *hxk2* mutants are defective in glucose repression mediated by Mig1p (Entian 1980) (reviewed in Refs. Gancedo 1998; Rolland et al., 2002). The mechanism by which Hxk2p participates in glucose repression remains obscure to date. In general, a strong correlation is found between the capacity of mutant Hxk2p to phosphorylate glucose or fructose and CCR by these two sugars (Ma et al., 1989; Rose et al., 1991). However, catalytic activity of Hxk2p might not be essential for glucose repression; rather, signal transmission might be linked to Hxk2p conformational changes induced by the sugar and ATP (Hohmann et al., 1999; Kraakman et al., 1999; Mayordomo and Sanz 2001).

In the filamentous fungus *A. nidulans*, hexose phosphorylation was previously studied in the D-fructose non-utilizing *frA1* ("fructokinase") mutant (Roberts 1963;Ruijter et al., 1996). This mutant lacks Hxk activity but, unlike the situation in *S. cerevisiae*, its glucose CCR of ethanol and L-arabinose catabolism appeared fully functional. Here, we have utilized three carbon utilization systems, ethanol (*alcA* and *alcR* genes), xylan (*xlnA*), and acetate (*facA*), to investigate the role of hexose kinases in CCR at the transcript level. The previously mentioned Hxk mutant (*frA1*, herein renamed *hxkA1*) as well as newly obtained Glk (*glkA4*) and hexose kinase double mutants (*hxkA*/ *glkA4*) were studied. We show that the two hexose kinases play parallel roles in glucose repression in the model organism *A. nidulans*.

Experimental procedures

A. nidulans Strains, Media, and Growth Conditions

A. nidulans strains used in this study are listed in Table 1. The references refer to the mutations relevant to this work. Other markers are in standard use (Clutterbuck 1993). Media composition, supplements, and basic growth conditions at 37 °C were as described by Cove (Cove 1966), using di-ammonium tartrate (5 mM) as the nitrogen source and the various carbon sources at 1% (w/v or v/v), unless stated otherwise. Conidiospores were obtained on solidified complete medium with either glycerol or sodium D-gluconate as the carbon source. Mycelia for enzyme assays were grown on glycerol minimal medium for 16 h and then transferred to fresh minimal medium containing 1% (v/v) ethanol and 1% (w/v) D-glucose or D-fructose and incubated for another 4 h prior to harvesting.

Table 1. *Aspergillus nidulans* strains used in this study

Strain	Genotype	References for characterized mutation or strain
BF054	*yA2 pabaA1*	
BF110	*biA1 creA*^{d} *30*	56, 66
C62	*pabaA1*	
CEA54	*pyrG89; wA1; pyroA4;* Δ*treA*::*neo*	67
G092	*yA2; pyroA4 hxkA1* (T1 (IV, VIII))	35, 36
NW193	*pabaA1; glkA4; hxkA2*	This work
NW298	*yA2; glkA4; pyroA4; hxkA1* (T1 (IV, VIII))	This work
NW299	*yA2; glkA4; pyroA4*	This work
NW300	*pabaA1; glkA4*	This work
NW301	*pabaA1 pyrG89; wA3; argB2 galA1; facA303; sB3; nicB8; riboB2*	This work
NW303	*glkA4; pyroA4 hxkA2;* Δ*treA*::*neo*	This work
NW330	*biA1 creA*^{d} *30; glkA4; hxkA2*	This work

The trehalase gene disruption is described in d'Enfert and Fontaine (1997). The diploid used for parasexual analysis was isolated from a heterokaryon of strains NW301 and NW303. Roman numerals indicate the chromosomes involved in the *hxkA1*-associated translocation.

Mycelia for the analysis of *alc* and *facA* transcription were grown for 10–12 h in minimal medium with glycerol as the carbon source and urea (5 mM) as the nitrogen source. For the analysis of *xlnA* transcription, sodium D-gluconate replaced glycerol and the incubation time was extended to 40 h. Induction was achieved by the addition of the inducer compounds specific for the three diagnostic systems examined, 2-butanone to 50 mM (final concentration) for *alc*, D-xylose to 50 mM for *xlnA*, or sodium acetate (pH 6.8) to 10 mM for *facA*, respectively. Cultures were harvested after 2.5 h of further incubation (inducing conditions). For repressed conditions, D-glucose or D-fructose was added simultaneously with the inducer to a final concentration of 1% (w/v) (i.e. 55 mM). Non-induced mycelia were grown in the initial growth media during the induction period.

Mutagenesis and Genetic Techniques

Conventional genetic techniques were employed (Clutterbuck 1974). Following UV mutagenesis of a suspension of 10^6 conidiospores of G092 per ml, the survival rate was ~25%. Selection for resistance to 50 mg/L 2-deoxy-D-glucose (2DOG) was done in the presence of 1% (v/v) glycerol as carbon source and 0.08% (w/v) sodium desoxycholate to reduce colony size. Resistant colonies were allowed to develop for 6 days at 37 °C, and mutants were purified using the same medium. Wild-type strain C62 was used to cross out the *hxkA1* (*frA1*) translocation in NW298 to yield single glucokinase (*glkA4*) mutants NW299 and NW300. Genetic mapping of the *glkA4* mutation was only possible in a Hxk-deficient background (see “Results”). 2DOG resistant strain NW193 (*glkA4 hxkA2*) was crossed to CEA54 to exchange auxotrophic markers to facilitate the formation of a diploid between the progeny strain NW303 and the tester strain NW301, utilized in parasexual analysis to localize the *glkA4* and *hxkA2* mutations (see Table 1).

Enzyme Assays

Cell-free extracts were prepared from frozen mycelia in liquid nitrogen as described previously (Ruijter et al., 1996). All enzyme assays were done at 25 °C. Glucose- and fructose-phosphorylating activities in crude extracts were determined as described by (Ruijter et al., 1996). To distinguish better between in vitro Hxk and Glk activities, assays were performed in both the absence and presence of the Hxk-specific inhibitor trehalose-6-phosphate (Blazquez et al., 1993). At 1 mM, the inhibitor reduced the measured Hxk activity to about 1/10 of its actual value (see Table 2, compare with activities in the Glk mutant *glkA4*).

Isolation of RNA and Northern Blot Analysis

Total RNA was isolated from about 250 mg of mycelial powder, obtained by grinding mycelia in liquid nitrogen, with RNA Plus extraction solution (Qbiogene) following the manufacturer's instructions. It was further purified by precipitation in 3 M sodium acetate, pH 6.0, for 2 h at -20 °C. The precipitate was collected by centrifugation, and residual salt was removed by conventional alcohol precipitation. Northern analysis was carried out with 15 µg samples of glyoxal-treated total RNA (Sambrook et al., 1989) using Hybond N membranes (Amersham Biosciences). Membranes were hybridized with 32P-labeled probes synthesized from DNA fragments from the cloned *A. nidulans* genes *alcA* (Panozzo et al., 1998), *alcR* (Felenbok et al., 1988), *xlnA* (Perez-Gonzalez et al., 1996), *facA* (Sandeman and Hynes 1989), and *acnA* (γ-actin) (Fidel et al., 1988). Autoradiographs were exposed for various time periods to avoid film saturation. Intensities of the hybridization signals were quantified using a Phosphor Imager (Amersham Biosciences). The γ -actin gene was used to normalize the data from a single membrane. For *alc* and *facA*, panels A and C of Figs. 1 and 4–7 originate from a single membrane, enabling direct comparison among all principal strains. For *xlnA*, panels B of Figs. 1 and 4–6 originate from a single membrane, allowing direct comparison between wild type and the three different hexose kinase mutants. All expression experiments were repeated at least twice.

Results

Only mutants impaired in both GlkA and HxkA activity cannot phosphorylate glucose.

To investigate the involvement of hexose phosphorylation in the signaling of D-glucose repression in filamentous fungi, we obtained new mutants lacking either Glk or both Glk and Hxk by classic means. D-Fructose non-utilizing Hxk-deficient *hxkA1* (*frA1*) mutant strains are unable to phosphorylate fructose but grow quite well on glucose by virtue of Glk activity (Ruijter et al., 1996). The selection of Glk mutations in a *hxkA1* strain was based on increased resistance to the toxic antimetabolite 2DOG in the presence of glycerol as sole carbon source. This glucose analogue is phosphorylated by either hexose kinase but cannot be catabolized any further (Brown and Romano 1969).

Among the 2DOG-resistant mutants, several were unable to grow on glucose. The glucose non-utilizing mutant exhibiting the lowest residual glucose phosphorylating activity

was chosen for further analysis. Fructose-utilizing (*hxkA*$^{+}$) progeny from an outcross grew normally on glucose and were tested for hexose phosphorylating activities in crude extracts as compared with wild type, the 2DOG-resistant glucose non-utilizing parent, and the single *hxkA1* mutant. Some of the glucose- and fructose-utilizing progeny clearly lacked Glk activity (see Table 2). The mutation resulting in the Glk lesion was designated *glkA4*. Other glucose and fructose non-utilizing mutants were selected for 2DOG resistance in an outcrossed *glkA4* single mutant. Parasexual analysis of one such strain allocated *glkA4* to chromosome III and the newly selected *hxkA2* mutation, as expected, to chromosome IV (results not shown).

We can conclude that only hexose kinase double mutants are unable to grow on glucose as sole carbon source. In contrast to the fructose non-utilizing Hxk mutants, the Glk mutant strains do not show any obvious nutritional deficiencies. Table 2 shows high levels of glucose and fructose phosphorylating activities in a *glkA4* strain that are decreased drastically in the presence of the Hxk inhibitor trehalose-6-phosphate. This strongly suggests that Hxk can compensate the lack of Glk activity, allowing *glkA4* mutants to grow normally on glucose. Similarly, Glk appears to compensate the absence of Hxk in *hxkA1* for glucose phosphorylation and growth because reasonable levels of glucose phosphorylation were measured in the absence and presence of trehalose-6-phosphate (Table 2). In agreement with nutritional phenotypes, only the double sugar kinase double mutant (*hxkA1*/*glkA4*) is unable to phosphorylate either hexose (Table 2).

Defining the conditions to trigger CCR using three different catabolic systems.

Ethanol utilization—The ethanol utilization (*alc*) pathway is convenient for studying the signaling of CCR because of its highly inducible structural (*alcA*) and regulatory (*alcR*) gene expression, the marginal levels of non-induced (constitutive) expression, and the high repressibility by CreA in the presence of repressing carbon sources like glucose, fructose, and D-xylose (Fig. 1A). Competition between the pathway-specific activator AlcR and the general repressor CreA occurs in the *alcA* and *alcR* genes under all conditions of growth; *alcR* expression is, in addition, subject to direct repression by CreA (Fillinger et al., 1995; Mathieu and Felenbok 1994; Mathieu et al., 2000; Panozzo et al., 1998). A further advantage is the availability of an efficient gratuitous inducer, 2-butanone (Flipphi et al., 2002).

Xylan degradation—The gene encoding xylanase A (X_{22}) in *A. nidulans*, *xlnA* (Perez-Gonzalez et al., 1996), was chosen as the second system (Fig. 1B). *xlnA* is one of the few genes in which a functional target site for CreA has been identified (Orejas et al., 1999). In addition, the *xlnR* regulatory gene, encoding the pathway-specific activator, is most likely subject to CreA-mediated repression (Orejas et al., 2001). For expression of *xlnA*, induction is absolutely required. The simplest inducer, D-xylose (the monomer of

Table 2. Phosphorylation of glucose and fructose in extracts of various *A. nidulans* strains

Relevant genotype	Carbon source	Phosphorylation activity			
		Glucose		Fructose	
		-T6P	+T6P	-T6P	+T6P
Wt	Glucose	438	189	582	96
	Fructose	431	153	622	88
hxkA1	Glucose	99	101	10	17
	Fructose	126	123	18	16
glkA4	Glucose	599	55	1084	135
	Fructose	667	54	1054	129
glkA4 hxkA1	Glucose	0	7	1	9
	Fructose	9	3	8	2

Phosphorylation of 1 mM glucose or 10 mM fructose was measured in extracts as described under "Experimental Procedures." Activities were determined in the absence and presence of 1 mM trehalose 6-phosphate (T6P) and are given in nmol (min.mg protein)$^{-1}$. Values are means from duplicate experiments. The transfer media contained ethanol (1 % v/v) in addition to the indicated sugar to avoid starvation response in the *hxkA1* and *glkA4*/*hxkA1* mutant strains.

xylan), is highly metabolizable and is strongly repressing at high concentrations (Arst and Cove 1973), as confirmed by transcript analyses in *alc* (M. Flipphi and B. Felenbok, unpublished data). This pentose requires specific transport (see below). The xylanase system is extremely sensitive to repression. D-Gluconate as carbon source for growth allows reproducible glucose-repressible, xylose-inducible expression of *xlnA* in wild type but, as will be shown below, nevertheless exerts significant repression.

Acetyl-CoA synthetase—The third system chosen is the *facA* gene, encoding acetyl-CoA synthetase catalyzing the first step of acetate catabolism in *A. nidulans* (Armitt et al., 1976;Sandeman and Hynes 1989). This gene features expression characteristics completely different from those of *xlnA* (Fig. 1C). Glycerol was used as growth substrate. As on many other carbon sources, *facA* is constitutively expressed to considerable levels on glycerol, but wild-type strains remain clearly inducible by acetate and glyoxylate (to between 2- and 3-fold the basal level). We utilized 10 mM acetate (pH 6.8) as inducer, because higher concentrations reduce general transcription efficiency as characterized by lower γ-actin transcript levels (Flipphi et al., 2002). The true inducer of *facA* expression is not acetate nor glyoxylate but more likely acetyl-CoA, a key intermediate of cellular metabolism (Hynes and Kelly 1977, M. Flipphi and B. Felenbok unpublished data). In contrast to the basal level, the acetate-induced *facA* expression appears completely repressible by glucose and fructose in the wild type. Although repression of induced *facA*

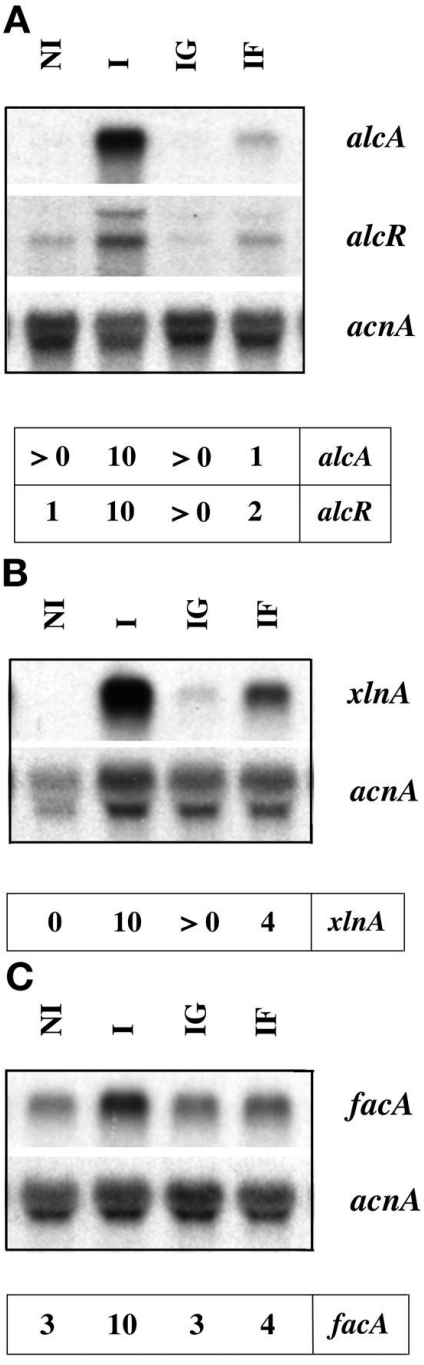


Fig. 1. Transcript analyses of CRR with three inducible and carbon catabolite-repressible diagnostic systems in an *A. nidulans* wild-type strain.

Mycelia were grown on either glycerol (A and C) or sodium D-gluconate (B) (non-induced conditions, NI), and the indicated effectors were added to cultures as either the inducer compound only (induced conditions, I) or both inducer and repressor compounds simultaneously (repressed conditions, IG for D-glucose and IF for D-fructose), as detailed for each of the individual systems under "Experimental Procedures." Growth culture conditions, RNA isolation, and Northern blots were as described under "Experimental Procedures." A, Northern blot analysis of the ethanol utilization pathway, i.e. the *alcA* and *alcR* genes induced by 50 mM 2-butanone. B, Northern blot analysis of xylanase A (X_{22}) gene (*xlnA*) expression induced by 50 mM D-xylose. C, analysis of the acetyl-CoA synthetase gene (*facA*) expression induced with 10 mM acetate. Northern blots were hybridized with 32P-labeled probes hybridizing to *alcA*, *alcR*, *xlnA*, and *facA* genes, respectively, and the -actin gene (acnA). The latter gene served as an internal control to normalize the amounts of mRNA. Quantification of the expression levels was done with a PhosphorImager and is given underneath. The transcript level under induced conditions was set arbitrarily at 10. The relative quantitative data are given to the nearest integer; any weaker expression levels revealed are indicated as > 0. The data from two independent experiments show a maximal variation of 25%.

expression appears to depend on CreA (see below), no functional analysis of CreA target sites has been reported.

The onset of induction is faster than that of carbon catabolite repression.

To establish appropriate conditions to study the effects of hexose kinase mutations on carbon catabolite repression, it was important to analyze the time course of induction versus repression for the CCR-regulated systems.

For *xlnA*, a short induction period appears desirable because the inducibility drops sharply with time when using a high concentration of xylose as inducer of medium-shifted mycelia (MacCabe et al., 1998). This feature could result from "self " CCR by the inducer compound, xylose, consistent with observations for xylanolytic genes in *A. niger*, for which the inducibility drops with increasing xylose concentration (de Vries et al., 1999). In the absence of medium shift, the inducibility does not decline drastically with time or with increasing xylose concentrations, although the *xlnA* transcript levels are lower than those reported by (MacCabe et al., 1998) (results not shown). Fig. 2A shows that an induction period of 1 h was not sufficient to observe any repression by fructose, whereas some *xlnA* repression could be observed after 2.5 h. Comparison of these two induction periods in wild type for the alc genes clearly shows that induction by 2-butanone had been established within 1 h, whereas repression by fructose and also that by glucose was far from complete at that time (Fig. 2B).

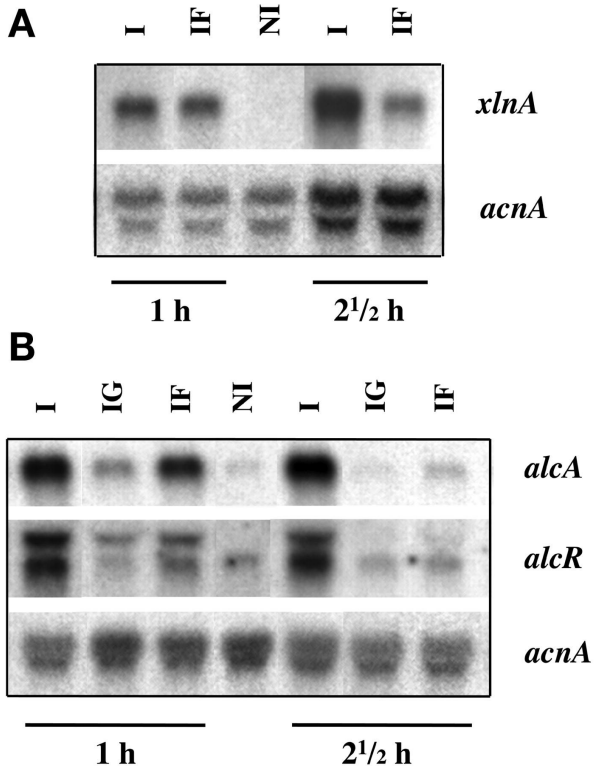
Induction thus occurs faster than repression. For the alc system, induction is a rather straightforward process, requiring the binding of AlcR to DNA targets in the presence of the physiological inducer, acetaldehyde (reviewed in Felenbok et al., 2001). Our results suggest that onset of transcriptional repression requires several steps such as sugar uptake, formation of a physiological repressor, post-translational modification of CreA, and possibly indirectly involved proteins such as CreB and CreC. For transcript analysis, we have chosen the longer time period of 2.5 h to properly evaluate induction and repression of all three systems.

