IDENTIFICATION OF THE mstE GENE ENCODING A GLUCOSE INDUCIBLE, LOW-AFFINITY GLUCOSE TRANSPORTER IN ASPERGILLUS NIDULANS
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The mstE gene encoding a low-affinity glucose transporter active during the germination of Aspergillus nidulans conidia on glucose medium has been identified. mstE expression also occurs in hyphae, is induced in the presence of other repressing carbon sources besides glucose, and is dependent on the function of the transcriptional repressor CreA. The expression of MstE and its subcellular distribution have been studied using a MstE-sGFP fusion protein. Concordant with data on mstE expression, MstE-sGFP is synthesised in the presence of repressing carbon sources and fluorescence at the periphery of conidia and hyphae is consistent with MstE location in the plasma membrane. Deletion of mstE has no morphologically obvious phenotype but does result in the absence of low-affinity glucose uptake kinetics, the latter being substituted by a high-affinity system.

For many filamentous fungi, the principal source of nutrients in the natural environment is dead and decaying plant material. Plant cell walls consist of two structural phases: the microfibrillar phase the bulk of which is cellulose, and the much more heterogeneous matrix phase of which pectins and hemicelluloses are among the major constituents (1). Organic carbon is obtained from these materials by virtue of the action of cellulosytic enzymes secreted by the fungus which digest these polymers resulting in the liberation of smaller compounds that can be assimilated. Studies in various filamentous fungi have shown that enzyme production is regulated in such a way that the nature of the activities secreted is appropriate for effective utilisation of the substrate available, and whilst the monosaccharides released are principal sources of carbon and energy they also play important roles in the induction and repression of gene expression (2 and refs therein). In this regard the identification and characterisation of the genes that encode catabolic enzymes and in particular their regulation have been major research interests for several decades, and relatively little attention has been focussed on the fundamental physiological process of monosaccharide uptake in the filamentous fungi.

Previous analyses of sugar uptake kinetics in the model filamentous fungi Aspergillus nidulans and Neurospora crassa provided evidence for the existence of energy consuming, carrier-mediated transport systems for D-glucose and some other sugars (3-7). In contrast, studies conducted by a number of groups in the model yeast Saccharomyces cerevisiae ultimately concluded that monosaccharide uptake was effected by at least two facilitated diffusion systems, whilst the uptake of disaccharides required the expenditure of energy (8, 9 and refs therein). The identification of hexose transporter genes in yeast commenced with the isolation of the sucrose non-fermenting mutant snf3 (10) followed by the characterisation of the SNF3 gene (11), and progressed by various means until its rapid conclusion upon analysis of the whole yeast genome data (12, 13). A total of 34 proteins comprise the S. cerevisiae sugar permease homologues. This group forms part of the Sugar Porter family of the Major Facilitator Superfamily (MFS), the latter originally having been conceived as a result of sequence comparisons between the then known permeases of almost exclusively...
bacterial or mammalian origin (14 and refs therein). Eighteen proteins form the yeast hexose transporter subgroup (Hxt1-17 and Gal2), and two (Snf3 and Rgt2) function as sensors of external glucose rather than transporters. The development of multiply deleted sugar transporter mutants and the subsequent reintroduction of individual transporter genes has greatly facilitated the characterisation of the gene products, and by such means it has been established that Hxt1-4, 6 and 7 and Gal2 are the major physiologically relevant hexose transporters in budding yeast. In addition, it has been shown that apart from glucose certain Hxts also mediate the uptake of D-fructose and D-mannose, and the D-galactose transporter Gal2 is able to transport glucose. Glucose uptake systems have also been described for other yeasts (15-18).

As regards the filamentous fungi, far less is known of the molecular genetics of sugar sensing and transport. Only four functional sugar transporters have been identified to date, encoded by: AmMst1 (19) and HXT1 (20) from the basidomycetes Amanita muscaria and Uromyces fabae respectively; gtI from the mycoparasitic fungus Trichoderma harzianum (21); and mstA from Aspergillus niger (22). All are MFS type high-affinity glucose transporters. A putative glucose sensor gene (rco-3) has been identified in N. crassa (23), and in A. nidulans three putative glucose transporter genes, mstA, mstB (accession numbers ANI251561, ANI278285 and our unpublished data) and hxtA (24) have been partially characterised. In a recent reappraisal of the kinetics of glucose uptake in A. nidulans using germinating conidia, two energy-requiring glucose transport systems were identified: a high-affinity glucose repressible system and one of low-affinity inducible by glucose (25). The present report is the first to provide evidence for the identification of a filamentous fungal gene encoding a low-affinity glucose transporter in glucose-germinating conidia.

EXPERIMENTAL PROCEDURES

Fungal strains and culture conditions - All strains used in this work are listed in Table 1. Genetic techniques, culture media and the obtention of conidia for inoculating cultures for either RNA isolation or glucose uptake experiments were obtained as described previously (25, 29). Carbon sources were added to cooled autoclaved medium to a final concentration of 0.5% (w/v) from filter-sterilized stocks. Supplemented minimal medium (SMM) was prepared by addition of the appropriate auxotrophic supplements.

Transformation of A. nidulans was carried out as detailed previously (30).

