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Cloning and characterization of a sialidase from the filamentous fungus, *Aspergillus fumigatus*

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Abstract A gene encoding a putative sialidase was identified in the genome of the opportunistic fungal pathogen, *Aspergillus fumigatus*. Computational analysis showed that this protein has Asp box and FRIP domains, it was predicted to have an extracellular localization, and a mass of 42 kDa, all of which are characteristics of sialidases. Structural modeling predicted a canonical 6-bladed β-propeller structure with the model's highly conserved catalytic residues aligning well with those of an experimentally determined sialidase structure. The gene encoding the putative Af sialidase was cloned and expressed in *Escherichia coli*. Enzymatic characterization found that the enzyme was able to cleave the synthetic sialic acid substrate, 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (MUN), and had a pH optimum of 3.5. Further kinetic characterization using 4-methylumbelliferyl α-D-*N*-acetylneuraminylgalactopyranoside revealed that Af sialidase preferred α2-3-linked sialic acids over the α2-6 isomers. No *trans*-sialidase activity was detected. qPCR studies showed that exposure to MEM plus human serum induced expression. Purified Af sialidase released sialic acid from diverse substrates such as mucin, fetuin, epithelial cell glycans and colominic acid, though *A. fumigatus* was unable to use either sialic acid or colominic acid as a sole source of carbon. Phylogenetic analysis revealed that the fungal sialidases were more closely related to those of bacteria than to sialidases from other eukaryotes.

Keywords Sialidase · Sialic acid · Phylogeny · Fungi *N*-acetylneuraminic acid · Expression

Abbreviations

bodipy-Lac	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-lactose
PCR	polymerase chain reaction
RT	reverse transcriptase
MEM	minimal essential media
IPTG	isopropyl β-D-thiogalactopyranoside
α2-3SMUG	α2-3 isomer of 4-methylumbelliferyl α-D-*N*-acetylneuraminylgalactopyranoside
α2-6SMUG	α2-6 isomer of 4-methylumbelliferyl α-D-*N*-acetylneuraminylgalactopyranoside
MU	4-methylumbelliferone
MUN	4-methylumbelliferyl α-D-*N*-acetylneuraminic acid
Ni-NTA	nickel nitrilotriacetic acid agarose
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
LB	Luria Bertani media.

Introduction

Aspergillus species are saprophytic fungi that are found in soil, water and decaying organic matter. There are greater than 200 species of *Aspergillus*; however, only *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus*

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nidulans and *Aspergillus terreus* cause invasive disease (invasive aspergillosis) in immuncompromised humans [30]. Although it is not the most prevalent *Aspergillus* species, *A. fumigatus* accounts for 53-68% of all *Aspergillus* infections [51] suggesting that it possesses specific virulence factors that promote host colonization. Immunocompromised patients such as those undergoing bone marrow and solid organ transplant, patients with hematological malignancies, HIV/AIDS patients and patients with chronic granulomatous disease, are most at risk of developing invasive aspergillosis [34, 39, 63]. Antifungal drugs are used to treat invasive aspergillosis; however, the current success rate averages only 53% [22] with mortality rates ranging between 20% and 84% for hematopoetic stem cell and solid organ transplant recipients, respectively [38]. Invasive aspergillosis is now a principal cause of death in immunocompromised patients in medical centers worldwide [32]. Due to the high incidence and severity of infection and lack of adequate antifungal therapy, understanding the mechanisms underlying pathogenesis by *A. fumigatus* is crucial.

Infection by *A. fumigatus* occurs primarily via inhalation of airborne conidiospores (conidia). As the first step in fungal colonization is thought to be the adhesion of conidia to lung tissue, several studies have attempted to identify adhesins present on the spore surface. Work from our laboratory has shown that *A. fumigatus* has surface sialic acids, which may mediate adhesion to basal lamina proteins [61]. Furthermore, pathogenic species of *Aspergillus* were shown to have a higher density of sialic acids compared to non-pathogenic species [62]. More recently, we have demonstrated that sialoglycoconjugates on the conidial surface mediate the uptake of spores into human Type II pneumocytes and cultured murine macrophages [60]. Although no sialic acid biosynthetic genes have been identified to date in the genome sequence of *Aspergillus*, sialic acid biosynthesis has been reported to occur *de novo* [62]. An alternative pathway for sialic acid acquisition in microbes is the uptake of pre-formed sialic acid via plasma membrane permeases [58]. Sialic acids are released from available glycans by the action of sialidase enzymes (*N*-acetylneuraminic acid hydrolase, EC 3.2.1.18); a family of exo-glycosidases that catalyze the hydrolytic cleavage of sialic acid residues in glyconjugates. Sialidases are widespread in nature and to date, they have been identified in viruses, bacteria and animals [1]. Sialidases play important roles in diverse biological processes such as lysosomal catabolism [21], muscle cell differentiation [49], cancer invasion and metastasis [35], nutrition and microbial pathogenesis [12]. Viral sialidases (neuraminidases) are important in the release of newly formed virus particles from the host cell thereby playing an important role in the life cycle of the virus [54]. In the bacterial pathogen *Pseudomonas aeruginosa*, sialidase was found to play a significant role in biofilm production leading pneumonia and bacteremia in a mouse model of the disease [53]. In another bacterial pathogen, *Vibrio cholerae*, sialidase is thought to convert complex gangliosides to GM_1, the receptor for cholera toxin, thereby increasing binding of the cholera toxin to host cells expressing the receptor and contributing to the pathogenicity of this organism [19]. Finally, *Streptococcus pneumoniae* uses two sialidases along with other exoglycosidases to obtain carbohydrates from host glycoproteins that are utilized as carbon sources required for growth [10].

We have recently discovered a gene encoding a putative sialidase in the genome sequence of *Aspergillus fumigatus* Af293. Here, we report the first successful cloning and expression of a fungal sialidase. Because of the importance of sialidases in microbial pathogenesis, the specific aims of the current research were: to clone and characterize the *A. fumigatus* sialidase and assess its substrate specificity and kinetic parameters, to monitor gene expression in various growth media, to determine the substrate specificity of the purified enzyme, to determine whether sialic acids are used for nutrition in *A. fumigatus*, and to determine the phylogenetic relationship of this enzyme with other bacterial and animal sialidases.

Experimental

Computational analyses

A 1221 base pair gene encoding a putative sialidase was identified in the *A. fumigatus* Af293 genome database available at The Institute of Genomic Research (TIGR) (http://www.tigr.org) (now the J. Craig Venter Institute) using a keyword search. The intron/exon structure of the gene was determined using the program GlimmerM (http://www.tigr.org/tdb/glimmerm/glmr_form.html) optimized for *A. fumigatus*. The derived protein sequence was then analyzed using the programs iPSORT (http://hc.ims.u-tokyo.ac.jp/iPSORT/) [6], SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) [7] and NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) to determine its localization, presence and length of signal peptide, and to identify potential N-glycosylation sites. Sequence alignments were performed using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Finally, the derived protein sequence was submitted to the PHYRE protein homology/analogy recognition engine (Imperial College of London, http://www.sbg.bio.ic.ac.uk/phyre/) [8], to generate a tertiary structural model using the sialidase from *Salmonella typhimurium* (Protein Data Bank (PDB) file: 3sil) as a template. Model quality was assessed using DeepView Swiss-PDB Viewer (http://ca.expasy.org/spdbv/)

[20] and images prepared using the molecular visualization system, PyMol (DeLano Scientific LLC, Palo Alto, CA).

A 1,020 bp upstream fragment of the putative sialidase was analyzed for regulatory sequences in the untranslated region both manually and with the program Match—1.0 Public, which uses a library of mononucleotide weight matrices from TRANSFAC 6.0 (http://www.gene-regulation.com) [33].

