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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 synthesized 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 morphological phenotype but results 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 cellulolytic enzymes secreted by the fungus that 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 utilization of the substrate available, and whereas the monosaccharides released are principal sources of carbon and energy, they also play important roles in the induction and repression of gene expression (Ref. 2 and references therein). In this regard the identification and characterization 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 focused 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, whereas the uptake of disaccharides required the expenditure of energy (Refs. 8 and 9 and references therein). The identification of hexose transporter genes in yeast commenced with the isolation of the sucrose non-fermenting mutant *snf3* (10) followed by the characterization 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),[4] the latter originally having been conceived as a result of sequence comparisons between the then known permeases of almost exclusively bacterial or mammalian origin (Ref. 14 and references 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 characterization 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).

Regarding 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, *gtt1* 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)[5] and *hxtA* (24) have been partially characterized. 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.

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[4] The abbreviations used are: MFS, major facilitator superfamily; GFP, green fluorescent protein; SMM, supplemented minimal medium; TM, transmembranal domain.
[5] J. V. Forment, M. Flipphi, D. Ramón, L. Ventura, and A. P. MacCabe, unpublished data.

TABLE 1

***A. 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
Wild type	*biA1*	CECT2544
creA^{d}1	*creA^{d}1 pabaA1*	Ref. 26
creA^{d}30	*creA^{d}30 biA1*	Ref. 27
SRF200	*pyrG89; ΔargB::trpCΔB; pyroA4*	Ref. 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

EXPERIMENTAL PROCEDURES

Fungal Strains and Culture Conditions—All strains used in this work are listed in Table 1. Genetic techniques, culture media, and obtaining conidia for inoculating cultures for either RNA isolation or glucose uptake experiments were 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 the 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 (*sup*E44, Δ*lac*U169 (ϕ80 *lac*ZΔM15, *hsd*R17, *rec*A1, *end*A1, *gyr*A96, thi-1, *rel*A1)) 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 Applied Science). DNA probes were labeled with [^{32}P]dCTP using the random hexanucleotide priming method. Southern blot analysis was carried out using Hybond-N$^+$ membranes (Amersham Biosciences). 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). Sequencing reactions were performed as outlined in the Ready Reaction DyeDeoxy Terminator Cycle sequencing Kit (PerkinElmer Life Sciences) and were analyzed using a 310 Applied Biosystems Automatic DNA Sequencer (ABI PRISM^{TM} 310 Genetic Analyzer, PerkinElmer Life Sciences). 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 unit of RNase-free DNase (Roche Applied Science) 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 dT_{18} oligonucleotide using Moloney murine leukemia virus reverse transcriptase (Amersham Biosciences). Two independent cDNA clones (in pGEM-T Easy) were obtained by reverse transcription-PCR, sequenced, and found to be identical. One of these was used 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^{TM} 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), yielding pENTR-mstE. Using the LR clonase enzyme mix (Invitrogen), this plasmid was recombined with psGFP1, a GATEWAY vector derived from pMT-sGFP that carries the *A. nidulans argB* gene as a selectable marker (33). psGFP1 was derived from pMT-sGFP by digesting with XbaI 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 kilobases 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) 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 the 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 hypothesized 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,[5] and *mstE*-deleted mutant strains resulting from single integrations of the linear transforming DNA (identified and characterized 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 titer of 5×10^6 conidia/ml, mixed, and subsequently distributed into the number of 300-ml lots required in 1 liter shake flasks. With the exception of samples taken before 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 was 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 hybridized. 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 the carbon source and for 2 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

