Aspergillus niger mstA encodes a high-affinity sugar/H^+ symporter which is regulated in response to extracellular pH

Patricia A. vanKUYK*[1,2], Jasper A. DIDERICH†‡[3], Andrew P. MacCABE§, Oscar HERERRO§, George J. G. RUIJTER*[4] and Jaap VISSER*[5]

*Molecular Genetics of Industrial Microorganisms. Wageningen University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands, †Swammerdam Institute for Life Sciences, BioCentrum, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands, ‡Department of Molecular Cell Physiology, Faculty of Earth and Life Sciences, Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands, and §Instituto de Agroquimica y Tecnologica de Alimentos, Consejo Superior de Investigaciones Cientificas, P.O. Box 73, Burjassot, Valencia 46100, Spain

A sugar-transporter-encoding gene, *mstA*, which is a member of the major facilitator superfamily, has been cloned from a genomic DNA library of the filamentous fungus *Aspergillus niger*. To enable the functional characterization of MSTA, a full-length cDNA was expressed in a *Saccharomyces cerevisiae* strain deficient in hexose uptake. Uptake experiments using ^{14}C-labelled monosaccharides demonstrated that although able to transport D-fructose (K_m, 4.5 ± 1.0 mM), D-xylose (K_m, 0.3 ± 0.1 mM) and D-mannose (K_m, 60 ± 20 μM), MSTA has a preference for D-glucose (K_m, 25 ± 10 μM). pH changes associated with sugar transport indicate that MSTA catalyses monosaccharide/H^+ symport. Expression of *mstA* in response to carbon starvation and upon transfer to poor carbon sources is consistent with a role for MSTA as a high-affinity transporter for D-glucose, D-mannose and D-xylose. Northern analysis has shown that *mstA* is subject to CreA-mediated carbon catabolite repression and pH regulation mediated by PacC. *A. niger* strains in which the *mstA* gene had been disrupted are phenotypically identical with isogenic reference strains when grown on 0.1–60 mM D-glucose, D-mannose, D-fructose or D-xylose. This indicates that *A. niger* possesses other transporters capable of compensating for the absence of MSTA.

Key words: CreA, filamentous fungus, monosaccharide transport, PacC, symport.

INTRODUCTION

In Nature aspergilli grow predominately on dead plant material such as compost, leaf litter in soil, and fruit. The major carbon substrate in soil which is available for the use of saprophytes is leaf litter [1]. The concentration of free sugars in soil is low; for example Chidthaisong et al. [2] found D-glucose to be the most abundant monosaccharide, with maximal concentrations of 90 μM. The major monosaccharides found in fruit are D-glucose and D-fructose, both of which can be found at concentrations ranging from 50 to 450 mM depending on the type of fruit analysed (for review see [3]). As aspergilli often encounter situations in which monosaccharides are present in low concentrations, an efficient means by which these sugars can be taken up from the environment is required. Little is known about sugar transport in filamentous fungi. In *Neurospora crassa*, *Aspergillus nidulans* and *Aspergillus niger* both high- and low-affinity glucose transport has been identified using transport assays [4–8]. Constitutive transport of D-glucose (K_m, 0.06 mM), D-galactose (K_m, 0.03 mM) and D-fructose (K_m, 0.4 mM) was identified in *A. nidulans* [7]. A gene, *mst1*, from the ectomychorrizal fungus *Amanita muscaria* encoding a hexose/H^+ symporter has been isolated and the corresponding protein characterized. Uptake experiments to determine the kinetic properties of *A. muscaria* MST1 revealed K_m values of 0.46 mM for glucose and 4.20 mM for fructose [9]. Northern analysis showed that *mst1* is constitutively expressed [10]. Mutations in the *N. crassa* gene *rco-3*, which encodes a hexose-transporter-like protein that functions as a glucose sensor, affects both high- and low-affinity glucose transport, glucose regulation of gene expression and carbon repression of conidiation [11].

The eukaryotic fungal system in which sugar transport has been most comprehensively studied is the budding yeast, *Saccharomyces cerevisiae*. The yeast hexose transporters (*HXT* family), as well as mammalian sugar transporters (GLUT family) and bacterial sugar transporters are all members of the MFS (major facilitator superfamily), a group that encompasses transporters which appear to share a common ancestral origin [12]. Some 34 sugar-transporter homologues are known in *S. cerevisiae* [12]. The function of 16 of these putative proteins is currently unknown, while the remainder are known to encode transporters for maltose, galactose, α-glucosides, inositol, hexoses or inorganic phosphate, proteins which sense extracellular sugar concentrations, or proteins involved in multidrug resistance [12–14]. The disruption of seven sugar-transporter-encoding genes (*hxt1–7*) and *gal2*, the galactose permease, renders *S. cerevisiae* unable to utilize D-glucose, D-fructose, D-mannose and D-galactose [9,15–17].

Preferential consumption of particular carbon sources is a phenomenon known as carbon catabolite repression, and is mediated by the negatively acting transcriptional regulator CreA [18]. The binding of CreA to the consensus binding site SYGGRG in *Aspergillus* promoters prevents or decreases transcription of genes subject to carbon catabolite repression [18]. Commonly these genes are involved in the metabolism of less

Abbreviation used: MM, minimal medium.

[1] To whom correspondence should be addressed (e-mail kuyk@rulbim.leidenuniv.nl).
[2] Present address: Leiden University, Institute of Biology Leiden, Clusius Laboratory, Department of Fungal Genetics and Metabolomics, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands.
[3] Present address: Kluyver Laboratory for Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands.
[4] Present address: Department of Clinical Genetics, Leiden University Medical Center, Building 1 P3-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands.
[5] Present address: Fungal Genetics and Technology Consultancy, P.O. Box 396, 6700 AJ Wageningen, The Netherlands.

Table 1 Fungal strains used in this study

DR, direct repeat.