General molecular techniques - Standard molecular techniques were as described in Sambrook and Russell (31). Escherichia coli DH5-α (supE44, ΔlacU169 [φ80 lacZΔM15, hsdR17, recA1, endA1, gyrA96, thi-1, relA1]) was used as the host strain. All genomic DNAs were prepared using the method of Specht et al. (32). PCR reactions were generally performed using the Expand High Fidelity PCR System (Roche, Penzberg, Germany). DNA probes were labelled with 32P-dCTP using the random hexanucleotide priming method. Southern blot analysis was carried out using Hybond-N+ membranes (Amersham Biosciences, Piscataway, New Jersey, USA). DNA sequencing was done using both standard (e.g. universal and reverse) and custom oligonucleotides. Vector templates for sequencing reactions were prepared using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, USA). Sequencing reactions were performed as outlined in the Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Wellesley, Massachussetts, USA) and were analysed using a 310 Applied Biosystems Automatic DNA Sequencer (ABI PRISM™ 310 Genetic Analyzer, Perkin Elmer). The sequences of the oligonucleotides used in this study are available on request.

mstE cDNA and genomic clones - Total RNA was isolated (see below) from wild type mycelia grown for 6 h in shake flask culture (37°C, 200 rpm) in SMM containing 1% (w/v) glucose as sole carbon source. RNA was made DNA-free by treating 1 μg of RNA with 1 U of RNase-free DNase (Roche) at 37°C for 30 min, and the DNase was subsequently denatured by heating at 75°C for 5 min. First strand DNA synthesis was primed off a dT18 oligonucleotide using Moloney murine leukaemia virus reverse transcriptase (Amersham Biosciences). Two independent cDNA clones (in pGEM-T Easy) were obtained by
RT/PCR, sequenced and found to be identical. One of these was taken and named pGEM-cMstE. The mstE gene containing the entire coding region was obtained by PCR using wild type genomic DNA as template and cloned into pGEM-T Easy yielding pGEM-mst903. This clone was also sequenced. Sequence data have been lodged in GenBank under accession number AJ812567.

Construction of the MstE-sGFP fusion - The last 1131 bp of the mstE CDS were amplified and cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA) yielding pENTR-mstE. Using the LR clonase enzyme mix (Invitrogen), this plasmid was recombined with psGFP1, a GATEWAY vector derived from pMT-sGFP which carries the A. nidulans argB gene as a selectable marker (33). psGFP1 was derived from pMT-sGFP by digesting with XbaI in order to eliminate the alcA promoter. Recombination between pENTR-mstE and psGFP1 yielded plasmid pMstE-sGFP which carries the argB gene and an in-frame fusion between the 3’ terminus of the mstE CDS and the sequence encoding sGFP. A. nidulans strain SRF200 (Table 1) was transformed with this plasmid and L-arginine prototrophs were selected.

Procedures for mstE deletion - Upstream and downstream sequences (>2 kb of each) flanking the mstE gene were amplified from A. nidulans wild type genomic DNA. A plasmid was constructed by sequentially cloning the two mstE flanking regions into pBluescript SK+ II (Stratagene, La Jolla, CA, USA) followed by insertion between them of a functional PCR-generated A. nidulans panB gene (34) obtained by an appropriately primed PCR off genomic DNA. A. nidulans strain AN032 was transformed with a gel-purified linear product of a PCR primed off the plasmid and D-pantothenate prototrophic transformants were selected on medium containing 1 M sorbitol as both osmotic stabilizer and carbon source. AN032 was chosen for mstE deletion as it carries the sorA3 mutation (affects high-affinity glucose uptake-25) and it was hypothesised that this could lead to an additive effect resulting in enhanced 2-deoxy-D-glucose resistance and hence a means to rapidly identify mstE-deleted transformants. This however was observed not to be the case (unpublished data), and mstE-deleted mutant strains resulting from single integrations of the linear transforming DNA (identified and characterised by PCR and Southern analysis) were obtained after elimination of the sorA3 mutation by out-crossing with AN033.

RNA isolation and northern analysis - For RNA preparations, mycelia were grown in SMM shake flask cultures in an orbital shaker (200 rpm) at 37°C. For a given culture condition the total amount of SMM needed was inoculated to a final titre of 5×10^6 conidia/ml, mixed and subsequently distributed into the number of 300 ml lots required in 1 litre shake flasks. With the exception of samples taken prior to 6 h of growth, mycelia were recovered by filtration using nytal mesh, rinsed with fresh SMM lacking a carbon source and rapidly pressed dry between pads of absorbent paper and frozen in liquid nitrogen. Samples taken 0-6 h after inoculation were recovered by low-speed centrifugation (3200 g) for 6 min at 4°C and the pellet immediately frozen in liquid nitrogen. All biological material was maintained at -70°C until use.

RNA isolation and northern blotting were performed as described previously (35). Agarose gels were loaded with 15 µg of total RNA per track. Membranes (Hybond-N, Amersham Biosciences) were stained with methylene blue as a loading control, washed and hybridised. All expression experiments were repeated at least twice.