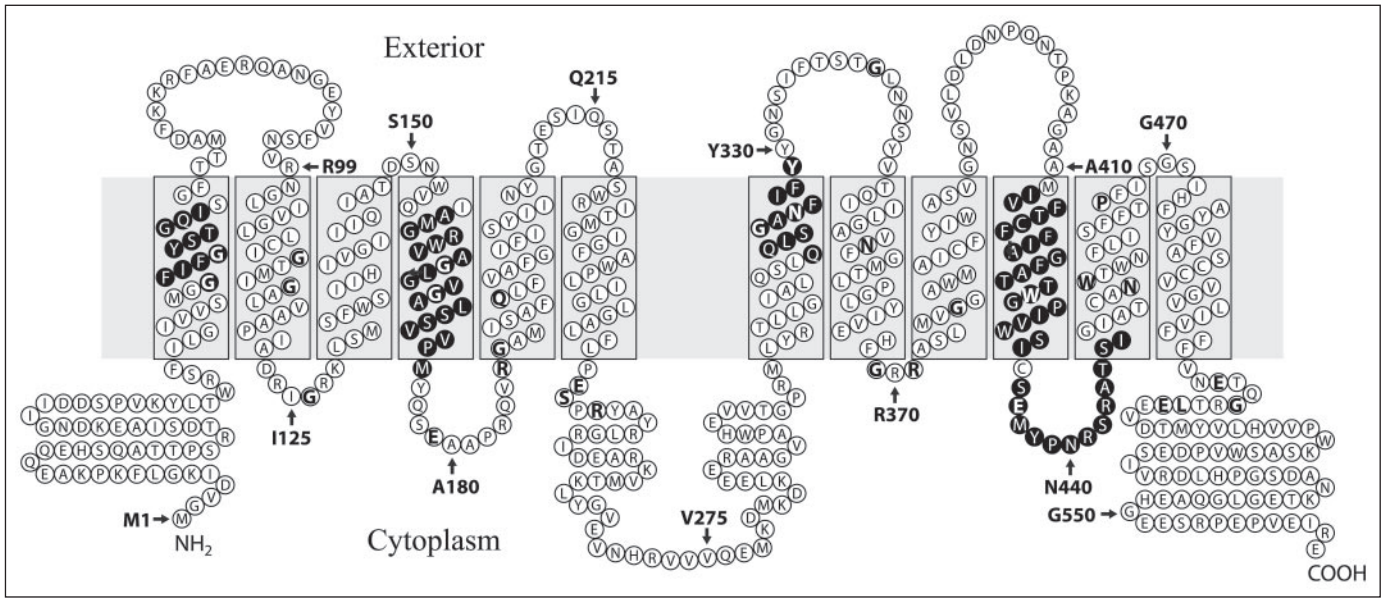

FIGURE 1. **Schematic representation of the topology predicted for the primary structure of MstE by the program TMHMM Version 2.0 (41).** Certain amino acids have been numbered adjacently for the reader's convenience. The five sugar porter family fingerprint sequences are shown on a *black background*. Amino acids conserved between fungal TM proteins that have been shown to function as sugar transporters or sugar sensors are shown in *bold*.

SMM lacking a carbon source, and fresh galactose-SMM was added. Fluorescence was visualized 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). Labeling of *A. nidulans* vacuoles was carried out with 7-amino-4-chloromethyl coumarin (Molecular Probes) 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 before filtration of [^{14}C]glucose-labeled 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-[U-^{14}C]fructose (11.7 GBq mmol^{-1}) was purchased from Amersham Biosciences. Glucose uptake rates in the presence of a 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 analyzed using SigmaPlot Version 8.02 (SSP Inc.).

RESULTS

Identification and Characterization of mstE—In a BLAST screening (37) of the Oklahoma University *A. nidulans* EST database 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 *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 *Kluyveromyces lactis* (62% similarity, 45% identity (38)) and Hxt3 from *S. cerevisiae* (59% similarity, 42% identity (39)), both of which are described as low affinity glucose transporters.

Using *mstE*-specific primers, a reverse transcription-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 a calculated molecular mass of 62 kDa) is encoded by a 1849-bp sequence interrupted by three introns (those previously predicted) and contains the five-element fingerprint (Ref. 40; PR00171; see Fig. 1) that identifies members of the sugar porter family of proteins (2.A.1.1), a subfamily of the MFS. Twelve putative TM α-helices are predicted to be distributed as two groups of six (Fig. 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 (Fig. 2) shows that MstE segregates apart from the characterized 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 *Schizosaccharomyces pombe*. The alignment of sequences revealed 31 positions in which identical amino acids occur (see Fig. 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, summarized in Table 2.

mstE Expression—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 (Fig. 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 utilization (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 with 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 Fig. 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 the sole carbon sources. Although 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 (Fig. 4). By contrast, no expression of *mstE* was detected in conidia growing on lactose (data not shown). To analyze 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 (Fig. 5*A*). 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 the 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).