Species	Strain	Genotype	Reference
A. niger	N402	*cspA1*	[59]
	NW176	*fwnA1*, *cspA1*, *hisD4*, *lysA7*, *creAd2*, *nicA1*, *pabA1*	[60]
	NW138	*fwnA6*, *nicA1 argB13*, *pacC*Δ::*argB*$_{(A.\ nidulans)}$	[43]
	NW283	*fwnA1*, *cspA1*, *lysA7 pyrA6*, *creAd4*	[60]
	NW324	*argB*Δ, *goxC17*, *pyrA6*, *leuA1*, *nicA1*	This study
	NW325	*argB*Δ, *goxC17*, *pyrA6*, *leuA1*, *nicA1*, pIM2104 (*argB*$^+$)	This study
	NW326	*argB*Δ, *goxC17*, *pyrA6*, *leuA1*, *nicA1*, *mstA*Δ::*argB*$^+$	This study
	NW327	*argB*Δ, *goxC17*, *pyrA6*, *leuA1*, *nicA1*, *mstA*Δ::*argB*$^+$	This study
S. cerevisiae	KY73	*MAT*a *hxt1*Δ::*HIS3*::Δ*hxt4 hxt5*::*LEU2 hxt2*Δ::*HIS3 hxt3*Δ::*LEU2*::*hxt6 hxt7*::*HIS3 gal2*Δ::*DR ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 GAL MEL*	[61]
	KY73.001	*MAT*a *hxt1*Δ::*HIS3*::Δ*hxt4 hxt5*::*LEU2 hxt2*Δ::*HIS3 hxt3*Δ::*LEU2*::*hxt6 hxt7*::*HIS3 gal2*Δ::*DR ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 GAL MEL* pYEX-BX (*URA3*)	This study
	KY73.004	*MAT*a *hxt1*Δ::*HIS3*::Δ*hxt4 hxt5*::*LEU2 hxt2*Δ::*HIS3 hxt3*Δ::*LEU2*::*hxt6 hxt7*::*HIS3 gal2*Δ::*DR ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 GAL MEL* pIM4904 (*mstA*$_{(A.\ niger)}$ *URA3*)	This study

favourable carbon sources. It can be expected that high-affinity sugar transporters are not produced in the presence of high concentrations of preferred monosaccharides and that the expression of the corresponding genes is controlled by CreA. Another wide-domain regulatory protein in *Aspergillus* is the pH-responsive regulator PacC, which has been implicated in the regulation of genes encoding transporter proteins, such as GABA (γ-amino-*n*-butyrate) permease and phosphate permease [19]. Thus it is possible that PacC also regulates sugar-transporter-encoding genes in *Aspergillus*.

The protein sequences of the *S. cerevisiae* HXT family of hexose transporters were used for the *in silico* screening of an *A. nidulans* expressed sequence tag library (http://www.genome.ou.edu/fungal.html), resulting in the identification of an *A. nidulans* gene (*mstA*) encoding a putative monosaccharide tranporter (A. P. MacCabe and D. Ramon, unpublished work). We have used the *A. nidulans mstA* gene to identify its *A. niger* homologue, designated *mstA*. The aims of this study were 4-fold. (i) Identify the conditions under which *A. niger mstA* is expressed. (ii) Determine the effects of the wide-domain regulators CreA and PacC on expression of *mstA*. (iii) Use *S. cerevisiae* as a tool to demonstrate the function of MstA. (iv) Discover if the disruption of *mstA* has a discernible phenotype.

EXPERIMENTAL

Strains and growth conditions

All *A. niger* strains used were derived from *A. niger* N400 (=CBS120.49) and are described in Table 1, as are all of the *S. cerevisiae* strains used. *A. niger* strain NW324 was transformed with approx. 4.5 μg of either circular pIM2104 (*argB*$^+$ control), resulting in strain NW325, or the linear 4.4 kb *Bam*HI insert of pIM4910 (Figure 1), resulting in the *mstA*Δ strains NW326 and NW327. The transformation procedure was conducted as described previously [20]. Cultures were grown in MM (minimal medium) containing 6.0 g/l $NaNO_3$, 1.5 g/l KH_2PO_4, 0.5 g/l KCl, 0.5 g/l $MgSO_4$ and trace elements [21], pH 6.0, with carbon sources as indicated in the text, in a rotary shaker at 250 rev./min and 30 °C. For the growth of strains with auxotrophic markers, the necessary supplements were added to the medium. To examine the effect of extracellular pH, media was buffered to pH 4.0 with 100 mM citrate or pH 8.0 with 100 mM Tris. In transfer experiments strains were pre-grown in MM (pH 6) containing 1 % (w/v) fructose as a carbon source, 0.5 % (w/v) yeast extract and 0.2 % (w/v) casamino acids. After 16 h mycelium was harvested and washed with MM without carbon source, and aliquots of 1.5 g (wet weight) were transferred to 70 ml of MM containing different carbon sources and incubated as indicated in the text.

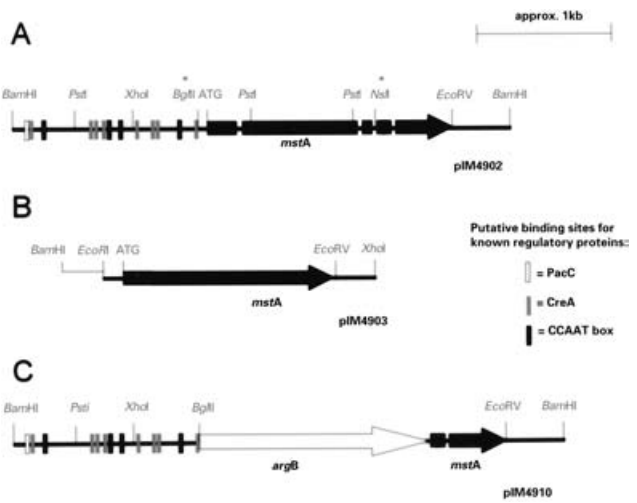

Figure 1 Schematic representation of clones containing *A. niger mstA*

(**A**) Diagram of the *mstA* genomic DNA fragment that was cloned into pBluescript to generate pIM4902. The coding region (solid arrow) is interrupted by four introns. Putative binding sites for known regulatory factors are marked (see key for details). *Site-directed mutagenesis was used to introduce the unique restriction sites *Bgl*II at nt 931 (−17 bp) and *Nsi*I at nt 2051 (+1122 bp) of the insert from pIM4902. The 1.1 kb *Bgl*II/*Nsi*I fragment of the mutagenized version of pIM4902 was replaced with a 2.6 kb *Bgl*II/*Pst*I fragment containing a functional copy of the *A. niger argB* gene, and the resulting plasmid was designated pIM4910 (see **C**). (**B**) The cDNA fragment containing the entire coding region of *mstA* in pBluescript SK+ was designated pIM4903. The *Xho*I and *Bam*HI sites in the polylinker were used to excise the *mstA* cDNA and clone it into pYEX-BX. The resulting yeast expression construct, pIM4904 (not shown), allows expression of *mstA* under control of the copper-inducible CUP1 promoter [62]. (**C**) Diagram of the construct used to disrupt *mstA*.