Inducer exclusion accounts for one apparent form of CCR of *xlnA* expression.

A very straightforward way to prevent the expression of an inducible catabolic system is by inducer exclusion, i.e. blocking entry of the inducer. Inducer exclusion can result from direct CreA-mediated repression of the gene encoding the specific permease of the repressible catabolic system. This is the principal mechanism of repression of the structural genes of proline catabolism in *A. nidulans* (Arst et al., 1990; Cubero et al., 2000).

The regulation of the *xlnA* gene illustrates inducer exclusion. 5 mM xylose is sufficient to induce *xlnA* in wild type, and, as expected, *xlnA* expression is completely prevented in the presence of 1% glucose (results not shown). This is, however, not related

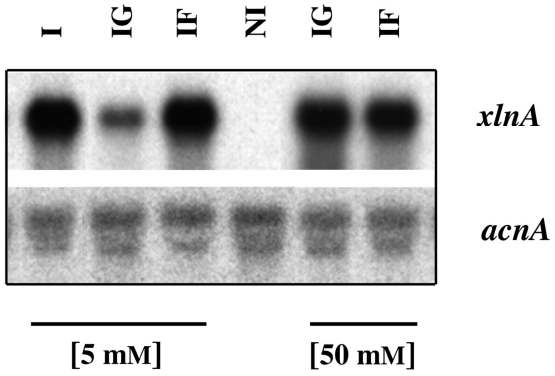
Fig. 2. Induction is established faster than repression.



Northern blot transcript analysis in a wild-type strain to determine an appropriate induction and repression period. A comparison between the impacts of sugar repression after 1 h (left) and 2.5 h (right) of exposure to both inducer and repressor compounds. A, Northern blot analysis of the repression by fructose of induced *xlnA* expression (IF). B, Northern blot analysis of the repression by glucose (IG) and fructose (IF) of the ethanol utilization pathway (*alcA* and *alcR*). All experimental details were as described in the legend to Fig. 1.

to any action of CreA because in the strongly derepressed mutant *creA^{d}30*, expression of *xlnA* is also prevented under these conditions (Fig. 3). The presence of fructose has no such effect. Furthermore, this phenomenon did not occur in the *creA^{d}30* strain when equimolar (50 mM) amounts of both xylose and glucose were present, although *xlnA* was fully repressed in wild type under these conditions (not shown). We therefore avoided inducer exclusion by employing the higher xylose concentration (50 mM). Presumably, the inducer exclusion is caused by a direct inhibition of xylose transport by the structurally related sugar glucose. Xylose inhibition of 2DOG transport in *A. nidulans* has been reported (Mark and Romano 1971).

Fig. 3. Evidence for inducer exclusion of xylose in the presence of glucose as repressor.



Northern blot analysis was performed in a derepressed *creA* ^{d}*30* strain in the presence of two xylose concentrations, 5 mM (left) and 50 mM (right). The repressor compound, glucose or fructose, was present at 55 mM (1% w/v). Experimental details were as described in the legend to Fig. 1

The effect of single hexose kinase lesions on CCR by glucose and fructose: hexokinase is required for full fructose repression.

The lack of hexokinase in the *hxkA1* mutant does not prevent glucose repression in any of the three systems at the transcript level (Fig. 4). However, the Hxk-deficient strain is clearly derepressed in the presence of fructose, the sugar that is neither phosphorylated nor catabolized by this mutant. This indicates that Hxk at least plays some role in the transmission of the repression signal for fructose. In *S. cerevisiae*, elimination of Hxk activity by the deletion of both the Hxk-encoding genes, HXK1 and HXK2, likewise leads to derepression on fructose (de Winde et al., 1996).

Interestingly, from the analysis in alc, clearly this derepression is far from complete (Fig. 4A). Partial derepression is consistent with the ability of *hxkA1* mutants to be suitable for selection of CCR-derepressed *creA* mutants such as *creA* ^{d}*30* (Arst et al., 1990). This strongly suggests that, in the absence of hexokinase, another factor, devoid of apparent fructose phosphorylating activity, can partly fulfil the regulatory function of Hxk with respect to CCR by fructose and its precursors.

One possible candidate for this regulatory factor would be glucokinase, a hexose phosphorylating enzyme with a narrower substrate range apparently constitutively produced (Table 2). However, the single Glk mutant *glkA4* exhibits wild-type repression by both fructose and glucose for all three systems (Fig. 5). It would thus appear that Glk integrity is not important for CCR in *A. nidulans*. In *S. cerevisiae*, Glk does not fulfil any regulatory

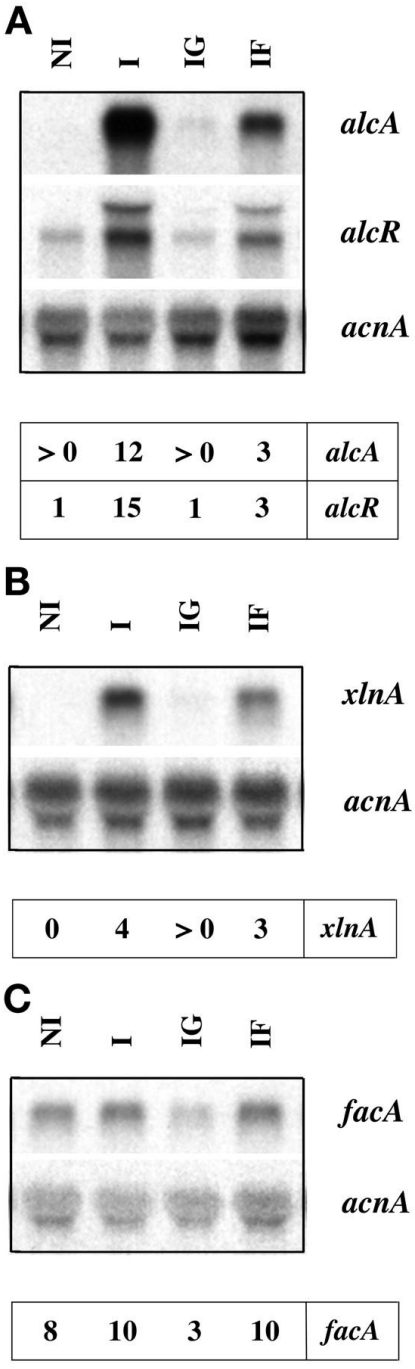


Fig. 4. The effect of a hexokinase lesion on CCR of the three systems: wild-type repression for glucose but derepression for fructose.

Transcript analyses of induction and repression of the three reporter systems in a hexokinase single mutant (*hxkA1*) strain. A, *alcA* and *alcR* transcripts. B, *xlnA* transcript. C, *facA* transcript. Quantification is relative to the wild-type levels under induced conditions as shown in Fig. 1. Experimental details were as described in the legend to Fig. 1.

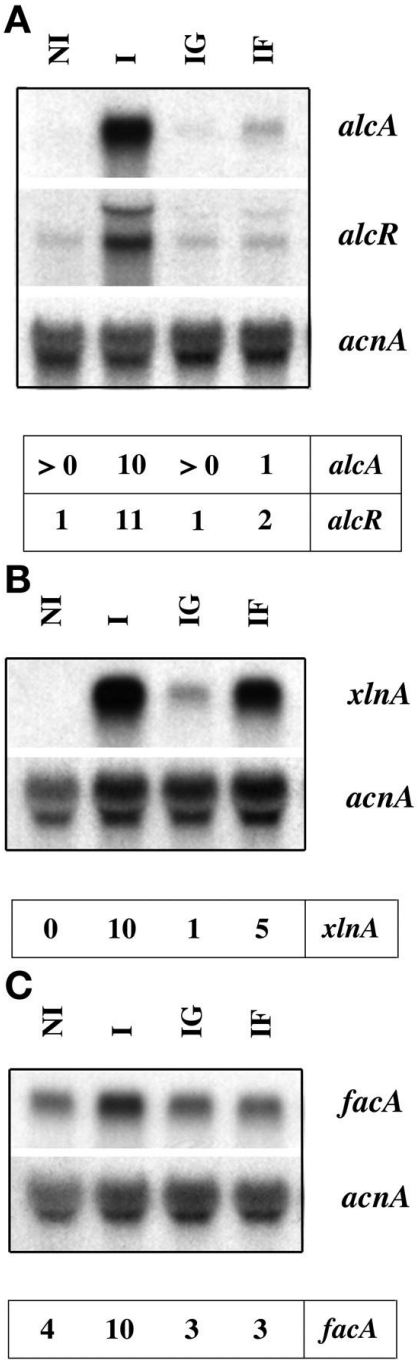


Fig. 5. Lack of glucokinase activity does not affect CCR repression characteristics of the three systems.

Transcript analyses of induction and repression of the three reporter systems in a glucokinase single mutant (*glkA4*) strain. A, *alcA* and *alcR* transcripts. B, *xlnA* transcript. C, *facA* transcript. Details were as described in the legends to Figs. 1 and 4.

function in either glucose or fructose repression, even when artificially overexpressed (Rose et al., 1991; de Winde et al., 1996; Ma et al., 1986).

Derepression in hexose kinase double mutants: hexokinase HxkA and glucokinase GlkA compensate each other for CCR by glucose.

In the *hxkA1*/*glkA4* double mutant all three systems are derepressed for both glucose and fructose (Fig. 6). The translocation-free *hxkA2*/*glkA4* double mutant gave identical results for the alc system derepression (not shown). This excludes the possibility that derepression involved a consequence of a translocation breakpoint other than the translocation associated with the *hxkA1* mutation. The derepression of the *alcR* and *alcA* genes on fructose is almost complete (80–100%), whereas on glucose it is about 50%. The *xlnA* and *facA* genes are completely derepressed both on fructose and glucose. However, an unexpected observation for *xlnA* is that its induced expression is decreased considerably. Furthermore, for *facA*, the basal level expression is increased, whereas the steady-state induced level remains similar to that in the wild type. The reason for this increased *facA* basal level is unknown.

Comparison of the derepression of the alc genes on fructose in the single *hxkA1* mutant and in both hexose kinase double mutants shows that derepression in the absence of both hexose phosphorylating enzymes is virtually complete, whereas that in the single hexokinase-deficient strain is only partial (Figs. 4A and 6A and data not shown). For signaling by the ketosugar, glucokinase appears to partially compensate the Hxk deficiency. Our results suggest that Glk is either directly or indirectly involved in fructose repression in *A. nidulans*, despite the fact that fructose is not a relevant catalytic substrate for the enzyme (see Table 2). A direct involvement of Glk in fructose repression in *A. nidulans* would unambiguously distinguish the regulatory function of hexose phosphorylating enzymes in CCR-related signal transmission from the catalytic activity with regard to this sugar.

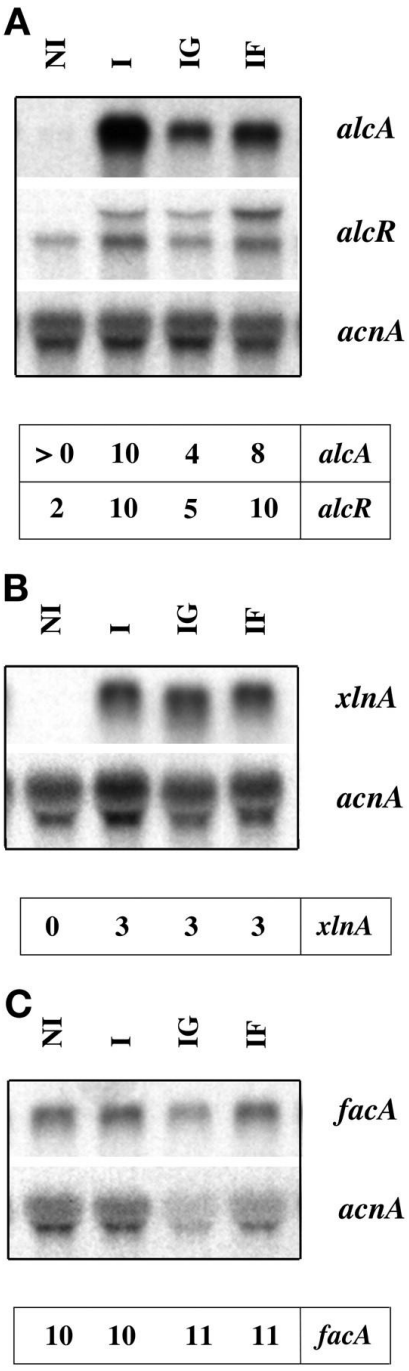
Derepression in hexose kinase double mutants put in perspective: comparison with the strongly derepressed *creA^{d}30* mutant.

We compared the levels of transcriptional derepression of the three diagnostic systems in the hexose kinase double mutants and in a *creA*-derepressed strain. Nearly complete derepression of the alc genes is achieved either in an extreme *creA* mutant such as *creA^{d}30* (Mathieu and Felenbok 1994) or by disrupting functional CreA targets in responsive promoters (Panozzo et al., 1997; Mathieu et al., 2000). The observed “superinduction” of *alcA* and *alcR* expression is a direct result of the absence of promoter binding competition between AlcR and CreA that normally occurs under all physiological conditions.

In Fig. 7, the superinduction in the *creA^{d}30* strain is evident for all three systems. In no case does the level of expression observed in the double hexose kinase mutant on glucose and fructose equal that in the *creA^{d}30* strain. For *facA*, the basal (non-induced)

Fig. 6. Derepression of induced expression in the three systems for both fructose and glucose in a hexose kinase double mutant.

Transcript analyses of a *glkA4*/*hxkA1* strain. A, *alcA* and *alcR* transcripts. B, *xlnA* transcript. C, *facA* transcript. Details were as described in the legends to Figs. 1 and 4.



level is elevated in the *creA* mutant, but the gene CreA-AlcR binding competition, whereas the more modest effect of the double hexose kinase deficiency suggests it does not. This indicates that hexose kinases are not involved in CreA-AlcR binding competition and thus that CreA can bind its target sites in the absence of sugar signaling.

For *xlnA*, superinduction in the *creA*^{d}*30* strain is striking, at least 100-fold greater than in the wild type (Figs. 7B and 8). Apparently, *xlnA* is extremely sensitive to CCR, not only by repressing compounds such as glucose and fructose but also by compounds generally considered non-repressing for other catabolic systems such as ethanol utilization, e.g. glycerol, D-gluconate and L-glutamate (Arst et al., 1973; Arst et al., 1977) (results not shown). We were in fact unable to find a completely derepressing, non-inducing carbon source for the analysis using *xlnA*. Gluconate was eventually selected because it allowed reproducible glucose-repressible and xylose-inducible *xlnA* expression in the wild type (see Fig. 1B). Hence, derepression observed in the hexose kinase double mutant appears to concern only the additional repression from glucose or fructose over the "basal" repression from gluconate and the inducer, xylose.

Reduced inducibility of *xlnA* in hexokinase mutants, an indication of positive involvement of hexokinase in xylose induction

An interesting observation is the considerable reduction (3-fold) in xylose-induced expression of *xlnA* in the single hexokinase and hexose kinase double mutants, but not in the glucokinase mutant (Figs. 1B, 4B, 5B, and 6B). Two hypotheses could explain this observation. First, *xlnA* repression by xylose might be elevated in mutants lacking Hxk, thereby decreasing the level of expression. Alternatively, xylose induction of *xlnA* might be lower because of the Hxk lesion. To distinguish between these hypotheses, we analyzed xylose-induced *xlnA* steady-state transcription in a derepressed triple mutant (*creA*^{d}*30*/*glkA4*/*hxkA1*). If the first hypothesis were correct, the inducibility should be restored to the level observed in the *creA*^{d}*30* single mutant, 100-fold higher than that in the wild type. In the second case, reduced inducibility should still be seen in the triple mutant. Revealingly, the xylose-induced *xlnA* level in the triple mutant is clearly lower than that in the single *creA*^{d}*30* mutant (Fig. 8), although the induced level of *xlnA* transcript is still elevated because of the absence of functional CreA (30-fold greater than in wild type) (Table 3). Hence, the reduced (3-fold) inducibility observed in Hxk mutants is maintained in a truly derepressed background. This suggests that Hxk has a CreA-independent, positive role in xylose induction of *xlnA*.

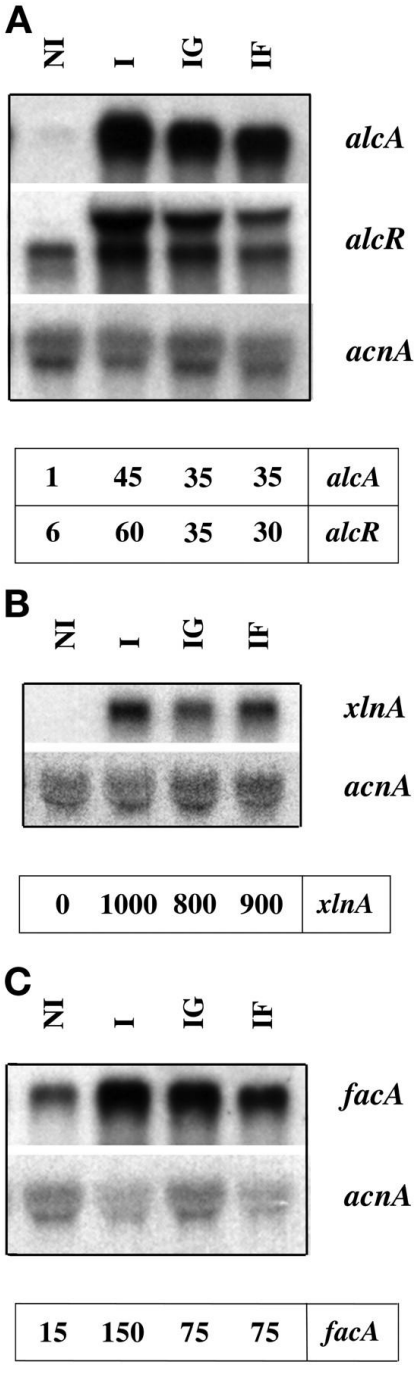


Fig. 7. Derepression and superinduction in absence of CreA.

Transcript analyses of induction and repression of the three systems in a strongly derepressed *creA* ^{d}*30* strain. A, *alcA* and *alcR* transcripts. B, *xlnA* transcript. C, *facA* transcript. Direct (single blot) comparison of *xlnA* expression in the *creA* ^{d}*30* strain with that in the other strains is not possible because of the considerable differences in expression level (see Table 3). The relative data below the *xlnA* Northern blot were determined using a dilution range of the *creA* ^{d}*30* xylose-induced RNA sample. Details were as described in the legends to Figs. 1 and 4.

Table 3. Relative induced levels of *xlnA* transcript in different mutant contexts

wt	*hxkA1*	*glkA4*	*glkA4* /*hxkA1*	*creAd30*	*creAd30* /*glkA4* /*hxkA1*
10	3	10	3	1000	300

Induction by 50 mM xylose was as described under "Experimental Procedures." The amounts of hybridized mRNA from different Northern blots (Figs. 1 and 4-8) were quantified by densitometry scanning with a PhosphorImager. Data correspond to: wild type (wt) in Fig. 1B; *hxkA1* in Fig. 4B; *glkA4* in Fig. 5B; *glkA4*/*hxkA1* in Fig. 6B; *creA* ^{d}*30* in Figs. 7B and 8; and *creA* ^{d}*30*/*glkA4*/*hxkA1* in Fig. 8. The values represent the amount of *xlnA* mRNA in each strain relative to the amount of -actin mRNA as an internal control. The amount of wild type *xlnA* mRNA was arbitrarily set at 10.