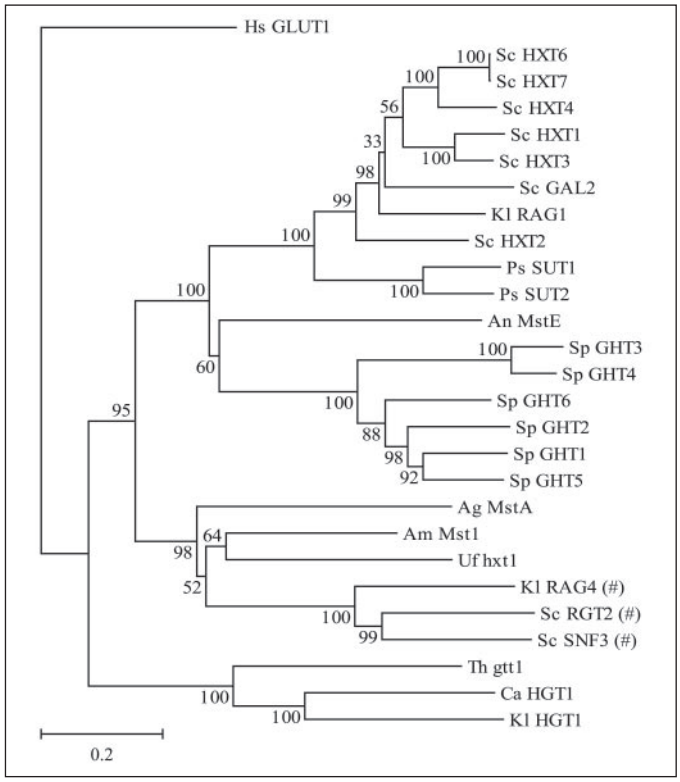

FIGURE 2. **Phylogenetic analysis of sequence similarities between MstE and physiologically functional fungal sugar transporters and sensors (#).** *A. muscaria* (*Am*), *A. nidulans* (*An*), *A. niger* (*Ag*), *Candida albicans* (*Ca*), *K. lactis* (*Kl*), *Pichia stipitis* (*Ps*), *S. cerevisiae* (*Sc*), *S. pombe* (*Sp*), *T. harzianum* (*Th*), and *U. fabae* (*Uf*). The *Homo sapiens* (*Hs*) glucose transporter GLUT1 was used as the out-group. 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.

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 with that seen at 12 h (shown only for glucose in Fig. 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 Fig. 5*B*, 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% (Fig. 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 in *A. nidulans* by binding to the consensus DNA sequence 5′-SYGGRG-3′ (50), and strains carrying derepressing *creA* mutant alleles (*creA*^{d}) 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*^{d} mutant conidia germinating in either glucose- or glycerol-containing media (25). Northern analysis of the expression of *mstE* was carried out on the wild type strain and the two *creA*^{d} mutants (*creA*^{d}*1* and *creA*^{d}*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 Fig. 6, a very low level of *mstE* transcript accumulation is discernable in the strain carrying the less severe *creA*^{d}*1* mutant allele, whereas no *mstE* mRNA is detectable in the phenotypically extreme *creA*^{d}*30* mutant strain grown in the presence of glucose. Indeed, no *mstE* transcript is detected in the *creA*^{d}*30* mutant grown in the presence of any of the repressing carbon sources: fructose, mannose, maltose, sucrose, or xylose (data not shown). These data indicate a role for the CreA protein in the expression of *mstE* in the presence of repressing carbon sources.