Fluorescence microscopy - Sterile coverslips were placed in a petri dish and spores were added in the appropriate SMM. Growth was carried out at room temperature overnight when 1% (w/v) glucose was used as carbon source, and for two days in the case of 1% (w/v) galactose. Media shift experiments were done by germinating conidia in glucose SMM on a 'Dantridish' (courtesy of Daniel Veith, Angewandte Mikrobiologie, Institut für Angewandte Biowissenschaften, Universität Karlsruhe, Germany) overnight at room temperature. Medium was subsequently removed by aspiration, the attached mycelia were rinsed with SMM lacking a carbon source and fresh galactose-SMM was added. Fluorescence was visualised with filter no. 9 (Zeiss, Jena, Germany) using an Axiophot microscope (Zeiss) and images
were captured with a high-resolution Orca ER camera (Hamamatsu, Munich, Germany). Labelling of *A. nidulans* vacuoles was carried out with CMAC (7-amino-4-chloromethyl coumarin; Molecular Probes, Invitrogen) as previously described (36).

Glucose uptake experiments - Glucose uptake measurements were done as described previously (25) except that filters were rinsed with 1.5 ml of ice cold 200 mM glucose immediately prior to filtration of $^{14}$C-glucose-labelled conidia. D-fructose uptake kinetics were determined using the appropriately modified protocol and measuring uptake rates at 10, 40, 100, 700, 2000 and 5000 µM fructose substrate concentrations. D-[^14]C]fructose (11.7 GBqmmol$^{-1}$) was purchased from Amersham Pharmacia. Glucose uptake rates in the presence of 100-fold excess of potential competing substrates were determined by linear regression of plots of the amounts of glucose taken up after 30, 60 and 90 s of incubation of 4 h glucose germinating conidia. All data were analysed using SigmaPlot v 8.02 (SSP Inc., UK).

RESULTS

Identification and characterisation of *mstE* - In a BLAST screening (37) of the Oklahoma University *A. nidulans* EST database (http://www.genome.ou.edu/fungal.html) with the primary structures of sugar transporters from a variety of organisms the lowest expectation (E) value (1e-40) noted occurred in an alignment between translations of tag o0f08a1 and a protein sequence (GenBank - AAB65790) which is described as corresponding to an *Aspergillus parasiticus* hexose transporter (Hxt1). Contig ANI61C903 was subsequently identified upon probing a low coverage *A. nidulans* genomic sequence database (http://www.cereon.com) *in silico* with the nucleotide sequence of this tag. By making comparisons between the potential translation products of ANI61C903 and the primary structures of known hexose transporters (e.g. the Hxt proteins of *S. cerevisiae*), and assuming classical intron/exon junctions, a hypothetical gene, *mstE*, containing three introns and encoding a 12 transmembranal (TM) domain protein (MstE) was deduced. After probing the NCBI database with the sequence of MstE the most similar proteins detected whose physiological functions have been experimentally determined were the hexose transporters RAG1 from *K. lactis* (62% similarity, 45% identity; 38) and Hxt3 from *S. cerevisiae* (59% similarity, 42% identity; 39), both of which are described as being low-affinity glucose transporters.

Using *mstE*-specific primers a RT/PCR product was obtained from RNA isolated from a 6 h glucose grown culture of wild type *A. nidulans*, conditions under which glucose transport is known to take place with low-affinity kinetics (25). The corresponding genomic clone was also obtained by PCR. The translation product MstE (562 amino acids and calculated molecular mass 62 kDa) is encoded by a 1849 bp sequence interrupted by three introns (those previously predicted) and contains the 5 element fingerprint (40; PR00171 - http://umber.sbs.man.ac.uk/dbbrowser/PRINTS/; see Figure 1) that identifies members of the sugar porter family of proteins (2.A.1.1 - http://www.tcdb.org/tcdb/index.php?tc=2.A.1.1), a subfamily of the MFS. Twelve putative TM α-helices are predicted to be distributed as two groups of six (Figure 1) as is common for proteins belonging to the MFS (42). Phylogenetic comparison with the primary structures of those fungal proteins for which experimental evidence of their physiological function as sugar transporters or sensors has been published (Figure 2) shows that MstE segregates apart from the characterised filamentous fungal sugar transporters of *A. muscaria*, *A. niger*, *T. harzianum* and *U. fabae* and the yeast sensor proteins, and instead is more closely related to the hexose transporters of *S. cerevisiae* and *S. pombe*. The alignment of sequences revealed 31 positions in which identical amino acids occur (see Figure 1), some of which correspond to residues that have been demonstrated to be involved in either substrate (sugar) binding or specificity in other sugar transporters – summarised in Table 2.

*mstE* expression - In order to accumulate information that could contribute to defining the function of its gene product, the expression of *mstE* was studied in total RNA isolated from germinating conidia and mycelia after various periods of shake flask liquid culture in the presence of distinct carbon sources (Figure 3). *mstE* mRNA abundance was very low (arabinose
and glycerol) or non-detectable (galactose, ethanol and lactose) on non-repressing sources whereas transcript levels were found to be abundant in 12 h mycelia growing in the presence of repressing carbon sources (fructose, glucose, maltose, mannose, sorbitol, sucrose and xylose). The relative repressiveness of carbon sources refers to their impacts on L-proline and acetamide utilisation (26). In germinating conidia (4 h) grown in the presence of glucose, mannose, sorbitol or sucrose greater abundance of the mstE transcript was seen compared to the other repressing carbon sources at the same time point. As cultures progressed (24 and 36 h) and the repressing carbon sources depleted, the levels of both the mstE and gpdA (constitutively expressed control) transcripts reduced significantly and/or disappeared (this is shown only for glucose in Figure 3 since the same effect was seen in all the other carbon sources); actin transcript abundance (not shown) paralleled that seen for gpdA. The only apparent exception to this was for mycelia growing in the presence of ethanol, for which a small amount of mstE transcript was detectable in the 24 and 36 h time points (data not shown).