Phylogenetic analysis

The phylogeny of selected sialidases was inferred using both distance and maximum likelihood techniques in PHYLIP (Phylogeny Inference Package) version 3.67 (distributed by the author, Department of Genome Sciences, University of Washington, Seattle) [14]. Data was gathered by submitting the predicted amino acid sequence of the putative *A. fumigatus* sialidase to Blastp [3]. Twenty-one bacterial, fungal or vertebrate sialidases with E values less than $4e^{-07}$ were selected for further analysis. The sequences used were as follows: XP_001266669.1, conserved hypothetical protein [*Neosartorya fischeri* NRRL 181]; XP_001214142.1, predicted protein [*Aspergillus terreus* NIH2624]; XP_001226947.1, hypothetical protein CHGG_09020 [*Chaetomium globosum* CBS 148.51]; YP_001107328.1, neuraminidase [*Saccharopolyspora erythraea* NRRL 2338]; NP_827111.1, neuramidase [*Streptomyces avermitilis* MA-4680]; NP_630638.1, neuramidase (secreted protein) [*Streptomyces coelicolor* A3 (2)]; ZP_02071388.1, hypothetical protein BACUNI_02826 [*Bacteroides uniformis* ATCC 8492]; YP_213298.1, putative sialidase [*Bacteroides fragilis* NCTC 9343]; Q02834.1, NANH_MICVI Sialidase precursor (Neuraminidase)(*Micromonospora viridifaciens*); AAI52169.1, Zgc:100806 [*Danio rerio*]; NP_001103203.1, hypothetical protein LOC100000684 [*Danio rerio* 2]; ABO30984.1, Neu3.1 [*Danio rerio* 3]; YP_001304276.1, glycoside hydrolase family 33, candidate sialidase [*Parabacteroides distasonis* ATCC 8503]; YP_001138502.1, hypothetical protein cgR_1608 [*Corynebacterium glutamicum* R]; NP_776547.1, sialidase 3 (membrane sialidase) [*Bos taurus*]; XP_001366978.1, PREDICTED: similar to sialidase 3 [*Monodelphis domestica*](Opposum); YP_001301367.1, glycoside hydrolase family 33, candidate sialidase [*Bacteroides vulgatus* ATCC 8482]; Q9UQ49.1, NEUR3_HUMAN Sialidase-3 (Membrane sialidase) (Ganglioside sialidase) (*N*-acetyl-α-neuraminidase 3); NP_057929.1, neuraminidase 3 [*Mus musculus*](Mouse1); BAE26342.1, unnamed protein product [*Mus musculus*](Mouse2); and NP_446462.1, neuraminidase 3 [*Rattus norvegicus*]. Sequences were aligned and formatted for PHYLIP analysis using Clustal X2.0. Maximum-Likelihood and Neighbour-Joining algorithms were employed using the Jones-Taylor-Thornton matrix and 100 replicates were used for bootstrapping. Unrooted bootstrap consensus trees were compiled and the output files visualized using the program, Dendroscope v1.2.4 [23]. Statistical tests of user trees were done in PHYLIP using the Kishino-Hasegawa test as implemented in proml [26].

The *A. fumigatus* sialidase sequence was also used to search all the predicted protein sequences for the fungi that have been sequenced or are being sequenced at the Broad Institute (http://www.broad.mit.edu/cgi-bin/annotation/fgi/blast_page.cgi) and by the Concordia University Fungal Genomics Project (https://fungalgenomics.concordia.ca/). Codon usage was analyzed by the Graphical Codon Usage Analyzer v2, Oct 2006 [17].

Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) was used to assess expression of the sialidase in *A. fumigatus*. To accomplish this, DNase-free total RNA was prepared from *A. fumigatus* mycelia cultured at 37°C for 6 h in minimal essential medium (MEM) (Life Technologies) supplemented with 10% (v/v) human serum (male) (Sigma) and 50 μM $FeCl_3$ using the Qiagen RNeasy kit (Qiagen, Mississauga, ON) and used as the template for first strand cDNA synthesis. First strand cDNA was synthesized by adding 4.5 μg RNA, 2 pmol oligo-dT primer, 10 mM dNTPs (Fermentas, Burlington, ON), 0.1 M DTT, 40 units RNAseOUT ribonuclease inhibitor, 4 μl 5X first strand buffer, 200 units Superscript II RNA polymerase (Invitrogen, Burlington, ON) and ribonuclease-free H_2O (Ambion, Austin, TX) for a total volume of 20 μl. The resulting solution was incubated for 60 min at 42°C. The cDNA product (2 μl) was then used as a template for PCR amplification by adding it to a solution consisting of 15 mM dNTPs, 10 pmol forward primer (5′-ATCAA CGACCCGGCC (Afsialcdna-F)) and reverse primer (5′-CTAATTGTTAGGACCATTCAGGATC (Afsialcdna-R)), 50 mM $MgSO_4$, 5 μl 10X Platinum Pfx PCR buffer, 1.25 units Platinum Pfx DNA polymerase (Invitrogen) and H_2O for a total volume of 50 μl.

Cloning and expression of the *Aspergillus fumigatus* sialidase in *Escherichia coli*

Standard molecular biology methods were performed as described by [48]. Plasmids were prepared using the Plasmid Mini kit according to the manufacturer's directions (Qiagen). Genomic DNA was obtained from *A. fumigatus* strain ATCC 13073 mycelia grown overnight in MYPD liquid broth (0.3%, w/v, yeast extract and malt extract; 0.5%, w/v, peptone; and 1%, w/v, glucose) using the phenol/chloroform method and was used as a template for

polymerase chain reaction amplification of the putative sialidase gene. PCR amplification of the putative sialidase gene was accomplished using the following PCR reagents: 15 pmol forward and reverse primers, 10 mM dNTPs, 2.5 units Taq DNA polymerase (Fermentas), 2 μg *A. fumigatus* genomic DNA, 4% DMSO, 5 μl 10X Taq buffer and H_2O for a total volume of 50 μl. Forward (5′-GGCATTACGGCTAGCATCAACGACCCGGCC (Afsial-F)) and reverse (5′-TACGCACCAGCGGCCGCCTAATTGTTAGGACCATTCAGGATC (Afsial-R)) primers (Invitrogen) were designed to amplify a fragment beginning 60 base pairs downstream of the transcription start site (to prevent amplification of the predicted signal peptide) and ending at the stop codon and included *NheI* and *NotI* restriction enzyme recognition sites (underlined) to facilitate cloning into the protein expression vector pET28A + (EMD Chemicals Inc., San Diego, CA). Following PCR amplification, the entire reaction volume was run on an agarose gel and the correct PCR product was excised and purified using the Qiaquick gel extraction kit (Qiagen).

The PCR-amplified sialidase gene was treated with restriction enzymes (*NheI*/*NotI* (Fermentas)) and ligated (with the addition of 5% DMSO to the ligation reaction) into the *NheI*/*NotI* site of pET28A+, in-frame with an N-terminal 6XHis tag creating the plasmid, pAfsialidase. The recombinant plasmid was used to transform *Escherichia coli* DH5α (Life Technologies, Gaithersburg, MD) on Luria-Bertani (LB) media supplemented with kanamycin (30 μl ml^{-1}) (BioShop Canada Inc., Burlington, ON) and kanamycin-resistant transformants screened for the presence of pAfsialidase by colony PCR using primers targeting the T7 promoter and terminator (Invitrogen), which flank the multiple cloning site of pET28A+. Correct gene amplification and insertion was verified in several clones by restriction analysis and DNA sequencing (Macrogen, Korea).

pAfsialidase containing a complete and correct in-frame sequence of the putative sialidase was isolated from the appropriate clones and used to transform the protein expression host *E. coli* Tuner (DE3) (EMD Chemicals Inc.). Protein expression in transformed host cells was accomplished by culturing *E.coli* Tuner (DE3) in 250 ml LB-kanamycin broth at 37°C with shaking at 200 rpm to an OD_{600} of 0.8. This was followed by inducing protein expression with isopropyl β-D-thiogalactopyranoside (IPTG) (BioShop Canada Inc.) at concentrations of 0.2 mM, 0.4 mM, or 1 mM at room temperature, 30°C or 37°C with shaking at 200 rpm for 6 or 24 h. Protein concentration was determined using the method of [9].