Discussion

Hexose kinase single mutants are not affected in glucose-mediated CCR

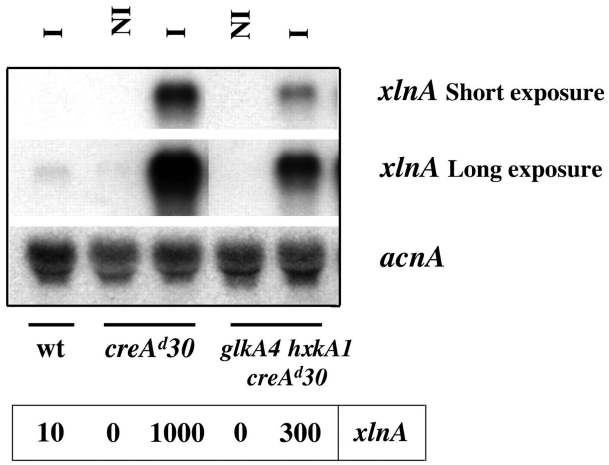
Carbon catabolite repression in the filamentous fungus *A. nidulans* appears related to the capacity to utilize a given carbon source. The three repressible systems monitored here, ethanol catabolism (*alcA* and *alcR*), xylanase A (*xlnA*), and acetyl-CoA synthetase (*facA*), respond in a quantitatively similar manner in the different hexose kinase mutant backgrounds.

hxkA mutants have derepressed steady-state transcript levels for the three systems vis-a-vis fructose while being unable to utilize it. On the other hand, *hxkA* mutants grow normally on glucose, whereas the three diagnostic systems remain repressed in its presence, in sharp contrast to the situation in *S. cerevisiae* where Hxk2p is essential for glucose repression

(but not for growth on glucose) (reviewed in Ganceno 1998 and Rolland et al., 2002). The hexokinase mutant produces glucokinase activity in the presence of either fructose or glucose (Table 2).

The single glucokinase (*glkA4*) mutant data support the argument that CCR correlates with carbon source utilization. Glk mutants do not exhibit nutritional deficiencies because they produce high levels of Hxk activity, enabling phosphorylation of both fructose and glucose. The Glk mutant shows an apparently wild-type transcriptional repression for all our diagnostic systems in the presence of either sugar. The two glucose-phosphorylating enzymes thus substitute each other functionally in the single mutants, both with respect to catalytic function and in establishing glucose-mediated carbon catabolite repression. In this respect, transcript analyses of the three systems correlate with the measured hexose kinase activities.

Fig. 8. Evidence for a positive role of hexokinase in xylose induction of *xlnA*.



Xylose-induced *xlnA* expression in the derepressed *creA* ^{d}*30* strain (middle) was compared with that in a triple mutant carrying both hexose kinase mutations as well as *creA* ^{d}*30* (*creA* ^{d}*30*/*glkA4*/*hxkA1*) (right). On the left, wild type. Details were as described in the legends to Figs. 1, 4, and 7.

Sugar phosphorylation is a critical step in CCR by hexose sugars

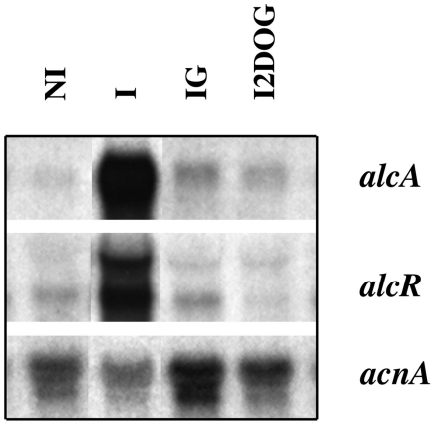
The observations in both hexose kinase single mutants are in agreement with previous work with the *hxkA1* mutant (Ruijter et al., 1996) and would suggest that hexokinase and glucokinase are not involved in CCR by glucose. However, both our double hexose kinase (hxkA/*glkA4*) mutants refute this conclusion because they show considerable transcriptional derepression for all three systems in the presence of either glucose or fructose. Derepression in both double mutants is total for *xlnA* and *facA*, whereas for alc it is greater on fructose than on glucose. For these double mutants, glucose is not a carbon source, and (in vitro) phosphorylation of both fructose and glucose is virtually abolished. The substantial residual repression of the alc genes on glucose in the double mutants might indicate involvement of a third protein, possibly capable of phosphorylating glucose in vivo although remaining undetected in vitro.

Overall, our results would implicate either glucose phosphorylation, the first step in glycolysis, or the catabolic flux initiated from it as essential for signaling glucose repression in *A. nidulans*. Two independent findings favor sugar phosphorylation as the critical step. First, *A. nidulans* pyruvate dehydrogenase-deficient (pdhA) strains are unable to grow on glycolytic substrates (Romano and Kornberg 1968, Romano and Kornberg

1969). In such glucose non-utilizing mutants, sugar phosphorylation still occurs. However, they provide a very amenable genetic background for the positive selection of derepressed *creA*d mutants (Arst and Bailey 1977; Bailey and Arst 1975). Second, our repression defective hexose kinase double mutants were isolated by positive selection for resistance to 2DOG, a glucose analogue that is phosphorylated without initiating catabolic flux (Mark and Romano 1971). Nevertheless, 2DOG represses induced expression of the alc genes at the transcript level as strongly as glucose does, as shown in Fig. 9. In *S. cerevisiae*, Hxk2p is involved in the regulation of glucose uptake and hxk2 mutants have a different expression spectrum for hexose transporters (Petit et al., 2000; reviewed in Ozcan and Johnston 1999). Interestingly, preliminary results show that both hexose kinase double mutants appear to have glucose uptake characteristics similar to those of wild type (A. P. MacCabe, M. Flipphi, B. Felenbok and D. Ramon, unpublished results). This indicates that the unphosphorylated sugar is unlikely to play a direct role in signaling carbon catabolite repression in *A. nidulans*.

The levels of derepression of the three systems in glucose derepressed hexose kinase double mutants are less than those seen in the *creA* ^{d}*30* strain, widely used as a reference for derepression. These results suggest that the CreA protein normally mediates repression from other metabolites in the hexose kinase double mutant, irrespective of the sugar kinase lesions. This is most evident for *xlnA* (Table 3). Several mechanisms, possibly acting in concert, might account for the 300-fold difference in induction: a total lack of repression from the carbon source gluconate and the inducer xylose, derepression of the

Fig. 9. 2DOG represses transcription of induced *alcA* and *alcR*.



Transcript analysis of a wild-type strain was performed using ethanol (50 mM) as the inducer. 2DOG and glucose were added at 1% (w/v). Details were as described in the legend to Fig. 1.

activator-encoding *xlnR* gene in addition to that of the structural *xlnA* gene, and altered xylose transport. To this latter end, we have shown that the reduced inducibility of *xlnA* in hexokinase mutants is not because of CreA-mediated repression (Fig. 8 and Table 3). In the derepressed triple mutant (*creA ^{d}30*/*glkA4*/*hxkA1*), a superinduction of *xlnA* is observed as expected from the *creA ^{d}30* mutation, but the wild-type xylose inducibility ratio is not restored. The positive involvement of Hxk in *xlnA* induction might occur at the level of xylose uptake by hexose transporters. The xylose-fermenting yeast Pichia stipitis takes up xylose and glucose with a common transport system (Weierstall et al., 1999). Here, we have provided evidence for inducer exclusion of xylose by glucose (Fig. 3), consistent with a common transporter for xylose and glucose in *A. nidulans*. This is the case for the *A. niger* sugar transporter MstA (P. A. vanKuyk, personal communication). Possible regulatory functions of the hexose kinases in the catabolism of glucose and xylose are currently under study.

CCR in *A. nidulans* differs fundamentally from glucose repression in *S. cerevisiae.*

In *A. nidulans*, glucokinase and hexokinase appear to play a mere catalytic role in CCR, specific for their substrates (repressing hexose sugars). The complete absence of (in vitro) glucose phosphorylating activity in our hexose kinase double mutants correlates with derepression of the three diagnostic systems for glucose and fructose. In contrast to Hxk2p in *S. cerevisiae*, neither hexose kinase exhibits a unique, general regulatory function in CCR in *A. nidulans*. A possible reason could be that glucose repression in yeast is specifically related to fermentation of glucose into ethanol (Diderich et al., 2001), whereas most filamentous fungi metabolize glucose almost uniquely via oxidative phosphorylation. Moreover, CCR in filamentous fungi can result not only from glucose, sucrose, or fructose but also from other carbon sources such as xylose and acetate (Arst and Cove 1973, M. M. Tanzer, H. N. Arst, Jr., A. R. Skalchunes, M. Coffin, B. A. Darveaux, R. W. Heiniger, and J. R. Shuster, unpublished data). These fundamental differences might be relevant to the lack of similarity between the respective CCR-mediating repressor proteins, Mig1p and CreA, beyond the DNA-binding domains (the two Cys2His2-zinc fingers). CRE1, the CreA homologue from the filamentous fungus Sclerotinia sclerotiorum, cannot complement a mig1 deletion in S. cerevisiae (Vautard et al., 1999b). In A. nidulans, CCR could be signalled independently for individual carbon sources and CreA might be the ultimate receptor of multiple converging signaling routes. In this respect, the signal transmission processes preceding transcriptional repression also seem to differ fundamentally between the two types of fungi.

Acknowledgements

We thank Dr. Christophe d'Enfert and Dr. John Clutterbuck for kindly providing several A. nidulans strains and Maarten Bax for technical assistance.

Note added in proof. Katz et al. (2000) have characterized the *Aspergillus nidulans xprF* gene whose conceptual translation product shares sequence similarity with hexose kinases but apparently has little or no hexose kinase activity. *xprF* mutations affect the regulation of extracellular proteases, probably in response to carbon starvation or carbon catabolite repression. Whether *xprF* mutations affect the regulation of other activities has not been reported, but because *xprF* is on chromosome VII, it is clearly distinct from *glkA* and *hxkA*.

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Chapter 5

Selection and characterisation of a xylitol-derepressed *Aspergillus niger* mutant that is apparently impaired in xylitol transport

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The first two authors have contributed equally to this work.

Appl Microbiol Biotechnol (2006) 73:881-886
DOI 10.1007/s00253-006-0527-y

Abstract

Aspergillus niger is known for its biotechnological applications, such as the use of xylanase enzyme for the degradation of hemicellulose. Depending on culture conditions, several polyols may also be accumulated, such as xylitol during D-xylose oxidation. Also during industrial fermentation of xylose for the production of fuel ethanol by recombinant yeast, xylitol is a by-product. We studied xylitol metabolism by isolating mutants that have impaired xylitol-mediated repression. Genetic and biochemical characterization revealed that one of these mutants was affected not only in xylitol-mediated carbon repression, but also had impaired xylitol transport.

Introduction

Aspergillus is a genus of mainly saprophytic fungi known for their biotechnological applications. Many of these applications are related to hemicellulose degradation, such as the use of xylanases. For the food industry, *Aspergillus niger* enzymes are particularly attractive because they are generally regarded as safe (Archer 2000). Another interest in this research field is related to the production of fuel ethanol from lignocellulose by recombinant yeast strains, expressing fungal hemicellulose-degrading enzymes (Den Haan and Van Zyl 2003; La Grange et al., 2001; Richard et al., 2003). Wild-type *Saccharomyces cerevisiae* does not metabolise D-xylose, but the introduction of pentose-catabolising activities from *Pichia stipitis* amended this problem (Kötter and Ciriacy 1993; Tantirungkij et al., 1993). However, xylitol production during D-xylose fermentation was a problem, which remained despite several strategies (reviewed by Jeffries and Jin 2004). In these fermentations, xylitol is formed as a result of cofactor imbalance, because in yeast D-xylose reductase can use both NADPH and NADH, whereas for the oxidation of xylitol only NAD+ is used.

A comparable situation exists in *A. niger*; however, only NADP-dependent xylose reductase is found in *A. niger* (de Groot et al., 2005; Witteveen et al., 1989). In *A. niger*, xylitol is an intermediate of both the D-xylose and L-arabinose catabolism, and it is produced in low amounts during growth on these pentoses (de Groot et al., 2005; Prathumpai et al., 2003; Witteveen et al., 1989). Another pentitol, L-arabitol, is found upon growth on L-arabinose, whilst D-arabitol is produced by several Aspergilli during growth on hexoses (Dijkema et al., 1985; Kelavkar and Chhatpar 1993; Ramos et al., 1999; Ruijter et al., 2004; Witteveen and Visser 1995).

Despite the importance of xylitol, only little information is available with regard to transport of xylitol or regulation of metabolism by xylitol. In this study, we investigated whether the mutant selection system used by de Groot et al. (2003) could be applied to identify functions involved in xylitol metabolism. We show that this selection system was capable of selecting a new mutant affected in xylitol-mediated repression, and detailed investigation of a xylitol-derepressed mutant shows it to be severely hampered in xylitol transport.

Materials and methods

Strains and growth conditions

The *A. niger* strains used for this study were derived from *A. niger* N400 (CBS 120.49) and are described in Table 1. Strains N402, N572, NW315, 689.1 and 740.1 have been deposited at the public fungal collection of the Centraalbureau voor Schimmelcultures in The Netherlands (http://www.cbs.knaw.nl/). NW315 was used for mutagenesis and N402 was used as a reference strain. Mycelium cultures were grown at pH 6 in minimal medium (MM) containing per litre 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 10 mg EDTA, 4.4 mg $ZnSO_4 \cdot 7H_2O$, 1.0 mg $MnCl_2 \cdot 4H_2O$, 0.32 mg $CoCl_2 \cdot 6H_2O$, 0.32 mg $CuSO_4 \cdot 5H_2O$, 0.22 mg $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 1.47 mg $CaCl_2 \cdot 2H_2O$ and 1.0 mg $FeSO_4 \cdot 7H_2O$ and carbon sources as indicated in the text. Culturing was done in a rotary shaker at 250 rpm and 30°C or in a 2.5-l jacketed Applicon bioreactor with pH controlled

Table 1. *A. niger* strains used in this study

Strain	Genotype	Reference
N402	*cspA1*	(Bos et al. 1988)
N572	*cspA1; xkiA1; nicA1*	(Witteveen et al. 1989)
NW148	*hisD4; lysA7 bioA1 cspA1; leuA1; metB10 argB15; pabA1; cnxC5; trpB2*	(de Groot et al. 2003)
NW315	*fwnA1; pyrA6 cspA1; xkiA1; nicA1*	(Witteveen et al. 1989)
689.1	*fwnA1; pyrA6 cspA1; xkiA1; nicA1; xtlA36*	This study
689.2	*fwnA1; pyrA6 cspA1; xkiA1; nicA1; xtlA46*	This study
689.3	*fwnA1; pyrA6 cspA1; xkiA1; nicA1; xtlA55*	This study
740.1	*lysA7 bioA1 cspA1; leuA1; nicA1; xtlA36*	This study, (Prathumpai et al. 2003)

at 5.0 and dissolved oxygen tension controlled at a minimum of 30%. For growth of strains with auxotrophic mutations, the necessary supplements were added to the medium.

Selection and genetic analysis of mutants

The selection of mutants was done similar to the method described by de Groot et al. (2003); instead of the use of L-arabinose as the repressing pentose, we used xylitol. Conidiospores were irradiated with UV light and 10^5 spores were spread on solid media plates containing 50 mM xylitol in combination with 50 mM sodium D-gluconate. The plates were incubated for 3 days at 30°C and colonies were purified on the same selective medium. The mutants were tested by replica plating for growth on D-xylose and combinations of xylitol, D-xylose or l-arabinose with either D-gluconate or L-alanine. Mutants that remained unable to grow on D-xylose but showed growth on the xylitol + D-gluconate and xylitol + L-alanine combinations were analysed in more detail. Genetic localisation of the pentose-derepressed mutations was determined by mitotic recombination using master strain NW148 (Bos et al., 1988). Complementation of the xylitol-derepressed mutations was tested using heterokaryons of the original mutants with strain 740.1 containing the *xtlA36* mutation.

Consumption and excretion experiments

The experiments were performed as biological duplicates. To analyse consumption of xylitol, strains 740.1 and N402 were pre-cultured on MM containing 2% xylose. After 16 h, mycelium was harvested and washed using 30°C MM by suction over a filter. Aliquots of 33–44 g wet weight were transferred to 2.2 l MM containing xylitol. Samples used for dry weight and polyol analysis were not washed. To investigate excretion of xylitol, strains N572 and 689.1 were pre-grown using MM containing 2% fructose. After 16 h, mycelium was harvested, washed and transferred to MM containing 2% xylose. Samples were taken as described above.

Polyol extraction and determination

Extraction of intracellular polyols was carried out as described previously (Witteveen and Visser 1995). Polyol and xylose concentrations were measured by high pH anion exchange chromatography (Dionex) with a Carbopac MA1 column using isocratic elution with 0.48 M NaOH.

Results

Isolation of *A. niger* mutants

We adapted the selection method described by de Groot et al. (2003) to obtain *A. niger* mutants altered in xylitol-mediated repression. The selection system uses a parental strain containing the *xkiA1* mutation, which lacks D-xylulose kinase activity. This strain is able to take up L-arabinose, L-arabitol, xylitol and D-xylose, but it is unable to metabolise them beyond D-xylulose, leading to accumulation of catabolites (Witteveen et al., 1989). Xylitol is able to repress the use of poorer carbon sources such as D-gluconate and L-alanine preventing an *xkiA1* strain from growing on a combination of this pentose and D-gluconate. The first selection applied was for mutants capable of growth on D-gluconate in the presence of xylitol. To avoid selection of mutants affected only in D-gluconate catabolism,

we considered only those mutants derepressed for the utilisation of both D-gluconate and L-alanine. Xylitol-derepressed mutants were selected in two petri dishes containing MM xylitol + D-gluconate, one containing 10^5 and the other containing 10^6 irradiated spores of NW315. From the plate with 10^5 spores, we isolated three xylitol-derepressed mutants. All three mutants were derepressed for both D-gluconate and L-alanine utilisation on combinations with xylitol, but not with D-xylose or L-arabinose.

Strain 689.1 was subjected to mitotic recombination with tester strain NW148 for three purposes: (1) to obtain information on the genetic localization of the *xtlA36* mutation; (2) to remove the *xkiA1* background from the *xtlA36* recombinants to determine the phenotype of the *xtlA36* mutation; and (3) to isolate an *xtlA36* recombinant with a different auxotrophic marker, which could then be used in complementation tests with the other *xtl* mutants.

Some of the 53 haploid recombinants we isolated from diploid 689.1//NW148 were unable to grow on 50 mM xylitol, whereas growth on 50 mM L-arabinose, L-arabitol and D-xylose was not affected. This phenotype, apparently resulting from the *xtlA36* mutation, enabled us to determine the presence of the *xtlA36* mutation in all 53 progeny, not just in the *xkiA1*-carrying ones. Recombination frequencies of the genetic markers on chromosome IV with *xtlA36* were 33% for leuA1 and 17% for *xkiA1*. Recombination frequencies with markers on other chromosomes were approximately 50%, which localised *xtlA36* on chromosome IV. The heterozygous diploid strain, derived from 689.1 and NW148, grew well on xylitol, demonstrating the *xtlA36* mutation to be recessive. This allowed us to perform complementation tests with the other two *xtl* mutations. Recombinant strain 740.1 (Table 1) was forced to form a heterokaryon with 689.2 (*xtlA*46) and 689.3 (*xtlA*55). Both heterokaryons grew on glucose, but not on xylitol, indicating that they are allelic. Because they were isolated from the same petri dish, it is not certain that these *xtlA* mutations are truly independent.