The mycelium was harvested by suction over a filter, washed with MM without a carbon source, dried between paper and frozen in liquid nitrogen. The mycelium samples were stored at − 70 °C. Growth of transformants was analysed by spot-inoculating approx. 10^2, 10^3 and 10^4 spores suspended in saline Tween [0.9 % (w/v) NaCl/0.005 % (v/v) Tween-80] on MM plates containing 0.1, 1.0, 5.0, 15.0 or 60.0 mM D-glucose, D-mannose, D-fructose or D-xylose.

Yeast strain KY73 was grown on YP [1 % (w/v) Bacto yeast extract/2 % (w/v) Bacto peptone] with 2 % (w/v) maltose at 30 °C. Other *S. cerevisiae* strains used were derived from strain KY73, and were transformed with plasmids based on pYEX-BX (Clontech). Plasmid transformations of yeast cells were carried out according to the quick and easy TRAFO protocol [22]. Yeast strains were grown at 30 °C in a rotary shaker at 250 rev./min, in YNB [0.67 % (w/v) Difco yeast nitrogen base] supplemented with 0.1 % (w/v) casamino acids and 0.2 mg/l tryptophan. The carbon sources used were as stated in the text. Unless stated otherwise, 0.5 mM $CuSO_4$ was used to induce expression from the CUP1 promoter.

Cloning and molecular characterization

Standard methods were used for other DNA manipulations, such as Southern analysis, library screening, subcloning, DNA digestions and plasmid DNA isolations [23]. *Escherichia coli* DH5αF′ was used for routine plasmid propagation. *E. coli* LE392 was used as a host for phage λEMBL3. The genomic library of *A. niger* N402 has been described previously [24]. pUC19 [25] and pBluescript SK [26] were used in routine DNA cloning. Chromosomal DNA was isolated as described previously [27]. DNA sequencing was carried out using the Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit (PerkinElmer) following the manufacturer's instructions, in an Applied Biosystems automatic DNA sequencer (ABI Prism 310 Genetic Analyser, PerkinElmer). Nucleotide sequences were analysed with computer programs based on those of Devereux et al. [28]. The GenBank accession number for the nucleotide sequence of *A. niger mstA* is AY081845.

The QuikChange™ site-directed mutagenesis kit (Stratagene) was used to introduce a *Bgl*II site and a *Nsi*I site into plasmid pIM4902 (see Figure 1). These enzyme sites were used to facilitate the construction of plasmid pIM4910, which was used for the disruption of *mstA*.

Total RNA was isolated from powdered mycelium using TRIzol® reagent (Life Technologies), according to the supplier's instructions. For Northern-blot analysis 3 μg of total RNA was incubated with 3.3 μl of 6 M glyoxal, 10 μl of DMSO and 2 μl of 0.1 M sodium phosphate buffer, pH 7, in a total volume of 20 μl for 1 h at 50 °C to denature the RNA. The RNA samples were separated on a 1.5 % agarose gel using 0.01 M sodium phosphate buffer (pH 7) and transferred to Hybond-N filters (Amersham Biosciences) by capillary blotting. Filters were hybridized at 42 °C in a solution of 50 % (w/v) formamide, 10 % (w/v) dextran sulphate, 0.9 M NaCl, 90 mM sodium citrate, 0.2 % (w/v) Ficoll, 0.2 % (w/v) polyvinylpyrrolidone, 0.2 % (w/v) BSA, 0.1 % (w/v) SDS and 100 μg/ml single-stranded herring sperm DNA. Washing was performed under stringent conditions with 30 mM NaCl, 3 mM sodium citrate and 0.5 % (w/v) SDS at 68 °C. Two fragments of *A. niger mstA* were used as probes: a 1.95 kb *Eco*RI/*Xho*I fragment from *mstA* cDNA clone pIM4903 was used for Northern analysis, and a 3.1 kb *Bam*HI fragment from the *mstA* genomic DNA clone pIM4902 was used in all other hybridization experiments. Other probes used were a 2.4 kb *Bam*HI fragment from *A. nidulans mstA* (GenBank accession number AN1251561) for screening the *A. niger* genomic and cDNA libraries, and a 0.7 kb *Eco*RI fragment from the gene encoding the 18 S rRNA subunit [29], used as an RNA loading control.

Construction and screening of an L-arabinose-/L-arabitol-induced cDNA library of *A. niger* NW176

A. niger NW176 was grown for 16 h, after which mycelia were harvested, washed with MM without carbon source, and aliquots of 3.0 g (wet weight) transferred to 100 ml of MM containing either 50 mM L-arabinose or 50 mM L-arabitol. Cultures were harvested 2, 4 and 8 h after transfer. Total RNA was isolated as described above. Poly(A)$^+$ mRNA was isolated from 1 mg of total RNA (comprising equal amounts of total RNA isolated from mycelia transferred to both carbon sources for all three time points) using the PolyATtract mRNA isolation system (Promega), according to the manufacturer's instructions. Approx. 5 μg of poly(A)$^+$ mRNA was used for the synthesis of cDNA using the ZAP-cDNA Synthesis Kit (Stratagene) according to the manufacturer's instructions. The resulting cDNA was used in the construction of the cDNA library using the ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene) according to the manufacturer's instructions. The resulting primary library consisted of 2.8×10^5 recombinant clones/ml. The library was amplified, titred and stored at − 70 °C as described by the manufacturer of the kit. The library was screened using the *A. nidulans mstA* probe described above. Washing was performed under conditions of moderate stringency with 300 mM NaCl, 30 mM sodium citrate and 0.5 % (w/v) SDS at 56 °C.

Functional analysis

In order to compare growth on D-glucose of the yeast strain expressing *mstA* with that of a strain harbouring the plasmid without insert, the yeast strains were pregrown for 48 h on YNB + 2 % maltose (w/v). Cells were centrifuged (10 min at 5000 ***g***), rinsed once in an equivalent volume of sterile MilliQ-purified water, and then resuspended to give a cell suspension of 5 % (wet weight). Subsequently 5 μl of undiluted, and 10^{-1}, 10^{-2} and 10^{-3} dilutions were spotted on to YNB plates containing 1 % (w/v) D-glucose, D-fructose, D-mannose or D-galactose, and 0, 0.01, 0.1 or 0.5 mM $CuSO_4$. Growth was examined after 4 days at 30 °C.