Protein purification

After protein induction, bacterial cells containing the expressed sialidase were cooled on ice and pelleted by centrifugation at 3,000*g* for 20 min at 4°C. The pelleted cells were then resuspended in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM imidazole, pH 8.0) at 2 ml/gram bacterial pellet (wet weight). Lysozyme (1 mg ml^{-1}) (BioShop Canada Inc.) was added to the bacterial suspension, which was then incubated on ice for 30 min. Following lysozyme treatment, the suspension was sonicated with four 10-second bursts at 200 W with 1 min cooling periods between bursts. The lysate was then centrifuged at 18,000 *g* for 30 min at 4°C to clear cellular debris. The cleared lysate containing the expressed 6XHis-tagged sialidase was added to 1 ml nickel nitrilotriacetic acid agarose (Ni-NTA) (Qiagen) equilibrated with lysis buffer and allowed to bind while shaking on a rotary shaker for 60 min at 4°C. Following binding, bound sialidase was eluted from Ni-NTA beads by batch purification; the bound beads were washed with 5 mL of wash buffer #1 (50 mM Tris-HCl, 100 mM NaCl, 20 mM imidazole, pH 8.0) twice and 5 mL of wash buffer #2 (50 mM Tris-HCl, 100 mM NaCl, 50 mM imidazole, pH 8.0) once, with each wash followed by centrifugation at 800 g for 3 min at 4°C. Sialidase is eluted in 5 ml elution buffer (50 mM Tris-HCl, 100 mM NaCl, 250 mM imidazole, pH 8.0), followed by centrifugation at 800 g for 3 min at 4°C, and the supernatant was collected. Residual Ni-NTA beads were removed by filtering at 4°C. Imidazole and non-specific proteins were removed by gel filtration chromatography (ÄTKAFPLC, GE Healthcare) with the clean-up buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0) at 4°C. Fractions collected were analyzed by 12% SDS-PAGE gel. Selected fractions containing only sialidase were concentrated by loading the fraction into a 30,000 molecular weight cut-off centrifugal filter (Millipore, Billerica, MA) and centrifuging at 3,000 *g* for 30 min at 4°C. The concentrated protein solution was frozen using liquid nitrogen and stored at -80°C.

Sialidase activity assays

The activity of enzyme preparations was determined by measuring cleavage of the synthetic sialic acid substrate, 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (MUN) (Sigma, Oakville, ON), as described previously [42]. Briefly, reactions were set up in 96-well plates by adding 200 μM MUN, crude cell lysate or purified sialidase and 40 mM sodium formate, pH 3.5 for a total volume of 100 μl. Plates were incubated at 37°C for 15 min followed by the addition of 200 μl cold stop solution (0.1 M glycine, 0.014 M NaCl, 25% ethanol, pH 10.7). Following the addition of stop solution, the amount of 4-methylumbelliferone released from MUN was determined using a Cary Eclipse fluorescence spectrophotometer at excitation and emission wavelengths of 365 and 450 nm, respectively. Fluorescence produced during the course of the reaction was related to the

concentration of MUN cleaved using a standard curve of 4-methylumbelliferone (Sigma).

To determine the *A. fumigatus* sialidase activity on biological substrates, the sialidase was incubated with either heat-inactivated A549 cells (a human Type II pneumocyte cell line) (ATCC), bovine mucin (Sigma), colominic acid (Sigma), fetuin (Sigma), and asialofetuin (Sigma) for 3 h in 40 mM sodium formate, pH 3.5, or in 16 mM sodium tartrate, pH 5.2. To quantify the amount of sialic acid released, the method described by Kolisis [27] and Simpson *et al.* [52] were used with some modifications and adapted for 96 well plates. The amount sialic acid released from each sialidase reaction was tested in triplicate and each test consisted of 90 μL of sample (0.5 mg/ml for each substrate except A549 cells, which were used at a concentration of 6.4 x 10^5 cells/reaction), 1 M Tris-HCl, 0.4 mM NADH, 0.1 units sialic acid aldolase, and 0.2 units lactate dehydrogenase (LDH). Prior to the addition sialic acid aldolase and LDH, the initial A_{340} was recorded spectrophotometrically. The tests were incubated for 1 h at 37°C and the final A_{340} was recorded. Quantification of the total sialic acid released was calculated as described by Kolisis [27].

pH rate profile

Optimal pH of the purified enzyme was determined by measuring the specific activity of the sialidase towards MUN in buffers of varying pH. Buffers used were 40 mM sodium formate (pH 3–3.5), 50 mM sodium acetate (pH 4–5.5) and 32.5 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6) (Sigma). The ionic strength of all buffers was maintained at 100 mM with NaCl and all buffers were prepared for use at 37°C.

Quantitative RT-PCR

Total RNA was obtained from *A. fumigatus* grown in MYPD for 24 h, two chemically defined media (Kafer's and Neilands') for 24 h, and MEM + 10% human serum (Sigma) for 2 h, 4 h, 6 h, and 24 h at 37°C. RNA samples were isolated using the RNeasy Plant RNA minikit (QIAGEN) and quantified via the NanoDrop ND-1000 Spectrophotometer. To remove any possible genomic DNA contamination, RNA used for cDNA first-strand synthesis was treated with DNase1 (Fermentas). Total first-strand cDNA was made using qScript cDNA Supermix (Quanta Bioscience) with random primers. β-tubulin forward primer (5'-TTC CGC AAT GGA CGT TAC CT) and reverse primer (5'-ACA GAG CGG TCT GGA TCT TCT), and sialidase forward primer (5'-CGG ACG CGA AGA AGT TCA AC) and reverse primer (5'-TGC CAG TGC CAT CAT TGA AG) (Invitrogen) were designed to amplify 148 bp and 153 bp fragments, respectively. The expected qPCR products were blasted against the *A. fumigatus* genome and other fungal genomes using WU-Blast2 (www.yeastgenome.org) to ensure sequence specificity. Quantitative real-time PCR (qPCR) was completed by using PerfeCTa SYBR Green SuperMix (Quanta Bioscience). To validate the use of the $2^{-\Delta\Delta CT}$ method [31], an efficiency test was completed by performing qPCR on a dilution series of cDNA from *A. fumigatus* grown in MYPD at 37°C for 24 h. The ΔC_T ($C_T$sialidase—C_Tβ-tubulin) was calculated for each cDNA dilution. A plot of the log cDNA dilution *versus* ΔCT was made and the absolute value of the slope was less than 1 (data not shown). This indicated that the efficiencies of the target and reference genes were similar and ΔΔCT calculation for the relative quantification of the sialidase gene could be used.

Kinetic analyses

Michaelis-Menten parameters were measured using MUN and an α2-3 isomer of 4-methylumbelliferyl α-D-*N*-acetylneuraminylgalactopyranoside (α2-3SMUG) [25]. Each 400 μl reaction was performed at 37°C by equilibrating the buffer, substrate (and for the α2-3SMUG kinetic determination, *Aspergillus oryzae* galactosidase (Sigma)) for 5 min in a heat block prior to addition of 9.6 μg purified sialidase. The progress of the reaction was continuously monitored for 10–15 min using a Cary Eclipse fluorescence spectrophotometer equipped with a Peltier temperature controller set to 37°C and excitation and emission wavelengths of 365 and 450 nm, respectively. For MUN, kinetic parameters were determined from 21 initial rate measurements using a substrate concentration range of 500 μM to 4000 μM. For α2-3SMUG, kinetic parameters were determined from 5 initial rate measurements using a substrate concentration range of 250 μM to 4000 μM. The rate *versus* substrate concentration data were fitted to the Michaelis-Menten equation using GraphPad (GraphPad Software Inc., San Diego, CA).

Trans-sialidase activity assay

The assay used to detect possible *trans*-sialidase activity was previously described in [2] and its supplementary materials. Briefly, *A. fumigatus* sialidase (45 μg) or *Trypanosoma cruzi trans*-sialidase (0.6 μg) were incubated with either MUN (Rose Scientific) alone, or MUN and bodipy-lactose in 100 μl of buffered solution for 30 s. The products were analyzed by thin layer chromatography using an ethyl-acetate/methanol/H_2O (7:2:1 v/v) solvent system and visualized under UV light at 350 nm. The image of the TLC was captured with a PowerShot A720IS digital camera (Canon).

