**hymA** (hypha-like metulae), a new developmental mutant of *Aspergillus nidulans*

Marvin Karos and Reinhard Fischer

**INTRODUCTION**

The life cycle of the filamentous fungus *Aspergillus nidulans* consists of a sexual and an asexual part. The asexual reproductive pathway has been used as a model system to study the molecular genetic control of morphogenesis in lower eukaryotes for more than 30 years (Clutterbuck, 1969; Timberlake, 1990, 1991, 1993; Clutterbuck & Timberlake, 1992). During asexual development of *A. nidulans*, hyphae differentiate after acquisition of developmental competence and specialized multicellular spore-forming structures, called conidiophores, are formed. First, a hyphal cell develops into a foot cell, which elongates into a stalk, about 100 μm in length. The stalk swells at the tip to a vesicle, which then produces two uninuclear cell types, metulae and phialides. The formation of metulae and phialides is accompanied by transition from hyphal growth to a budding-like proliferation. Phialides continuously bud off spores (conidia); many thousand conidia are produced by one conidiophore.

Martinelli & Clutterbuck (1971) mutagenized *A. nidulans* and compared the number of auxotrophs with the number of developmental mutants. From this ratio it was estimated that the number of developmental genes is at least 150. Strains with a defect in vegetative growth and in developmental processes were not considered. In an alternative approach, the appearance of differentially expressed transcripts during asexual development was studied; from these data the number of involved genes was re-estimated to be 1200 (Timberlake, 1980; Clutterbuck & Timberlake, 1992). Genes expressed in hyphae and in conidiophores were neglected. Subsequent deletion studies revealed that some of these genes, discovered by reverse genetics, were dispensable for conidiophore production and had a very subtle or no phenotype under laboratory conditions (Aramayo *et al*., 1989).

More than 12 developmental genes of *A. nidulans* have been analysed at the molecular level. Some of these are transcriptional activators, including flbD, brlA, abaA and...
probably wetA, which form a linear signal-amplifying pathway (Clutterbuck, 1969; Boylan et al., 1987; Adams et al., 1988; Sewall et al., 1990; Marshall & Timmerlake, 1991; Prade & Timmerlake, 1993; Andrianopoulos & Timmerlake, 1994; Wieser & Adams, 1995). Others, including medA and stuA, act as modifiers in the main cascade (Miller et al., 1991, 1992; Gems & Clutterbuck, 1994). In addition, a set of structural genes, including wA, yA, dewA and rodA, were identified as targets of the transcription factors (Aramo & Timmerlake, 1990, 1993; Mayorga & Timmerlake, 1990, 1992; Stringer et al., 1991; Stringer & Timmerlake, 1994). Finally, genes such as apaA or apaB couple vegetative functions (e.g. nuclear migration) to developmental processes (Clutterbuck, 1994; Fischer & Timmerlake, 1995). Mutants defective in one of these developmental genes display an abnormal conidiophore phenotype, but in most cases vegetative growth is not affected.

In fluffy mutants, such as fbbA or fbbD, hyphae produce cotton-like aerial mycelia but fail to produce further developmental structures. The fluffy genes have therefore been recognized as "early genes" in the developmental process, leading to activation of the transcriptional activator brlA. In bristle mutants (brlA), development stops after the formation of stalks. The zinc-finger protein BrlA directly activates the transcription factor AbaA, a leucine-zipper protein with an ATTS/TEA DNA-binding motif. In abacus mutants (abA), phialides are elongated and separte; they fail to differentiate and to produce spores. In wet mutants (wetA), conidia are formed but are unstable and lyse after a short time. The function of the modifier genes medusa (medA) and stunted (stuA) is less well established. Defects in some structural genes such as white (wA), yellow (yA), rodlessless (rodA) and dew (dewA) lead to abnormalities in pigmentation or cell wall morphogenesis. apa mutants (apaA and apaB) produce metulae which remain anucleate and thus do not proceed with the developmental programme. In addition to the genes mentioned, many more components are expected to be necessary for the production of conidiophores.