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 1° structure and proposed role	Equivalent position in MstE	Reference
H. sapiens GLUT1	Glucose transport	Gln-161; Trp-412 substrate binding Gln-282 glucose binding	Gln-194; Trp-455 Gln-319	44, 45 46
S. cerevisiae Hxt2	Glucose transport	Phe-431 glucose specificity	Phe-422	47 and references therein
S. cerevisiae Gal2	Galactose transport	Trp-455; Tyr-446 galactose specificity	Trp-431; Phe-422	47 and references therein
S. pombe GHT1 (& 2,5,6)	Glucose transport	Phe-379; Tyr-388 suggested glucose specificity	Phe-422; Trp-431	18
S. pombe GHT3 (& 4)	Gluconate transport	Tyr-379; Trp-388 suggested gluconate specificity	Phe-422; Trp-431	18

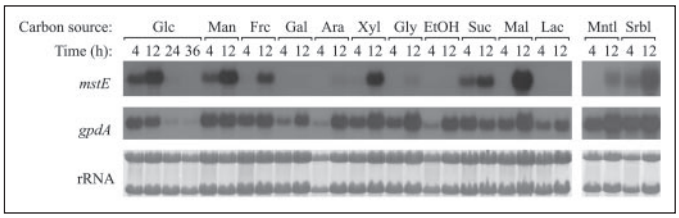

FIGURE 3. **Northern blot analysis of the expression of the *mstE* gene in the wild type strain growing in the presence of different carbon sources: D-glucose (*Glc*), D-mannose (*Man*), D-fructose (*Frc*), D-galactose (*Gal*), L-arabinose (*Ara*), D-xylose (*Xyl*), glycerol (*Gly*), ethanol (*EtOH*), sucrose (*Suc*), maltose (*Mal*), lactose (*Lac*), D-mannitol (*Mntl*) and D-sorbitol (*Srbl*).** Blots were stained with methylene blue before hybridization to visualize the 18 S and 28 S 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).

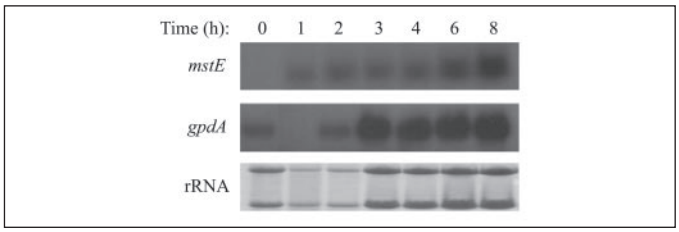

FIGURE 4. **Northern blot analysis of the expression of the *mstE* gene in wild type conidia incubated in SMM with glucose 0. 5% as carbon source.** *Lane 0* indicates RNA isolated directly from dormant wild type conidia harvested from a complete medium spore plate (25).

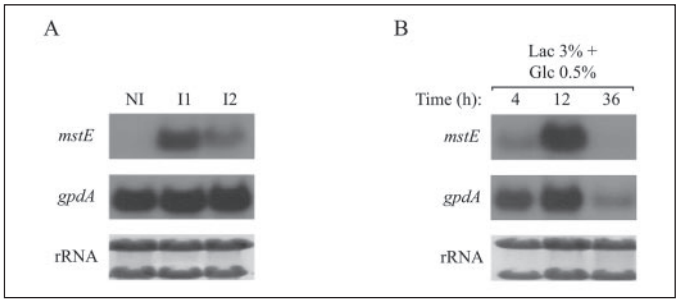

FIGURE 5. **Northern blot analysis of the expression of the *mstE* gene in the wild type strain growing on SMM with lactose (0. 5%) for 14 h (*NI*) and 1 h (*I1*) and 2 h (*I2*) after induction with glucose (0.5%) (*A*) and SMM with lactose (*Lac*, 3%) plus glucose (0.5%) 4, 12, and 36 h after inoculation of conidia (*B*).**

Subcellular Localization of MstE—To examine the cellular location of MstE, the protein was C-terminal-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 (Fig. 7*A*). Additional experiments (data not shown) carried out in the presence of other *mstE*-inducing compounds yielded the same results as those seen for glucose, whereas 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.