Biochemical analysis of the *xtlA36* mutation

D-xylose and L-arabinose are catabolised via xylitol (Witteveen et al., 1989). Because *xtlA36* strain 740.1 grows like wild type on D-xylose and L-arabinose, it is unlikely that the growth defect on xylitol is caused by a dramatic change in the intracellular pentose metabolism. Therefore, we conducted assays to test whether xylitol was taken up (Fig. 1). During the experiment, the biomass of the wild-type N402 at 60 mM xylitol increased from 0.065±0.003 to 0.075±0.004 g/l, but at 6 mM xylitol the biomass remained constant at 0.071±0.005 g/l. The biomass of the *xtlA36* strain remained constant at 0.070±0.005 and 0.060±0.003 g/l in the 60- and 6-mM xylitol cultures, respectively. In the first 30 h, there was an almost constant decrease of the xylitol concentration in all four cultures, after which the xylitol consumption leveled off. Inspection of the xylitol consumption rate over these 30

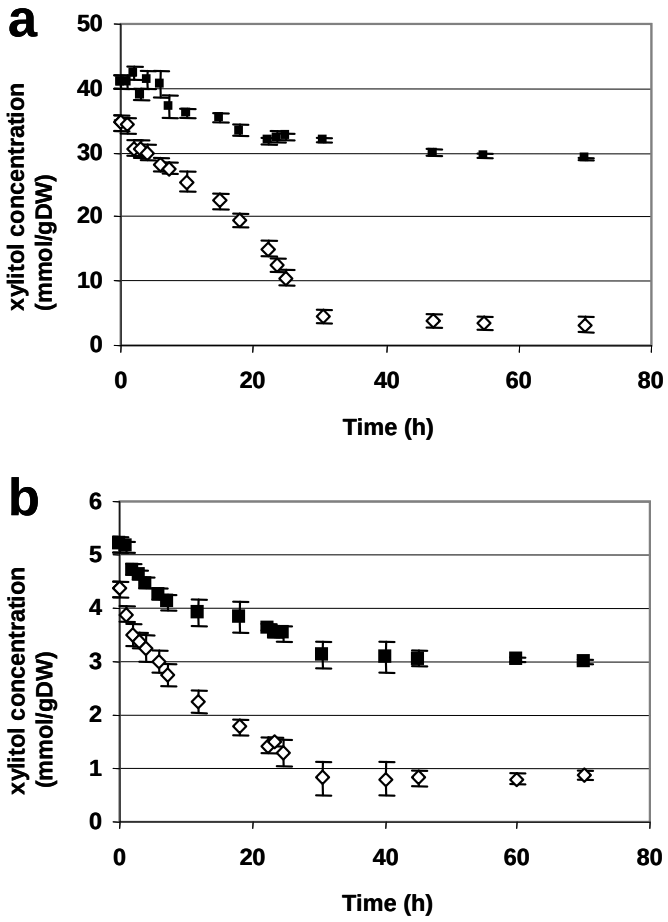

Fig. 1. Xylitol consumption in strains N402 (diamond, reference strain) and 740.1 (black square, *xtlA36*) at initial concentrations of 60 mM (a) and 6 mM (b)

h reveals a clear difference between the reference strain and the *xtlA36* mutant. With an initial concentration of 60 mM xylitol, the xylitol consumption rate of the reference strain was 0.92 mmol g-1 DW h-1. The *xtlA36* mutant consumed xylitol with a rate of 0.37 mmol g-1 DW h-1 over the first 30 h. At a lower initial concentration of 6 mM xylitol, there was also a clear difference between the two strains. The xylitol consumption rate during the first 30 h of the reference strain was 0.10 mmol xylitol g-1 DW h-1, whereas that of the *xtlA36* mutant was 0.06 mmol xylitol g-1 DW h-1.

To investigate whether the *xtlA36* mutation would also affect the excretion of

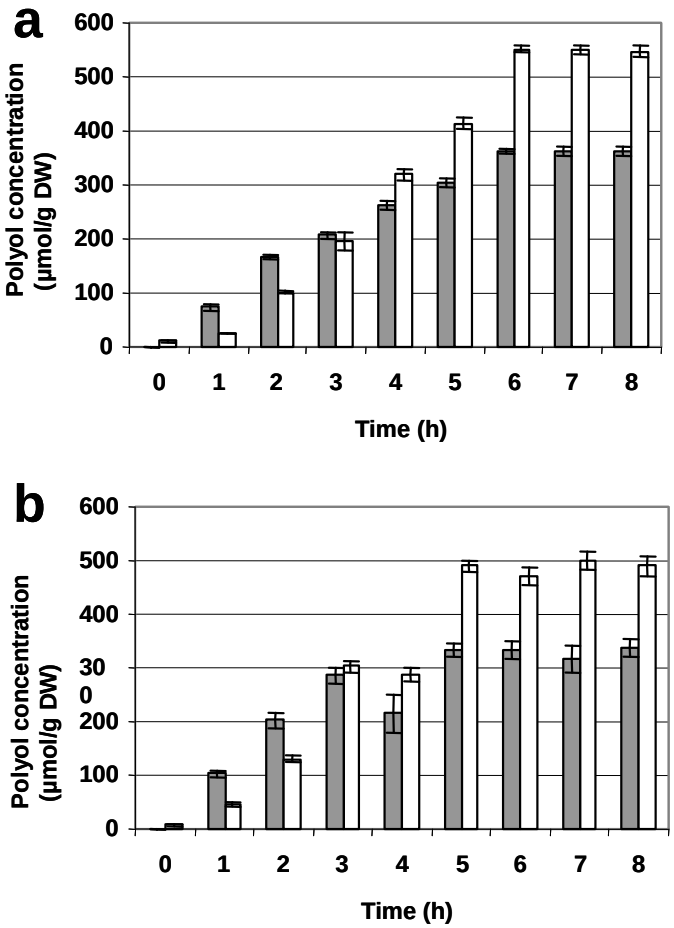

Fig. 2. Intracellular arabitol (white bars) and xylitol (grey bars) accumulation in strains a N572 (*xkiA1*) and b 689.1 (*xkiA1 xtlA36*) on 2% D-xylose

xylitol, we used the *xkiA1* background. With D-xylose as the carbon source, the lack of xylulose kinase in this background causes accumulation of xylitol and arabitol (Witteveen et al., 1989 and Fig. 2). The biomass remained constant at 0.66±0.02 g/l for 689.1 and 0.62±0.02 g/l for N572. We observed excretion of up to 2 mM of xylitol in the *xkiA1* mutant N572, but no significant excretion in the *xkiA1 xtlA36* strain 689.1 (Fig. 3). In the last two samples, the arabitol excretion was higher in the *xkiA1 xtlA36* strain than in the *xkiA1* strain. The polyol accumulation (Fig. 2) in this experiment was comparable between the two strains.

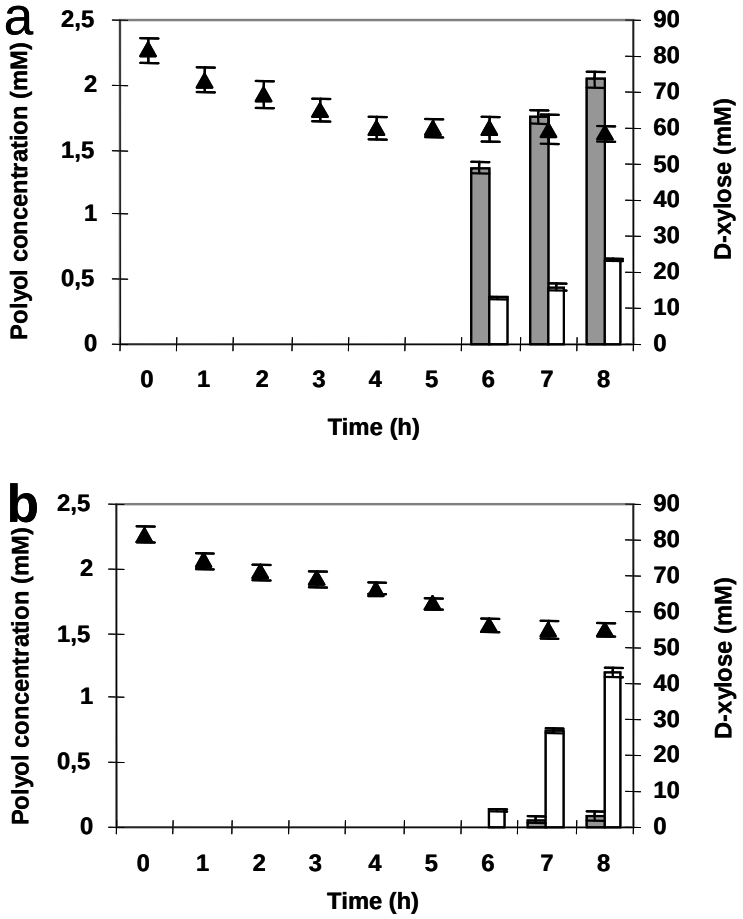

Fig. 3. D-xylose (black triangles) consumption and arabitol (white bars) and xylitol (grey bars) excretion by strains a N572 (*xkiA1*) and b 689.1 (*xkiA1 xtlA36*) using a initial concentration of 2% D-xylose

Discussion

The selection method described in this report proves useful for identifying new genes involved in xylitol metabolism and xylitol-mediated repression. We identified a new mutant affected in repression of gluconate and alanine utilisation exerted by xylitol and in the import and export of xylitol. The *xtlA36* mutation causes a partial xylitol non-utilising

phenotype. The difference in consumption rate between the *xtlA36* mutant and the reference strain clearly shows that the transport of xylitol into the cell is impaired. This can be explained either by another xylitol importer present, which is not affected by the *xtlA36* mutation, or by a partial effect on a single xylitol transporter.

The polyol excretion experiment shows a marked difference between the excretion of xylitol by the *xkiA1* reference strain and the *xkiA1 xtlA36* double mutant. Because of the *xkiA1* mutation, these strains cannot metabolise any of the substrates from the L-arabinose/D-xylose metabolism beyond D-xylulose. However, the metabolites can be taken up and converted, causing an extreme accumulation of the polyols arabitol and xylitol (Witteveen et al., 1989). It appears that the *xtlA36* mutation prevents the fungus from excreting xylitol produced, but does not reduce the excretion of arabitol compared to the reference strain. A similar intracellular level of both polyols is maintained in the *xtlA* strain and the reference strain. Altogether, these data suggest that the *xtlA36* mutation leads to a severe reduction in xylitol transport and, therefore, could probably be located in a gene encoding the major xylitol transporter. This putative transporter would be responsible for both the import and export of xylitol as by the single *xtlA36* mutation both consumption and excretion are impaired. Also in yeast, bi-directional transporters are found, for example, in the case of glucose (Jansen et al., 2002) and glycerol transport (Karlgren et al., 2004).

It is not difficult to understand why an uptake mutant is isolated with our selection system. In the case of glucose repression, mutants no longer capable of phosphorylating glucose have reduced carbon catabolite repression (Flipphi et al., 2003), indicating that a glucose metabolite is involved in this pathway. In our study, we looked at xylitol-mediated repression, but we do not know which compound or metabolite is the key signal to xylitol-mediated repression. Alternatively, accumulation of xylitol and L-arabitol in the *xkiA1* strain might cause such changes in the anabolic (NADPH/NADP+NADPH) and catabolic (NADH/NAD+NADH) reduction charge that this interferes with the consumption of gluconate and alanine, which requires these reduced cofactors. The types of mutants one can expect to find using our selection method affect xylitol uptake and catabolism to the final metabolite(s) that influence the signaling pathway to pentose-mediated repression. We indeed identified a mutation, *xtlA36*, affecting xylitol transport. Previously, in a screen using L-arabinose instead of xylitol to isolate mutants able to grow both on gluconate and alanine, this selection system resulted in the isolation of the signaling mutants *araA4* and *araB3*, which are surprisingly diverse in their phenotype (de Groot et al., 2003). They are involved in the regulation of the arabinose catabolic pathway as well as the extracellular enzymes involved in arabinan degradation and have reduced growth on L-arabinose and L-arabitol in a xkiA + background. We did not find these types of mutations, nor did we find mutations affecting enzymes in the catabolic pathways leading from xylitol. An explanation

for this could be redundancy in xylitol catabolism (Witteveen et al., 1989; de Groot et al., 2005).

In conclusion, we demonstrated this mutant selection technique to allow the identification of a new function in xylitol catabolism, and it is likely that the *xtlA* gene encodes a transporter involved in xylitol uptake and excretion. In *A. niger*, this mutation can prevent xylitol excretion, a finding particularly of interest in ethanol production in yeast.

Acknowledgements

We thank Marieke Escher for technical assistance. This work was supported by grants from the European Union (EUROFUNG), contract numbers BIO4CT96-0535 and QTRK3-199-00729 to J.V.

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Chapter 6

Linking the *Aspergillus niger* physical map to the genetic map

Peter J.I. van de Vondervoort

Parts of this chapter are included in "Genome sequence of *Aspergillus niger* strain CBS 513.88: a versatile cell factory" by Pel et al., 2007 Nature Biotech. 25:221-231. DOI 10.1038/nbt1282

Abstract

Genetic recombination is an important tool in *Aspergillus niger* strain breeding. The genome sequence of *A. niger* can be used to increase the knowledge of its genetics. A first and important step is to link the physical map to the genetic map. Using data from parasexual analysis and chromosome hybridisations, 33.9 megabase of genome sequence was assigned to the eight chromosomes. Together with other data, the physical map was linked to the genetic map and the position and orientation of all 19 supercontigs was deduced. Synteny with the genome of *A. nidulans* and a high quality annotation of the *A. niger* genome allowed the assignment of 67 genetic markers to their probable corresponding genes.

Introduction

Aspergillus niger is a key organism for the production of proteins, primarily enzymes. DSM sequenced the complete 33.9 megabase genome of *A. niger* CBS 513.88. The 7.5-fold coverage random sequencing of carefully selected large insert BAC's allowed the assembly of the DNA sequence data into 19 large so-called supercontigs, providing a detailed physical map. Despite the lack of a sexual cycle in *A. niger*, recombination is possible by means of the parasexual cycle. Using parasexual recombination, various mutations have been assigned to a linkage group and for some the linear gene order relative to the centromere has been determined providing a genetic map (Figure 1, Debets et al., 1993). For some of the fungi that have been sequenced, high quality genetic maps are available, such as for *Aspergillus nidulans, Magnaporte grisea* and *Neurospora crassa*. Cloned genetic markers could be used to assemble the sequence information onto their genetic maps (Clutterbuck 1997, Galagan et al., 2003, Dean et al., 2005). Of some other fungi being sequenced, the genetic map is not very detailed, such as of *A. flavus* (Papa 1976), *A. oryzae* and *A. fumigatus*. This problem was amended in *A. fumigatus* and *A. oryzae* by

Fig 1. Genetic map of *A. niger*.

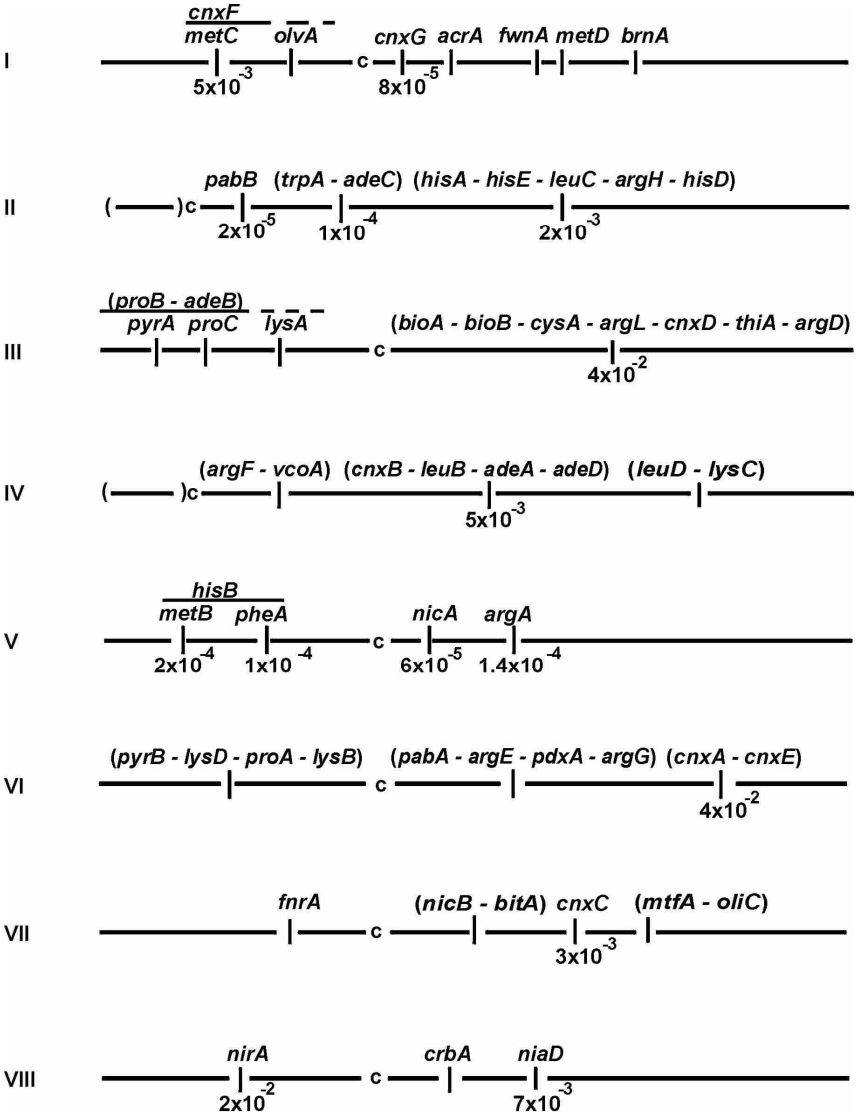


Figure reproduced from Debets et al. (1993) with permission.

optical mapping (Nierman et al., 2005, Machida et al 2005). In *A. niger* the genetic map available is not sufficient to link the physical map to it completely. Additional information provided by expression analysis and synteny with *A. nidulans* proved to be sufficient to link all 19 supercontigs to the genetic map.

Result and discussion

Assigning the supercontigs to the linkage groups.

A first step to link the physical map to the genetic map is the assignment of all 19 supercontigs to a specific linkage group. Previously, the electrophoretic karyotype of *A. niger* strain CBS 120.49 was estimated to contain 37 mega base pairs located on eight chromosomes (Verdoes et al., 1994a). For the few available *A. niger* markers with known DNA sequence, the genetic and supercontig location were compared. To complete this comparison more cloned genes were assigned to a chromosome of strain CBS 120.49 using pulsed field electrophoresis in combination with Southern analysis. In total, the locations of 72 genes have been assigned and compared (Table 1). All 72 genes unambiguously linked the 19 supercontigs to one chromosome, indicating that there are no major differences between *A. niger* CBS 513.88 and CBS 120.49 due to translocations. Comparing the size of the physical map with the predicted size deduced from the electrophoretic karyotype, only for chromosomes VI and VII there is a large difference (Table 2). The difference for chromosome VI can be explained by the presence of the ribosomal RNA gene cluster, which consists of about 70 copies in repeats of 7 kb. The difference for chromosome VII might be caused by a large deletion in strain CBS 513.88 or lack of sequence information.

Establishing the orientation of the supercontigs

Four different methods were used to establish the orientation of supercontigs on the chromosomes. At first we searched for telomeric repeats. In *A. nidulans* common telomeric repeats of the hexamer TTAGGG were found (Bhattacharyya and Blackburn 1997), while for *A. oryzae* TTAGGGTCAACA repeats were found (Kusumoto et al., 2003). In our sequences we found only one telomeric repeat, consisting of a sixteen fold repeat of TTAGGGTT, where in two cases a T was missing. This telomeric repeat was located in BAC end EQ03278.p1, positioning it on the end of supercontig An04 on chromosome VI. Apparently, this sequencing method is unfavourable for detecting telomeres.

