

A broader role for AmyR in *Aspergillus niger*: regulation of the utilisation of D-glucose or D-galactose containing oligo- and polysaccharides

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Abstract AmyR is commonly considered a regulator of starch degradation whose activity is induced by the presence of maltose, the disaccharide building block of starch. In this study, we demonstrate that the role of AmyR extends beyond starch degradation. Enzyme activity assays, genes expression analysis and growth profiling on D-glucose- and D-galactose-containing oligo- and polysaccharides showed that AmyR regulates the expression of some of the *Aspergillus niger* genes

encoding α - and β -glucosidases, α - and β - galactosidases, as well as genes encoding α -amylases and glucoamylases. In addition, we provide evidence that D-glucose or a metabolic product thereof may be the inducer of the AmyR system in *A. niger* and not maltose, as is commonly assumed.

Keywords *Aspergillus niger* · AmyR · Polysaccharide degradation · Gene regulation

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Introduction

AmyR was the first transcriptional activator of the GAL4 type identified in filamentous fungi that is involved in the degradation of plant polysaccharides (Petersen et al. 1999). Its role in starch degradation has been studied in detail in different *Aspergillus* species, such as *Aspergillus nidulans* and *Aspergillus oryzae*, and AmyR was shown to activate expression of glucoamylase, α -amylase and α -glucosidase in the presence of starch or maltose (Gomi et al. 2000; Petersen et al. 1999; Tani et al. 2001). The amylolytic system has also been studied in *Aspergillus niger*, and a recent study compared the expression of genes related to starch degradation using micro-array analysis (Yuan et al. 2008) but did not study in detail other genes that may be under AmyR control.

Induction of the amylolytic system in *Aspergillus* is reported to be caused by the presence of starch or its disaccharide building block, maltose (Barton et al. 1972; Carlsen and Nielsen 2001; Pedersen et al. 2000; Santerre Henriksen et al. 1999), and the latter compound is often suggested to be the low-molecular-weight inducer of the system. However, the actual nature of the inducer has not been studied in detail. It has also been claimed that

iso-maltose is the inducing compounds (Kato et al. 2002), but no conclusive evidence has been presented to verify this. D-glucose has been shown to repress the expression of the amyolytic genes (Tsukagoshi et al. 2001) but was also reported as an inducer for the amyolytic genes of *A. oryzae* (Carlsen and Nielsen 2001). However, no studies have been reported in which low levels of D-glucose were tested for induction of the amyolytic system in *Aspergillus*.

A role in both induction and repression has been observed previously for D-xylose and the xylanolytic system of *A. niger* (de Vries et al. 1999b). In this fungus, D-xylose induces the xylanolytic regulator XlnR that activates expression of xylanolytic and cellulolytic genes. The concentration of D-xylose in the medium affects the expression level of the genes. High D-xylose concentrations result in activation of the carbon catabolite repressor protein CreA that represses the expression of the XlnR-regulated genes.

Although XlnR was originally described as a xylanolytic activator, later studies indicated that it also controlled genes that were not involved in xylan degradation and utilization. These genes encoded enzymes involved in cellulose, xyloglucan and galactomannan degradation (de Vries et al. 1999a; Gielkens et al. 1999; Hasper et al. 2002).

Here, we present a study into the *A. niger amyR* gene in which we constructed deletion and multicopy strains. Using these strains, we have found that D-glucose induces the amyolytic system depending on the concentration in the medium. In addition, it became clear that AmyR has a broader physiological role than starch degradation in that it controls the production of D-glucose and D-galactose releasing enzymes.

Materials and methods

Strains and media

All *A. niger* strains used are derivatives of N402 (FGSC A733; Table 1). Inocula for shake flask cultivations were obtained from cultures grown on complete medium agar plates (de Vries et al. 2004). Minimal medium (MM; de Vries et al. 2004) was inoculated with harvested conidia to give a concentration of 1.0×10^6 conidia per milliliter. Supplements to complement the auxotrophic requirements were added when required and were 200 mg/l leucine, 122 mg/l uridine and 1 mg/l nicotinic acid (niacin). The liquid pre-cultures contained 2% D-fructose as the sole carbon source. Sugars were not autoclaved but filter-sterilised and added to the autoclaved medium. Maltose, D-glucose (G8270, 99.5%), D-fructose, lactose and D-xylose were from Sigma. Soluble starch was from Difco. Cultures

were grown at 30°C, with 250 rpm rotary shaking. Precultures were cultivated for 16 h, mycelia were washed with MM (without carbon source), and 1.5 g wet weight filamentous mycelium was transferred to shake flasks containing 70 ml MM with the carbon sources, as stated in the results. Transferred mycelium was then incubated at 30°C with 250 rpm rotary shaking for 2–24 h (see “Results” for details of specific experiments) before mycelia were harvested and/or culture filtrate samples taken. *A. niger* transformations were performed as described previously (Kusters-van Someren et al. 1991).