mstE expression was also studied in RNA isolated from dormant wild type conidia and conidia germinating for between 1 and 8 h in parallel cultures in SMM containing either glucose or lactose as sole carbon sources. Whilst transcripts of mstE were not found in dormant conidia (0 h), mstE expression was detectable 1 h after inoculation of conidia into glucose SMM (activation phase – 49) and accumulated with time (Figure 4). By contrast, no expression of mstE was detected in conidia growing on lactose (data not shown). To analyse the glucose inducibility of mstE, glucose (0.5%) was added to a 14 h culture of wild type mycelia growing on SMM in the presence of 0.5% lactose (Figure 5A). Whereas mstE accumulation occurs over a prolonged period in continuous cultures containing glucose - or other repressing carbon sources - the rapid accumulation of mstE transcript after addition of glucose (1 h) to lactose-grown mycelia was followed by a rapid diminution over the course of the following hour despite the presence of 0.25% glucose in the medium (0.3% glucose after 1 h of induction and 0.25% after 2 h of induction).

It has been noted above that the amount of the control gpdA mRNA accumulated in wild type mycelia is very reduced by the 24 h time point compared to that seen at 12 h (shown only for glucose in Figure 3 but also observed for the other carbon sources). To test whether this effect is related with exhaustion of the carbon source, conidia were inoculated into SMM supplemented with both 3% lactose and 0.5% glucose and samples were taken after 4, 12 and 36 h of inoculation. Glucose was found to be completely depleted by 36 h but the residual lactose concentration was 0.5%. As can be observed in Figure 5B, the expression patterns of both gpdA and mstE were the same as those seen for mycelia growing in medium containing glucose as sole carbon source at an initial concentration of 0.5% (Figure 3). Thus some factor other than carbon source depletion must be responsible for this reduction in the accumulation of the two mRNAs.

Expression of mstE in creA derepressed mutants -

The transcription factor CreA is responsible for mediating carbon catabolite repression (CCR) in A. nidulans by binding to the consensus DNA sequence 5'-SYGGRG-3' (50), and strains carrying derepressing creA mutant alleles (creA<sup>d</sup>) show differing degrees of derepression for repressing carbon sources such as glucose depending on the particular allele carried (27, 50, 51). Previous studies on glucose uptake by germinating conidia (4 h culture) have demonstrated the existence of two distinct transport systems, one of high glucose affinity and one of low affinity. The latter is expressed in wild type conidia germinating in the presence of glucose but is not detected in creA<sup>d</sup> mutant conidia germinating in either glucose- or galactose-containing media (25). Northern analysis of the expression of mstE was carried out on the wild type strain and the two creA<sup>d</sup> mutants (creA<sup>d</sup>1 and creA<sup>d</sup>30) used in the previous study, grown for 4, 12 and 36 h on media containing either glucose or galactose as sole carbon sources. As can be seen in Figure 6, a very low level of mstE transcript accumulation is discernable in the strain carrying the less severe creA<sup>d</sup>1 mutant allele whereas no mstE mRNA is detectable in the phenotypically extreme creA<sup>d</sup>30 mutant strain grown in the presence of glucose. Indeed, no mstE transcript is detected in the creA<sup>d</sup>30 mutant grown in the presence of any of the repressing carbon sources: fructose, mannose, malatose, sucrose or xylose (data not shown). These data indicate a role for the
phenotypes is an essential element in the
conidia - The characterisation of mutant
Glucose uptake in germinating
plasma membrane to the vacuole.
inducing conditions, being relocated from the
the MstE protein is modified upon shifting to non-
This indicates that the subcellular distribution of
fluorescence that was initially associated with the
incubation progressed the localization of
medium. The mycelia were then periodically
were subsequently transferred to galactose
mstE (confirmed by Southern blotting) were grown in
transformant (AN077) carrying a single copy of
examine the consequences of a change from
the plasma membrane.
Subcellular localization of MstE - In order to
examine the cellular location of MstE the protein
was C-terminally tagged with sGFP (a plant
adapted version of GFP). The design of the fusion
construct was such that the production of sGFP-
tagged MstE should only occur in those A.
nidulans transformants in which homologous
integration of the plasmid has taken place at the
genomic mstE locus. In all those transformants
where fluorescence was seen this was localized at
the conidial and hyphal periphery when grown in
glucose SMM (mstE expression conditions).
Fluorescence was also associated with septa and
vacuoles, the latter being the only organelle
distinguishable by phase contrast light microscopy
in A. nidulans mycelia. No fluorescence was
however detected when transformants were grown
in galactose SMM (Figure 7A). Additional
experiments (data not shown) carried out in the
presence of other mstE-inducing compounds
yielded the same results as those seen for glucose,
whilst the non-inducing carbon sources failed to
yield fluorescence. These results demonstrate that
MstE is produced in the presence of glucose or
other repressing carbon sources and is localized in
the plasma membrane.