In this paper we describe the isolation of a new developmental mutant with a specific block in conidiophore development at the metula stage, possibly due to a defect in the transition from hyphal to budding-like growth.

### METHODS

**Aspergillus nidulans strains, Escherichia coli plasmids and growth conditions.** All _A. nidulans_ strains used in this study are listed in Table 1. Supplemented minimal and complete media for _A. nidulans_ were prepared as described by Käfer (1977). Standard genetic techniques for strain construction were used, essentially as described by Käfer (1977). _A. nidulans_ colonies were incubated on complete agar medium until the hyphae of two different strains had grown together. A small agar piece from this region was transferred to minimal agar medium, where only the heterokaryon could grow. For induction of the sexual cycle, plates were sealed with tape and incubated for 2 weeks until the fruit bodies had formed.

_E. coli_ strains, and the constructed plasmids and cosmids used in this study, are listed in Table 2.

**Molecular techniques.** Standard DNA transformation procedures for _A. nidulans_ (Yetlon et al., 1984) and _E. coli_ (Sambrook et al., 1989) were used. In _A. nidulans_ transformation experiments _KpnI_ (20 U) was added, which increased the transformation efficiency. Genomic DNA was extracted from the fungus essentially as described by Timmerlake & Marshall (1989). DNA was isolated with TRIzol (Gibco BRL), according to the manufacturer's protocol. DNA and RNA analyses (Southern and Northern hybridization) were performed as described by Sambrook et al. (1989).

For plasmid rescue, 20 μg SIK5 genomic DNA was digested to completion with _XhoI_, purified with the Wizard Minipreps DNA purification system (Promega) and religated in a 200 μl volume overnight at 16 °C. Competent _E. coli_ XL1-blue cells (New England Biolabs) were electrotransformed with 2 μl of the ligation mixture. The rescued plasmid was used to isolate the border sequences of the integration site.

**Staining and microscopy.** For examination of conidiophores by fluorescence microscopy, microscope slides were point-inocu-

### Table 1. _A. nidulans_ strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>FGSC26</td>
<td>biA1; veA1</td>
<td>Fungal Genetics Stock Center, Kansas, USA</td>
</tr>
<tr>
<td>GR95</td>
<td>suA1 adE20, yA2, adE20; acrA1; galA1; pyraA4; fusA1303; zB3; nicB8; riboB2; veA1</td>
<td>J. Clutterbuck, Glasgow, UK</td>
</tr>
<tr>
<td>RMSO11</td>
<td>pabaA1, yA2, bargB::trpCAB; veA1, trpC801</td>
<td>Stringer et al. (1991)</td>
</tr>
<tr>
<td>GR5</td>
<td>pyrG89; wA3; pyraA4; veA1</td>
<td>G. May, Houston, TX, USA</td>
</tr>
<tr>
<td>G623</td>
<td>biA1; nicC10; veA1</td>
<td>J. Clutterbuck, Glasgow, UK</td>
</tr>
<tr>
<td>SMK8</td>
<td>pyrG89; wA3; pyraA4; hynA::pMK1; veA1</td>
<td>Transformation of GR5 with pMK1</td>
</tr>
<tr>
<td>K5</td>
<td>pabaA1; pyraA4; hynA::pMK1; veA1</td>
<td>Progeny of SMK8 × RMSO11</td>
</tr>
<tr>
<td>SIK5</td>
<td>pabaA1; pyraA4; hynA::pMK1; veA1</td>
<td>Progeny of K5 × GR95</td>
</tr>
<tr>
<td>SIK8</td>
<td>pabaA1, yA2; bargB::trpCAB; veA1</td>
<td>Progeny of SIK5 × RMSO11</td>
</tr>
<tr>
<td>SAA1</td>
<td>yA2, pabaA1; bargB::trpCAB; abaA1</td>
<td>A. Andrianopoulos, Melbourne, Australia</td>
</tr>
<tr>
<td>SR1</td>
<td>pyrG89; wA3; pyraA4; apsB6; veA1</td>
<td>This work</td>
</tr>
<tr>
<td>SRF30</td>
<td>pabaA1, yA2, wA3; bargB::trpCAB; pyraA4; apsA::pyr4; veA1</td>
<td>Fischer &amp; Timmerlake (1995)</td>
</tr>
</tbody>
</table>
Table 2. E. coli plasmids and cosmids used in this study