Preferred carbon source utilization of *A. fumigatus*

To determine the preferred carbon source use by *A. fumigatus*, 10^6 *A. fumigatus* spores were inoculated per well in 96-well plates containing chemically defined media with either glucose, colominic acid, or sialic acid as a carbon source, or no carbon source. All carbon sources were tested in triplicate. Media were adjusted to ~pH 6 using K_2HPO_4 prior to inoculation. A parafilm overlay was added to each well to prevent cross contamination of spores from the different wells of the plate. Plates were placed in a moist chamber and incubated at 37°C. Growth of the fungus was monitored spectrophotometrically at A_{620nm}.

Sialic acid detection by lectin binding

A. fumigatus conidia were inoculated into minimal media and grown for two weeks at 37°C. During the two-week period, the cultures were replaced with fresh media every three days by first centrifuging the cultures, removing the supernatant and resuspending the mycelia in fresh minimal media. Spores were also inoculated into MYPD broth and incubated for 24 h at 37°C. At the end of the incubation period, mycelia were harvested by filtration, washed and incubated with the sialic acid-specific lectin, *Limax flavus* agglutinin (LFA) conjugated to Texas Red (EY Laboratories) as described previously by [62]. Controls were incubated in the same way except that the LFA was omitted from the incubation. Fungi were viewed under 400X magnification with a fluorescence microscope (Zeiss) equipped with an LED lightsource (Colibri) with an excitation wavelength of 590 nm. Images were captured with the Zeiss Axiocam MRm.

Results

Computational analyses

Analysis of the intron/exon structure of the putative sialidase gene using GlimmerM revealed no potential introns in this sialidase. Examination of the amino acid sequence revealed that the sialidase has the canonical FRIP sequence at the N-terminal region, one Asp box (SXDXGXXT/W) and a predicted mass of 42 kDa. Further analysis of the protein sequence with iPSORT, SignalP and NetNGlyc revealed that this putative sialidase has an extracellular localization, a 20 amino acid signal peptide and one low scoring N-glycosylation site at Asn 215. The protein sequence (without the 20 amino acid signal peptide) was then submitted to the PHYRE protein homology/analogy recognition engine to generate a three dimensional model of this sialidase using the sialidase from *S. typhimurium* as a template. The model generated displayed the canonical 6-bladed β-propeller structure consistent with sialidases. Moreover, structural alignment using the sialidases from *S. typhimurium* and *M. viridifaciens* as templates resulted in good overall structural homology with root mean square deviations of only 0.58 Å and 1.42 Å, respectively. An analysis of the Ramachandran plot of the model revealed that only 8 amino acids fell out of the allowed range of phi and psi angles (data not shown). Finally, structural and sequence alignments of the model with other bacterial and animal sialidases showed that catalytic amino acids Asp 92, Glu 260 and Tyr 370 (*M. viridifaciens* numbering) as well as the highly conserved arginine triad of the model align well with those of other sialidases (Fig. 1).

Regulatory sequences were identified in the promoter sequence of the *A. fumigatus* sialidase (Fig. 2). The eukaryotic consensus sequence GGNCAATCT (GGCCAATCT) is located 669 bp upstream of a TATAAA box. Analysis by the Match1.0 program also revealed a STRE motif (AGGGGA) 62 bp upstream of the CAAT box. STRE has been shown to bind C2H2 transcription factors that regulate gene expression in response to stress [50]. The STRE-binding transcription factor Seb1 is a C2H2 transcription factor in filamentous fungi that regulates response to osmotic stress [41]. A Seb1-like transcription factor has been identified in the genome sequence of *A. fumigatus* suggesting that the sialidase may be upregulated under stress.

Finally, a single GATA element was found in the upstream region of the sialidase. Trans-acting zinc-finger DNA binding proteins (the GATA proteins) regulate gene expression by binding to these elements in responsive genes; however, binding is significantly weaker when only one GATA element is present [15] suggesting that this element may have only a minor role in controlling gene expression.

Gene expression, sialidase cloning and protein expression

RT-PCR was used to determine whether the putative sialidase gene in *A. fumigatus* was expressed. Using a total RNA extract as a template, RT-PCR amplified an 1,161 bp cDNA product consistent with the expected size of the *A. fumigatus* cDNA product thus providing evidence that this sialidase is expressed in *A. fumigatus* (Fig. 3a). To amplify the *A. fumigatus* sialidase gene for cloning into a protein expression vector, primers were designed to amplify the gene beginning 60 base pairs downstream of the gene start site to avoid amplification of the signal peptide, which, along with the N-terminal 6XHis tag, may have been cleaved after extracellular secretion by the protein expression host. The amplified fragment was cloned into pET28A+, which was used to transform *E. coli* DH5α (Fig. 3b). Restriction digest and sequencing were used to verify correct amplification and