Molecular methods

DNA manipulations and molecular biology techniques were performed using standard methods (Sambrook et al. 1989) and *Escherichia coli* DH5 α . Gel extractions were performed using the QIAGEN QIAquick gel extraction kit.

RNA analysis was performed as described previously (de Vries et al. 2002). Internal fragments of *lacA* (A00968, An01g12150; Kumar et al. 1992), *aglC* (AJ251873, An09g00260/270; Ademark et al. 2001), *agdA* (An04g06920; Pel et al. 2007), *glaA* (X00548, An03g06550; Boel et al. 1984), *amyA* (An04g06930) and *amyR* (An04g06910; Pel et al. 2007) were used as probes for expression analysis. A 0.7-kb EcoRI fragment from the gene encoding the 18S rRNA subunit (Melchers et al. 1994) was used as an RNA loading control.

Sequence analysis

Nucleotide sequences were analysed with computer programs based on those of Devereux et al. (Devereux et al. 1984). Sequence alignments were performed by using the Blast programs (Altschul et al. 1990) at the server of the National Center for Biotechnology Information, Bethesda, USA (<http://www.ncbi.nlm.nih.gov/blast/>). Synteny analysis was performed at the AspGD website (<http://www.aspgd.org/>) using the Sybil programme.

Enzyme assays

Extracellular hydrolytic activities were assayed using 0.01% substrate, 20–40 μ l sample and 25 mM sodium acetate pH 5.0 in a total volume of 100 μ l. The mixtures were incubated for 1 h at 30°C after which the reaction was stopped by adding 100 μ l 0.25 M Na₂CO₃. Absorbance was measured at 405 nm in a microtiter plate reader. The activity was calculated using a standard curve of *p*-nitrophenol. The substrates used for enzyme assays were all obtained from Sigma and were *p*-nitrophenol- α -arabinofuranoside, *p*-nitrophenol- α -xylopyranoside, *p*-nitrophenol- β -xylopyranoside, *p*-nitrophenol- α -galactopyra-

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

Strain	Genotype	Reference
N402	<i>cspA1</i>	Bos et al. (1988)
NW249	$\Delta argB, pyrA6, leuA1, nicA1$	vanKuyk et al. (2004)
UU-A049.1	$\Delta argB, pyrA6, leuA1, nicA1, argB^+$	This study
UU-A101.1	$\Delta argB, pyrA6, leuA1, nicA1, \Delta amyR:: argB^+$	This study
UU-A001.13	$\Delta argB, pyrA6, leuA1, nicA1, argB^+, mcamyR$	This study
UU-A001.24	$argB\Delta, pyrA6, leuA1, nicA1, argB^+, mcamyR$	This study

noside, *p*-nitrophenol- β -galactopyranoside, *p*-nitrophenol- α -glucopyranoside, *p*-nitrophenol- β -glucopyranoside, *p*-nitrophenol- α -fucopyranoside, *p*-nitrophenol- β -fucopyranoside, *p*-nitrophenol- β -glucuronoside and *p*-nitrophenol- β -mannopyranoside to measure α -arabinofuranosidase, α - and β -xylosidase, α - and β -galactosidase, α - and β -glucosidase, β -glucuronidase and β -mannosidase, respectively.

Results

Identification and cloning of *amyR*

A lambda phage clone was isolated from an *A. niger* gDNA library using a fragment of the *A. oryzae amyR* gene as a probe. A 4.3-kb *NsiI* fragment was subcloned into pGEM-11 and designated pJG01. Sequence analysis showed that this fragment contained the coding region of *A. niger amyR* as well as the 5'-flanking region and part of the *A. niger agdA* gene. Further analysis using the Sybil tool at www.aspgd.org (Crabtree et al. 2007) demonstrated that the amyolytic cluster (*amyA–aglA–amyR*) is highly conserved in all eight sequenced Aspergilli, with only a small insertion downstream of *amyR* in *Aspergillus terreus* (Suppl. Fig. 1). In all these species except *A. nidulans*, the proteolytic regulator PrtT (Punt et al. 2008) lies directly upstream from the cluster.