<table>
<thead>
<tr>
<th>Plasmid/cosmid</th>
<th>Construction</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRG1</td>
<td>Contains pyr4 gene from Neurospora crassa</td>
<td>Waring et al. (1989)</td>
</tr>
<tr>
<td>pMK1</td>
<td>Contains pyr4 gene with pBluescript KS(−) as vector</td>
<td>This work</td>
</tr>
<tr>
<td>pMK2</td>
<td>Rescued plasmid out of genomic DNA from A. nidulans strain SIK5; incomplete pMK1 with border sequences of the integration site</td>
<td>This work</td>
</tr>
<tr>
<td>pMK4</td>
<td>5 kb XhoI fragment containing the complete hymA gene in pBluescript KS(−)</td>
<td>This work</td>
</tr>
<tr>
<td>pDC1</td>
<td>A. nidulans argB gene in pC20R</td>
<td>Aramayo et al. (1989)</td>
</tr>
<tr>
<td>CMK1</td>
<td>Cosmid, PUI as vector, with a 40 kb insert of genomic Aspergillus DNA, which contains the complete hymA gene and the argB gene as selection marker</td>
<td>B. Miller, Idaho, USA; this work</td>
</tr>
</tbody>
</table>

RESULTS

Transformation of A. nidulans with plasmids frequently leads to non-homologous integration of the vector into the genome, thereby disrupting the gene at the integration locus. This phenomenon, often a disadvantage in specific knock-out experiments which are dependent on homologous integration events, was used as a method to mutagenize A. nidulans. The advantages of this approach are the potential for disrupting any gene and the tagging of the integration site by the transformed plasmid. Consequently, this method allows quick access to the gene of interest (Fig. 1) (Kahmann et al., 1995). The approach is particularly suitable for studying developmental genes, because loss-of-function mutations in these genes are usually not lethal.

Isolation of a new developmental mutant

In a transformation experiment of the uracil and uridine auxotrophic but conidiating A. nidulans strain GR5 with the pyr4-containing plasmid pMK1, approximately 500 ura− transformants were obtained. The majority of the transformants produced conidiophores like the wildtype. In some strains conidiation was markedly reduced and only a few did not conidiate at all. The phenotypes of the latter group were frequently unstable after restreaking. However, we were able to colony-purify one stable aconidial strain, named K5, with a slightly reduced growth rate (Fig. 2a). This strain was subsequently crossed to a wild-type and the progeny were analysed. Conidiating and non-conidiating colonies were found at a ratio of 1:1. This suggested that the defect was due to a single gene. However, Southern blot analysis revealed that the genome of the strain harboured several integrations of the plasmid (result not shown). Therefore three sequential crosses of progeny mutants with wild-type strains were performed. Finally, we obtained a strain, SIK8, with a single integration (result not shown). The mutant phenotype (Fig. 3b) still was inherited in a Mendelian manner. In order to test whether in SIK8 an unknown gene had been hit, the strain was crossed to several known A. nidulans developmental mutants. In all cases hyphae of different strains fused and formed stable heterokaryons with nuclei of both parent strains. All heterokaryons conidiated, suggesting cross-complementation of the mutations. In addition, among the progeny analysed after sexual spore formation, wild-type colonies were found as expected for a non-linked gene. These results, together with the phenotypic characteristics, suggested that in the mutant an unknown gene was disrupted; this gene was named hymA (hypha-like metulae).