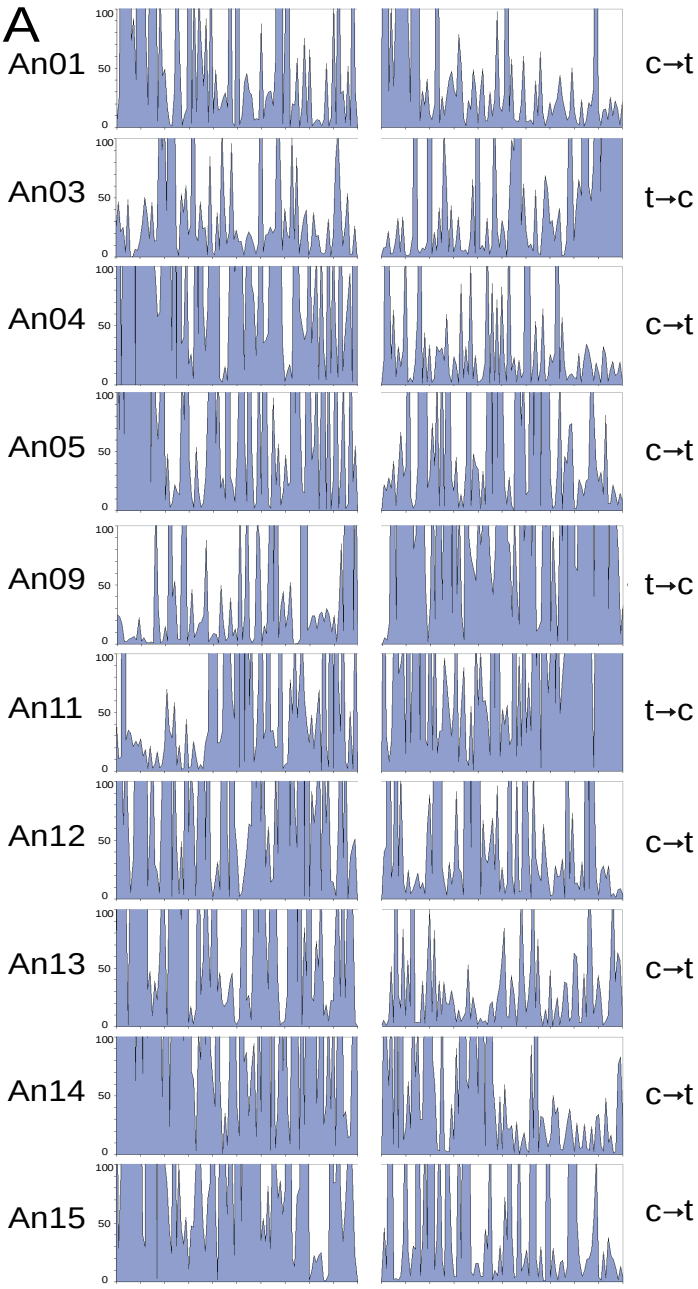
The second method uses the high degree of synteny between *Aspergillus* species near the centromeres. For *A. nidulans* a high quality genetic map is available, which has been linked to the physical map (Clutterbuck 1997). Although synteny with *A. niger* was very weak near the telomeric regions of the *A. nidulans* scaffolds, synteny was remarkably

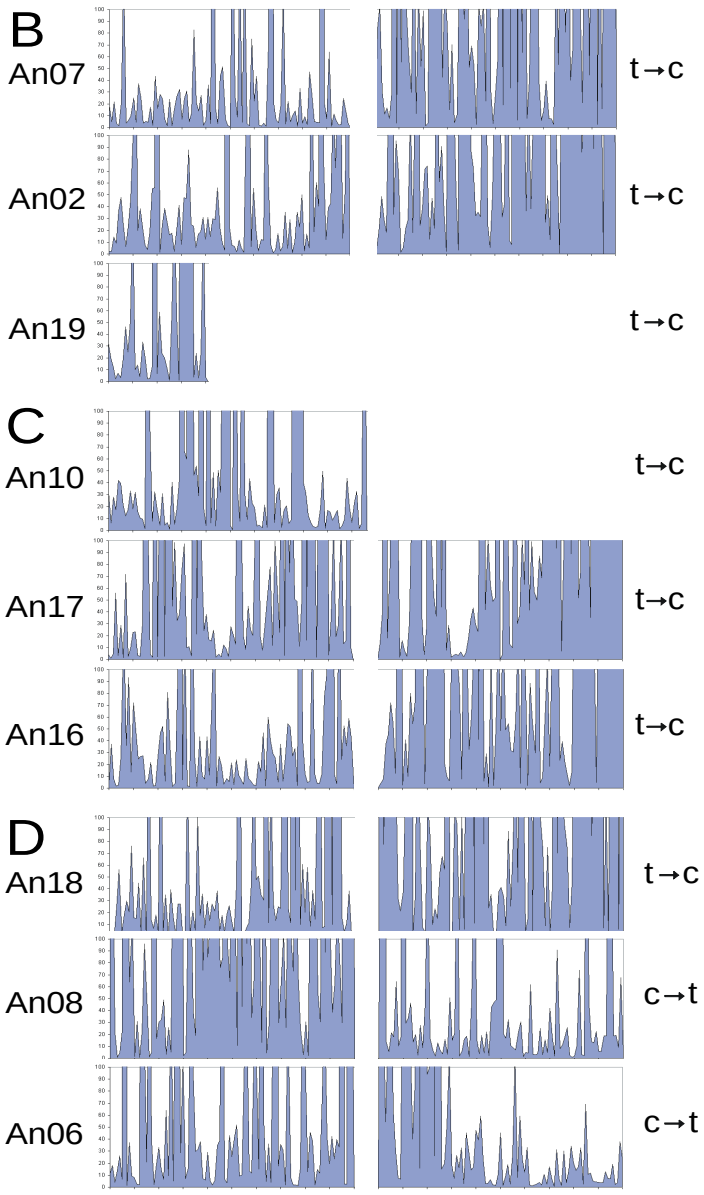
high near the centromeres. On one side of 16 *A. niger* supercontigs there is a high degree of synteny with the *A. nidulans* scaffolds closest to the centromeres, representing eight pairs of supercontigs allocated to each linkage group, leaving three small supercontigs with unknown orientation.

The third method used to determine the orientation of the supercontigs uses expression data obtained by Affymetrix microarray analysis. When plotting the expression of the first and last 100 genes of the supercontigs on a 0 to 100 scale, a reduced expression of genes located near the telomeres can be seen on the telomeric ends of the chromosomes I, II, III, VI and VII (Fig 2a), resembling the telomeric position effect described in yeast (Gottschling et al., 1990). For the supercontigs spanning a complete chromosome arm, the average expression level of the 10 genes closest to the centromeres is 458, while the average expression level of the 10 genes closest to the telomeric ends is 27. Looking at expression on either side of the three other supercontig pairs (An02 and An07, An16 and An17, An08 and An18) and the three remaining supercontigs (An19, An10, An06), the most likely position of the telomeric ends was determined (Fig 2b 2c and 2d).

A last step to align the physical map to the genetic map was to determine which supercontigs represent the left or the right arms. For three chromosomes we could make a direct link using cloned genetic markers *niaD, nicB, nirA* and *pyrA* (Table 2). This way the orientation of the supercontigs representing linkage groups III, VII and VIII could be concluded. To determine the orientation of the supercontigs for the other five chromosomes, we tried to find out which genes correlate to some of the genetic markers. The types of markers were investigated were mutations in amino acid or vitamin biosynthesis, cnx cofactor synthesis and conidia pigmentation. For most of the various biosynthetic routes, the number of markers identified in *A. niger* (listed in Table 3) is comparable to the situation in *A. nidulans*. Although only part of these markers are cloned, the high degree of saturation of the biosynthetic markers, in combination with synteny, allowed us to deduce the genetic location of the *A. niger* markers from the known location of the *A. nidulans* markers. The possible genes corresponding to the *A. niger* markers were found by orthologs of cloned *A. nidulans* markers, through synteny with the genetic regions of uncloned but accurately mapped markers, and by the Funcat annotation (Ruepp et al., 2004) of the *A. niger* genes. In Table 4, for 67 genetic markers putative corresponding genes are listed. For only few of the listed markers the correspondence is experimentally proven. The number of putative identified markers that helped determine the orientation of the supercontig pairs is listed in Table 2 as the “nid” value. Altogether, this resulted in the alignment of all 19 supercontigs to the genetic map of *A. niger* (Table 2).

Fig 2. Telomere positioning effect on supercontig ends





Expression level of the first and last 100 genes on each supercontig plotted on a scale of 0 to 100. The centromeric side (c) of the supercontigs was identified by a high level of synteny with *A. nidulans* near-centromeric genes, revealing reduced expression at the telomeric side of the supercontigs (t) (Panel A). Panels B, C and D show the expression data for supercontigs belonging to chromosome IV, V and VIII respectively.

Table 1. Physical and genetic mapping of cloned *A. niger* genes.

Gene	Genbank no	Linkage group	Super-contig	Orf name	reference CHEF *1) or parasexual analysis *2)
abfA	L29005	II	1	An01g00330	this study *1)
pepB	M68871	II	1	An01g00530	(van den Hombergh et al. 1997) *1, 2)
xyrA	AF219625	II	1	An01g03740	this study *1)
*cpcA *3)*	X99215	II	1	An01g07900	this study, (Wanke et al. 1997) *2)
kexB	Y18127	II	1	An01g08530	this study *1)
xlnD	Z84377	II	1	An01g09960	this study *1)
pgaI	X58892	II	1	An01g11520	this study *1)
cbhB	AF156269	II	1	An01g11660	this study *1)
pgaE	Y14386	II	1	An01g14670	this study *1)
goxC	X16061	II	1	An01g14740	(Swart et al. 1990; Witteveen et al. 1993) *2)
creA	L03811	IV	2	An02g03830	(Ruijter et al. 1997) *2)
pkaC	X94399	IV	2	An02g04270	(Staudohar et al. 2002) *2)
pgaB	Y18805	IV	2	An02g04900	this study *1)
mpdA	AY081178	IV	2	An02g05830	this study *1) (Ruijter et al. 2003) *2)
pepE	U03278	IV	2	An02g07210	(van den Hombergh et al. 1997) *1, 2)
pacC	X98417	IV	2	An02g07890	(van den Hombergh et al. 1996) *1, 2)
aglB	Y18586	IV	2	An02g11150	this study *1)
hxkA	AJ009973	IV	2	An02g14380	this study *1)
pelB	X65552	VI	3	An03g00190	this study *1)
axhA	Z78011	VI	3	An03g00960	this study *1)
rDNA	X78538	VI	3	An03e03200	(Verdoes et al. 1994a) *1)
pmeA	X54145	VI	3	An03g06310	this study *1)
glaA	AY250996	VI	3	An03g06550	(Verdoes et al. 1994a) *1)
glcA	AY955284	VI	4	An04g04890	(Witteveen et al. 1990) *2)
acuA	X16990	VI	4	An04g05620	(Ruijter et al. 1999) *2)
pgaC	A21180	VII	5	An05g02440	this study *1)
aglA	X63348	VIII	6	An06g00170	(Verdoes et al. 1994a) *1)
xkiA	AJ305311	IV	7	An07g03140	(vanKuyk et al. 2001) *1) (Witteveen et al. 1989) *2)
pepC	M96758	IV	7	An07g03880	this study *1)
pepF	X79541	IV	7	An07g08030	this study *1)
eglB	X54145	IV	7	An07g08950	this study *1)
rodA	DQ349135	IV	7	An07g03350	this study *1)
cbhA	AF156268	IV	7	An07g09330	this study *1)
trpC	X07071	VIII	8	An08g06080	(Debets et al. 1990) *2)
acuB	U56097	VIII	8	An08g06580	(Ruijter et al. 1999) *2)
cprA	Z26938	VIII	8	An08g07840	(Verdoes et al. 1994a) *1)
niaD (R)	M77022	VIII	8	An08g11170	(Debets et al. 1990) *2)
faeA	Y09330	I	9	An09g00120	(de Vries et al. 1997) *2)
abnA	L23430	I	9	An09g01190	this study *1)
rgaeA	AJ242854	I	9	An09g02160	this study *1)
pgaD	Y18806	I	9	An09g03260	this study *1)
bphA	X52521	I	9	An09g03500	(Verdoes et al. 1994a) *1) (Boschloo et al. 1990) *2)
pepD	L19059	I	9	An09g03780	(van den Hombergh et al. 1997) *1, 2)

Table 1 continued.

Gene	Genbank no	Linkage group	Super-contig	Orf name	reference CHEF *1) or parasexual analysis *2)
prtF *4)	AJ567910	V	10	An10g00820	(van den Hombergh et al. 1995) *2)
plyA	AJ276331	V	10	An10g00870	this study *1)
pelC	AY839647	VII	11	An11g04030	this study *1)
bipA	Y08868	VII	11	An11g04180	(Van Gemeren et al. 1997)
nicB (R)	5)	VII	11	An11g10910	(Verdoes et al. 1994a) *1) (Verdoes et al. 1994b) *2)
rhgA	X94220	III	12	An12g00950	this study *1)
pyrA (L)	X96734	III	12	An12g03570	(Verdoes et al. 1994a) *1) (Bos et al. 1989) *2)
axeA	A22880	III	12	An12g05010	this study *1)
pgaX	A39428	III	12	An12g07500	this study *1)
glkA	X99626	III	12	An12g08610	this study *1)
areA	X81998	III	12	An12g08960	this study *1) (Lenouvel et al. 2001) *2)
faeB	AJ309807	III	12	An12g10390	this study *1)
alcB	AY955276	II	13	An13g00950	this study *1)
eglA	AJ224451	I	14	An14g02760	this study *1)
argB	M19158	I	14	An14g03400	(Lenouvel et al. 2002) *1, 2)
rhgB	X94221	I	14	An14g04200	this study *1)
pelA	X60724	I	14	An14g04370	this study *1)
pepA	U03507	I	14	An14g04710	(Verdoes et al. 1994a) *1) (van den Hombergh et al. 1997) *2)
aguA	AJ290451	I	14	An14g05800	this study *1)
abfB	L23502	III	15	An15g02300	this study *1)
pgaII	X58893	III	15	An15g05370	this study *1)
xlnR	AJ001909	III	15	An15g05810	(van Peij et al. 1998) *1, 2)
gpdA	X99652	V	16	An16g01830	(Verdoes et al. 1994a) *1)
pkaR	AJ296317	V	16	An16g03740	(Staudohar et al. 2002) *2)
pgaA	Y18804	V	16	An16g06990	this study *1)
gatA	AY955283	V	17	An17g00910	this study *1)
nirA (L)	M68900	VIII	18	An18g02330	this study *1) (Debets et al. 1990) *2)
bglA	AF121777	VIII	18	An18g03570	this study *1)
pelD	M55657	IV	19	An19g00270	this study *1)

*1) genetic localisation determined by Southern blot hybridization of CHEF gels

*2) genetic localisation by parasexual analysis of mutants or gene-disruptants

*3) the *cpcA* disruption was mapped to linkage group II by screening progeny for phleomycin resistance (results not shown)

*4) the *oahA* gene corresponds with the *prtF* mutation

*5) personal communication T. Goosen

(L) (R) = if known, the location of the gene on the left (L) or right (R) arm is given

Table 2. The chromosomal location and tentative orientation of the supercontigs.

Chromo-some		Super-contig	Mapped genes linking chromosomes to contigs		Orientation	Orientation evidence*		Supercontig size	Total size	Chromosome size
			by CHEF hybridization	by parasexual analysis		reciprocal	to genetic map			
I	L	14	*eglA argB rhgB pelA pepA aguA*	*argB pepA*	← c	s, e	nid 1	1.73	3.32	3.5
	R	9	*faeA abnA rgaeA pgaD bphA pepD*	*bphA pepD*	c ←	s, e	nid 1	1.59		
II	L	13	*alcB*		← c	s, e		0.91	4.53	5.0
	R	1	*abfA pepB xyrA kexB xlnD pgaI cbhB pgaE*	*cpcA pepB goxC*	c →	s, e	nid 7	3.62		
III	L	12	*rhgA pyrA axeA pgaX glkA areA faeB*	*pyrA areA*	← c	s, e	nig 1	2.56	4.44	4.1
	R	15	*abfB pgaII xlnR*	*xlnR*	c →	s, e		1.88		
IV	L	7	*xkiA pepC pepF eglB rodA cbhA*	*xkiA*	→ c	s, e		2.34	6.06	6.6
	R	2	*pgaB mpdA pepE pacC aglB hxkA*	*creA pkaC mpdA pepE pacC*	c ←	s	nid 6	3.62		
	R	19	*pelD*		c ←←	e		0.10		
V	L	10	*plyA*	*prtF*	→→ c	e		0.26	2.98	3.5
	L	17	*gatA*		→ c	s	nid 3	0.59		
	R	16	*gpdA pgaA*	*pkaR*	c ←	s, e	nid 1	2.13		
VI	L	4		*glcA acuA*	← c	t, s, e	nid 3	2.52	4.13	5.2
	R	3	*pelB axhA rDNA pmeA glaA*		c ←	s, e	nid 2	1.61		
VII	L	5	*pgaC*		← c	s, e		0.60	3.32	4.1
	R	11	*pelC bipA nicB*	*nicB*	c ←	s, e	nig 1	2.72		
VIII	L	18	*nirA bglA*	*nirA*	→ c	s, e	nig 1	1.54	5.14	5.0
	R	8	*cprA*	*trpC acuB niaD*	c →	s	nig 1	2.92		
	R	6	*aglA*		c →→	e		0.68		

The proposed position of the supercontigs on the left arm (L) or right arm (R) of the chromosomes (I-VIII) are indicated. The arrows indicate the orientation of the supercontigs relative to the centromere (c). Orientation evidence is based on telomeric sequences (t), expression data showing telomeric positioning effect (e) and genetically mapped and identified *A. niger* genes (nig). The nig value indicates the number of *A. niger* genes mapped to the chromosome arm. The nid value is the number of orthologous *A. nidulans* genes corresponding to *A. niger* markers that confirm the proposed orientation. Total size refers to the sum of the supercontig sizes whereas the chromosome size is based on the predictions by Verdoes et al. (1994). All sizes are in Mb.

Table 3. Genetic markers of *A. niger*. The position on the chromosome relative to the centromere is indicated if known (L, left arm; R, right arm).

Linkage group							
I	II	III	IV	V	VI	VII	VIII
acrA (R)	*adeC (R)*	*adeB (L)*	*adeA (R)*	*argA (R)*	*argE (R)*	*adeF*	*crbA (R)*
brnA (R)	*argH (R)*	*adeE*	*adeD (R)*	*hisB (L)*	*argG (R)*	*bitA (R)*	*crnA*
cnxF (L)	*fpaC*	*adeG*	*argF (R)*	*metB (L)*	*argK*	*cnxC (R)*	*crnB*
cnxG (R)	*fpaD*	*argD (R)*	*cnxB (R)*	*nicA (R)*	*cnxA (R)*	*fnrA (L)*	*niaD (R)*
cysB	*fpaE*	*argL (R)*	*cysC*	*pheA (L)*	*cnxE (R)*	*lysF*	*nirA (L)*
fpaB	*fpaG*	*bioA (R)*	*fpaF*	*pheB*	*lysB (L)*	*mtfA (R)*	*pyrD*
fwnA (R)	*hisA (R)*	*bioB (R)*	*hisC*	*serA*	*lysD (L)*	*nicB (R)*	*pyrE*
metC (L)	*hisD (R)*	*choA*	*leuA*		*pabA (R)*	*oliC (R)*	*trpB*
metD (R)	*hisE (R)*	*cnxD (R)*	*leuB (R)*		*pabC*		*trpC*
olvA (L)	*hisF*	*cysA (R)*	*leuD (R)*		*pabD*		*trpE*
ntrA	*hisG*	*fpaA*	*lysC (R)*		*pdxA (R)*		
	hisI	*hisH*	*ntrB*		*proA (L)*		
	leuC (R)	*lysA (L)*	*ntrC*		*proE*		
	leuE	*lysE*	*ntrD*		*pyrB (L)*		
	pabB (R)	*pdxB*	*thiB*				
	pyrC	*proB (L)*	*trfA*				
	trpA (R)	*proC (L)*	*vcoA (R)*				
		proD					
		pyrA (L)					
		thiA (R)					

Acr, acriflavin resistance; *ade*, adenine; *arg*, arginine; *bit*, bitertanol resistance; *bio*, biotin; brn, brown-coloured conidiospores; *cho*, choline; *cnx*, nitrate non-utilising, chlorate resistance; *crb*, carbendazim resistance; *crn*, nitrate utilising, chlorate resistance; *cys*, cysteine; *fnr*, fenarimol resistance; *fpa*, p-fluoro-phenylalanine resistance; *fwn*, fawn-coloured conidiospores; *his*, histidine; *leu*, leucine; *lys*, lysine; *met*, methionin; *mtf*, methylthiofanate; *nia*, nitrate reductase, chlorate resistance; *nic*, nicotinamide; *nir*, nitrate utilisation regulatory gene; *ntr*, nicotinamide or tryptophan; *oli*, oligomycin resistance; *olv*, olive-green conidiospores; *pab*, p-aminobenzoic acid; *pdx*, pyridoxin; *phe*, phenylalanine; *pro*, proline; *pyr*, pyrimidine; *ser*, serine; *thi*, thiamine; *trf*, triforine resistance; *trp*, trypthophan; *vco*, vinchlozolin resistance.