Construction and analysis of disruption and multicopy *amyR* strains

A deletion construct (pAmyRd) was made by introducing a *PstI* site 800 bp downstream of the endogenous *PstI* site of pJG01. Plasmid pJG01 was then digested with *BamHI/NsiI* and the liberated fragment cloned into pGEM9. The resulting construct (pJG10) was digested with *PstI/BamHI*, and a *BglII/PstI* fragment containing the *argB* gene of *A. niger* was inserted. Insertion of this construct at the *amyR* locus would result in the removal of 1,600 bp, consisting of approximately 600 bp 5'-region and 1,000 bp coding region (including the zinc finger). This fragment was used to transform *A. niger* NW249. In addition, pJG01 was used in a co-transformation with pIM2101 (containing the *A. niger*

argB gene (Lenouvel et al. 2002)) to obtain *amyR* multicopy strains in *A. niger* NW249. Transformants were screened for their ability to grow on starch. Selected transformants from the disruption transformation with reduced growth and from the multicopy transformation with improved growth on starch were used for Southern analysis and demonstrated that multiple disruption and multicopy transformants were obtained (data not shown). Based on this analysis, UU-A001.24 was selected as *amyR* multicopy (overexpression) transformant and UU-A101.1 was selected as an *amyR* disruptant.

The influence of AmyR extends beyond amyolytic genes

Strains UU-A001.24 (*amyR* overexpression) and UU-A101.1 (*amyR* disruptant) together with UU-A049.1 (reference strain) were grown overnight in complete medium (CM) with 2% fructose and transferred to MM with the carbon sources indicated in Fig. 1 for 6 h. Culture filtrate samples were used to determine the presence of extracellular enzymes. No activity was detected on any of the carbon sources for α - or β -fucosidase, α -xylosidase, α -arabinofuranosidase, β -mannosidase or β -glucuronidase (data not shown). However, α - and β -galactosidase and α - and β -glucosidase activity were detected on D-glucose, lactose, maltose and starch (Fig. 1). Compared with the reference strain (UU-A049.1), these activities were lower in the *amyR* disruptant and 2- to 15-fold increased in the *amyR* multicopy strain.

Based on recent *A. niger* genome annotations (Coutinho et al. 2009; Pel et al. 2007), genes encoding α -amylase (AMY), glucoamylase (GLA), α -glucosidase (AGD), β -glucosidase (BGL), α -galactosidase (AGL) and β -galactosidase (LAC) were analysed for their expression in an *A. niger* wild-type and *amyR* disruptant on maltose using previously published micro-array data (Yuan et al. 2008). This demonstrated that AmyR controls only a small subset of these genes (Suppl. Table 1), but this subset included two BGL-, two AMY-, two AGD-, two GLA-, two AGL and one LAC-encoding genes, supporting the activity assays performed in our study. All these genes contained at least one putative AmyR binding site in their promoter.