Phenotype of the hymA mutant

The hymA mutant strain was compared to an A. nidulans wild-type strain with respect to hyphal growth and conidiophore development using phase-contrast, fluorescence and scanning electron microscopy.

The mycelia of mutant strains were more branched than wild-type mycelia, leading to the compact colony phenotype of hymA strains (Fig. 2b, c). Other morphological or cell biological abnormalities were not observed in mutant hyphae. However, a dramatic difference between mutant and wild-type was found in conidiophore development.

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**A. nidulans** wild-type conidiophores consist of five different cell types (see Introduction), which are sequentially produced during the differentiation process. The pigmented asexual spores lead to green-coloured colonies. In contrast, colonies of SIK8 appeared brown on agar plates, because differentiation of the mutant conidiophores stopped after formation of a stalk, a vesicle and metulae. The latter were elongated in comparison to wild-type metulae, which are defined by a precise cell length and width (Fig. 3a, b). The metula-structures in the SIK8 mutant were quite variable in length (10–20 μm). The phenotype was independent of the growth medium. Interestingly, some older metulae produced a spore-like structure at their tips, comparable to conidia generated by hyphae in liquid media when grown under nutrient limitation or after forced expression of brlA (Marhoul & Adams, 1995; Skromne et al., 1995). SIK8 conidiophores were also compared to conidiophores of *aps* mutants, in which development is also blocked at the metula stage, and to *abaA* mutants, which have a defect in phialide differentiation. However, in *aps* and *aba* strains the metulae are essentially like the wild-type in morphology (Fig. 3c, d).

To characterize the SIK8 phenotype further, conidiophores of wild-type, SIK8 and *apsA* strains were analysed with respect to nuclear distribution and septum formation (Figs 4 and 5). Whereas wild-type and *aba* metulae contained a single nucleus (Fig. 4a, d) and *aps* metulae were anucleate (Fig. 4c), in SIK8 the metulae contained several nuclei and were compartmentalized by septa at different positions (Figs 4b and 5a–c). In some cases, a cell wall very close to the vesicle surface was observed (Fig. 5b). Several compartments were multinucleate. Thus, the metulae of SIK8 resembled hyphae rather than true metulae (hence the gene name *hymA*: hypha-like metulae). The idea was supported by the study of double mutants.

![Figure 1](image-url)  **Fig. 1.** Scheme of insertional mutagenesis and rescue of the integrated plasmid.

![Figure 2](image-url)  **Fig. 2.** (a) Colonies of wild-type *A. nidulans* (FGSC26) and mutant strain K3 grown on complete medium for 2 d at 37 °C. (b, c) Colony margins of the wild-type (b) and the mutant strain (c). Bars, 1 cm (a) and 500 μm (b; same magnification in c).
A new developmental mutant of \textit{A. nidulans}

\textbf{Fig. 3.} Scanning electron microscopic analysis of (a) a wild-type, (b) a hymA, (c) an apsA and (d) an abaA mutant conidiophore of \textit{A. nidulans}. Bar, 5 \mu m (a–c) and 10 \mu m (d).

\textbf{Fig. 4.} Nuclear distribution in conidiophores of (a) a wild-type, (b) a hymA, (c) an apsA and (d) an abaA mutant of \textit{A. nidulans}. Nuclei were visualized by DAPI fluorescence microscopy. Bar, 5 \mu m. M, metulae; P, phialide.

\textbf{Fig. 5.} Comparison of (a–c) different hymA conidiophores with (d) a wild-type \textit{A. nidulans} conidiophore. Cell walls were stained with Calcofluor. (b) Sometimes a septum was found close to the vesicle (arrowhead). (b, c) In older hymA conidiophores spore-like structures were observed at the tip of the hypha-like metulae (arrowhead in c). Bar, 10 \mu m.