Table 4. Proposed correlation between genes and genetic markers

genetic marker	chromosome	possible corresponding orf
adeA or D	IV R	An02g02700 or An02g04020
adeB	III L	An12g04800
adeC	II R	An01g13920 or An01g08090
adeE or G	III	An15g00570 or An15g01760
argA	V R	An16g04970
argB	I	An14g03400
argD	III R	An15g02360
argF	IV R	An02g07250
argH	II R	An01g06560
bioA	III R	An15g02000
bioB	III R	An15g01980
cnxA or E	VI R	An03g02920
cnxB	IV R	An02g13370
cnxD	III R	An15g05720
cnxG	I R	An09g03020
crbA	VIII R	An08g03190
cysA	III R	An15g05170
cysB	IV	An09g02800 or An09g06710
cysC	IV	An02g10750
fwnA	I R	An09g01860 or An09g05730
hisA, D or E	II R	An01g11930 or An01g12570
hisB	V L	An17g01640
hisC	IV	An02g14890
hisF, G or I	II	An13g01080
hisH	III	An15g00610
leuA	IV	An07g07930
leuB or D	IV R	An02g03250 or An02g06150
leuC	II R	An01g14130 or An01g13160
leuE	II	An01g14130 or An01g13160
lysA	III R	An15g00350
lysB or D	VI L	An04g05420, An04g05260 or An04g06210
lysC	IV R	An02g07500
lysE	III	An15g02490
metB	V L	An17g00630
metC	I L	An14g00930
metD	I R	An09g05860
niaD	VIII R	An08g11170
nicB	VII R	An11g10910
nirA	VIII L	An18g02330
olvA	I L	An14g05350
pabA	VI R	An03g03130
pabB	II R	An01g00870
pabC or D	VI	An04g00170
pdxA	VI	An03g04280
pheA	V L	An17g02200
pheB	V	An17g02200
proA	VI L	An04g02800
proE	VI	An04g02800
pyrA	III L	An12g03570
pyrB	VI L	An04g08330
pyrC	II	An01g10650
pyrD or E	VIII	An08g07420
trpA	II R	An01g07260
trpB, C or E	VIII	An08g06060

Materials and methods

Genetic localisation of genes

The genetic localization was performed on CBS 120.49 derived strains. The cloned genes were localised using pulsed field electrophoresis followed by Southern blotting as described by Verdoes et al., 1994a. Parasexual analysis was used to determine the genetic location of *dcpcA* by linkage of the phleomycin resistance to *hisD4* as described by Debets et al. (1990).

Expression data

Gene expression was studied using DSM proprietary Affymetrix arrays, hybridized with RNA samples from an glucose-ammonium fed-batch after day 2 and day 4. The average values for these 2 timepoints were used for the plots in Figure 2.

Acknowledgements

I would like to thank Harry Kools for his help using his *A. niger A. nidulans* synteny plots, Herman Pel, Hein Stam and Jaap Visser for help with Table 2 and Fons Debets for providing Table 3 and Figure 1.

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Chapter 7

Identification of a mitotic recombination hotspot on chromosome III of the asexual fungus *Aspergillus niger* and its possible correlation with an open chromatin structure.

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Part of this Chapter is published in Current Genetics
DOI 10.1007/s00294-007-0143-0

Abstract

Genetic recombination is an important tool in strain breeding in many organisms. We studied the possibilities of mitotic recombination in strain breeding of the asexual fungus *Aspergillus niger*. By identifying genes that complemented mapped auxotrophic mutations, the physical map was compared to the genetic map of chromosome III using the genome sequence. In a program to construct a chromosome III specific marker strain by selecting mitotic crossing-over in diploids, a mitotic recombination hotspot was identified. We tested the effect of temperature on benomyl induced haploidization and found that the highest tested temperature, 37 °C, appeared most effective. The effect of recombination enhancing agents on mitotic recombination frequencies was also examined. A two-fold increase of single crossover events using neomycin and an three-fold increase in double crossover events using 5-azacytidine was found. In addition, these experiments also confirmed the position of the recombination hotspot. Analysis of the mitotic recombination hotspot revealed some physical features that indicate an open chromatin structure and a possible correlation with purine stretches.

Introduction

Aspergillus niger fermentation products have many uses in food applications, for example organic acids and enzymes (Archer 2000). Production processes have been thoroughly optimized by improvements in process technology (Naidu and Panda, 1998; Schügerl, 2000) and by strain breeding (Punt et al., 2002). Classical improved strains have been obtained using random mutagenesis, recombination and genetic modification based on transformation of protoplasts.

Because of its haploid nature, *A. niger* production strains can be improved by subsequent rounds of mutation and selection. Genetic recombination is more troublesome because *A. niger* lacks a sexual cycle. An alternative is recombination using the parasexual cycle, which first was described for *Aspergillus nidulans* (Pontecorvo et al., 1953). The parasexual cycle starts with a heterokaryon, which can be obtained via anastomosis or protoplast fusion. Using double markers, a heterokaryon can be selected and maintained on synthetic medium. A heterozygous diploid, formed by the fusion of the two different nuclei, can be selected as a prototroph. Through chemically induced loss of chromosomes, this diploid is reduced to an unstable aneuploid, finally giving rise to stable haploids. Essentially in this way complete chromosomes are exchanged between the starting strains. Markers with less than 25% recombination are believed to be located on the same chromosome. Using the parasexual cycle, 89 genetic markers were assigned to a linkage group and several marker strains were constructed (Bos et al., 1993).

Although in *A. niger* mitotic crossing-over of homologous chromosomes occurs at low frequencies, it is the only method used to determine the gene order on a linkage group. In a heterozygote diploid, all markers distal to a crossover become homozygous. Debets et al. (1993) used several genetic markers to select such partially homozygous diploids, thereby constructing a genetic map of *A. niger* which shows the gene order of 60 markers.

For the improvement of strain properties, such as enzyme production, the construction of a diploid may result in improved isolates (Khattab and Bazaraa, 2005; Loera and Cordova, 2003; Montiel-Gonzalez et al., 2002). By completing the parasexual cross, also recombinants with higher enzyme production than the parental strains have been isolated (Ball et al., 1978; Das 1980; Das and Ghosh, 1989). This strategy was also successfully used for genetically modified production strains (Bodie et al., 1994). Furthermore, the parasexual cycle can be used for recombination of less compatible strains via protoplast fusion. Heterokaryons that would not be formed otherwise can be obtained this way, and haploidization followed by selection has resulted in the isolation of improved strains (Rubinder et al., 2000).

Usually recombinants are selected randomly, after which their performance is compared to their parents. By first assigning mutations or disruptions to a linkage group, recombination of unlinked properties can be achieved in an orchestrated manner (Swart et al., 1990; van den Hombergh et al., 1997b).

Recombination of linked mutations is troublesome because of the low rate of mitotic crossing-over in *A. niger*. Meiotic crossing between mitotically linked markers is much more frequent and provides a means to establish reliable genetic maps. Possibly an increased rate of mitotic crossing-over could approach the advantages of meiotic recombination. In several organisms mitotic crossing-over has been induced using heat

shock, chemicals or irradiation (Becker et al., 2003; Davies et al., 1975; Hilton et al., 1985; Jansen 1964; Klinner et al., 1984; Sermonti and Morpurgo, 1959; Whelan et al., 1980).

With the *A. niger* genomic sequence available (Pel et al., 2007), the genetic map can be improved by cloning a number oa markers, thus linking the genetic map to the physical map. Using such linked markers, we investigated mitotic crossing-over via selection of partially homozygous diploids. Also crossing-over during haploidization and induced crossing-over was studied and results were compared to the physical map. For this purpose we chose to study markers on chromosome III for several reasons; many markers are available on both arms, on the left arm the cloned markers *pyrA* and *areA* are present and in parasexual crosses the linked markers *bioA1* and *lysA7* were found to have recombination frequencies around 15% (Bos et al., 1988). Furthermore, the sequence information for chromosome III, located on supercontigs 12 and 15 adds up to 4.5 Mb (Pel et al., 2006), while the estimated size by CHEF gels is 4.1 Mb (Verdoes et al., 1994a). This indicates that the sequence information for this chromosome must be close to complete. Using the improved genetic map of chromosome III, we constructed specific marker strains with which we studied the induction and consequence of induced mitotic crossing-over.

Methods

Strains

The *A. niger* strains used were derived from CBS 120.49 and were kindly provided by the Laboratory of Genetics, Wageningen University, the Netherlands. The strains used to construct diploids and new master strains are listed in Table 1. Strains used for the complementation of genetic markers are N495 (*adeB2; nicA1*) for *adeB*, N521 (*fwnA1; adeE8*) for *adeE8*, N658 (*lysA7 argD6; nicA1*) for *argD* and *lysA*, N660 (*bioA1 argL2; nicA1; pabA1*) for *argL*, N687 (*fwnA1; proC3 lysA7 cysA2*) for *cysA* and *lysA*, N705 (*fwnA1; cnxD6; leuA1; nicA1*) for *cnxD*, N733 (*hisD4; adeG13*) for *adeG13*, N884 (*bioA1 lysE28)* for *bioA* and *lysE*, N885 (*bioA1 hisH8; nicB5*) for *hisH*, N901 (*olvA1; bioB2; argI15 metB10*) for *bioB*.

PCR cloning and complementation of genetic markers.

Open reading frames (ORFs) selected for complementation of markers were cloned by using a forward primer 200 bases 5' of the start codon, and a reversed primer 200 bases 3' of the stop codon. The PCR was performed on genomic DNA of *A. niger* CBS 513.88, using pfu-polymerase (Promega). After the last cycle of amplification, Amplitaq (Applied Biosystems) and dATP were added, incubated at 72°C and 1 µl of the PCR mix was used in the pGEMT easy cloning kit (Promega). Of twenty different clones plasmid DNA was isolated and pooled. Transformations were performed essentially as described by Kusters-van Someren et al. (1991). A mixture of 1 µg of the pooled PCR-clones and 5 µg of an *ama1* containing plasmid (Verdoes et al., 1994b) was used for PEG-mediated transformation of 10^6 protoplasts. Transformants appearing were tested for their residual markers. The successful transformations were repeated using plasmid DNA from single clones.

Table 1. Strains used for the construction of diploids and two new marker strains

Diploid	Strain	Markers on III	Other markes
dp 1	N705	*cnxD9*	*metB10*
	N932	*lysA7 argD6*	*fwnA25; nicA1*
dp 2	N430	*argD6*	
	N720	*cnxD6*	*fwnA1; leuA1; nicA1*
dp 3	N893	*pyrA5 hisH8 bioA1*	*fwnA1; pheA1*
	N933	*argD6 cnxD6*	*nicA1*
dp 4	N934	*lysA7 cnxD9*	*fwnA25; nicA1*
	N935	*pyrA5 hisH8 bioA1*	*phenA1*
New marker strains	N936	*pyrA5 argD6 cnxD6*	*nicA1*
	N937	*pyrA5 hisH8 bioA1 cnxD6*	*fwnA1; nicA1*

Selection for mitotic crossing-over and segregation of diploids

Diploids were selected as described (Bos et al., 1989). Selection of homozygous diploids was done by growing single cell colonies of diploids on minimal medium (MM) with sodium nitrate as single nitrogen source and stab inoculation of these colonies on selective complete medium (CM) containing 150 mM $KClO_4$ and proper supplements for all markers present in the diploids. Haploidization of diploids was done on CM plates with 0.88 mg/L D/L-benomyl unless stated otherwise. The chemicals added to the benomyl plates as effectors were used at a concentration that caused only a slight reduction of growth or sporulation of both diploid dp4 and haploid N935 when used on CM. For neomycin (neo) that was 0.55 mM, for butyric acid and ethidium bromide (etb) that was 10 µM, and for 5-azacytidine (5ac) that was 0.25 mM. The UV dose used was 20 J/mm^2 and resulted in 30% kill in haploid N935.

Microarray data.

The microarray data of all ORFs examined in this study are averaged data of day3 and day5 expression levels using GeneChips for a glucose fed-batch culture of CBS 513.88 (Pel et al., 2007). The expression levels were normalized to an average of 100.

Results

Linking the genetic map to the physical map

A first goal was to identify genes complementing known genetic auxotrophic marker genes on Chromosome III and to link the position of these genes on the physical map with the genetic map. Candidate genes for the corresponding biosynthetic routes were selected from supercontigs 12 and 15, comprising Chromosome III specific contigs (Pel et al., 2007) by using the FunCat annotation (Ruepp et al., 2004) (Table 2). A second criterion for the

Table 2. Complementation of genetic markers with selected genes and their position on supercontig 12 and 15

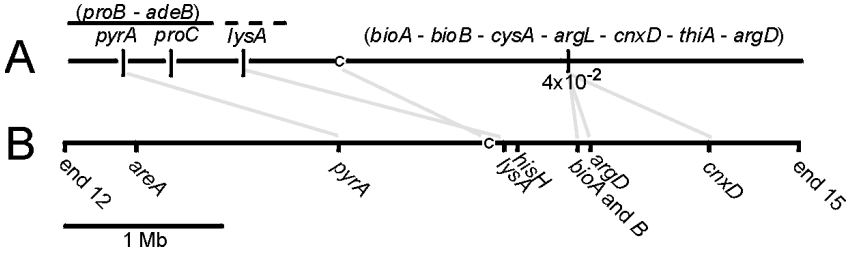
ORF name	Possible corresponding markers	Complementation found of mutation	No complementation of mutations	Position of the ORF (kb)
End of An12				2562
An12g08960	*areA*	*areAd*		2131
An12g07690	*lysA or lysE*		*lysA7*	1845
An12g03570	*pyrA*	*pyrA6*		907
An12g01280	*argD or argL*		*argD6 L25*	354
An12g01110	*cysA*		*cysA2*	319
An12g00320	*adeB E or G*		*adeB2 E8 G13*	76
centromere				0
An15g00350	*lysA or lysE*	*lysA7*		94
An15g00610	*hisH*	*hisH8*		174
An15g01980	*bioA or bioB*	*bioB2*	*bioA1*	534
An15g01990	*bioA or bioB*		*bioA1 B2*	535
An15g02000	*bioA or bioB*	*bioA1*	*bioB2*	539
An15g02340	*argD or argL*		*argD6 L25*	612
An15g02360	*argD or argL*	*argD6*	*argL25*	618
An15g05170	*cysA*		*cysA2*	1220
An15g05720	*cnxD*	*cnxD6*		1334
End of An15				1877

selection of genes was presence of their transcripts in a GenChip hybridization experiment using RNA isolated from a glucose fed-batch fermentation (Pel et al., 2007). With two of the markers, *pyrA* and *areA*, already identified (Goosen et al., 1987; Lenouvel et al., 2001), 14 remaining candidates were cloned as genomic PCR clones. Using these clones, complementation was found for the genetic markers *bioA, bioB, lysA, hisH, argD,* and *cnxD*. In all cases, the auxotrophic mutant was complemented by only one of the candidate genes (Table 2). With 8 cloned markers we compared the physical map of chromosome III with the genetic map described before (Fig. 1, Debets et al., 1993). This comparison adds extra information to the genetic map of chromosome III is in accordance with the previously published genetic map (Debets et al., 1993), except for the position of the *lysA* marker.

Recombination of the genetic markers

The genetic map of Debets et al. (1993) gives limited information on the order of the markers because they were positioned individually, with the ones on the right arm at a crossing-over frequency of 4 % from the centromere. This positioning of markers was established by selection for mitotic crossing-over in heterozygous diploids. The strains

Figure 1. Linking the genetic and physical map of chromosome III.

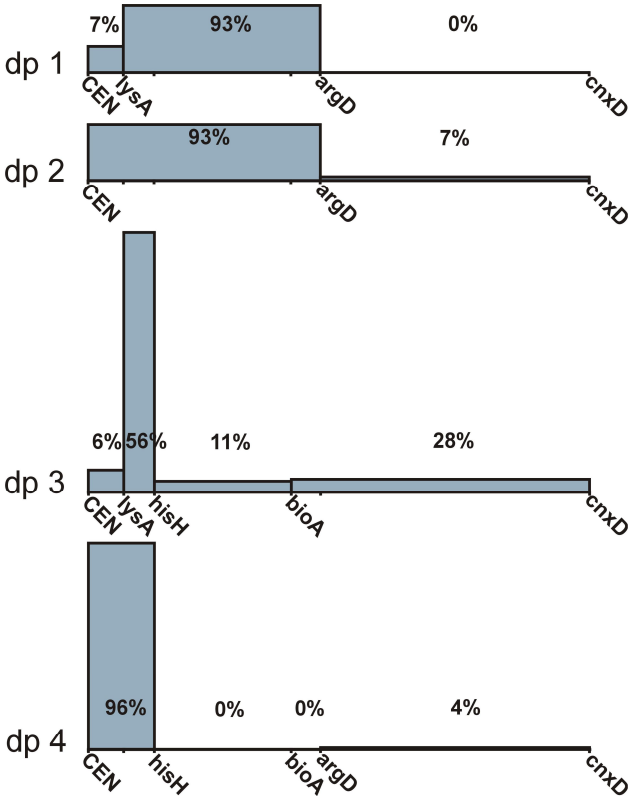


The panel A shows the genetic map of linkage group III as published by Debets et al. (1993). Markers with unknown gene order are shown in parentheses and/or above each other. Panel B shows the physical map drawn to scale, consisting of supercontigs 12 and 15 of the *A. niger* genome sequence (Pel et al., 2007). The location of identified genetic markers is indicated. The centromere (c) is located in a gap of unknown size between the positions 0 of supercontig 12 and 15. For convenience the size of the gap is depicted as 0 bases.

available at the beginning of our study contained only a few of the cloned genetic markers.

Therefore, additional marker strains were constructed containing several auxotrophic mutations which allowed the determination of genetic distances between these markers. Recombination of linked markers requires crossing-over, which is a rare event. Selection for crossing-over events is possible when using a recessive marker located more closely to the telomere than the other markers, as is the case for *cnxD* (Debets et al., 1990). The first recombination performed was the *cnxD9* mutation with *argD6* using diploid 1 (Table 1). From this heterozygous diploid, 15 independent homozygous *cnxD9* diploids were selected on chlorate. The colonies appearing on the chlorate plates were carefully checked for a sector-like growth, in order to be certain that the homozygous diploids arised from independent cross-overs. These diploids were analysed by haploidization on benomyl, and 14 of them appeared to have had a mitotic crossover between *lysA7* and *argD6*, one diploid had a crossover between *lysA7* and the centromere, but no crossover between *argD6* and *cnxD9* was found (Figure 2). In a second recombination experiment using diploid 2 (Table 1) with *cnxD6* and *argD6* we found one diploid with a crossover between *argD* and *cnxD*. The other 12 selected homozygous *cnxD6* diploids resulted from crossovers between the centromere and *argD6* (Figure 2). Of the first crossed-over diploid N933 was isolated, which was subsequently used for the construction of diploid 3.

Figure 2. Frequency of crossing-over between centromere and *cnxD*.



In four diploids homozygous *cnxD6/9* diploids were selected on chlorate. The regions of crossover are delimited by the markers used in the four diploids and are positioned between the centromere (CEN) and *cnxD*. The frequency of the crossing-over in a region correlates with the surface of the box depicted above that region.

Effect of benomyl concentration and temperature on haploidization

There are several reports on factors influencing mitotic crossing-over in asexual cycles, and we examined a number of them, single or in combination. These possible effectors were applied in the haploidization stage of the parasexual cycle, which was induced by benomyl. Because temperature shock is reported to cause chromosome loss in *Candida albicans* (Hilton et al., 1985) and the benomyl concentration is rather critical for optimal haploidization, we tested a possible influence of benomyl concentration and different temperatures on mitotic crossing-over frequency. As expected, the benomyl concentration did not have much effect on the frequency of mitotic crossover. Only a slight increase in

mitotic recombination was found at 37 °C compared to the lower temperatures (Table 3). A remarkable difference is that at 37 °C haploidization was much more efficient compared to 25 °C and 28 °C (data not shown). All plates at 37 °C gave well-growing black and white sporulating haploid sectors, with hardly any dark brown sporulation, which is characteristic for diploids. At 25 and 28 °C however, the highest benomyl concentration resulted in much fewer haploid sectors and at the lowest benomyl concentration a lot of diploid sporulation could be observed. We therefore chose 37 °C as the standard temperature at which the remaining haploidization experiments were carried out. In this experiment, all 13 recombination events between the markers on chromosome III took place between *lysA7* and *argD6* and none between *cnxD9* and *argD6*.