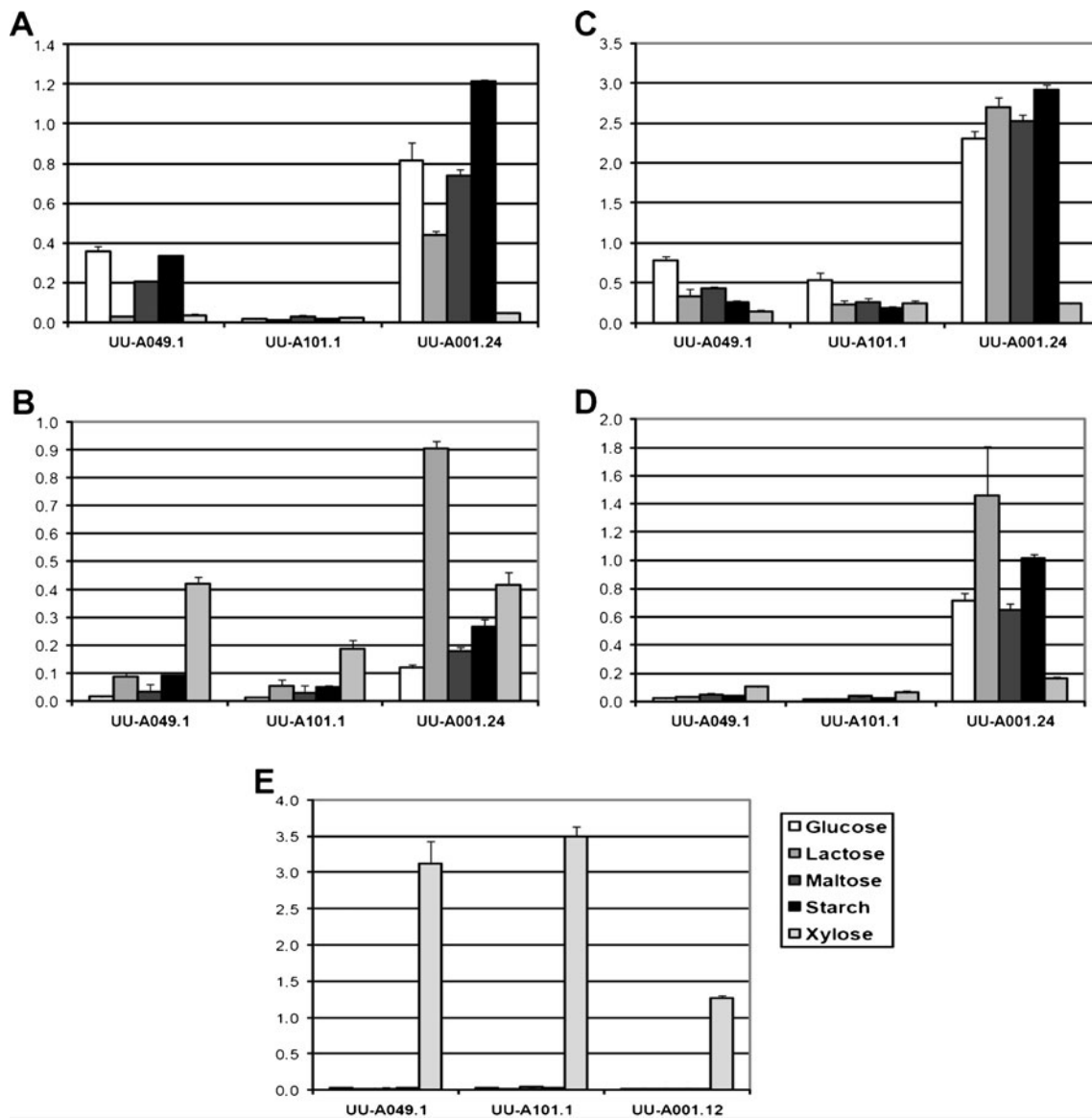


Fig. 1 Extracellular activities in UU-A049.1 (reference strain), UU-A101.1 ($\Delta amyR$) and UU-A001.24 (*amyR* multicopy strain) on five carbon sources (25 mM for mono- and disaccharides, 1% for starch). Activity (nanomoles per minute per milliliter culture filtrate) was

assayed in culture filtrates obtained 6 h after transfer. Enzyme activities measured were **A** α -glucosidase (AGD), **B** β -glucosidase (BGL), **C** α -galactosidase (AGL), **D** β -galactosidase (LAC), and **(E)** β -xylosidase (BXL)

To confirm these data, a transfer experiment was performed in which UU-A049.1, UU-A001.24 and UU-A101.1 were pre-grown O/N in CM with 2% fructose. Mycelial aliquots were transferred to 25 mM D-glucose, 25 mM maltose and 1% starch and incubated for the time points indicated in Fig. 2. Northern analysis was performed for the three genes of the AmyR cluster (*amyR*, *agdA*, *amyA*), the main glucoamylase (*glaA*) and genes encoding an α -galactosidase (*aglC*; Ademark et al. 2001) and a β -galactosidase (*lacA*; de Vries et al. 1999a; Kumar et al. 1992). Expression of *glaA*, *amyA* and *agdA*

on maltose and starch was reduced in the *amyR* disruption strain (UU-A101.1) and increased in the *amyR* multicopy strain (UU-A001.24; Fig. 2). A similar effect was observed for *lacA* and *aglC*, although the expression of the genes peaked at the latest and earliest timepoint, respectively.

A similar expression pattern was observed for *glaA*, *agdA* and *amyA* on D-glucose, and expression was highest at the latest time point (Fig. 2). Expression of *aglC* and *lacA* was not reduced in the *amyR* disruptant compared to the reference on D-glucose but was increased in the *amyR* multicopy strain.

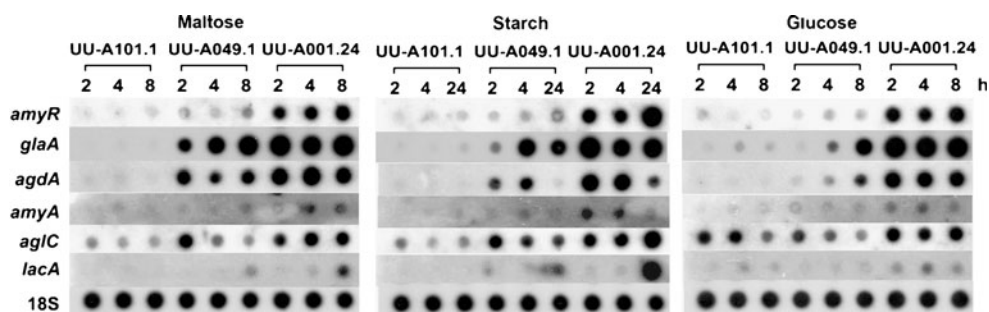


Fig. 2 Expression analysis of *amyR* (amyolytic activator), *glaA* (glucoamylase), *agdA* (α -glucosidase), *amyA* (α -amylase), *aglC* (α -galactosidase) and *lacA* (β -galactosidase) in UU-A049.1 (ref), UU-