\textbf{Analysis of double mutants}

\textit{hymA} and \textit{apsB} mutant strains specifically stop development at the metula stage, whereas \textit{abaA} mutants fail to differentiate their phialides (Sewall et al., 1990; Clutterbuck, 1994). Double mutants between \textit{hymA} and \textit{abaA}, and between \textit{hymA} and \textit{apsB}, were constructed and phenotypically studied using phase-contrast, DAPI and Calcofluor fluorescence microscopy (results not shown).

The \textit{hymA/abaA} double mutant displayed a \textit{hymA} phenotype, indicating that the action of \textit{hymA} was required before production of abacus structures. Surprisingly, \textit{hymA/apsB} double mutants also showed a \textit{hymA} conidiophore type, with all metulae structures nucleate. This result suggested that in the absence of the \textit{hymA} function, ‘normal’ metulae could not be formed. Instead hypha-like ‘metulae’ arose from the vesicles.
Isolation of the \textit{hymA} gene

In order to isolate the \textit{hymA} wild-type gene advantage was taken of the mutagenesis method in which the vector marked its integration site (Fig. 1). Although the restriction enzyme \textit{KpnI} was added in the transformation experiment, the integration event did not occur via this site and could not be explained in Southern blot analyses (results not shown). This suggested a non-homologous integration. In order to rescue the integrated plasmid, genomic DNA from SIK8 was isolated and restricted with \textit{XhoI}. After religation of the restriction fragments \textit{pMK2} was recovered in \textit{E. coli}. To detect the border sequences of the integration site of SIK8 in \textit{pMK2} this plasmid was compared with \textit{pMK1}. \textit{pMK2} was analysed by restriction enzyme digestion and hybridization of the fragments in a Southern blot with \textit{pMK1}. DNA fragments visible after ethidium bromide staining in the gel but not detectable with the probe were isolated. In a triple digest of \textit{pMK2} with \textit{KpnI}, \textit{XhoI} and \textit{EcoRV} a 1 kb fragment was isolated with these attributes. In order to isolate the complete wild-type copy of \textit{hymA} the 1 kb fragment was used to screen a wild-type cosmid library of \textit{A. nidulans} (PUI library, kindly provided by B. Miller, Moscow, ID, USA). Of 50000 clones, 12 hybridized to the specific probe and all 12 clones contained the same 50 kb cosmid, \textit{CMK1}. To test this cosmid for complementation of a \textit{hymA} mutant strain, SIK8 was transformed with \textit{CMK1}; this resulted in 35 transformants, 33 of which conidiated like the wild-type. This high frequency of complementation suggested that this cosmid was ectopically integrated and hence contained the entire \textit{hymA} gene. This was confirmed by crosses of five transformants with wild-type \textit{A. nidulans} strains in which colonies with \textit{hymA} phenotypes could be recovered. The complementing activity was finally localized on a 5 kb \textit{XhoI} restriction fragment. With this fragment as a probe, a 1-8 kb transcript was detected in wild-type RNA. In \textit{hymA} mutant strains the 1-8 kb transcript was shifted to 1-5 kb, indicating that this mRNA was encoded by the \textit{hymA} gene and disrupted by the vector (Fig. 6). The \textit{hymA} transcript was detected in hyphae before and after acquisition of developmental competence, as well as in developing conidiophores (result not shown). Since in \textit{hymA} mutant strains a shorter mRNA was still detectable it remains to be shown whether the observed phenotype is the phenotype of the null mutant. However, the truncated transcript also contained vector sequences, indicating that a significant part of \textit{hymA} transcript is missing in the mutant.