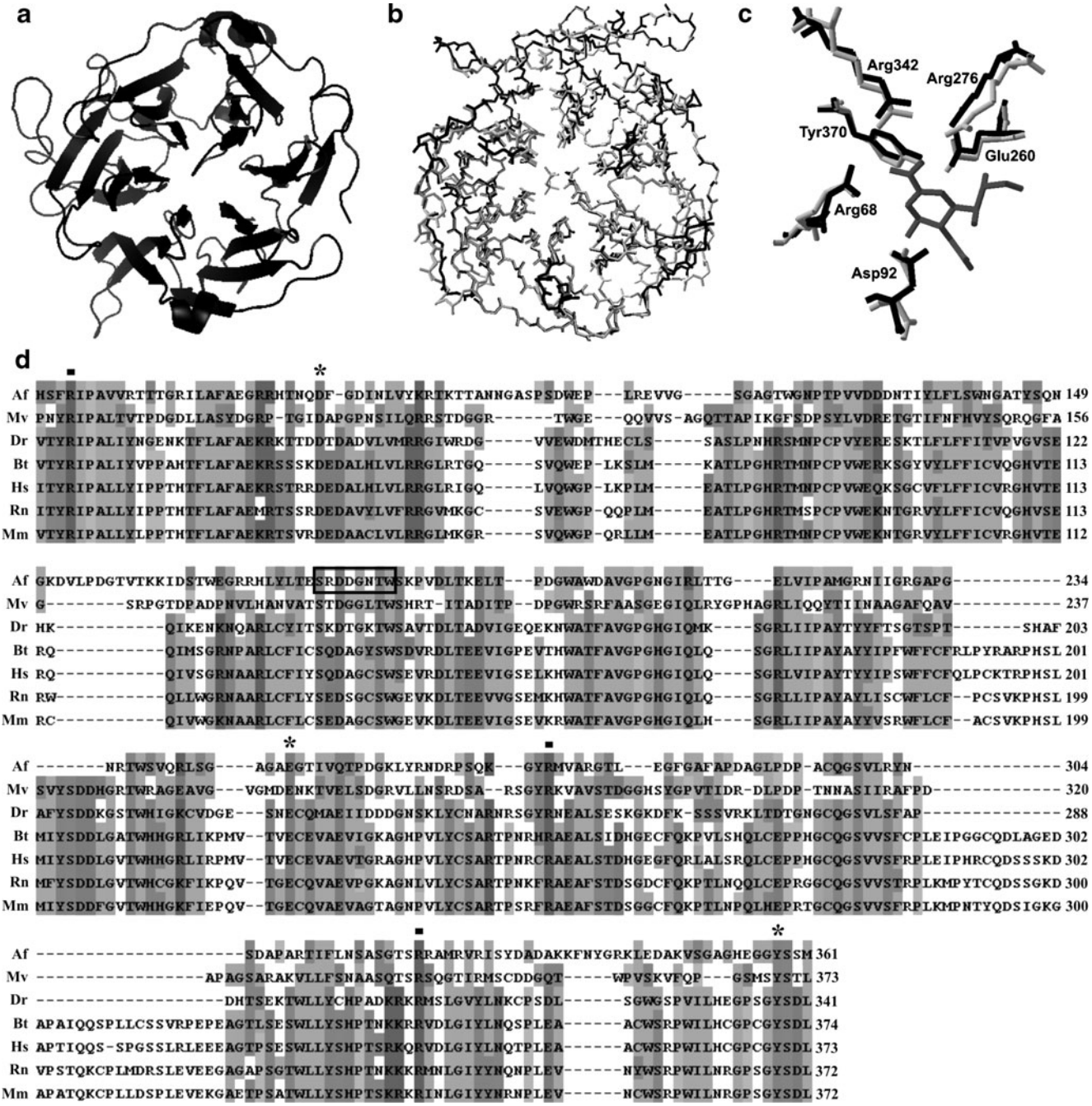

Fig. 1 Structural modeling and sequence alignment of the *A. fumigatus* sialidase. **a** The putative sialidase was predicted by PHYRE to fold into a 6-bladed β-propeller. **b** Structural superposition of the putative *A. fumigatus* sialidase (black) on the sialidase from *M. viridifaciens* (grey) (PDB: 1eus). **c** Superposition of the *A. fumigatus* model active site onto that of *M. viridifaciens.* It can be seen that catalytic residues (Asp 92, Glu 260 and Tyr 370) as well as the arginine triad (Arg 68, Arg 276 and Arg 342) (*M. viridifaciens* numbering) align well with those of the *A. fumigatus* sialidase model. **d**) Sequence alignment of the *A. fumigatus* sialidase with sialidases from *M. viridifaciens* (Mv), *Danio rerio* (Dr), *Bos taurus* (Bt), *Homo sapiens* (Hs), *Rattus norvegicus* (Rn) and *Mus musculus* (Mm). Catalytic residues (Asp 92, Glu 260 and Tyr 370), marked with *, and the arginine triad (Arg 68, Arg 276 and Arg 342), marked with ■, are well conserved among these sialidases. The conserved Asp box (**SXDXGXT**) is outlined

insertion of the gene into pET28A + (data not shown). Sequencing revealed a single synonymous base difference T_{996} to C_{996} (maintaining an aspartic acid at this position) between the cloned gene and the gene sequence available for strain 293 in the *A. fumigatus* genome database. The recombinant plasmid was then used to transform the protein expression host, *E. coli* Tuner. Successful transformation was detected by colony PCR (data not shown) and by monitoring

Fig. 2 Promoter sequence of the putative *A. fumigatus* sialidase. A 978 bp upstream sequence revealed the presence of eukaryotic promoter elements (caat and tata boxes) as well as the STRE motif (agggga) found in many fungi. A single gata sequence was identified in the upstream sequence that may act as a weak binding site for gata transcription factors

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tctggcgaggaatcttgtgcttcatgtcatgcctagctcaagaagtcccccagaactgcc
ctttccctctacttctcgtctgagtctctacgtacactcaagagtttgagtcctgatttg
ctggtacaactggaaatcaatgctacgggaacacttgggatgattggcactcgtgcctag
acagagattagccaagggga acctgaccacgcaatatgtcgtgacgatgtccctggttgc
tcgactcgcattttgtggccaatcttggaagacttatgtgcttggcaagggattctcgga
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gcttcaagtcggggctgtatagcagacgacagttgcagtgttcgtgaccttcgtgacctt
cgtgactgattcaatgtatatccttggcagactcgcagcacccgtccggctccacctgta
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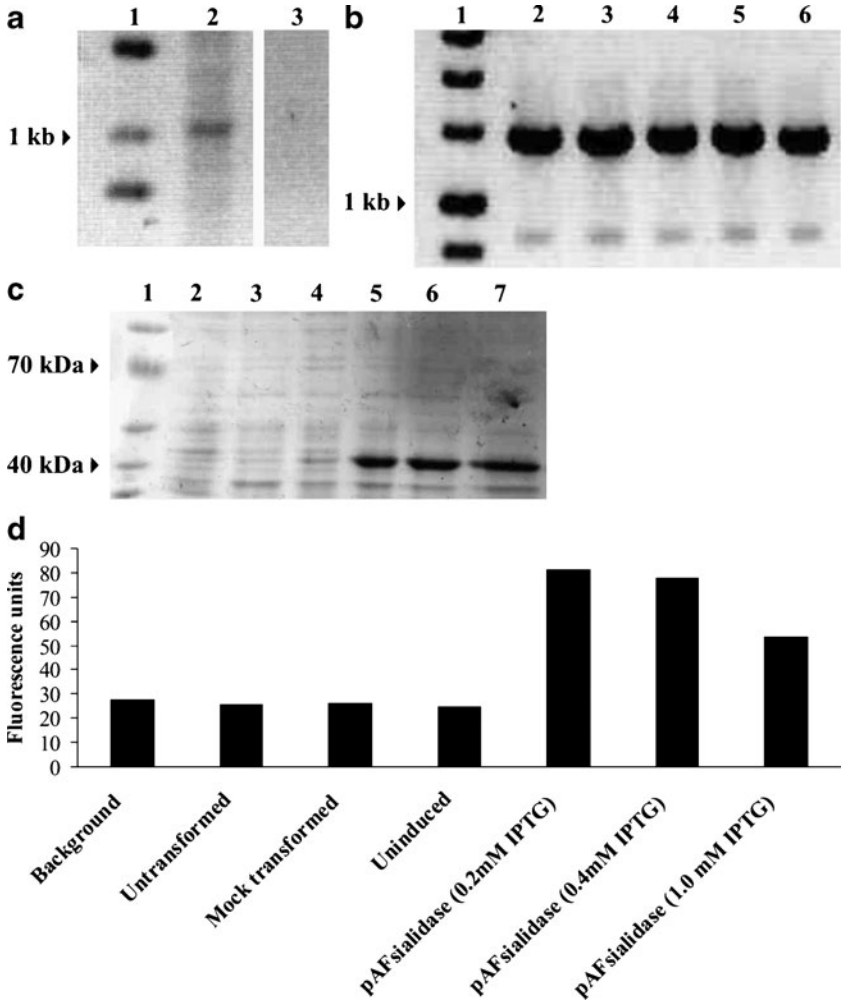

Fig. 3 pAfsialidase transformed *E. coli* Tuner expresses an active sialidase. **a** RT-PCR using total RNA from *A. fumigatus* as a template yields an 1,161 bp cDNA band consistent with the sialidase gene product indicating the sialidase is expressed in *A. fumigatus* under experimental conditions. Lane 1 represents the DNA ladder, lane 2 is the *A. fumigatus* sialidase RT-PCR product and lane 3 is the negative RT-PCR control. **b** Colony PCR of transformed *E. coli* yields a 1,475 bp band (1,161 bp (sialidase gene) + 314 bp (flanking region)) showing that the *A. fumigatus* sialidase gene is successfully cloned into the pET28A + protein expression vector. Lane 1 represents the DNA ladder and lanes 2–6 are the colony PCR products of the 5 selected kanamycin-resistant clones. **c** Transformation of the *E. coli* Tuner protein expression strain with pAfsialidase and induction by IPTG (0.2 mM–1.0 mM) produced a 42 kDa band consistent with the predicted mass of the *A. fumigatus* sialidase. Lane 1 represents the protein ladder, lane 2 is the lysate from untransformed *E. coli* Tuner, lane 3 is the lysate from mock (pET28A+) transformed cells, lane 4 is the lysate from transformed but uninduced cells and lanes 5–7 represent cell lysates from Tuner transformed with pAfsialidase and induced with varying concentrations of IPTG. **d** Crude bacterial cell lysates transformed with pAfsialidase and induced with IPTG have activity toward the synthetic sialic acid substrate, MUN. Importantly, untransformed, mock transformed and uninduced *E. coli* Tuner controls do not express a 42 kDa band or have activity toward MUN