A101.1 (Δ *amyR*) and UU-A001.24 (*amyR* multicopy strain) during growth on 25 mM maltose, 1% starch and 25 mM D-glucose at various time points (in hours)

Influence of AmyR on growth of *A. niger* on oligo- and polysaccharides

To study the physiological effect of this putative broader role for AmyR, a growth experiment was performed using UU-A049.1, UU-A001.24 and UU-A101.1 on a variety of carbon sources, including controls (xylose, D-glucose) and several oligo- and polysaccharides containing α - or β -linked D-glucose or D-galactose residues (Fig. 3). Reduced growth was observed for the *amyR* disruptant (UU-A101.1) on maltose, starch, lactose, melibiose, raffinose, melezitose and carrageenan compared with the reference strain (UU-A049.1). No clearly improved growth could be seen on most of these substrates for the *amyR* multicopy strain (UU-A001.24), with the exception of lactose, where some increase in vegetative growth was visible, but especially a significant increase in sporulation was observed. Growth and, in particular, sporulation of the reference strain and the *amyR* multicopy strain on carrageenan was poor on these substrates. No increase in sporulation was observed in the *amyR* multicopy strain compared with the reference strain.

No difference in growth was observed between the strains using sucrose, D-xylose or D-glucose as a carbon source and only a slight reduction in growth of the *amyR* disruptant on cellobiose and Guar gum. Furthermore, no difference in growth was observed on chitin, *N*-acetylglucosamine and inulin (data not shown).

Increased concentrations of D-glucose results in altered morphology in *amyR* multicopy strains

Early in the growth experiments, an unusual morphology (dense colonies with reduced sporulation) was observed for the *amyR* multicopy strain on maltose and starch. In this strain, it can be expected that maltose is very quickly converted extracellularly to D-glucose by the overproduction of glucoamylase, and therefore a second growth experiment was performed at different D-glucose concentrations to determine whether this morphology was indeed caused by the D-glucose level. The reference (UU-A049.1)

and two multicopy strains (UU-A001.24 and UU-A001.13; a second multicopy strain was included to verify that this is a general effect) were grown on MM plates with D-glucose ranging from 0.5 to 400 mM. No significant difference was observed between the strains at D-glucose concentration of 25 mM or lower. At 50 mM, a small difference can be observed in that the mycelium of the *amyR* multicopy strains is denser. At 100 mM and higher, the same morphology is observed as seen for maltose in the multicopy strains: dense mycelium and reduced sporulation (Fig. 4).

Discussion

AmyR has been studied in detail in several *Aspergilli*, with emphasis on genes involved in starch and maltose hydrolysis, although a recent study (Yuan et al. 2008) already indicated that other genes may also be controlled by AmyR. Analysis of enzyme activity in our study provided clear indications for a broader role of this regulator, in that we observed strongly elevated levels of both α - and β -glucosidase as well as α - and β -galactosidase in the *amyR* multicopy strain compared with the reference. The reduction in enzyme activity in the *amyR* deletion strain was less obvious, possibly due to the fact that the activities in the reference were already very low. Analysis of publicly available micro-array data of an *A. niger* wild-type and *amyR* disruptant during growth on maltose (Yuan et al. 2008) demonstrated that some, but not all genes encoding these enzymes (Coutinho et al. 2009), were downregulated in the disruptant strain, suggesting regulation by AmyR. All the downregulated genes contained at least one putative AmyR binding site, which would be a pre-requisite for direct AmyR regulation. For one putative α -galactosidase encoding gene (An04g02700), no putative AmyR binding site was identified in the DSM genome strain (CBS 513.88) (Coutinho et al. 2009). However, a putative binding site was detected in the genome of *A. niger* ATCC 1015 that was sequenced by JGI (Andersen et al. 2011). As we used the