For the sugar-transport experiments (both hexose consumption measurements and zero *trans*-influx assays) yeast strains were grown for 40 h (approx. D_{600}, 2.0). Cells were pelleted by centrifugation (10 min at 4000 ***g***), washed in ice-cold 0.1 M phosphate buffer (pH 6.5), and resuspended to give a 10 % wet weight/volume suspension in 0.1 M phosphate buffer (pH 6.5). Cells were kept on ice until required. To initiate consumption experiments an aliquot of the cell suspension was added to an equal volume of pre-warmed (30 °C) buffer containing the sugar of interest (final concentration, 10 mM), and incubated at 30 °C and 250 rev./min in an orbital shaker. Samples taken at 1, 5, 10, 30, 60 and 90 min were stopped using perchloric acid (final concentration, 3.5 %, v/v). KOH was used to neutralize the samples prior to determining hexose concentrations. Glucose and fructose (using hexokinase, phosphoglucoisomerase and glucose-6-phosphate dehydrogenase) were determined as described previously [30]. Extracellular mannose was determined by high-performance anion-exchange chromatography system

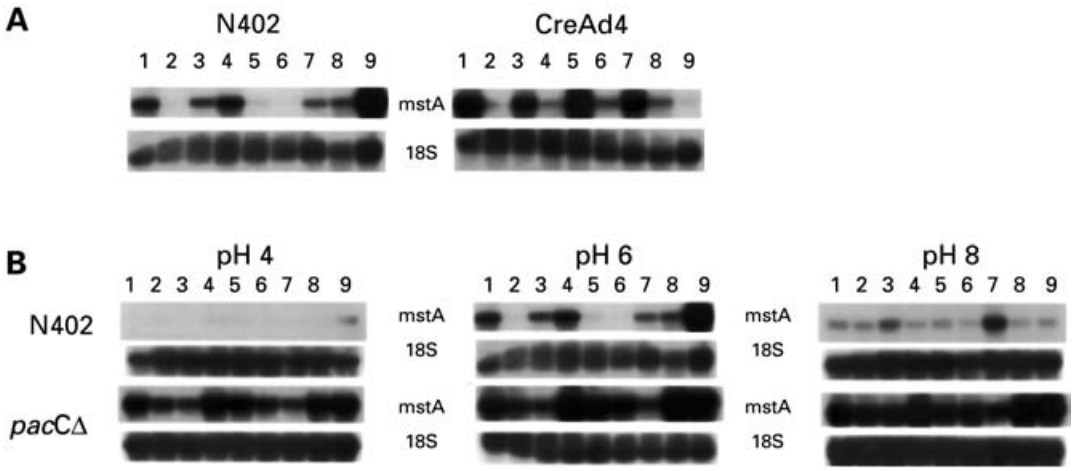

Figure 2 Regulation of *mstA* expression

Expression levels of *mstA* were determined in mycelia harvested 2 h after transfer. (**A**) Expression of *mstA* was compared between a reference strain (N402) and a *creAd4* strain (NW283) transferred to media at pH 6. The media contained the following carbon sources; lanes 1, 15 mM D-glucose; lanes 2, 15 mM D-fructose; lanes 3, 15 mM D-mannose; lanes 4, 15 mM sorbitol; lanes 5, 60 mM D-glucose; lanes 6, 60 mM D-fructose; lanes 7, 60 mM D-mannose; lanes 8, 60 mM sorbitol; lanes 9, no carbon source. (**B**) Expression of *mstA* was compared between a reference strain (N402) and a *pacC* disruption strain with a constitutive phenotype (NW138) at pH 4, 6 and 8. The carbon sources were as for (**A**). The results shown for N402 at pH 6 are the same as those in (**A**).

(Dionex Corp., Sunnyvale, CA, U.S.A.) equipped with a pulsed amperometric detector. Samples were injected into a Carbopac PA 100 column (25 mm × 4 mm; Dionex Corp) and eluted with 0.05 M NaOH for 10 min at a flow of 1 ml/min. Concentrations were calculated using D-mannose as a standard.

Zero *trans*-influx of ^{14}C-labelled D-glucose, D-fructose, D-mannose and D-xylose during a 5 s incubation at 30 °C was determined according to Walsh et al. [31]. For experiments done at pH 5, 0.1 M phthalic acid (pH 5.0) was used instead of phosphate buffer. Enzfitter software (version 1.05; Biosoft) was used to determine the apparent kinetic parameters of the transport protein for the different monosaccharides by non-linear regression analysis.

Proton uptake during sugar transport was monitored by recording pH changes in yeast suspensions as described previously [32,33], using a VIT90 Video Titrator and SAM90 sample station (Radiometer, Copenhagen, Denmark). The suspension was mixed using a magnetic stirrer at 30 °C. pH was lowered to 5.0–5.1 by pulses of 10 mM HCl from commencement of the individual experiments.

RESULTS

Cloning and molecular characterization of the *A. niger mstA* gene

Screening of the *A. niger* genomic DNA library with a fragment from the *A. nidulans mstA* gene resulted in seven hybridizing phage clones. A 3.1 kb *Bam*HI fragment was cloned into pBluescript SK+ resulting in a plasmid designated pIM4902. Sequence analysis showed that within this fragment is a gene (*mstA*) with a high degree of similarity to sugar-transporter-encoding genes.

Sequence analysis demonstrated that *mstA* has a coding region of 1823 bp that is interrupted by four introns and encodes a protein of 532 amino acids in size. The putative MSTA protein was predicted to have 12 membrane-spanning domains using both the TMHMM 1.0 [34] and SOSUI [35] programs. The derived amino acid sequence shows the greatest similarity to MST1 from the ectomycorrhizal fungus *A. muscaria* (45% identity) [10], RCO-3 from *Neurospora crassa* (40% identity) [11], a RAG4 from *Kluyveromyces lactis* (40% identity) [36], Snf3-p (41% identity) [37] and Rgt2-p from *S. cerevisiae* (40% identity) [38,39].

Within the 930 bp of promoter region sequenced there are a number of consensus target sequences for known DNA-binding regulatory factors (see Figure 1). These are: a single putative PacC-binding site [40] at −918 bp; four putative CCAAT boxes [41] at −839, −531, −479 and −204 bp; and eight putative CreA-binding sites [42] at −902, −612, −597, −556, −407, −329, −308 and −126 bp relative to the start codon.

Regulation of expression of *A. niger mstA*

To determine the conditions under which *mstA* is expressed, RNA from a reference *A. niger* strain (N402) grown under a variety of conditions was tested by Northern analysis. We found that *mstA* is expressed in mycelia transferred to conditions of carbon starvation, and poor (de-repressing) carbon sources such as sorbitol, glycerol, D-galactose and L-rhamnose (Figure 2A). Expression levels of *mstA* detected in RNA samples from mycelia transferred to repressing carbon sources such as D-glucose, D-fructose and D-mannose are elevated in the *creAd4* strain in comparison with the reference strain. This indicates that carbon catabolite repression of *mstA* is mediated by CreA. In the sample from the *creAd4* strain transferred to starvation conditions, a decrease in transcript levels, compared with the reference strain, was observed. The reason for this decrease in expression is not clear at this point, but the result has been confirmed using different *creA*-derepressed strains.