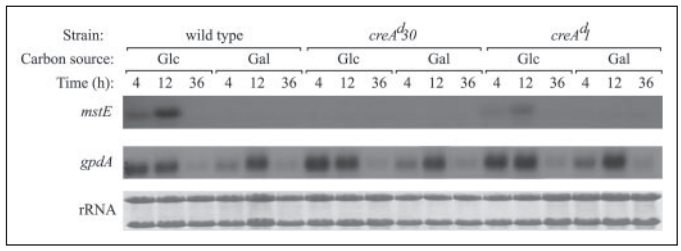

FIGURE 6. **Northern blot analysis of the expression of the *mstE* gene in *creA*^{d} mutants.** All samples were collected from cultures on SMM media with Glc or Gal as carbon source (0.5%) (note that galactose was used in this analysis rather than glycerol since a very slight *mstE* signal is detectable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

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, whereas that associated with vacuoles increased (Fig. 7*B*). 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 characterization 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 prototrophic 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 (Δ*mstE*). 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 xylose as sole carbon sources (data not shown).

Because 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 Δ*mstE 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 (Fig. 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 glucose uptake has been shown to be present in wild type conidia (25). Although the control strain AN057 (*mstE*$^+$) showed similar low affinity kinetics to those seen previously for wild type conidia (K_m = 1.54 mM, V_{max} = 0.22 nmol of glucose s^{-1} per 5×10^7 conidia), glucose uptake by both Δ*mstE* strains was effected by a system of considerably greater affinity, having a K_m 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 Δ*mstE sorA3* double

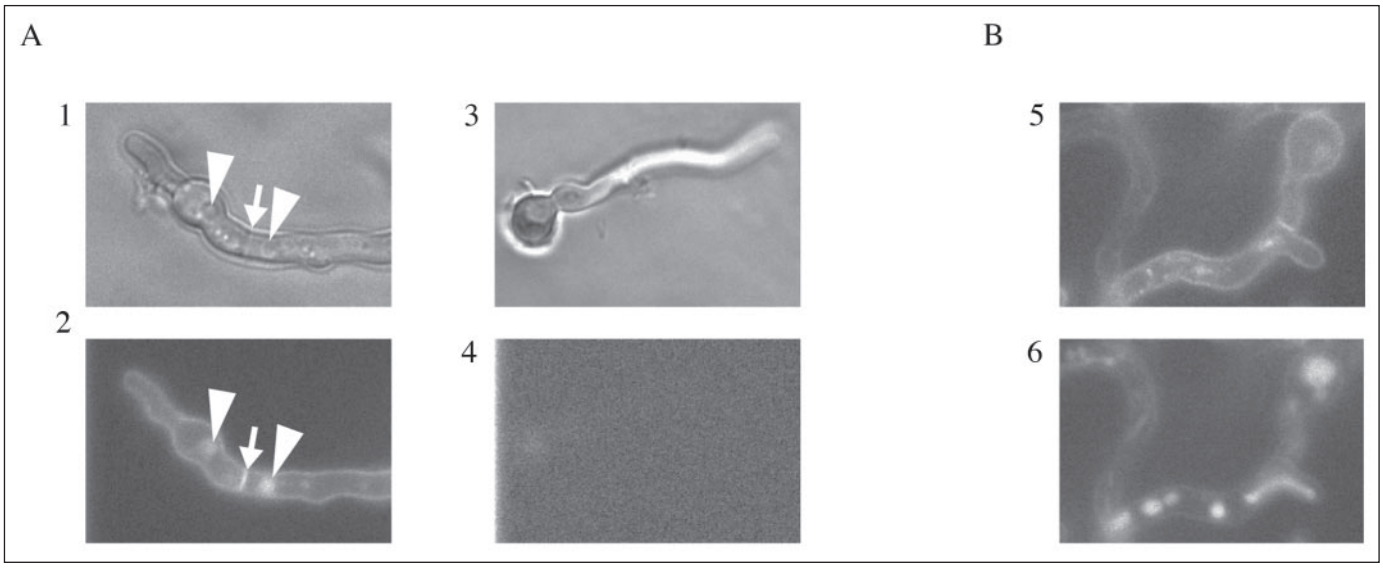

FIGURE 7. **Localization of MstE-sGFP *in vivo*.** *A*, strain AN077 was grown on SMM plus glucose (*mstE* expression conditions, *images 1* and *2*) or on SMM plus galactose (no expression of *mstE*, *images 3* and *4*) and observed with green fluorescence filters (images 2 and 4) or phase contrast (*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 *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 7-amino-4-chloromethyl coumarin (data not shown).