Mapping of the \textit{hymA} gene

The 5 kb \textit{XhoI} restriction fragment was also used as a probe to identify the corresponding cosmids in an ordered cosmid library, kindly provided by R. Prade and J. Arnold (Athens, GA, USA) (Xiong \textit{et al.}, 1996). The fragment hybridized to the three overlapping cosmids SL19F08, RL11H09 and RL30F06 localized at the very end of the left arm of chromosome VI (information available on the Internet: http://fungus.genetics.uga.edu: 5080/Physical_Maps.html). The next mapped gene in the vicinity of these cosmids is \textit{orlA} and the closed marker to \textit{orlA} on the genetic map of \textit{A. nidulans} is \textit{nicC}, 8 cM from \textit{orlA}. However, linkage between \textit{hymA} and \textit{nicC} was not observed in a genetic analysis.

\textbf{DISCUSSION}

Asexual development in \textit{A. nidulans} is defined through the onset of transcription of a number of regulatory as well as structural genes. Regulation of gene function is thereby triggered at the transcriptional or post-transcriptional level (Timberlake, 1993). In addition, genes required for vegetative growth and during development at specific stages have been recognized as important components (Fischer & Timberlake, 1995; Goldman & Morris, 1995; Morris \textit{et al.}, 1995). We have isolated a novel gene, \textit{hymA}, required for hyphal growth and differentiation. Mutation of \textit{hymA} by insertional mutagenesis slightly reduced vegetative growth in comparison to wild-type and led to a specific block in conidiophore development after formation of metulae. Further differentiation and the production of phialides and spores was not observed. Phenotypic studies of \textit{hymA} mutant strains revealed that the metulae resembled hyphae rather than true metulae.

Two possible functions of \textit{hymA} are suggested. First, the pleiotropic changes of conidiophore morphology in \textit{hymA} strains could be explained by \textit{hymA} being involved in differentiation of metulae. \textit{hymA} could be a transcriptional activator, responsible for the expression of metula-specific genes. A failure of metula maturation would lead
to hypha-like structures. Thus the function of \( \text{hmA} \) in metulae would be comparable to the function of \( \text{abaA} \) in phialides (Andrianopoulos & Timberlake, 1994). However, the reduction of growth rate and the presence of the \( \text{hmA} \) transcript throughout the entire life cycle could not be accounted for entirely by this hypothesis.

Secondly, a molecular function of \( \text{hmA} \) is suggested from the analysis of a double mutant between an \( \text{apsB} \) and a \( \text{hmA} \) strain. In \( \text{apsB} \) strains metulae are anucleate and in \( \text{hmA} \) strains they are nucleate. Therefore it would be expected that the double mutant \( \text{hmA/apsB} \) would produce anucleate metulae. However, the double mutant displayed a \( \text{hmA} \) phenotype. This result could be explained if \( \text{aps} \) (or wild-type) strains generate metulae by a different mechanism to the hypha-like metulae in \( \text{hmA} \) strains. In wild-type conidiophores a transition from hyphal to budding growth occurs as metulae are formed. \( \text{hmA} \) could be involved in this budding process. Hence, \( \text{hmA} \) could encode a protein necessary for e.g. bud site selection, like \text{BEM1} in \text{Saccharomyces cerevisiae} (Leberer et al., 1995). Another possibility for the molecular function of \( \text{hmA} \) in budding-like processes could be a regulatory one. \( \text{hmA} \) could be involved in the transition from hyphal to budding growth as a transcriptional activator of budding specific genes. The \( \text{hmA} \) gene of \text{Aspergillus nidulans} might thus be related to genes of \text{S. cerevisiae} involved in the transition from budding to pseudohyphal growth (Gimeno et al., 1992; Fink & Gimeno, 1994; Fink et al., 1994). The requirement for \( \text{hmA} \) in hyphal growth still remains unexplained. In either case, \( \text{hmA} \) as a structural component or as a regulator, the absence of bud formation could explain why hypha-like structures would grow out of the vesicle. Because these structures do not differentiate into metulae the developmental programme cannot proceed.

Further molecular analysis of the \( \text{hmA} \) gene is needed to elucidate its molecular functions in vegetative growth and during asexual development.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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