An experiment was also conducted to
examine the consequences of a change from mstE-
inducing to non-inducing conditions. Conidia of a
transformant (AN077) carrying a single copy of
pMstE-sGFP integrated at the mstE locus
(confirmed by Southern blotting) were grown in
glucose medium to yield young mycelia which
were subsequently transferred to galactose
medium. The mycelia were then periodically
examined by fluorescence microscopy. As the
incubation progressed the localization of
fluorescence that was initially associated with the
plasma membrane was seen to deplete whilst that
associated with vacuoles increased (Figure 7B).
This indicates that the subcellular distribution of
the MstE protein is modified upon shifting to non-
inducing conditions, being relocated from the
plasma membrane to the vacuole.

Glucose uptake in germinating mstE-deleted
conidia - The characterisation of mutant
phenotypes is an essential element in the
identification of gene function. A gene
replacement strategy was therefore designed to
obtain mstE complete loss-of-function mutants. A
linear deletion cassette comprising the panB gene
(34) flanked by the mstE upstream and
downstream sequences was used to transform a D-
pantothenate auxotroph (AN032). Two of the D-
pantothenate protrophic transformants (AN027
and AN028) were shown to carry single-copy
integrations of the deletion cassette at the mstE
locus with the consequent deletion of this gene
(AnmstE). No differences in growth were evident
between these strains and a panB-complemented
mstE+ transformant (AN030 – ectopic integration
of the deletion cassette) in plate tests on solid
media containing either ethanol, galactose,
glucose, glycerol, fructose, lactose, maltose,
mannitol, mannose, sorbitol, sucrose or xyllose as
sole carbon sources (data not shown).

Since AN032 carries the sora3 mutant
allele which is known to affect high-affinity
glucose uptake (25), transformants AN027 and
AN028 were crossed to a sora+ strain (AN033)
and two independent AnmstE sora+ offspring were
isolated (AN047 and AN048). Comparison by
light microscopy of the germination characteristics
of the conidia of these two strains to that of a non-
deleted control strain (AN057) of similar genetic
background (strain AN057 is progeny of the same
cross that yielded AN032) revealed no differences.
Glucose uptake kinetics (Figure 8) were measured
for conidia of each of the latter three sora+ strains
germinating in media containing glucose as carbon
source, conditions under which low-affinity
uptake has been shown to be present in
wild type conidia (25). Whilst the control strain
AN057 (mstE+) showed similar low-affinity
kinetics to those seen previously for wild type
conidia (Km = 1.54 mM, Vmax = 0.22 nmols
per 5 x 107 conidia), glucose uptake by both
AnmstE strains was effected by a system of
considerably greater affinity, having a Km value
(~50 µM) close to that measured for the high-
affinity glucose uptake system expressed in wild
type conidia germinating in the presence of
glycerol. Measurement of glucose uptake kinetics
in the AnmstE sora3 double mutants AN027 and
AN028 (data not shown) also revealed loss of the
low-affinity component compared to the sora3
single mutant control (AN030) and yielded Km
values similar to that reported for the sorA3 single mutant (25).

These results show that MstE is involved in low-affinity glucose uptake in germinating conidia, and indicate that an alternative system of high glucose affinity substitutes the low-affinity component in mstE deletion mutants.

mstE inducers and MstE substrates - Northern analysis has shown that expression of the mstE gene is induced by a variety of carbon sources, including fructose and the linear polyol sorbitol. In order to examine the possibility that MstE plays a role in fructose uptake, transport of this ketose was compared between conidia of the mstE-deleted strain (AN047) and those of AN057 (mstE+), germinating in strongly mstE-inducing sorbitol medium. Whilst measurement of the kinetics of glucose uptake showed the expected loss of low-affinity uptake by the mstE-deleted strain, fructose uptake kinetics were observed to be the same for both strains (data not shown) indicating that mstE does not play a physiologically relevant role in the uptake of this sugar.

Substrate competition experiments carried out under conditions of mstE expression provide a means to identify additional potential substrates of MstE. Hence, the rate of glucose uptake by glucose-germinating wild type conidia was measured at the Km value of low-affinity uptake (25) in the presence of 100-fold excess of those carbon sources used to investigate mstE induction (Figure 3) - with the exception of ethanol. As expected the presence of excess non-radioactively labelled glucose showed very strong competition, reducing 14C-glucose uptake to just 1-2% that of the control to which no competitor was added. Excess mannose reduced glucose uptake to about 13% that of the control, whilst galactose and xylose reduced uptake to around 70%. None of the remaining compounds competed effectively with 14C-glucose (data not shown).

These data indicate that mannose could well constitute a physiologically relevant additional substrate for MstE, whilst the inducing compounds fructose and sorbitol are not substrates for this transporter.