As we expected MSTA to be located in the cytoplasmic membrane and therefore exposed to the environment, we also wanted to determine if there were any effects of extracellular pH on the expression of this gene. Differences in expression are apparent for the reference strain grown at pH 6.0 and transferred to different pH conditions (Figure 2B), with *mstA* being most highly expressed at pH 6.0, whereas reduced levels of expression were observed at both pH 4.0 and 8.0. In an *A. niger pacC*-disruption strain [43], which results in a truncation of PacC and constitutive expression

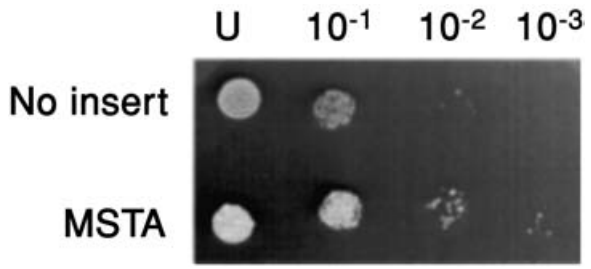

Figure 3 Growth of the yeast strain expressing *mstA* compared with a strain harbouring the plasmid without insert

Different dilutions of the strains were grown on YNB + 1 % D-glucose and 0.5 mM $CuSO_4$. Growth was examined after 4 days at 30 °C. U, undiluted.

of PacC-regulated genes, elevated levels of expression of *mstA* at pH 4, 6 and 8 were observed. The level of expression observed in the *pacC* mutant strain still varied depending on the carbon source. With the exception of the sample from carbon starvation conditions at pH 6, the mRNA levels detected in the samples from the *pacC* mutant strain were higher than those observed in the corresponding sample from the reference strain. This result indicates that the sole putative PacC-binding site identified in the 930 bp of the *mstA* promoter that were sequenced is probably functional, and that PacC activates *mstA* expression.

Functional analysis of MSTA in *S. cerevisiae*

To determine the function of *A. niger* MSTA, a full-length cDNA clone was isolated by probing an L-arabinose-/L-arabitol-induced cDNA library of *A. niger* NW176 with a genomic DNA fragment containing *mstA*. A full-length clone, designated pIM4903, was sequenced to confirm the intron locations (Figure 1). A 1.95 kb *Bam*HI/*Xho*I fragment containing the entire insert of pIM4903 was cloned into pYEX-BX digested with *Bam*HI/*Sal*I resulting in the *mstA* yeast expression construct pIM4904.

The *S. cerevisiae* strain KY73 was transformed with pYEX-BX and pIM4904, which resulted in the yeast strains KY73.001 and KY73.004, respectively (Table 1). Growth of these strains was tested on plates containing 1 % (w/v) D-glucose, D-fructose, D-mannose or D-galactose, and 0.1 % D-glucose. A copper-dependent partial complementation of growth was observed for the strain expressing *mstA* when grown on D-glucose (Figure 3), D-fructose or D-mannose, but not for D-galactose (results not shown). No difference in growth was observed on 0.1 % D-glucose compared with 1 % D-glucose. This indicates that MSTA is expressed in *S. cerevisiae*, and functions as a plasma-membrane-localized sugar transporter.

Extracellular hexose consumption experiments confirmed that the expression of MSTA in *S. cerevisiae* strain KY73 (KY73.004) enabled it to consume D-glucose, D-fructose and D-mannose, while the reference strain (KY73.001) showed no significant consumption of these three hexose sugars (Figure 4A). Zero *trans*-influx assays were used to determine some kinetic properties of MSTA produced in *S. cerevisiae* at pH 6.5. The data obey Michaelis–Menten kinetics, as determined by the kinetic data-fitting program Enzfitter. Apparent K_m values of $25 \pm 10\ \mu$M and $60 \pm 20\ \mu$M were determined for D-glucose and D-mannose respectively, using non-linear regression analysis. This indicates that MSTA has a 'very high affinity' for these sugars. $MSTA_{(A.\ niger)}$ has a 'high affinity' for D-xylose with an apparent K_m of 0.3 ± 0.1 mM, and a 'moderate affinity' for D-fructose with

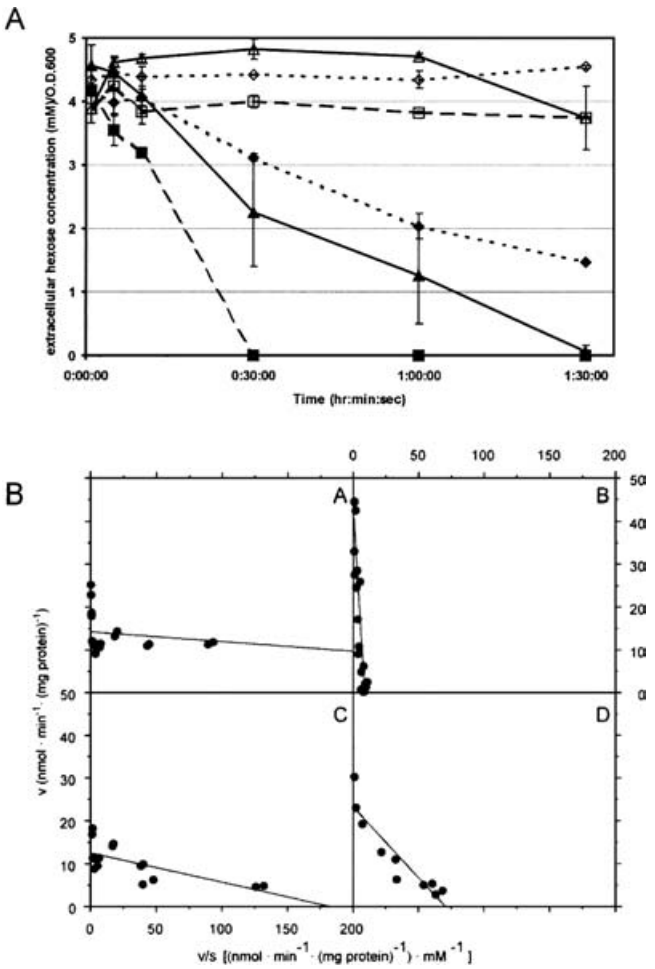

Figure 4 Functional analysis studies of *A. niger* MSTA expressed in *S. cerevisiae*