The effect of recombination enhancing agents on mitotic recombination frequencies

In several yeasts and filamentous fungi, chemicals and irradiation have been shown to induce mitotic crossover. UV light could increase mitotic crossing-over by causing single and double strand breaks. Ethidium bromide (etb) and neomycin (neo) might influence recombination through their intercalating activity, where neomycin is reported to be an efficient stabilizer of DNA triplexes (Arya et al 2001). The methylation inhibitor 5-azacytidine (5ac) and the deacetylation inhibitor butyric acid (bta) (Selker 1998) might influence crossover frequencies by their chromatin modifying activity (Toth et al., 2004). Moreover, 5-azacytidine was reported to increase mitotic recombination and point mutations (Zimmerman and Scheel 1984). To determine if this was also the case in *A. niger*, we tested some of the described chemicals and UV light. For this we used diploid 3 because it is heterozygous for 5 markers which are nicely divided over chromosome III.

Table 3. The influence of benomyl concentration and temperature on crossing-over

	1.1 µg/L D/L-benomyl	0.94 µg/L D/L-benomyl	0.83 µg/L D/L-benomyl	average
25°C	1 / 21	1 / 17	0 / 13	4%
28°C	0 / 2	0 / 13	2 / 20	6%
30°C	1 / 21	2 / 17	0 / 16	6%
37°C	3 / 21	2 / 18	1 / 17	11%
average	8%	8%	5%	

The number of recombinants found between *lysA7* and *argD6* per total of haploids tested at different haploidization temperatures and benomyl concentrations. Average recombination frequencies are calculated for the three different temperatures used and for the three different benomyl concentrations used.

On average, 25 recombinants were tested per condition, which included single effectors and combinations of them (Table 4). In total 509 haploids were tested of which 25 were found to be recombinants of the linked markers. It is very difficult to estimate the accuracy of the

Table 4. Recombination of linked markers found after haploidization of diploid 3 under different conditions

CMben +:	Number and type of cross-overs	No haploids screened	Recombinant %
-	2a	29	7
30°C	1a	33	3
UV	0	28	0
etb	0	33	0
neo	1a	20	5
bta	2b 1abd	31	10
5ac	0	31	0
UV + etb	1a	33	3
UV + neo	2a	16	13
UV + bta	0	9	0
UV + 5ac	1a 2bc	15	20
etb + neo	1a	24	4
etb + bta	0	28	0
etb + 5ac	0	29	0
neo + bta	1a 1cd	29	7
neo + 5ac	2a 1b 1cd	23	17
bta + 5ac	0	26	0
UV + etb + bta	0	36	0
UV + etb + 5ac	1a 1ab	20	10
UV + neo + bta	0	1	0
UV + neo + 5ac	1a 1b 1cd	15	20
total	14a 4b 1ab 2bc 3cd 1abd	509	5

Crossing-over between linked markers is compared for single, double and triple effector combinations. The number of recombinants is indicated per region where a crossover has occurred. These regions are coded a, b, c, or d, based on the marker position on the physical map: *pyrA*-a-*hisH*-b-*bioA*-c-*argB*-d-*cnxD* (see Fig. 3). For example, 1a 2bc / 15 means that out of 15 haploids one had a crossover in region a (i.e. 1a) and two haploids had a crossover in region b and a crossover in region c (i.e. 2bc). Instead of a double crossover, also a gene conversion could have occurred.

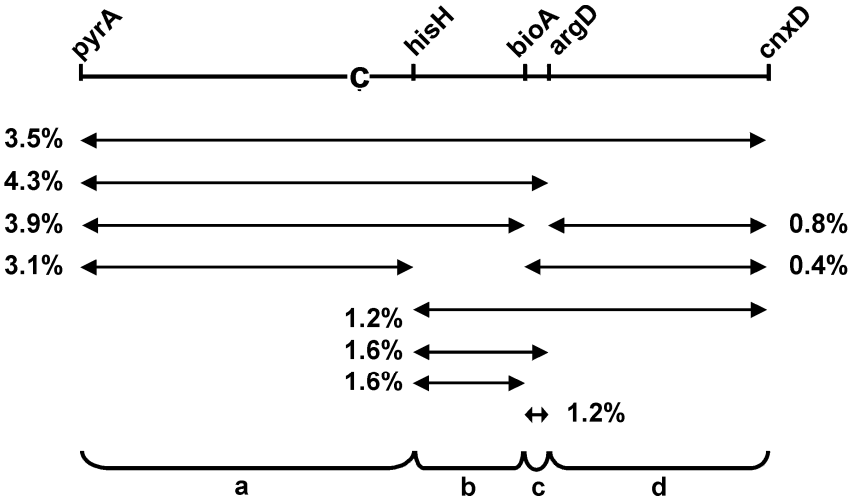
mitotic recombination frequencies found. Several recombinants isolated could be the result of one crossing-over event, or might be independent. Also, the number of haploids tested for each effector combination is limited. Of single effectors tested, only the use of bta results in a higher crossing-over frequency compared to the control. However, in combination with other effectors, no increase is found. The highest mitotic recombination frequencies were found with UV+5ac, UV+neo+5ac and with neo+5ac (20%, 20% and 17%, respectively). Statistical evidence, however, of these data is quite low because of the limited number of recombinants examined. To have a better indication of which effector provokes an increased recombination frequency, the data of all conditions under which an effector was included was pooled (Table 5). These pooled data suggests that neo, 5ac and to a lesser extent UV increase mitotic recombination in *A. niger*. Amongst the recombinants, six are listed to have resulted from a double crossover and one from a triple crossover. It is possible that these recombinants are not the result of multiple crossover event, but that they are the result of gene conversion, especially so because the double crossover spans only one marker. Gene conversion may also be responsible for some of the 18 “single crossover” recombinants. Besides an increase in mitotic recombination, also the distribution of the recombination events is of interest. From the complete progeny the linkage of all chromosome III markers was calculated (Figure 3). Clearly the recombination frequency between the markers does not resemble the physical distance on the chromosome. Although the physical distance between *pyrA* and *hisH* is comparable to the distance between *hisH* and *cnxD*, in the first region the recombination frequency is twice as high as in the latter.

Table 5. Pooled mitotic crossing-over averages upon haploidization of diploid 3 under different conditions.

Condition	No of haploids screened	% cross-over			Type and frequency of cross-overs
		Single	Double	Triple	
all	509	3.5	1.2	0.2	14a 4b 1ab 2bc 3cd 1abd
30°C	33	3.0	0.0	0.0	1a
-	31	6.5	0.0	0.0	2a
UV	163	4.0	2.3	0.0	6a 1b 1ab 2bc 1cd
etb	203	1.5	0.5	0.0	3a 1ab
Neo	128	7.8	2.3	0.0	8a 2b 3cd
Ba	159	1.9	0.6	0.6	1a, 2b, 2cd, 1abd
5ac	149	4.4	3.1	0.0	5a 2b 1ab 2bc 2cd

The results of Table 4 are pooled calculated for each separate effector. The crossovers are given as percentages. Type and number of crossovers are given as in Table 4.

Figure 3. Recombination percentages between genetic markers on chromosome III in cross 3.



Only part of the improved genetic map of chromosome III from Figure 1 is depicted. Recombination frequencies of the markers in this region are depicted beside the area in which mitotic crossover or gene conversion has given rise to these recombinations. The lengths of the arrows represent physical distances and do not correlate well with genetic distances.

Delimiting the recombination hotspot

Apparently, in *A. niger* there is a bias for mitotic crossover in certain regions of the genome (Fig. 2 and 3). It is striking that of the 18 single crossovers, 14 have occurred between *pyrA* and *hisH* and the other four between *hisH* and *bioA* (Table 4). These regions together overlap the region between *lysA* and *bioA* where the previously reported recombinations were found (Bos et al., 1988). Also this region overlaps with the region between *lysA* and *argD* where in the experiment with diploid 1 and 2 a much higher number of crossovers was found than between *argD* and *cnxD* (Fig. 2). To further examine the distribution of mitotic crossovers we analysed 26 homozygous *cnxD9* diploids selected from diploid number 3 and 18 homozygous *cnxD9* diploids selected from diploid number 4. In the first case 25 crossovers occurred between the centromere and *hisH8* and only one between *argD6* and *cnxD9*. In the latter case only one crossover occurred between the centromere and *lysA7*, 10 between *lysA7* and *hisH8*, two between *hisH8* and *bioA1* and five between *bioA1* and *cnxD9* (Figure 2). This clearly shows that there is a recombination hotspot in the 80 kb region between *hisH* and *lysA*.

Analysis of the recombination hotspot

Possibly the region between *hisH* and *lysA* contains genetic elements that increase crossing-over. We searched for microsattellite repeats (Majewski and Ott, 2000) and polyA stretches, using the pattern search on the Biomax server (Frishman et al., 2003). Furthermore, the expression profiles of all ORFs on supercontig 15 were examined, using the microarray data published by Pel et al. (2007) (Fig. 4A and B) and the Genome Atlas of supercontig 15, kindly provided by Dr. D. Ussery using previously developed tools (Skovgaard et al., 2002; Wanchanthuek et al., 2006) (Fig. 4C and D). We found a short polyA stretch and three GA stretches and at the telomeric side of the *hisH* gene a CT repeat. Looking at expression across supercontig 15 on a scale of 0 to 3000, there is no apparent abnormality near the mitotic recombination hotspot (Fig. 4A). There is a large region from 990kb to 1240kb where gene expression seems to be low. With the expression plotted on a scale from 0 to 100, it becomes clear that within the mitotic recombination hotspot, particularly around *hisH*, the lowest transcribed ORFs have a higher transcription compared to the lowest transcribed ORFs outside this region (Fig. 4B). Another feature deviating along the complete *lysA-hisH* region is a more negative stacking energy, indicating a more stabilized region (Skovgaard et al., 2002) and a low AT percentage. The position preference, a measure for flexibility (Baldi et al., 1996) is only slightly higher near *hisH*. There are a few simple repeats in the *lysA-hisH* region and there are multiple purine or pyrimidine stretches of minimal 10 bases ((Y)10 vs (R)10) around *hisH*, coinciding with an elevated GC skew (G-C)/(G+C) and AT (A-T)/(A+T) skew. A zoom-in of the *lysA-hisH* region is shown in Figure 4D.

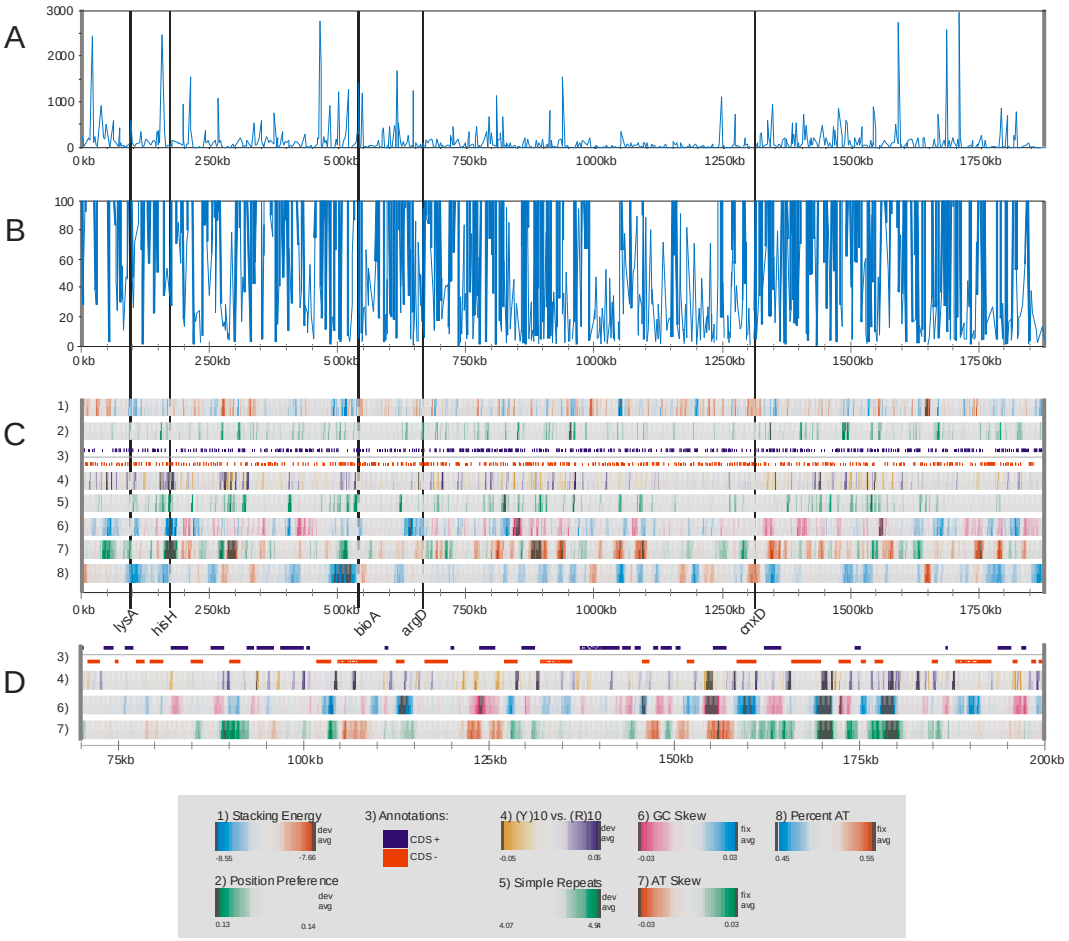
Occurrence of purine stretches in supercontig 15

Purine stretches inside ORFs are known to block transcription via the formation of an intramolecular triple helix (Bidichandani et al., 1998; Grabczyk and Fishman, 1995) while purine stretches were also reported to occur more often in fungal genomes than could be expected from random sequences (Ussery et al., 2002). Therefore, we had a closer look at the purine stretches. Inside the coding DNA located in the region between *lysA* and *hisH*, the largest purine stretches are 17 and 16 bases, respectively. Outside the coding DNA there are 6 purine stretches of 32 to 65 bases long, and 2.7 kb at the centromeric side of *hisH*, an 80 bp purine stretch containing only two pyrimidines.

Discussion

To link the genetic map to the physical map, thirteen ORFs were selected to possibly complement eleven markers. Six of those genes indeed complemented a corresponding marker, which is indicative for the high quality of the genome annotation. Six of these

Figure 4. Genome atlas and expression profile of supercontig 15.



Expression of all genes on supercontig 15 are plotted in two scales (A and B). The genome atlas of supercontig 15 according to (C) shows repeats, structural parameters and parameters directly related to the base composition. The region studied for mitotic recombination is located between the centromere (left of 0 kb) and *cnxD* (1334 kb). The recombination hotspot is located between *lysA* (94 kb) and *hisH* (174 kb).

markers and two already cloned markers *areA* and *pyrA*, were positioned on the genetic map by Debets et al. (1993; see also Figure 1). The position found for *lysA* on the right arm is not in agreement with the expected position left of the centromere. The experimental evidence for the left arm position is mainly provided by selection for mitotic crossing-over on fluoro-orotic acid (FOA) in a diploid heterozygous for *pyrA5* and *lysA7* (F. Debets, personal communication). FOA is very mutagenic compared to chlorate and possibly induced additional crossovers that led to a false positioning of *lysA*. In our results with *cnxD* selected crossovers in diploids 1 and 3 we found further evidence for *lysA* to be located on the right arm (Fig 2). The resulting physical map can be seen as an improved genetic map because more markers are placed in the correct physical order. The genetic distance of these markers, however, is still unclear.

The concentration of benomyl needed for haploidization is rather critical. If it is too low, the diploids will grow too well, and if it is too high, even haploids will not grow. This problem seemed to occur less at a temperature of 37 °C compared to 25 °C. Benomyl binds to heterodimeric tubulin (Davidse 1986) causing microtubule depolymerization, which in mitosis leads to disjunction. A cell with just one aneuploid nucleus with less than 8 chromosomes will not be viable, while an aneuploid nucleus with more than 8 chromosomes will fall back to a haploid. Aneuploidy in diploid nuclei will lead to a nucleus with less than 2n chromosomes, which will reduce its genome to 1n, and a nucleus with more than 2n. The latter will fall back to the initial diploid state. Although this explains why haploid sectors remain homogeneous and diploid sectors do not, it does not explain why at certain benomyl concentrations, diploids are severely inhibited in growth while haploids grow happily. There are a several proteins known that bind microtubules and thereby influence benomyl sensitivity (Rischitor et al., 2004; Weil et al., 1986). Possibly, some of these factors are expressed differently in a diploid compared to a haploid at 37 °C causing the temperature effect on haploidization efficiency.

In our analysis of mitotic crossover frequencies, we found a lower frequency of recombination of the used linked markers than the reported 15% for *lysA bioA* recombinations (Bos et al., 1988). A large difference in the methods used here and in previous reports is that in our case all recombinants were purified and rescreened. Of the effectors tested, the highest increase in single crossover was found using neomycin, increasing the average crossing-over from 3.5% to 7.8%. Possibly neomycin acts through stabilizing DNA triplexes (Alya et al., 2001), thereby promoting the first steps in the Meselson-Radding model for mitotic recombination initiated by single stand breaks, and the double strand break repair model (Orr-Weaver and Szostak, 1985). Combinations with other intercalating agents could increase the recombination frequency further.

Examining the types of crossover, it is striking that all of them occur on both sides of only one marker. Probably, most of these recombinations have not occurred by means of

a double crossover, but rather through a gene-conversion. In the theory of a single strand break leading to a heteroduplex of homologous chromosomes, this heteroduplex can resolve into a mitotic crossover or into a gene conversion (Orr-Weaver and Szostak 1985). In the model of the Holliday junction, this junction progresses over the chromosomes and causes gene conversion. Depending on how far it progresses before being resolved, it could have altered one of the few markers used. In case of a crossover, the markers on one side of the crossover are found to be of one parental type and vice versa. Especially using 5ac, a three times higher frequency of double crossover events has been found, while the frequency of single crossovers increased less than a factor two. A possible explanation is that de-acetylation of the DNA leads to a less compact chromatin structure, where a Holliday junction can progress more easily, increasing the chance of changing a marker and thus being detected.

It appears possible to increase mitotic crossover during haploidization using effectors. Especially the combination of 5ac with neomycin improves recombination frequencies of linked markers. Unfortunately, deriving the gene order from these recombinants can easily lead to a wrong conclusion. The recombination frequency between the markers does not correlate to their genetic distance or order (Fig. 3). Also a previous prediction of the linear gene order of *areA*, *pyrA*, *lysA* and *bioA*, based on haploid recombinants was erroneous (van den Hombergh et al., 1997a).

A series of mitotic recombinations to combine multiple genetic markers into one single master strain proved much more difficult than expected because of the presence of a mitotic recombination hotspot in the region between *lysA* and *hisH* (Fig. 2). There are several examples of increased recombination in genetic regions, both during mitosis and meiosis. Meiotic recombination hotspots are caused by increased double-strand breaks in defined genomic regions (Nishant and Rao 2006). In yeast, mitotic recombination in general occurs more often near the centromere (Minet et al., 1980) and a mitotic recombination hotspot is also proposed to act via double-strand breaks (Neitz and Carbon 1987), while *HotI* stimulates mitotic recombination in adjacent sequences (Steward 1989). In *A. nidulans* there is a position dependent difference between meiotic recombination and mitotic recombination. Using sexual crosses, genetic distances near the centromeric region appear larger than the physical distances (Aleksenko et al., 2001; Espeso et al., 2005), while the opposite is found when using mitotic recombination (Käfer 1977). Although there seems to be a preference for mitotic crossing-over near the centromere, we conclude that the 80 kb region between *lysA* and *hisH* contains a mitotic recombination hotspot, because most mitotic crossovers events occur in that region and not in the 94kb centromere-*lysA* region (Fig. 2).