DISCUSSION

Several lines of evidence support the implication of MstE in glucose inducible low-affinity glucose uptake and specifically its identification as the low-affinity transporter of glucose in germinating A. nidulans conidia. Firstly, the mstE transcript encodes a protein belonging to the sugar porter subfamily of the MFS of secondary transporters, and phylogenetic analysis indicates a closer relationship between MstE and the relatively low substrate affinity yeast hexose transporters (Km values in the millimolar range) than to those filamentous fungal glucose transporters studied to date (Km values in the micromolar range) or the yeast glucose sensors. Secondly, northern data show that the expression of mstE coincides with the expression of the low-affinity glucose uptake component in germinating conidia - mstE transcript is present in 4 h glucose-germinating conidia but absent in conidia germinating in glycerol medium. Thirdly, the glucose inducibility of mstE is evidenced by the rapid appearance of its transcript both in glucose-germinating conidia and also upon addition of glucose to a young but established culture (14 h) of lactose-growing mycelia in which the mstE transcript is otherwise not detected. Fourthly, coincident with the previously demonstrated requirement of a functional CreA transcription factor for expression of the low-affinity glucose uptake component (25), mstE expression is negatively influenced by loss-of-function mutations in the creA gene. Fifthly, the expected location for a nutrient uptake system at the plasma membrane is evidenced by the concentration of sGFP-tagged MstE at the hyphal periphery. Sixthly, conidia from strains in which the MstE coding sequence has been deleted fail to manifest low-affinity glucose uptake kinetics when germinating in glucose medium.

In addition to expression in the presence of glucose, northern blot analysis reveals a relationship between the repressiveness of a carbon source (26, 51) and mstE transcription: little or no mstE transcript accumulates upon germination/growth on non-repressing media whereas progressive accumulation occurs in the presence of repressing carbon sources. This observation may have implications for both the potential substrate range of the protein product and/or the identity of the coinducer of mstE transcription. Considering the former, certain yeast
and filamentous fungal glucose transporters have been shown to transport other sugars (20, 22, 52, 53). Indeed, earlier studies on A. nidulans mycelia provided evidence for the existence of various uptake systems exhibiting different affinities for different sugars (5). However, two points are noteworthy regarding the induction of mstE by fructose (moderate induction) and by sorbitol (strong induction): i) fructose transport in A. nidulans appears to be uniquely mediated by a highly specific fructose uptake system which does not transport, nor is affected by, glucose or other sugars (5), and ii) sorbitol whilst being equally as effective as glucose as an inducer of mstE is structurally quite distinct from it and hence highly unlikely to be taken up by the same transporter. Since the means available to kinetically characterise MstE substrate range are currently limited - on the one hand a yeast strain deleted for mstE appears to be uniquely mediated by a highly specific fructose uptake system which does not transport, nor is affected by, glucose or other sugars (5), and on the other no A. nidulans transport mutants consequently unable to grow on sugars are available – the possible uptake by MstE of carbon sources other than glucose was assessed indirectly via substrate competition and directly in the specific case of fructose. These analyses have shown mannose to be a potential physiological substrate for MstE whereas fructose and sorbitol are not. Thus there is no absolute equivalence between being an inducer of mstE and a substrate for transport by MstE. Intuitively, the inducibility of mstE by both substrates of MstE as well as compounds that are not transported by it, is more consistent with the possibility that the inductive event be related to some common metabolic consequence of the utilisation of repressing carbon sources rather than the existence of a battery of different sensors to detect and respond appropriately to the latter or differentiate their degrees of repressiveness. In this context it is noteworthy that mstE expression is dependent on the presence of functional CreA since its induction is severely impaired in creAd mutants. Recent studies of several CreA repressible systems (35, 54) have shown that catabolic hexose phosphorylation is a key event in the signalling of CreA-mediated repression by both glucose and fructose. Since growth in the presence of either of these sugars results in mstE expression, some aspect of the metabolism of the glycolytic/gluconeogenic intermediate glucose-6-phosphate may constitute the common metabolic consequence of the utilisation of repressing carbon sources, the signalling of which results in both the expression of mstE, and CCR involving CreA. Such a mechanism would also explain mstE induction and CCR on xylose.

The requirement of function of the transcriptional repressor CreA for mstE induction suggests mstE is normally repressed by the product of a gene which is itself subject to CreA-mediated CCR thus, paradoxically, leading to mstE derepression in the presence of repressing carbon sources. In this regard hexose transporter expression in S. cerevisiae is subject to transcriptional repression in the absence of glucose mediated by binding of the zinc binuclear cluster protein Rgt1 to HXT gene promoters and its recruitment of the Ssn6-Tup1 repressor complex (55). Interestingly, BLASTs of Rgt1 against the genomes of several filamentous fungi (A. nidulans, Fusarium graminearum, Magnaporthe grisea, N. crassa, Stagonospora nodorum and Trichoderma reesei) detect single hits of about 65% identity which are limited to the DNA-binding region of Rgt1 (our unpublished data). BLASTs of the sequences of yeast proteins known to interact with Rgt1 and modulate its function (Mth1 and Std1) fail to show hits in these filamentous fungal genomes. Thus, proteins containing Rgt1-like zinc binuclear clusters are encoded in filamentous fungal genomes but there is no evidence for their functional equivalence to Rgt1. Apart from a role for CreA, the possibility cannot be discounted that mstE could be subject to additional regulation by a specific positively-acting transcription factor. Analysis of the function of the mstE promoter is required to provide further detail on the nature of mstE regulation.