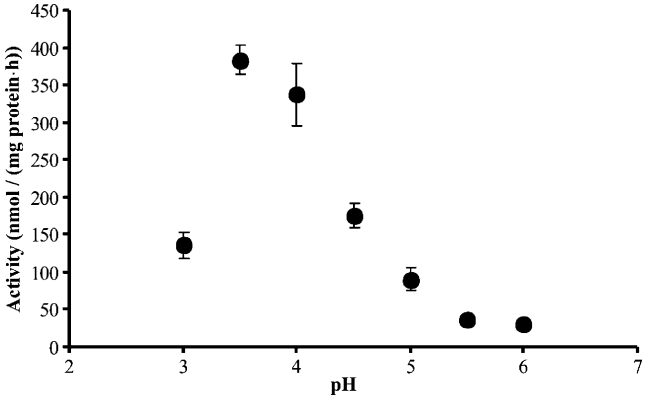

Fig. 4 The purified *A. fumigatus* sialidase has a pH optimum of 3.5. To measure the optimum pH of the sialidase, activity toward MUN was determined at 37°C in buffers ranging in pH from 3–6. Buffers used were 40 mM sodium formate (pH 3–3.5), 50 mM sodium acetate (pH 4–5.5) and 32.5 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6). Ionic strength of the buffers was maintained at 100 mM with NaCl

the expression of the putative sialidase by SDS-PAGE and by assessing the activity of the crude bacterial cell lysate toward the sialidase substrate, MUN. Transformed *E. coli* Tuner induced with IPTG produced a protein with a molecular mass of 42 kDa, consistent with the predicted mass of this sialidase, and the crude lysate had activity toward MUN. The protein expression and enzyme activity were solely attributable to the cloned *A. fumigatus* gene because untransformed, mock (pET28A+) transformed and transformed but uninduced *E. coli* Tuner crude lysates did not produce 42 kDa bands and were not able to cleave MUN (Fig. 3c and d). Several expression conditions were assessed to maximize protein expression and enzymatic activity and it was found that inducing protein expression with 1 mM IPTG for 24 h at 30°C was optimal (data not shown). Furthermore, the addition of bovine serum albumin, $CaCl_2$, $MgCl_2$ or $MnCl_2$ did not increase enzyme activity in the MUN activity assay (data not shown). Finally, the optimal pH of this enzyme was determined using affinity purified sialidase to cleave MUN in buffers ranging in pH from 3 to 6. The data obtained indicated that the *A. fumigatus* sialidase has a pH optimum of 3.5 (Fig. 4).

Quantitative RT-PCR

Table 1 contains the expression data that are the mean of triplicate runs conducted for each sample of RNA from the different growth conditions. The normalized expression level of sialidase in MYPD and in two minimal media (Kafer and Neiland) was approximately the same. In contrast, when conidia were grown in MEM plus human serum, sialidase gene expression increased 3–5 fold within the first 4 h of incubation, a period which corresponds to conidial swelling and germination. Thereafter, expression levels decreased almost 20-fold.

Enzyme kinetics

Michaelis-Menten kinetic parameters of the *A. fumigatus* sialidase were measured using MUN and an α2-3 isomer of 4-methylumbelliferyl-α-*D*-*N*-acetylneuraminylgalactopyranoside as substrates [25]. The *A. fumigatus* sialidase had a K_m of 3,300±380 μM and a catalytic efficiency (k_{cat}/K_m) of 22.3±3.0 M^{-1} s^{-1} for MUN. The α2-3 linkage was preferentially cleaved over substrates with an α2-6 linkage; the *A. fumigatus* sialidase had a K_m of 3,100±780 μM and catalytic efficiency (k_{cat}/K_m) of 0.144±0.001 M^{-1} s^{-1} toward α2-3SMUG (Table 2).

Table 1 Relative *Aspergillus fumigatus* sialidase gene expression values under different growth conditions

Growth condition[a]	Sialidase avg. C_T	β-tubulin avg. C_T	ΔC_T [b]	$\Delta\Delta C_T$ [c]	Normalized sialidase amount relative to β-tubulin ($2^{-\Delta\Delta CT}$) (range)[d]
MYPD, 24 h	26.18	19.52	6.67	0	1 .00 (0.78–1.27)
Kafer's MM, 24 h	28.36	21.73	6.63	-0.03	1.02 (0.79–1.33)
Neilands' MM, 24 h	26.31	19.41	6.89	0.24	0.86 (0.32–2.29)
MEM + human serum, 2 h	32.03	26.91	5.12	-1.54	2.93 (1.75–4.93)
MEM + human serum, 4 h	28.23	23.83	4.40	-2.26	4.80 (2.76–8.34)
MEM + human serum, 6 h	31.75	22.22	9.52	2.87	0.14 (0.08–0.24)
MEM + human serum, 24 h	32.46	22.68	9.78	3.12	0.12 (0.10–0.13)

[a] Data reported are the mean of triplicate runs conducted for each sample of RNA from the different growth conditions. β-tubulin was used as a reference gene to normalize data

[b] ΔC_T = C_T value of gene of interest (sialidase)—C_T value of reference gene (β-tubulin)

[c] $\Delta\Delta C_T$ = ΔC_T test condition - ΔC_T calibrator condition. The calibrator condition is MYPD for 24 h at 37°C

[d] Fold change value is calculated as $2^{-\Delta\Delta CT}$

Table 2 Kinetic parameters of selected sialidases

Sialidase	Substrate	K_m (μM)	K_{cat} (S^{-1})	K_{cat}/K_m ($M^{-1}s^{-1}$)	Specific activity (nmol mg $protein^{-1}h^{-1}$)	References
A. fumigatus	MUN	3,300±380	0.0743±0.0050	22.3±3.0	383.7±19.1[a]	
	α2-3SMUG	3,100±780	0.0004±0.0001	0.144±0.001	6.7±2.1[d]	current work
	α2-6SMUG	n.d.	n.d.	n.d.	2.2±0.4[d]	
HsNeu2	MUN	440			3,060[a,c]	[37, 56]
Neu3	MUN	143			250.7[a]	[22, 36]
C. perfringens	MUN	165±0.024			1.7×10^{7}[b]	[28]
	MUN	35±6	44.0±0.1	$(1.3\pm0.2)\times10^{6}$		
M. viridifaciens	α2-3SMUG	380±70	0.20±0.02	530±150		[26]
	α2-6SMUG	240±30	0.069±0.003	290±50		

[a] Assays were conducted using 200 μM MUN

[b] Assay was conducted using 1 mM MUN

[c] Enzyme expressed and purified from Cos7 cells

[d] Assays were conducted using 500 μM SMUG substrate

nd denotes not determined

Substrate profile

Af sialidase was able to release sialic acids from a wide variety of natural substrates including bovine salivary mucin, colominic acid (a homopolymer of sialic acid with α2-8 ketosidic linkages), bovine fetuin, a serum glycoprotein containing both α2-6 and α2-3-linkages in a ratio of ~3:2, and glycoproteins and glycolipids from thermally-denatured human lung epithelial cells (A549 pneumocyte cell line, containing both α2-3 and α2-6-linkages) (Fig. 5). In both pH conditions, the asialofetuin control contained no released sialic acids, as expected. The substrate profiles at pH 3.5 are shown in Fig. 5. At pH 5.2, activity was higher with A549 cells and fetuin (data not shown) suggesting that, even though the enzyme activity is lower at pH 5.2, the substrate was more accessible.

Despite the ability of the sialidase to release sialic acid from these substrates, *A. fumigatus* was unable to use either colominic acid or sialic acid as a sole source of carbon (data not shown).

Trans-sialidase activity

Trans-sialidase activity was not detected with the Af sialidase. Figure 6 shows that incubation of *A. fumigatus* sialidase with bodipy-lac, a fluorescent substrate for *trans*-sialylation and

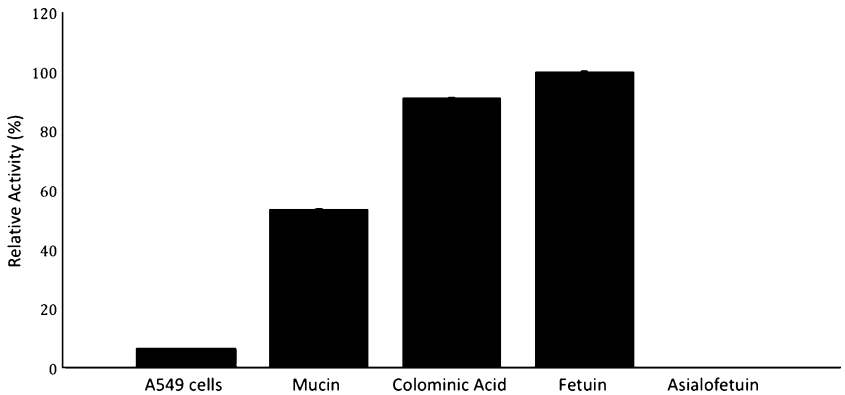

Fig. 5 Sialidase activity with sialylated substrates. Enzymatic activity of *A. fumigatus* sialidase was measured with heat-inactivated A549 cells (a human pneumocyte cell line), bovine mucin, colominic acid, fetuin, and asialofetuin. All substrates were tested at the pH optimum for the enzyme as described in Materials and Methods. The specific activity of each enzyme was measured against the specific activity of fetuin (42 nmol/mg protein/hr) to calculate the relative activity of the enzyme towards each biological substrate