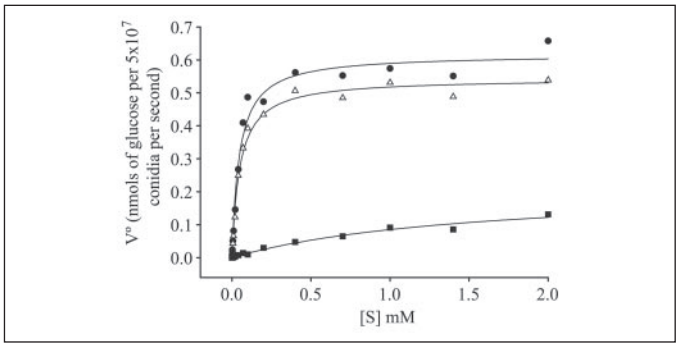

FIGURE 8. **Michaelis-Menten plots of glucose uptake rate *versus* glucose concentration for AN047 (△), AN048 (●), and AN057 (■) conidia germinated in 1% glucose media.** *Solid lines* represent the best fits for the data points.

mutants AN027 and AN028 (data not shown) also revealed loss of the low affinity component compared with the *sorA3* single mutant control (AN030) and yielded K_m 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. 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. Although 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 K_m value of low affinity uptake (25) in the presence of a 100-fold excess of those carbon sources used to investigate *mstE* induction (Fig. 3), with the exception of ethanol. As expected the presence of excess non-radioactively labeled glucose showed very strong competition, reducing [^{14}C]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, whereas galactose and xylose reduced uptake to around 70%. None of the remaining compounds competed effectively with [^{14}C]glucose (data not shown). These data indicate that mannose could well constitute a physiologically relevant additional substrate for MstE, whereas 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. First, 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 (K_m values in the millimolar range) than to those filamentous fungal glucose transporters studied to date (K_m values in the micromolar range) or the yeast glucose sensors. Second, 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. Third, the glucose inducibility of *mstE* is evidenced by the rapid appearance of its transcript both in glucose-germinating conidia and also upon the addition of glucose to a young but established culture (14 h) of lactose-growing mycelia in which the *mstE* transcript is otherwise not detected. Fourth, 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. Fifth,

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. Sixth, 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 that does not transport nor is affected by glucose or other sugars (5), and (ii) sorbitol, although 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. Because the means available to kinetically characterize MstE substrate range are currently limited (on the one hand, a yeast strain deleted for all hexose transporter genes failed to be complemented upon expression of the *mstE* cDNA,[5] and on the other hand, 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 utilization 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 *creA*^{d} mutants. Recent studies of several CreA repressible systems (35, 54) have shown that catabolic hexose phosphorylation is a key event in the signaling of CreA-mediated repression by both glucose and fructose. Because 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 utilization of repressing carbon sources, the signaling of which results in both the expression of *mstE* and carbon catabolite repression involving CreA. Such a mechanism would also explain *mstE* induction and carbon catabolite repression 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 that is itself subject to CreA-mediated carbon catabolite repression, 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 grisae*, *N. crassa*, *Stagonospora nodorum*, and *Trichoderma reesei*) detect single hits of about 65% identity that are limited to the DNA binding region of Rgt1.[5] 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 localization 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 with the longitudinal orientation of hyphae. Given that the expression of the *mstE-sgfp* gene fusion is driven by the native *mstE* promoter, the visualization 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 (www.membranetransport.org). The importance of transporters belonging to the MFS group is indicated by their representing ~40% of the total number of transporter proteins. Automatic annotation of the *A. nidulans* genome data 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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Membrane Transport, Structure, Function, and Biogenesis:

Identification of the *mstE* Gene Encoding a Glucose-inducible, Low Affinity Glucose Transporter in *Aspergillus nidulans*

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