As expected for a nutrient uptake system, the localisation of sGFP-tagged MstE is consistent with its presence in the plasma membrane. Vacuolar fluorescence probably results from some level of membrane protein turnover even under mstE inducing conditions since shifting mycelia from inducing to non-inducing media resulted in the steady accumulation of fluorescence in vacuoles concomitant with depletion at the mycelial periphery. The A. nidulans purine transporter UapC has similarly been shown to be redistributed from the plasma membrane to the
vacuole upon transfer to nitrogen repressing conditions (36). The enhanced fluorescence associated with septa, also seen for UapC, is related to the presence of the plasma membrane on each face of the septum (56) and the transverse orientation of this structure in the viewing field compared to the longitudinal orientation of hyphae. Given that the expression of the mstE-sgfp gene fusion is driven by the native mstE promoter, the visualisation of MstE-sGFP has in addition yielded independent data corroborating the observations made on mstE gene expression.

The potential transporter complements for two filamentous fungi (Aspergillus fumigatus and N. crassa) have recently been deduced from their genomic sequences (http://www.membranetransport.org/). The importance of transporters belonging to the MFS group is indicated by their representing approximately 40% of the total number of transporter proteins. Automatic annotation of the A. nidulans genome data (http://www.broad.mit.edu/annotation/fungi/aspergillus/) has assigned 102 putative gene products to the so-called "sugar (and other) transporter" family (Pfam: PF00083). Definition of the properties and functions of membrane proteins involved in sugar assimilation will provide key data to improve our understanding of carbohydrate metabolism in the filamentous fungi, and can be expected to provide potential targets for both biotechnological manipulation of metabolism and the design of novel anti-fungal strategies.

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1The abbreviations used are: CCR, carbon catabolite repression; CMAC, 7-amino-4-chloromethyl coumarin; GFP, green fluorescent protein; MFS, major facilitator superfamily; SMM, supplemented minimal medium; TM, transmembranal domain.

FIGURE LEGENDS

Figure 1. Schematic representation of the topology predicted for the primary structure of MstE by the program TMHMM v2.0 (41). Certain amino acids have been numbered adjacently for the reader’s convenience. The five sugar porter family fingerprint sequences are shown on a black background. Amino acids conserved between fungal TM proteins that have been shown to function as sugar transporters or sugar sensors are shown in bold.

Figure 2. Phylogenetic analysis of sequence similarities between MstE and physiologically functional fungal sugar transporters and sensors (#). *Amanita muscaria* (Am), *Aspergillus nidulans* (An), *Aspergillus niger* (Ag), *Candida albicans* (Ca), *Kluyveromyces lactis* (Kl), *Pichia stipitis* (Ps), *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *Trichoderma harzianum* (Th) and *Uromyces fabae* (Uf). The *Homo sapiens* (Hs) glucose transporter GLUT1 was used as the outgroup. MEGA3 software (43) was used to carry out the analysis. Bootstrap values are adjacent to each internal node, representing the percentages of 1000 bootstrap replicates. The scale represents amino acid replacements per residue.

Figure 3. Northern blot analysis of the expression of the *mstE* gene in the wild type strain growing in the presence of different carbon sources: D-glucose (Glc), D-mannose (Man), D-fructose (Frc), D-galactose (Gal), L-arabinose (Ara), D-xylose (Xyl), glycerol (Gly), ethanol (EtOH), sucrose (Suc), maltose (Mal), lactose (Lac), D-mannitol (Mntl) and D-sorbitol (Srbl). Blots were stained with methylene blue prior to hybridisation to visualise the 18S and 28S rRNA bands as a loading control, and for comparative purposes transcript corresponding to the strongly and constitutively expressed *gpdA* gene (encodes glyceraldehyde 3-phosphate dehydrogenase - 48) was probed as an internal mRNA control. All carbon sources were initially present at 0.5% (w/v) except for EtOH which was present at 0.5% (v/v).

Figure 4. Northern blot analysis of the expression of the *mstE* gene in wild type conidia incubated in SMM with glucose 0.5% as carbon source. Lane marked ‘0’ indicates RNA isolated directly from dormant wild type conidia harvested from a complete medium spore plate (25).

Figure 5. Northern blot analysis of the expression of the *mstE* gene in the wild type strain growing on: A) SMM with lactose (0.5%) for 14 h (NI), and 1 h (I1) and 2 h (I2) after induction with glucose (0.5%); B) SMM with lactose (3%) plus glucose (0.5%), 4, 12 and 36 hours after inoculation of conidia.

Figure 6. Northern blot analysis of the expression of the *mstE* gene in *creA* mutants. All samples were collected from cultures on SMM media with glucose (Glc) or galactose (Gal) as carbon source (0.5%).
(N. B. Galactose was used in this analysis rather than glycerol since a very slight \textit{mstE} signal is detectable in wild type mycelia growing for 12 h on glycerol whereas no \textit{mstE} accumulation is evidenced in Figure 3 either for germinating conidia or mycelia on galactose).