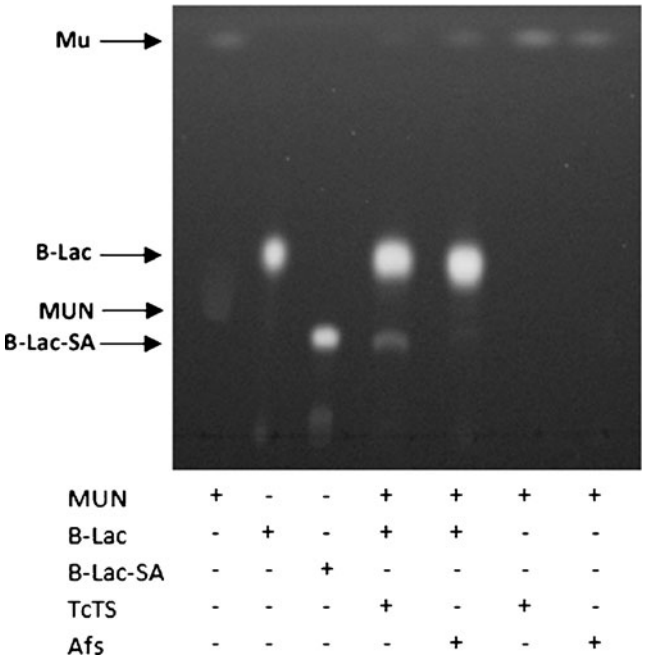

Fig. 6 *Trans*-sialidase activity analysis of *A. fumigatus* sialidase. *A. fumigatus* sialidase (AfS) was incubated with MUN, or with MUN and bodipy-lactose. Sialidase activity cleaves the sialic acid from MUN releasing the fluorescent product 4-methylumbelliferone (MU). *Trans*-sialidase activity results in the addition of sialic acid to the acceptor, bodipy-lactose (B-Lac) forming the more polar product, bodipy-lactose-sialic acid (B-Lac-SA). Recombinant *T. cruzi trans*-sialidase formed B-Lac-SA (and some MU as expected); however, no B-Lac-SA was formed by *A. fumigatus* sialidase. The faint band in the AfS lane that runs slightly higher than B-Lac-SA is MUN, confirmed by its purple fluorescence not evident in the black and white photo

MUN generated MU but produced no sialylated derivative (bodipy-Lac-SA). These data indicate that *A. fumigatus* sialidase was able to remove terminal Sia residues but was unable to catalyze their transfer to the acceptor substrate. The positive control, recombinant *trans*-sialidase from *Trypanosoma cruzi*, produced the bodipy-Lac-SA derivative, as expected.

De novo synthesis of sialic acids

In a previous study, we reported that *A. fumigatus* was able to synthesize sialic acids when grown on solid media containing no sialic acid, suggesting that sialic acid biosynthesis occurred *de novo* [62]. We wished to confirm this finding using synthetic liquid media to ensure that no sialylated compounds could have contributed to sialic acids in the fungus. Figures 7 and 8 show that *A. fumigatus* mycelia bind the sialic acid-specific lectin, LFA, when grown in liquid minimal medium containing only glucose as a carbon source. Similar results were obtained with growth in complex medium, MYPD. Staining intensity in MYPD was greater, confirming our previous finding that sialic acid density is enhanced when the fungus is grown in this medium [62]. Thus, *de novo* biosynthesis of sialic acids occurs in *A. fumigatus.*

Phylogenetic analysis

The phylogeny of the fungal sialidase was investigated using both Neighbour-Joining distances inferred from protdist and Maximum Likelihood analysis of protein evolution; both resulted in the same placement of the sialidase from *A. fumigatus*. The consensus tree generated from the maximum likelihood analysis is shown in Fig. 5. Many studies have shown that the Fungi are the sister group to animals and form part of the crown group of eukaryotes along with plants and animals [29, 59]. The four fungal species formed a separate cluster from both the animal and bacterial sialidases. The closest relative to *A. fumigatus* was *N. fischeri*; this reflects the known close phylogenetic relationship between these organisms [13]. However, our analyses also revealed that the fungal sialidases are more closely related to sialidases from Actinobacteria belonging to the order Actinomycetales, in particular, *Streptomyces coelicolor*, *Streptomyces avermitilis* and *Saccharopolyspora erythraea*. Although it is in a different genus, *S. erythraea* biology closely resembles that of these streptomycetes [24]. Interestingly, sialidases from two other Actinobacteria, *M. viridifaciens* and *C. glutamicum*, clustered separately with other Bacteria, the Bacteroidetes (*B. vulgatus, P. distasoni, B. fragilis* and *B. uniformis*) (Fig. 5) and the Planctomycetes (data not shown). To determine whether the data statistically supported this separation of Actinomycetales, we performed a Kishino-Hasegawa test for two trees; the original tree *versus* one in which the *S. avermitilis, S. coelicolor* and *S. erythraea* sialidases were constrained to be in a clade with *M. viridifaciens* and *C. glutamicum*. The results showed that this tree was significantly worse (Diff logL=-12) than the tree shown in Figure 5. Consistent with the finding that the fungal sialidase has no *trans*-sialidase activity, the *trans*-sialidase protein of *Trypanosoma cruzi* formed a unique branch that diverged from the *Bacteroides* spp., distinct from the *C. glutamicum/M. viridifaciens* sialidases (data not shown).

Only three other species of Fungi, *A. terreus*, *C. globosum* and *N. fischeri* (shown on tree) gave significant blastp hits when searched against the *A. fumigatus* sialidase. The candidate pool included fungi being sequenced by both the Broad Institute and the Concordia University Fungal Genomics project (a total of 40 species representing zygomycetes, chytridiomycetes, ascomycetes and basidiomycetes). In addition to *N. fischeri*, the genomes of several other *Aspergillus* species have been