Fig. 3 Growth profiles of UU-A049.1 (reference strain), UU-A101.1 ($\Delta amyR$) and UU-A001.24 (*amyR* multicopy strain) on a variety of carbon sources. Carbon source concentrations were 25 mM monosaccharides (D-glucose, D-xylose), 12.5 mM disaccharides (maltose, sucrose, lactose, cellobiose, melibiose), 7.57 mM trisaccharides (raffinose, melezitose), 1% polysaccharides (starch, carrageenan)

laboratory strain N402, this result suggests that this strain resembles ATCC 1015 with respect to the promoter of this gene. A similar difference was observed previously for the acetyl xylan esterase encoding gene *axeA* (R.P. de Vries, unpublished data). In both N402 and ATCC 1015, the promoter of this gene contains an XlnR consensus sequence, while CBS 513.88 does not. Also, several of the genes whose expression is not affected in the *amyR* disruption strain contain putative AmyR binding sites in their promoter. This confirms that the presence of such a sequence is no direct indication for regulation by AmyR, as was concluded previously for AmyR, XlnR and other regulators of *Aspergillus* (Coutinho et al. 2009). However, the absence of such binding sites strongly suggests that the gene is not (directly) regulated.

These data were confirmed for one α -galactosidase encoding gene (*aglC*) and one β -galactosidase encoding gene (*lacA*) using Northern analysis. On maltose and starch these two genes followed a similar pattern as the established AmyR regulated genes *glaA*, *amyA* and *agdA*. However, some differences were observed. The expression of *lacA* was only observed at late time points (8 h on maltose and 24 h on starch), while *aglC* expression peaked at 2 h, and the other genes were expressed on all time points. The reduced expression of *aglC* at later time points is not due to a reduction in expression compared with the pre-culture, as micro-array data from another study (R.P. de Vries, unpublished results) demonstrates lower *aglC* expression for the pre-culture than after 2 h on maltose or glucose. The expression of *aglC* in the *amyR* deletion strain was higher than that observed for the other genes tested, suggesting an AmyR-independent induction of *aglC*. Expression of *aglC* on D-glucose was observed previously (Ademark et al. 2001) and was shown to be independent of the major carbon catabolite repression system CreA (Ruijter and Visser 1997) while D-galactose induction of this gene was not observed (Ademark et al. 2001).

Expression of *agdA* in the reference strain on glucose was only observed after prolonged incubation, which correlates well with detectable α -glucosidase activity during growth of this strain on glucose as these activities were measured after 6 h. The activity level is higher than expected based on the expression of *agdA*, but this is likely due to the presence of additional α -glucosidases in the medium that are also produced under this condition.

Regulation of α - and β -glucosidase and α - and β -galactosidase activity by AmyR was further supported by growth profiling. Reduced growth of the *amyR* deletion