(**A**) Consumption of extracellular hexoses by the *S. cerevisiae* strain expressing MSTA. Open symbols represent KY73.001 (no insert) and filled symbols represent KY73.004 (expressing *A. niger* MSTA). Sugars tested were D-glucose (▲, △), D-fructose (◆, ◇) and D-mannose (■, □). Error bars show the S.D. of the duplicate results at each time point. (**B**) Eadie–Hofstee representation of zero *trans*-influx of ^{14}C-labelled monosaccharides by MSTA expressed in *S. cerevisiae* KY73. Sugars tested were D-glucose (A), D-fructose (B), D-mannose (C) and D-xylose (D). Theses graphs are representative of at least three independent experiments with comparable results. *v* is the zero *trans*-influx of the monosaccharide and *s* is the extracellular monosaccharide concentration.

an apparent K_m of approx. 4 ± 1.0 mM. The V_{max} values are dependent on the culture (level of copper induction) at the time of harvest. The V_{max} for D-glucose transport is approx. 15 nmol/min per mg of protein, and the V_{max} values for D-mannose and D-xylose are comparable with the V_{max} for D-glucose. The V_{max} of D-fructose transport appears to be relatively higher than the other sugars, approx. 35 nmol/min per mg of protein. Glucose transport was also examined at pH 5.0 (results not shown), and the values determined for the kinetic parameters, K_m and V_{max}, were similar to the values determined at pH 6.5. A graphical representation of affinity for the different sugars is given in Figure 4(B). Eadie–Hofstee plots were chosen for this representation to allow a visual comparison of the affinities for the different sugars (slope of graphs).

The addition of D-glucose to a suspension of *S. cerevisiae* cells producing MSTA (KY73.004) resulted in an increase in the pH

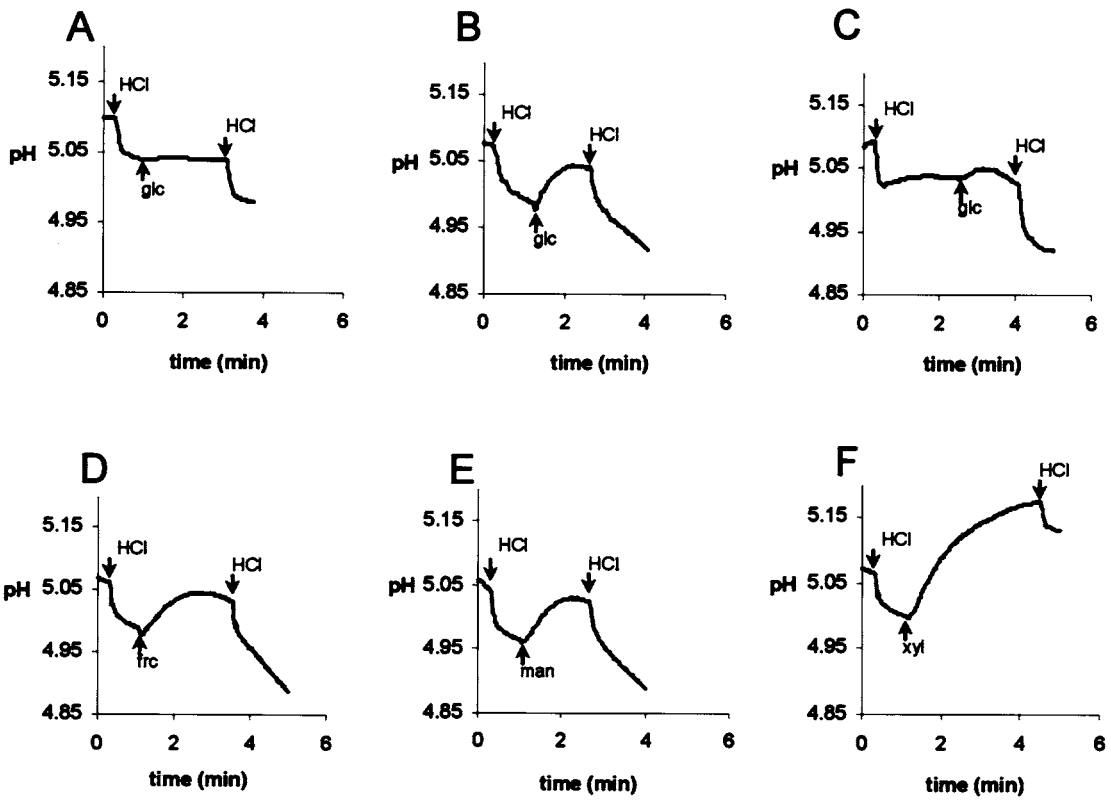

Figure 5 pH change associated with sugar transport

The pH of a 2 ml suspension containing 20 % wet weight (w/v) of *S. cerevisiae* cells in MilliQ-purified water was recorded continuously as described in the Experimental section. Arrows indicate the addition of 5 μl of 10 mM HCl, or sugar to the final concentration stated below. The graphs are representative of the results obtained for *S. cerevisiae* strain KY73.001 containing the vector without insert (**A**), and KY73.004, which expresses *A. niger* MSTA (**B**–**F**). The sugars added were 5 mM D-glucose (glc; **A**), 0.5 mM D-glucose (**B** and **C**), 50 mM D-fructose (frc; **D**), 5 mM D-mannose (man; **E**) and 50 mM D-xylose (xyl; **F**). The suspension used in (**C**) was pre-incubated with 1 mM 2,4-dinitrophenol for 15 min prior to the addition of D-glucose.

of the suspension (Figure 5B), which was not observed for cells containing the plasmid without insert (KY73.001; Figure 5A). The addition of maltose to the suspension of KY73.001 did result in an increase in pH, due to the activity of the endogenous maltose-transport system [32]. This shows that the absence of a response by this strain to the addition of D-glucose is due to the lack of an active transport system for D-glucose. An increase in pH of the KY73.004 cell suspension was observed upon the addition of 0.5 mM (Figure 5B) and 5.0 mM D-glucose (result not shown), 50 mM D-fructose (Figure 5D), 5 mM D-mannose (Figure 5E) and 50 mM D-xylose (Figure 5F). The proton production, which is concomitant with metabolism, occurs within 1 min of sugar addition and can be observed as the plateau and/or decrease in pH that occurs prior to the addition of HCl (Figures 5B, 5D and 5E). As *S. cerevisiae* is unable to metabolize D-xylose, the magnitude of the pH increase observed is greater than for sugars which can be metabolized (as the cells do not excrete protons in the case of D-xylose). This increase in pH was substantially reduced in cells pre-incubated, for 15 min prior to the addition of glucose, with 1 mM 2,4-dinitrophenol, which dissipates proton gradients across membranes (Figure 5C). These results are consistent with MSTA being a sugar/H^+ symporter.