The expression graphs and the genome atlas (Fig. 4) visualize some interesting features of the mitotic recombination hotspot. Over the whole *lysA*-*hisH* region there is a

high basic transcription, indicating an open chromatin structure (Fig. 4b). A high AT percentage compared to the rest of the supercontig causes an increased stacking energy, which indicates a more stable helix (Ornstein et al., 1978). Around *hisH*, both GC-skew and AT-skew are higher, coinciding with purine stretches (Ussery et al., 2002). In some plants and fungi, an elevated GC-skew near the transcription start sites of highly transcribed genes was found (Fujimori et al., 2005). As mentioned before, purine stretches can have a drastic effect on transcription. The *lysA-hisH* region contains purine stretches outside coding DNA, whereas inside the ORFs no apparent purine stretches are found. Additionally, the region of low transcription from 990 to 1240 kb contains very few purine stretches (Fig. 4) and is situated between the markers *argD* and *cnxD*, which show very little recombination. Ussery et al. (2002) reported that in microbial eukaryotic chromosomes, the purine tracks occur more often than would be expected from random sequences, and are localized mainly in non-coding regions. Also, a relation between mitotic recombination frequencies and transcription levels (Saxe et al., 2000; Aguilera, 2002; Grewal and Elgin, 2002) may be relevant in the *lysA-hisH* region.

All together, it is not clear whether the deviating physical properties of the DNA cause the mitotic recombination hotspot, and if so, which of them is most important. However, the connection between noted properties could be through elevated basic transcription, causing or caused by an open chromatin structure that could lead to an increased mitotic recombination in the *lysA-hisH* region. Especially the presence of purine stretches outside coding DNA, and the absence of purine stretches inside coding DNA seem to correlate with basic transcription and mitotic recombination. However, we cannot exclude that the mitotic recombination hotspot could also be caused by a yet unidentified genetic element.

Acknowledgement

The authors wish to thank Fons Debets for help with the recombination of *cnxD* and *argD*, Dave Ussery for making the genome atlas and Mark Arentshorst for technical assistance.

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Summary

Aspergillus niger has many uses in food biotechnology. It is used for the production of homologous and heterologous proteins, mainly enzymes and for the production of organic acids. Especially in the production of heterologous proteins, several problems are encountered such as the need for efficient transformation systems, the effect of CCR on induction of protein production and efficient use of resources, morphologic and genetic instability and undesired metabolite accumulation. In studies of these problems, often mitotic recombination has been used and several solutions have been found in the form of mutations. To examine the possibility for recombination of these mutations, their genetic and physical location is of importance as well as genetic properties of their chromosomal surroundings that influence mitotic crossing-over. A short introduction to these topics is given in **Chapter 1** and recent findings concerning these aspects are presented in Chapters 2 to 7.

In Chapter 2 we describe the *argB* transformant selection system. Although the *argB* gene of *A. nidulans* was shown to complement the *argI13* and *argI15* mutations in *A. niger*, it appeared that arginine requirement was not restored completely. Disruptions and characterisation of the *argB* gene of *A. niger* showed that in the *argI* mutants probably not the ornithine decarboxylase gene was mutated, but possibly a mitochondrial transport function. The *A. nidulans* gene, however, was able to restore this defect because of its cytosolic location, avoiding the necessity of transport of its substrate into the mitochondrion, or transport of the product out of the mitochondrion.

Strain instability is a problem, especially in continuous cultures, used to determine strain characteristics such as productivity at defined specific growth rates. Apart from copy number loss of genetically modified strains, also productivity and conidiation can be lost in continuous cultures. In **Chapter 3** we used a Variomixing bioreactor, especially designed to reduce wall growth, to study morphological stability in both glucose and ammonium limited continuous cultures of *A. niger*. In an ammonium limited culture at constant dilution rate, aconidial mutants appeared after 110 hours. One such aconidial or “fluffy” mutant, *fluA1* was isolated and genetically characterized. Its genetic location on chromosome III does not correspond to the position of orthologs of known *A. nidulans* fluffy mutants. The *fluA1* mutant has an increased radial growth rate of 30% explaining its appearance in the culture. The mutation is recessive, and a nitrate reductase (*niaD*) variant of the mutant was shown to conidiate again in a forced heterokaryon with a *fluA+* strain carrying several auxotrophic markers. Conidia harvested from this material were demonstrated to be an appropriate inoculation source for a liquid culture of the *fluA1* strain as the *fluA1* strain

again quickly dominated the culture while the other strain and the diploid decreased in content. Using this technique, the use of an aconidial strain to increase morphologic stability could be examined.

Chapter 4 addresses regulation of carbon catabolism. Saprophytic fungi such as *A. niger* can produce a wide variety of enzymes useful for the degradation of plant cell walls. These plant cell wall components contain different combinations of sugars and some sugars are metabolised more easily than others. Two important regulation mechanisms ensure that the appropriate extracellular enzymes are produced, i.e. specific induction and carbon catabolite repression (CCR). Of all sugars, glucose is the most repressing one. The signaling pathway leading from glucose to the CCR compound exerting the repression on targeted genes, CREA, possibly starts with glucose phosphorylation. We studied the role of hexose phosphorylation in glucose repression in *A. nidulans*. Using mutants, we showed that one hexokinase together with one glucokinase are responsible for glucose phosphorylation. Absence of glucose phosphorylation activity reduces the effect of glucose repression, but does not abolish it. In *Saccharomyces cerevisiae* hexokinase II plays an additional role in CCR signaling. Besides hexose phosphorylation its presence is required in a nuclear localised complex to allow CCR signaling. The two *A. nidulans* hexose phosphorylating enzymes were equally important in establishing CCR, their importance apparently limited to their hexose phosphorylating activity. However, genome sequencing of both *A. niger* and *A. nidulans* showed that other hexokinase orthologs are present, and one of their gene products might play the same role as HXKII in yeast. Also the mechanism of xylanase repression by glucose was demonstrated to work via inducer exclusion.

In **Chapter 5** the isolation and characterization of an *A. niger* xylitol transport mutant is described. Polyols play an important role in fungal physiology and they are found in mycelium and conidia under several conditions. Their proposed roles are in carbon storage, cofactor balance, osmoregulation and to overcome heat and oxidative stress. In *A. niger*, xylitol is an intermediate of both the D-xylose and L-arabinose catabolism, and it is secreted in low amounts during growth on these pentoses. In a genetic screen for mutants with reduced xylitol mediated carbon catabolite repression, a mutant was isolated with impaired xylitol transport. Genetic characterization showed that the *xtlA36* mutation, located on chromosome IV, is responsible for reduced growth on xylitol, probably by reduced xylitol uptake. More dramatic is the effect of this mutation on xylitol secretion. During growth on D-xylose, hardly any xylitol is secreted, a characteristic that would be very useful for yeast ethanol production on hemicellulose.

The linkage of the physical map with the genetic map is described in **Chapter 6**. The *A. niger* genome sequencing of CBS 513.88 resulted in 19 supercontigs of genetic information that together make up the 8 chromosomes of *A. niger*. The proposed orientation of the 19 supercontigs on the genetic map was deduced in several steps. First, all 19 supercontigs were assigned to a chromosome by parasexual analysis of mutants or by Southern blot analysis of electrophoretically separated chromosomes. In the next step, the reciprocal orientation of the supercontigs was established through their high synteny to *A. nidulans* at the centromeric sides of the supercontigs and through the telomeric positioning effect on transcription. The last step was to link the physical map of the chromosomes to the genetic map. This was done using mapped genes with known sequence and using synteny with *A. nidulans* to deduce the genes corresponding to genetic markers of *A. niger*. Of the 11 gaps in the chromosomal sequences, eight of them constitute centromeric regions. They are probably lacking because their highly repetitive sequences make cloning very difficult.

In **Chapter 7**, the genetic map of chromosome 3 was improved by cloning some of the genetic markers. Mitotic recombination frequencies across the right arm of the chromosome were established in heterozygous diploids during normal mitosis and during benomyl induced haploidization. Neomycin and 5-azacytidine were found to have an effect on recombination of linked markers in benomyl induced haploidization. The selection of mitotic crossing-over in heterozygous diploids revealed a mitotic recombination hotspot between *lysA* and *hisH*. This genomic region was analyzed using expression data and a genome atlas and we found an elevated basic transcription and the presence of large purine stretches between the genes, especially near *hisH*. Possibly, the occurrence of purine stretches between genes result in an open chromatin structure, causing an increase in crossing-over frequency.

Samenvatting

Aspergillus niger kent vele toepassingen in de voedingsindustrie. Deze schimmel wordt gebuikt voor de productie van homologe (soorteigen) en heterologe (soortvreemde) eiwitten, voornamelijk enzymen, en voor de productie van organische zuren zoals citroenzuur. Bij de productie van zowel homologe als heterologe eiwitten komen meerdere problemen voor. Een aantal van die problemen zijn de behoefte aan meerdere efficiënte transformatie systemen, suboptimale inductie door het effect van koolstof kataboliet repressie, morfologische en genetische instabiliteit en ongewenste metaboliet vorming. Bij de bestudering van deze problemen is in dit proefschrift vaak gebruik gemaakt van mutagenese en mitotische recombinatie om oplossingen daarvoor te vinden. Om de mogelijkheid voor recombinatie van dit soort mutaties te onderzoeken, is het van belang de genetische en fysieke locatie te weten, alsmede de genetische eigenschappen van het betreffende chromosoom. In **Hoofdstuk 1** wordt een korte inleiding gegeven over deze onderwerpen en in de **Hoofdstukken 2 t/m 7** worden in meer detail recente bevindingen gepresenteerd.

In **Hoofdstuk 2** wordt het *argB* transformatie selectie systeem beschreven. De *argI13* en *argI15* mutaties van *A. niger* worden gecomplementeeerd door het *argB* gen van *Aspergillus nidulans*, echter het opheffen van de arginine behoefte is niet volledig. Na disruptie en karakterisatie van het *argB* gen van *A. niger* bleek dat de *argI* mutaties niet in het ornitine transcarbamylase gen liggen, maar in een ander gen dat mogelijk codeert voor een mitochondriale transporter van ornitine of citrulline. Terwijl in *A. niger* het ornitine transcarbamylase waarschijnlijk werkzaam is in het mitochondrium, blijft het *A. nidulans* enzym mogelijk voor tenminste een deel in het cytosol. Daar zou het in staat kunnen zijn de arginine synthese te herstellen in de *argI* mutanten doordat het transport van substraat of product over het mitochondriale membraan althans gedeeltelijk overbodig wordt.

Een stam is niet altijd even stabiel, iets wat vooral merkbaar is in continu cultures. Deze worden vaak gebruikt om bij constante groeisnelheden stam eigenschappen als productiviteit vast te stellen. Stam instabiliteit uit zich bij genetisch gemodificeerde stammen in verlies van kopie aantal en verminderde productiviteit of verlies van het vermogen tot sporulatie. In **Hoofdstuk 3** gebruikten we een zogenaamde Variomixing bioreactor, een speciaal ontwerp om wandgroei te voorkomen, om daarin de morfologische stabiliteit in continu cultures van *A. niger* te bepalen. In een ammonium gelimiteerde culture bij constante verdunningssnelheid verscheen na 110 uur een niet-sporulerende stam. Eén zo'n niet-sporulerende oftewel "wollige" mutant is geïsoleerd en de verantwoordelijke mutatie *fluA1* is genetisch gekarakteriseerd. De genetische locatie van *fluA1*op chromosoom

III komt niet overeen met de positie van de othologen van de bekende *A. nidulans* wollige mutanten. De radiale groeisnelheid van de *fluA1* mutant is verhoogd met 30% wat verklaart dat de mutatie naar voren komt in de culture. De mutatie is recessief, en een nitraat reductase (*niaD*) variant van de mutant kon weer sporuleren in een geforceerd heterokaryon met een *fluA+* stam die meerdere auxotrofe markers had. Sporen afkomstig van dit heterokaryon bleken een geschikt entmateriaal voor een vloeibare culture van de *fluA1* stam omdat deze weer snel de overhand kreeg, terwijl de andere stam en diploiden snel uit de vloeibare culture verdwenen. Gebruik makend van deze techniek zou men het nut van een niet-sporulerende stam in het verhogen van morfologische stabiliteit kunnen onderzoeken.

Hoofdstuk 4 gaat over regulatie van koolstof metabolisme. Saprophytische schimmels als *A. niger* produceren een breed scala aan enzymen die dood plantaardig materiaal kunnen afbreken. De celwanden van planten zijn opgebouwd uit meerdere componenten die verschillende combinaties van suikers bevatten. *A. niger* kan sommige van deze suikers gemakkelijker opnemen en verbranden dan andere suikers. Er zijn twee belangrijke mechanismen die ervoor zorgen dat de suikers in een bepaalde volgorde gebruikt worden. Deze mechanismen zijn specifieke inductie en koolstof kataboliet repressie (carbon catabolite repression, CCR). Van alle suikers is glucose het meest represserend. Het eiwit dat uiteindelijk de repressie op al de onderdrukte genen uitoefent is CREA. Het signaal van glucose naar CREA zou kunnen beginnen bij glucose fosforylering. We bestudeerden daarom de rol van hexose fosforylering bij glucose repressie in *A. nidulans*. Met behulp van mutanten lieten we zien dat één hexokinase en één glucokinase verantwoordelijk zijn voor alle glucose fosforylatie. Afwezigheid van glucose fosforylerende activiteit vermindert het effect van glucose repressie, maar doet het niet verdwijnen. In *Saccharomyces cerevisiae* speelt een derde hexokinase, HXKII, nog een rol in de signaal transductie van glucose repressie. Behalve hexose fosforylering is het enzym nodig in een celkern gelocaliseerd complex om CCR tot stand te brengen. In *A. nidulans* echter, zijn de twee hexose fosforylerende enzymen even belangrijk in het tot stand brengen van CCR. Blijkbaar is hun belang beperkt tot hexose fosforylering. Daarentegen blijkt uit de genoom sequenties van zowel *A. niger* als *A. nidulans* dat er nog andere hexokinase orthologen aanwezig zijn, en misschien vervult één van deze enzymen eenzelfde rol als HXKII in gist. Naast deze resultaten blijkt het mechanisme van xylanase repressie door glucose te werken door het verhinderen van de opname van de xylanase inducerende suiker D-xylose.

In **Hoofdstuk 5** wordt de isolatie en karakterizering van een xylitol transport mutant van *A. niger* beschreven. Polyolen vervullen een belangrijke rol in de fysiologie van schimmels. Ze worden onder diverse condities aangetroffen in mycelium en sporen en spelen een rol in koolstof opslag, cofactor balans, osmoregulatie en in het weerstaan van hitte en oxidatieve

stress. In *A. niger* is xylitol een intermediair van zowel het D-xylose als het L-arabinose catabolisme, en het wordt in kleine hoeveelheden uitgescheiden tijdens groei op deze pentoses. In een selectie voor mutanten met verminderde xylitol afhankelijke koolstof kataboliet repressie, is een mutant geïsoleerd met een defect in het xylitol transport. Bij genetische karakterizering bleek dat de verantwoordelijke mutatie, *xtlA36*, is gelegen op chromosoom IV. Deze mutatie veroorzaakt verminderde groei op xylitol, waarschijnlijk door verminderde xylitol opname. Nog duidelijker is het effect van de *xtlA36* mutatie op xylitol uitscheiding. Tijdens groei op D-xylose wordt er nauwelijks xylitol uitgescheiden, een eigenschap die heel nuttig zou kunnen zijn voor ethanol productie uit hemicellulose door gist.

Het koppelen van de fysieke kaart met de genetische kaart van *A. niger* is beschreven in **Hoofdstuk 6**. De DNA sequentie bepaling van het *A. niger* genoom van stam CBS 513.88 resulteerde in 19 supercontigs met genetische informatie die samen de 8 chromosomen van *A. niger* vertegenwoordigen. De veronderstelde orientatie van deze 19 supercontigs ten opzichte van de genetische kaart was bepaald in drie stappen. Als eerste stap is van alle 19 supercontigs bepaald op welk chromosoom ze thuishoren door middel van parasexuele analyse van mutaties in een bekend gen of door Southern blot analyse van electroforetisch gescheiden chromosomen. Vervolgens is de reciproke orientatie vastgesteld door gebruik te maken van de hoge mate van synteny met *A. nidulans* aan de centromeer zijde van de supercontigs. Ook de telomerische kant van de supercontigs kon worden bepaald doordat genen aan de telomeer zijde veelal een verlaagde transcriptie vertonen. De laatste stap was het koppelen van de fysieke kaart van de chromosomen aan de genetische kaart met behulp van genetisch gelocaliseerde markers met bekende sequentie of waarvan herleid kon worden welke sequentie ze hadden. Dit laatste gebeurde met behulp van de genetische kaart van *A. nidulans* in combinatie met synteny tussen de regio's waar vergelijkbare markers lagen. Van de 11 gaten in de chromosomale sequenties behelsden acht daarvan centromeren. Zij ontbreken waarschijnlijk in de bekende genoom sequentie omdat ze waarschijnlijk herhalingen bevatten die het erg moeilijk maken zo'n gebied te kloneren.

In **Hoofdstuk 7** is de genetische kaart van chromosoom III verbeterd door een aantal markers te kloneren. Van de rechterarm werden mitotische recombinatie frequenties bepaald zowel in een heterozygote diploid gedurende normale mitose als gedurende benomyl geïnduceerde haploidizatie. Neomycine en 5-azacytidine bleken een verhogend effect te hebben op recombinatie van gekoppelde markers tijdens benomyl geïnduceerde haploidizatie. Tijdens selectie van mitotische crossing-over in heterozygote diploiden bleken de meeste mitotische recombinaties in een klein gedeelte van het chromosoom plaats te vinden. Dit gedeelte van het chromosoom liggend tussen *lysA* en *hisH* werd

geanalyseerd met behulp van expressie data en met een zogenaamde genoom atlas. We vonden in dit gebied een verhoogde basale transcriptie en de aanwezigheid van lange purine stretches tussen de genen, vooral in de nabijheid van *hisH*. Mogelijkerwijs veroorzaakt de aanwezigheid van die purine stretch een open chromatine structuur, die op zijn beurt een verhoging van de crossing-over frequentie veroorzaakt.

Curriculum vitae

Peter van de Vondervoort is geboren op 23 juli 1963 in Someren. In 1980 behaalde hij zijn HAVO diploma op het College Asten Someren en begon hij aan de laboratoriumopleiding HBO-A medisch microbiologisch analist in Eindhoven. Na twee jaar behaalde hij het HBO-A diploma en ging hij naar het Van 't Hoff instituut in Rotterdam om in 1984 het HBO-B diploma medisch chemisch analist te behalen. Na 1 jaar in het laboratorium van Van Ansem's Voedingscentrale in Milheeze te hebben gewerkt begon hij in september 1985 bij het Laboratorium voor Erfelijkheidsleer van de Landbouw Hogeschool in Wageningen als analist op zijn eerste STW project. Tot begin 2001 heeft hij aan meerdere extern gefinancïerde projecten gewerkt, voornamelijk aan *Aspergillus niger*. In deze tijd is de naam van de LH veranderd in Wageningen Agrarische Universiteit en later in Wageningen Universiteit. De werkgroep waar hij in die periode werkzaam was werd zelfstandig onder de naam Moleculaire Genetica van Industriële Micro-organismen. Vanaf mei 2001 was hij werkzaam bij het Laboratorium voor Fytopathologie binnen het *Phytophthora infestans* onderzoek. Tegelijk was hij van december 2003 tot eind 2006 gastmedewerker bij de Universiteit Leiden. Vanaf 1 november 2006 is hij werkzaam als associate scientist bij DSM Gist in Delft, bij de genetica afdeling van DSM Food Specialties.

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Mutagenesis and mitotic recombination in *Aspergillus niger* Peter J.I. van de Vondervoort 2007

ISBN 978-90-9022318-6