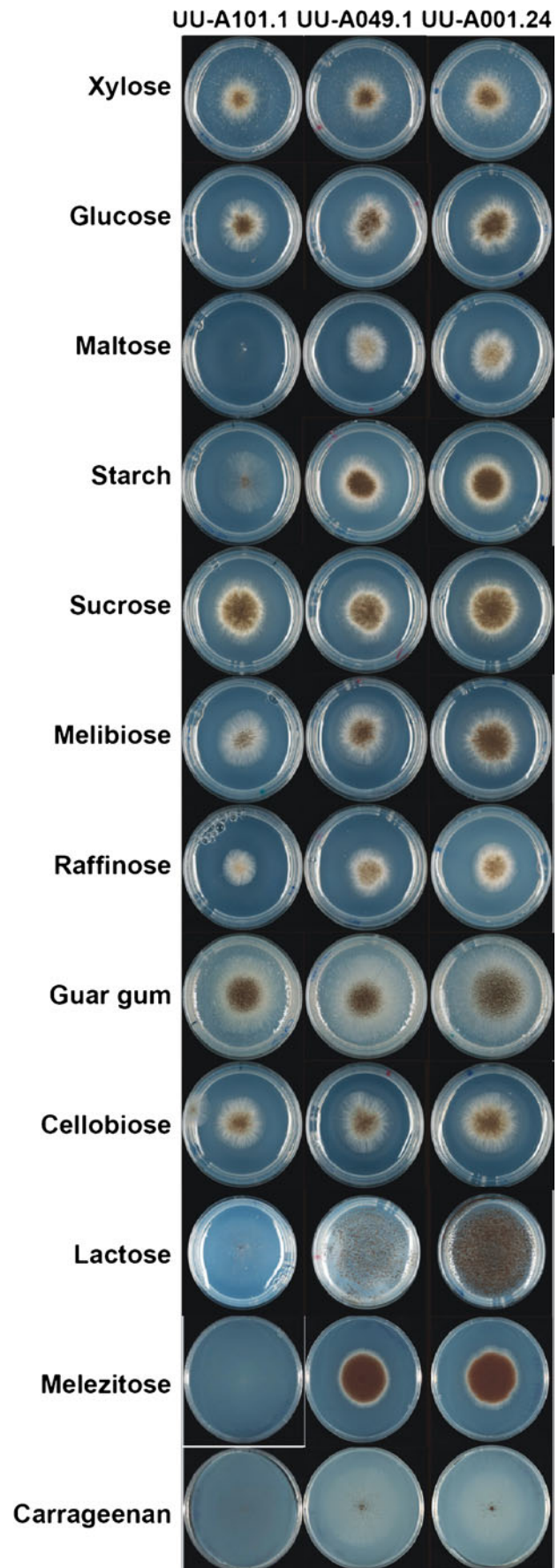
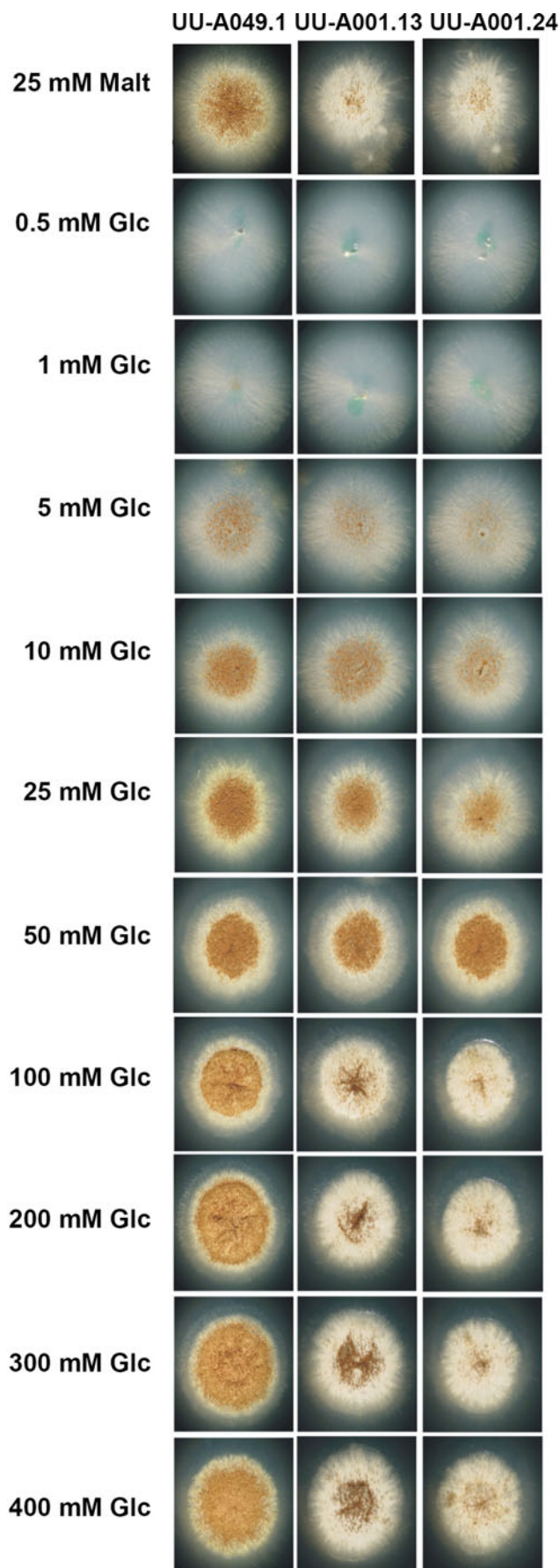


Fig. 4 Growth differences of UU-A049.1 (reference strain), and UU-A001.13 and UU-A001.24 (*amyR* multicopy strains) on different concentrations of D-glucose (Glc). Maltose (Malt) is included at the top of the figure for comparison

strain compared with the reference was observed on substrates containing α - (starch, maltose, melibiose, melezitose, raffinose, sucrose) and β -linked D-glucose (cellobiose) as well as α - (melibiose, raffinose, carrageenan) and β -linked D-galactose (lactose, carrageenan), while improved growth on several of these substrates was observed for the *amyR* multicopy strain. This provides in vivo evidence for a function for AmyR in utilization of these oligo- and polysaccharides by *A. niger*. This situation is similar to that for another *Aspergillus* transcriptional regulator related to polysaccharide degradation: XlnR. This protein was first described as a xylanolytic regulator (van Peij et al. 1998b) but was later shown to also regulate genes involved in cellulose degradation (van Peij et al. 1998a) and galactomannan degradation (de Vries et al. 1999a).