Construction and analysis of an *A. niger mstA*Δ strain

To determine if *A. niger* possesses more than one transporter capable of transporting D-glucose, D-mannose, D-fructose or D-xylose at low sugar concentrations, an *mstA*-disruptant strain was constructed (see Figure 1C for construct used). Transformants were selected on minimal media containing appropriate supplements and 0.95 M sucrose as both osmotic stabilizer and carbon source. Of 31 transformants screened by Southern analysis, nine showed hybridization patterns consistent with replacement of the endogenous copy of *mstA* with the disruption construct (results not shown). Further Southern analysis of two of these transformants, NW326 and NW327, showed that they were indeed *mstA*Δ strains (Figure 6). Plate tests, in which reference strain NW325 was compared with *mstA*Δ strains NW326 and NW327, showed no obvious difference in growth between these strains when incubated on solid MM containing 0.1, 1.0, 5.0, 15.0 or 60.0 mM D-glucose, D-mannose, D-fructose or D-xylose (see Figure 7 for a representative example of the results obtained).

DISCUSSION

The protein most similar to *A. niger* MSTA is the hexose transporter MST1 from the ectomychorrizal fungus *A. muscaria* [10]. Uptake kinetics obtained for MST1 expressed in *S. cerevisiae* showed that this transporter has a preference for glucose (K_m, 0.46 mM) over fructose (K_m, 4.20 mM) [9]. Transport of glucose and fructose by *A. muscaria* MST1 was found to be inhibited by the protonophore CCCP (carbonyl cyanide *m*-chlorophenyl-

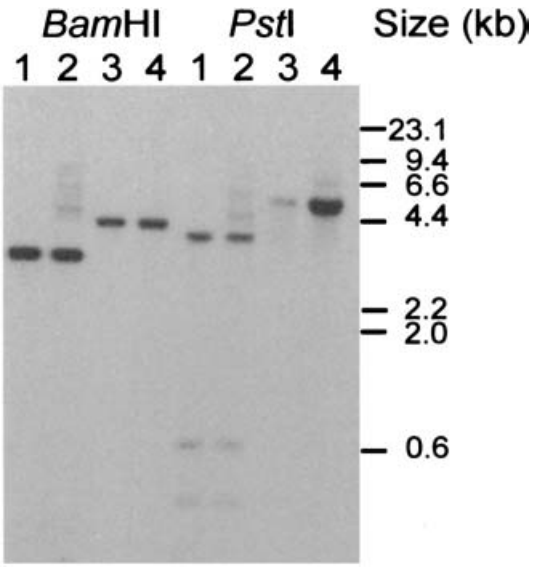

Figure 6 Southern analysis confirming the construction of two *mstA*Δ strains

Strains used are the parental strain NW324 (lane 1), an isogenic *mstA*+ strain NW325 (lane 2), and the disruptant strains NW326 (lane 3) and NW327 (lane 4). The *Bam*HI digest shows the increase in the size of the hybridizing fragment from 3.1 to 4.6 kb. The *Pst*I digest shows loss of the two *Pst*I internal fragments of 0.54 and 0.82 kb, and an increase in the size of the remaining fragment that hybridizes (see Figure 1 for restriction map of this region for the genomic DNA, pIM4902, and disruption construct pIM4910). The probe used was the *EcoR*I/*Xho*I fragment from the cDNA clone pIM4904. Size markers (lambda *Hin*dIII) are indicated on the right. Additional poorly hybridizing high-molecular-mass bands (*Bam*HI digest lane 2; *Pst*I digest lanes 2 and 4) are due to incomplete digestion of the DNA in these samples.

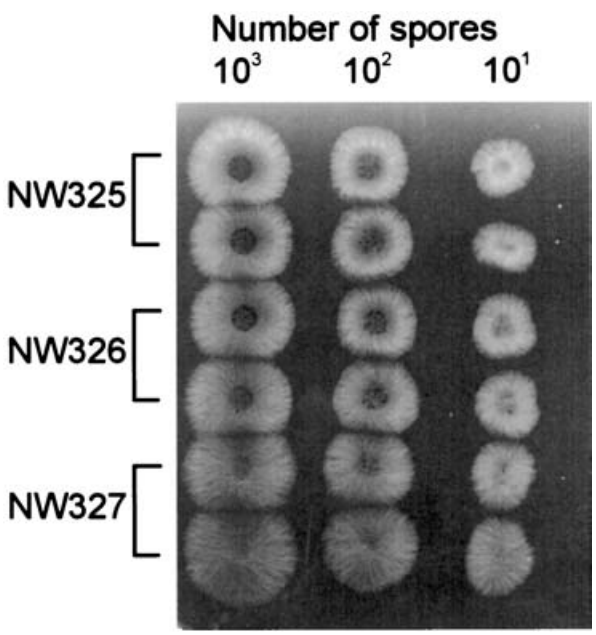

Figure 7 Growth of two *mstA*-disruptant strains (NW326 and NW327) compared with the reference strain NW325

10^3, 10^2 and 10^1 spores, suspended in sterile saline Tween, were spotted in duplicate on to MM plates containing 1.0 mM D-glucose as the sole carbon source.

hydrazone), and MST1 was therefore proposed to encode a monosaccharide/H^+ symporter [9]. Like MST1, MSTA also appears to be a monosaccharide/H^+ symporter with a higher affinity for D-glucose than for D-fructose. Based on its expression in *S. cerevisiae*, the apparent K_m of MSTA for D-fructose (K_m, 4.5 ± 1.0 mM) is similar to the apparent K_m value for fructose reported for MST1. The apparent K_m of MSTA for D-glucose of 25 ± 10 μM is almost 20-fold lower than that reported for MST1 [$K_{m(glucose)}$, 0.46 mM]. In addition MSTA is also able to transport D-mannose and D-xylose. Although *mstA* encodes a monosaccharide/H^+ symporter with amino acid sequence similarity to known monosaccharide transporters, it is its higher apparent affinity for D-glucose which distinguishes it from previously characterized fungal monosaccharide transporters.