\textbf{Figure 7.} A) Localization of MstE-sGFP \textit{in vivo}. Strain AN077 was grown on SMM plus glucose (\textit{mstE} expression conditions, images 1 and 2) or on SMM plus galactose (no expression of \textit{mstE}, images 3 and 4), and observed with green fluorescence filters (images 2 and 4) or phase contrast (images 1 and 3). Vacuoles (arrowheads) and a septum (arrow) are marked in images 1 and 2. Image 4 has been overexposed to show the complete absence of fluorescence. B) Time lapse analysis of the distribution of MstE-sGFP upon shifting from \textit{mstE}-inducing to non-inducing conditions. Image 5 shows the predominance of fluorescence at the periphery and septum of a young hypha immediately after the aspiration of glucose medium and its replacement by galactose medium (0 h). After 4 h of incubation in galactose medium the same hypha (image 6) shows practically complete redistribution of fluorescence to the vacuoles. The identification of vacuoles was confirmed using CMAC – data not shown.

\textbf{Figure 8.} Michaelis-Menten plots of glucose uptake rate \textit{vs} glucose concentration for AN047 (Δ), AN048 (●) and AN057 (●) conidia germinated in 1% glucose media. Solid lines represent the best fits for the data points.
Table 1. Aspergillus nidulans strains used in this work. The ~ symbol is used to indicate the presence of the associated allele where the location and/or copy number of that allele in the genome is unknown.

| Strain   | Genotype                                      | Origin   |
|----------|-----------------------------------------------|----------|
| wt       | biA1                                          | CECT2544 |
| creA1    | creA1 pabaA1                                  | 26       |
| creA30   | creA30 biA1                                   | 27       |
| SRF200   | pyrG89; ΔargB::trpCΔB; pyroA4                 | 28       |
| AN027    | uaZ11 ΔmstE::panB sorA3 pabaA1; panB100; riboB2 | This study |
| AN028    | uaZ11 ΔmstE::panB sorA3 pabaA1; panB100; riboB2 | This study |
| AN030    | uaZ11 sorA3 pabaA1; panB100; riboB2; ~panB     | This study |
| AN032    | uaZ11 sorA3 pabaA1; panB100; riboB2            | This study |
| AN033    | uaZ11; argB2; panB100                         | This study |
| AN047    | uaZ11 ΔmstE::panB pabaA1; panB100             | This study |
| AN048    | uaZ11 ΔmstE::panB pabaA1; panB100             | This study |
| AN057    | uaZ11 pabaA1                                  | This study |
| AN077    | pyrG89 mstE-sgfp argB; ΔargB::trpCΔB; pyroA4   | This study |
Table 2. Amino acids in MstE for which a role in substrate binding/specificity has been proposed in other sugar transporter proteins.

| Transporter protein | Transporter function | Position of amino acid in 1º structure and proposed role | Equivalent position in MstE | Reference |
|---------------------|----------------------|----------------------------------------------------------|-----------------------------|-----------|
| H. sapiens GLUT1    | glucose transport    | Q161; W412 substrate binding                             | Q194; W455                 | 44, 45    |
|                     |                      | Q282 glucose binding                                     | Q319                        |           |
| S. cerevisiae Hxt2  | glucose transport    | F431 glucose specificity                                 | F422                        | 46        |
| S. cerevisiae Gal2  | galactose transport  | W455; Y446 galactose specificity                         | W431; F422                 | 47 and refs therein |
| S. pombe GHT1 (& 2,5,6) | glucose transport | F379; Y388 suggested glucose specificity | F422; W431                 | 18        |
| S. pombe GHT3 (& 4) | gluconate transport | Y379; W388 suggested gluconate specificity               | F422; W431                 |           |
Figure 1
Figure 2
Figure 3

| Carbon source: | Glc | Man | Frc | Gal | Ara | Xyl | Gly | EtOH | Suc | Mal | Lac | Mntl | Srbl |
|----------------|-----|-----|-----|-----|-----|-----|-----|------|-----|-----|-----|------|------|
| Time (h):      | 4   | 12  | 24  | 4   | 12  | 4   | 12  | 4    | 12  | 4   | 12  | 4    | 12   |
| mstE           |     |     |     |     |     |     |     |      |     |     |     |      |      |
| gpdA           |     |     |     |     |     |     |     |      |     |     |     |      |      |
| rRNA           |     |     |     |     |     |     |     |      |     |     |     |      |      |
Figure 5

A

|       | NI | I1 | I2 |
|-------|----|----|----|
| mstE  |    |    |    |
| gpdA  |    |    |    |
| rRNA  |    |    |    |

B

| Time (h): | 4 | 12 | 36 |
|-----------|---|----|----|
| mstE      |   |    |    |
| gpdA      |   |    |    |
| rRNA      |   |    |    |

Lac 3% + Glc 0.5%
Figure 6

| Strain: |          | creA<sup>b</sup> |            | creA<sup>d</sup> |
|--------|----------|------------------|------------|------------------|
|        | Glc      | Gal              | Glc        | Gal              | Glc        | Gal              |
| Time (h): | 4 12 36 | 4 12 36          | 4 12 36    | 4 12 36          | 4 12 36    | 4 12 36          |
| mstE   | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) | ![Image](image5) | ![Image](image6) |
| gpdA   | ![Image](image7) | ![Image](image8) | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| rRNA   | ![Image](image13) | ![Image](image14) | ![Image](image15) | ![Image](image16) | ![Image](image17) | ![Image](image18) |
Figure 8
Identification of the mstE gene encoding a glucose inducible, low-affinity glucose transporter in Aspergillus nidulans
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