Several reports have provided evidence for different inducing compounds of the amyolytic system in *Aspergillus*. Most commonly, maltose is suggested to be the inducer of AmyR (Barton et al. 1972; Carlsen and Nielsen 2001; Pedersen et al. 2000; Santerre Henriksen et al. 1999), but also glucose (Carlsen and Nielsen 2001). Another study claimed that the inducer is iso-maltose (Kato et al. 2002), but a conclusive study has so far not been published. *A. niger* has been shown to produce high levels of glucoamylase in the presence of maltose or starch (Barton et al. 1972; Gouka et al. 1997a, b; Pedersen et al. 2000; Schrickx et al. 1995). In natural biotopes, high levels of maltose are uncommon, and it is therefore unlikely that the maltose liberated by *A. niger* during hydrolysis of starch is able to avoid the high levels of glucoamylase and get imported into the cell. It is more likely that (nearly) all maltose is hydrolysed extracellularly to D-glucose. Our study demonstrates that AmyR-regulated genes in *A. niger* are induced during growth on low levels of D-glucose, as their expression increases during the cultivation, which is similar to what was shown previously in *A. oryzae* (Carlsen and Nielsen 2001). As D-glucose has been shown to act as a repressor through the carbon catabolite repressor protein CreA (Ruijter and Visser 1997), the expression levels are likely a balance between induction through AmyR and repression through CreA. A previous study showed a similar effect for D-xylose and XlnR, in which higher D-xylose concentrations resulted in reduced expression of xylanolytic genes, mediated by CreA (de Vries et al. 1999b). In most studies reported previously (reviewed in Tsukagoshi et al. 2001), D-glucose is considered a repressing rather than an inducing carbon source. However, usually 1–3% (67–200 mM) of D-glucose is used in these studies, which are concentrations at which the repression through



CreA probably overrules induction through AmyR. These data suggests that in *A. niger* D-glucose or a metabolic product thereof is the inducer of AmyR as was shown previously for *A. oryzae* (Carlsen and Nielsen 2001). Higher induction of the AmyR-regulated genes on maltose and starch than on D-glucose supports this hypothesis, as growth on these substrates would give a gradual release of D-glucose, resulting in very low steady-state levels. Based on studies in other Aspergilli, differences in the possible inducing compounds may exist between species of this genus. The presence of the recently discovered *mal*-cluster (Hasegawa et al. 2010; Vongsangnak et al. 2009) in *A. oryzae* that consists of a putative second maltose-responsive regulator, a maltose transporter and a maltase, suggests that, in this species, at least part of the maltose is transported into the fungal cell. This cluster is also present in *Aspergillus flavus*, *Aspergillus clavatus*, *Neosartorya fischeri* and *Aspergillus fumigatus* but not in *A. niger*, *A. nidulans* and *A. terreus* (Suppl. Fig. 2), suggesting a different approach to starch utilisation in these two groups of Aspergilli.

The increased hydrolysis of maltose to D-glucose in the *amyR* multicopy strains is supported by similar morphology of these strains on maltose and on high concentrations of D-glucose. This morphology is likely due to the presence of a high intracellular concentration of D-glucose or a metabolite thereof as it is not observed in the reference strain when it is exposed to high extracellular D-glucose concentrations. This implies that D-glucose transport is upregulated in the multicopy strains, resulting in this higher intracellular concentration of D-glucose or a metabolic product of D-glucose. Analysis of the expression of the three *A. niger* D-glucose transporters reported previously (Jorgensen et al. 2007; vanKuyk et al. 2004) in the published AmyR micro array data set (Yuan et al. 2008) demonstrated that two of these genes (both encoding high affinity D-glucose transporters) are downregulated in the *amyR* deletion strain, suggesting control by AmyR. However, considering the presence of multiple putative D-glucose transporters in the *A. niger* genome (Pel et al. 2007), more functional data is needed on these other putative D-glucose transporters before any firm conclusion can be drawn. A similar growth phenotype was observed for strains under secretion stress (Carvalho et al. 2011), but it is unlikely that this can explain our results. It has been well-documented that AmyR-regulated genes are subject to carbon catabolite repression (Tsukagoshi et al. 2001), indicating that an increase in glucose concentration would reduce expression of these genes, resulting in lower protein production and likely also reduced rather than increased secretion stress

In conclusion, our study has shown that the influence of AmyR in *A. niger* extends beyond starch hydrolysis and

suggests a role for D-glucose or a metabolic product thereof as the inducer of the AmyR system in this fungus.

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