The glucose-uptake systems described for *S. cerevisiae* are a low-affinity system (K_m, 15–20 mM) and a high-affinity system (K_m 1–2 mM), which is glucose-repressed [44,45]. These values are the combined effects of individual transporters which can be classified as low affinity, e.g. Hxt1-p and Hxt3-p [$K_{m(glucose)}$, 50–100 mM], moderately low affinity, e.g. Hxt2-p and Hxt4-p [$K_{m(glucose)}$, approx. 10 mM] and high affinity, e.g. Hxt6-p, Hxt7-p and Gal2-p [$K_{m(glucose)}$, 1–2 mM] [16,46]. The Ght hexose transporters of *Schizosaccharomyces pombe* also prefer glucose over fructose, and the individual transporter $K_{m(glucose)}$ values are described as either low affinity (2–8 mM) or high affinity (0.4–0.6 mM) [47]. Previous studies of *A. niger* glucose-transport systems identified a high-affinity system (K_m, 0.26 mM) in mycelia grown in 1 and 15% glucose (w/v), and a low-affinity system (K_m, 3.67 mM) only observed in mycelia grown on 15% glucose (w/v) [8,48]. With a significantly lower K_m value for glucose, MSTA could be described as a 'very-high-affinity' glucose transporter. The existence of such a very-high-affinity glucose-transport system indicates that *A. niger* is capable of efficient uptake of very low amounts of glucose from its environment.

Based on derived amino acid sequence, MSTA also shows similarity to *K. lactis* RAG4 [36], *N. crassa* RCO-3 [11] and *S. cerevisiae* SNF3-p and RGT2-p [49,50], all of which function as sensors of extracellular glucose. Common to these four proteins is an extended C-terminus that resides in the cytoplasm, and is believed to confer the signal-transduction function of these sensor proteins [51]. An amino acid motif, with the consensus sequence DLGNGLXLNXYNRGPPSXXXXS [51], is common to the C-terminal tails of the yeast proteins, but is not found in the *N. crassa* glucose sensor [11,36]. Studies of the *S. cerevisiae* sensors have shown that they are not able to transport detectable levels of glucose [52]. Although it is possible that the partial complementation of the *S. cerevisiae* strain by MSTA is due to activation of normally silent endogenous transporters, we believe there is no real evidence to support the assertion that MSTA is a sensor. Unlike the known glucose sensors, MSTA does not have an extended C-terminal tail. Also we have detected transport capabilities for a number of sugars, indicating that MSTA is not a sensor. Additional evidence is that the *A. niger mstA*Δ strain lacks the major defects which would be expected if MSTA was a sensor.

Expression under carbon starvation conditions and in the presence of 'poor' carbon sources is consistent with a role for MSTA as a very-high-affinity glucose transporter. *mstA* is subject to CreA-mediated carbon catabolite repression, ensuring that MSTA is only produced in the presence of very low concentrations of, or in the absence of, preferred carbon sources such as D-glucose, D-mannose, D-xylose and D-fructose. As shown for other permease-encoding genes, such as *A. nidulans gabA* [53], PacC mediates their expression in response to ambient pH. The expression of *mstA* in the reference strain at pH 4, 6 and 8 requires cautious interpretation. In this study we see that *mstA* is expressed

predominantly at pH 6 and poorly or not at all at both pH 4 and 8. One interpretation is that *mstA* is a gene expressed specifically at 'neutral' pH values. Such an interpretation also implies that pH regulation in filamentous fungi is more complex than the proposed model where pH-regulated genes are expressed under either acidic or alkaline conditions [40]. An alternative explanation for this result centres on the fact that *A. niger* grows better at acidic pH values. Growth of *A. niger* N402 on plates buffered to pH 8 is poor when compared with its growth on media buffered to either pH 3 or 6 (P. A. vanKuyk, unpublished work). The pH of soil is usually 4.0–8.5 [1], while compost pH ranges from 4.4 to 9.4 [54] and the pH of fruit such as prickly pear and sweet cherries has been found to be acidic [55,56]. The natural habitat of *A. niger* is therefore often acidic, and in addition *A. niger* is also known to acidify the medium it grows on [57,58]. Thus, for *A. niger* pH 6 may represent an 'alkaline' pH, and *mstA* would therefore be considered an alkaline-expressed gene. The transfer of *A. niger* mycelia to pH 8 may not only affect induction of gene expression, it could also result in changes in the physiology of the mycelia and possibly affect parameters such as mRNA stability. Therefore poor expression of *mstA* at pH 8 probably reflects a stressed physiological state rather than the effect of gene-specific regulation.

Disruption of *mstA* does not result in a discernible phenotype when this strain is grown on 0.1–60 mM D-glucose, D-fructose, D-mannose or D-xylose. Such a result indicates that under the conditions tested MSTA is not essential for growth or survival. Considering the situation in *S. cerevisiae*, in which at least six genes must be disrupted before the ability to transport glucose (as measured by the ability to grow on glucose) is abolished [14,16], it is not surprising that the disruption of a single monosaccharide-transporter-encoding gene does not affect growth of *A. niger* on the monosaccharides tested. In Nature *A. niger* is a soil-borne fungus, and its main carbon source is derived from the degradation of plant-cell-wall polysaccharides. Consequently, it is able to utilize a wide variety of monosaccharides. It seems reasonable to expect that the genome of *A. niger* encodes proteins able to transport these sugars, and that there is more than one protein able to transport commonly encountered sugars such as the hexoses D-glucose, D-fructose and D-mannose and the pentoses D-xylose and L-arabinose. It is probably a reflection of the environmental niche of *A. niger*, in which low sugar levels are found in the environment, that it has the capacity to use active transport for the uptake of D-glucose, D-mannose, D-xylose and D-fructose, enabling these sugars to be taken up against a concentration gradient. The ability of the *mstA*Δ strains NW326 and NW327 to grow on D-glucose, D-fructose, D-mannose and D-xylose shows that *A. niger* possesses other transporters capable of catalysing the uptake of these monosaccharides. The growth tests were conducted in a concentration range of 0.1–60.0 mM, which is within the range in which we would expect MSTA to be functioning for D-fructose and D-xylose uptake. Based on the expression patterns, these concentrations may be too high for MSTA be expressed. To test its role at lower sugar concentrations, continuous cultures will be necessary.

It is anticipated that the genome sequence of *A. niger* will reveal a large number of genes encoding putative sugar transporter functions. The key to gaining a thorough understanding of sugar transport in filamentous fungi will be in determining the function of the individual proteins, and the role each protein plays in sugar transport in the whole organism. Functional analysis in suitable host strains, such as *S. cerevisiae* KY73, and the production of *A. niger* strains in which multiple sugar-transporter-encoding genes have been disrupted, will be useful tools in elucidating this complex but fundamental process.

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