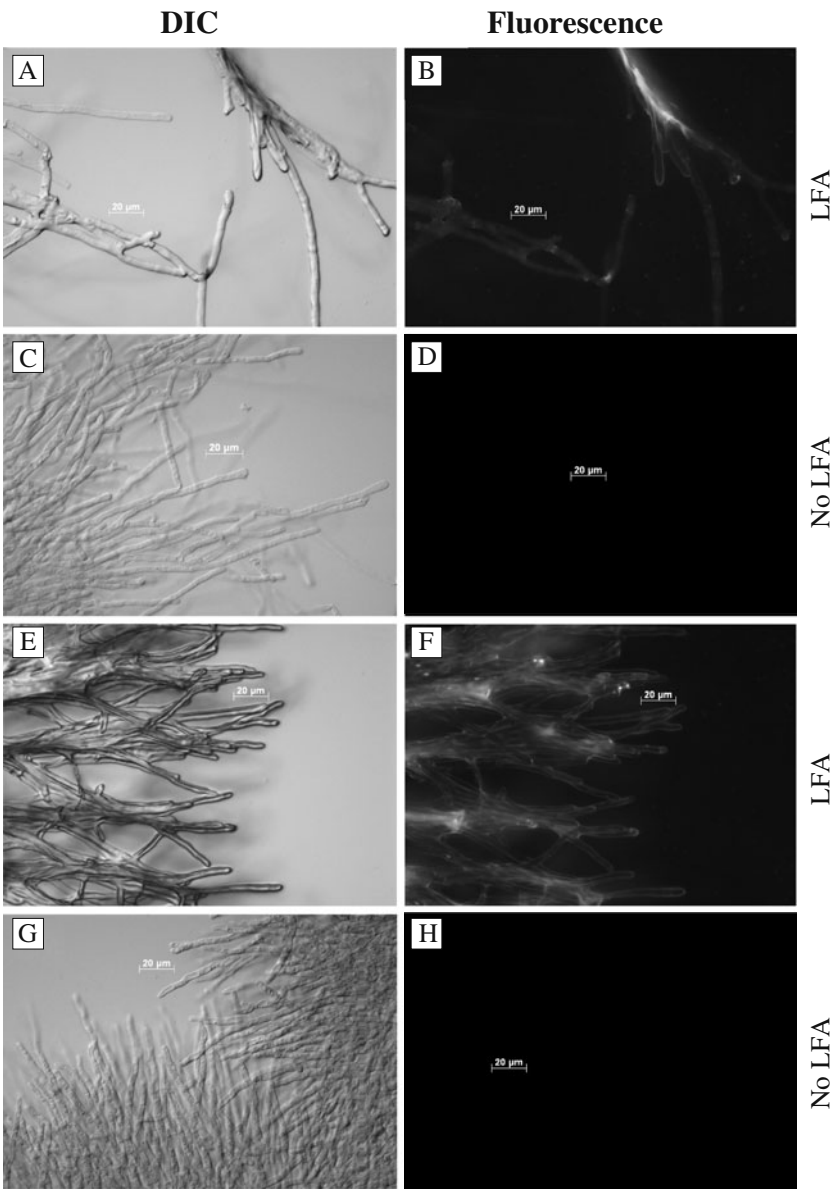

Fig. 7 *A. fumigatus* was grown in minimal media where glucose was the only carbon source **a**-**b** and in MYPD **e**–**f**. After washing, mycelia were incubated with LFA bound to Texas Red and processed for fluorescence microscopy as described in Materials and Methods. Controls were treated identically except that LFA-TR was omitted (minimal media, **c**–**d**; MYPD, **g**–**h**)

sequenced; however, other than *A. terreus*, we found no significant matches, including from *Aspergillus nidulans* (data not shown). The *A. nidulans* genome has two proteins that have sialidase domains and one protein has two Asp boxes. However, these genes are unlikely to encode sialidases: no FRIP sequences are present, and the top models generated by PHYRE did not align well to the *M. viridifaciens* sialidase as only 189 and 304 atoms were aligned in each protein (compared to thousands of atoms in the *A. fumigatus* sialidase) and there was no alignment of catalytic residues (data not shown). Many glycosyl hydrolases as well as other non-homologous proteins possess Asp-box repeats [11] and these sequences may encode other glycosyl hydrolases.

Discussion

We have successfully cloned and expressed a sialidase from *Aspergillus fumigatus*, which represents the first cloning of a fungal sialidase. Initial computational analysis indicated a protein mass of 42 kDa, the presence of conserved FRIP and Asp-box domains and folding into the canonical 6-bladed β-propeller, characteristics which are present in all known sialidases [45]. Molecular modeling revealed that the active site of the *A. fumigatus* sialidase aligned well with that of the experimentally determined structure of the sialidase from the bacterium, *M. viridifaciens* (1eus). Tyr 370, Asp 92 and Glu 260 of the *M. viridifaciens* sialidase, which function as the nucleophile, general base and general

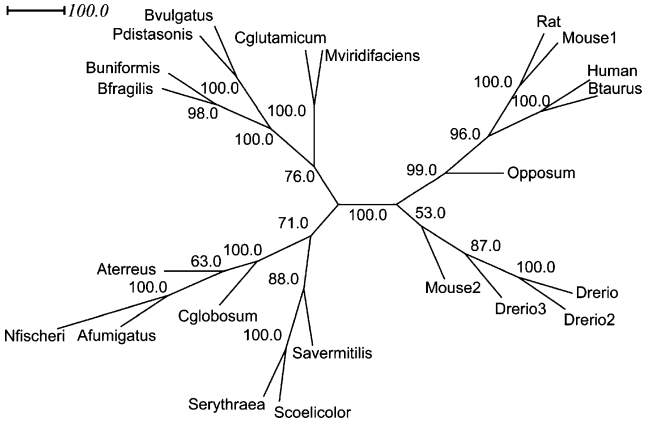

Fig. 8 Unrooted bootstrap consensus tree from maximum-likelihood analysis of the amino acid sequences of 22 sialidases from Bacteria, Animalia and Fungi. The mammalian and fish sialidases (all sequences on the right split at 100% bootstrap) form a cluster distinct from the Bacterial and Fungal sialidases. Despite the fact that Fungi are more closely related to animals than bacteria, the fungal sialidases (*N. fischeri, A. fumigatus, A. terreus* and *C. globosum*) are more closely related to a subset of bacterial sialidases from the Actinobacteria, *S. avermitilis*, *S. erythraea* and *S. coelicolor*

acid, respectively, correspond with Tyr 338, Asp 64 and Glu 229 of the *A. fumigatus* sialidase model. Furthermore, the highly conserved arginine triad, which functions to stabilize the sialic acid carboxylic acid is also conserved further supporting the quality of this model [45]. The fungal sialidase was enzymatically active when tested using standard substrates.

The kinetic parameters K_m and k_{cat} and specific activity of the *A. fumigatus* sialidase toward MUN are lower than those reported for the bacterial sialidases from *C. perfringens* and *M. viridifaciens* (Table 2) [25, 28]; however, the specific activity of the *A. fumigatus* sialidase is similar to that of the human ganglioside sialidase Neu3 [21, 36]. One possible explanation for the lower activity of the fungal sialidase compared to bacterial sialidases could be that the substrate (MUN) used for these assays is not the natural substrate for this enzyme. For example, human ganglioside sialidase, Neu3, preferentially acts on the ganglioside GD1a with a lower K_m value (98 μM for GD1a compared to 143 μM for MUN) and a fivefold greater specific activity (120 nmol mg protein^{-1} h^{-1} for MUN compared to 600 nmol mg protein^{-1} h^{-1} for GD1a) than it does for MUN [28]. Furthermore, Neu3 preferentially cleaves terminal α2-8-linked sialic acids from GD3 over GD1b despite their similar terminal trisaccharides (Neu5Ac(α2-8)Neu5Ac(α2-3)Gal), illustrating the important role that the entire substrate molecule plays in the recognition process [21]. Crystallization of the *A. fumigatus* sialidase and structural analysis will likely provide more information regarding substrate recognition and catalytic mechanism. Alternatively, the decreased activity may be due to a lack of proper posttranslational modification due to the expression of the eukaryotic *A. fumigatus* sialidase in bacteria. There is a potential, albeit low scoring, N-glycosylation site at Asn 215; glycosylation at this site may be important for proper enzyme folding and function. No N-glycosylation has been detected in the human cytosolic sialidase, Neu2 [37]; however, elimination of glycosylation at one of the four glycosylation sites in the neuraminidase from the Newcastle disease virus was found to significantly decrease neuraminidase activity and this corresponded to a decreased virulence of the virus [40]. Finally, bacterial sialidases have an important role in nutrition by supplying the bacterium with both carbon and nitrogen. Because we have shown that *A. fumigatus* cannot use sialic acid as a sole source of carbon, the sialidase is likely to play a more important role in removal of terminal sialic acid from glycoproteins and glycolipids.

Sialidases have been isolated and characterized from a wide variety of eukaryotes and prokaryotes. As far as we are aware, sialidase activity of cells or culture filtrates has been reported for only a handful of fungi including *Sporothrix schenckii* [57] and its teleomorph, *Ophiostoma stenocera* [44], as well as *Fonsecaea pedrosoi* [4]. Saito and Yu [47] summarized the characteristics of microbial sialidases and included fungal sialidases in the title; however, no true fungi were included in their study. Another study indicated that *Candida albicans* possessed sialidase activity [46]; however, this was later refuted by others [44]. In support of this, we did not identify a sialidase gene in the *C. albicans* genome in our study. Unfortunately, genome sequences for *Ophiostoma sternocera* and *Fonsecaea pedrosoi* are not yet available so we could not include these in our phylogenetic analysis. In our study, fungal sialidases form a unique cluster more closely related to a subset of prokaryotic sialidases than to enzymes from other eukaryotes. Furthermore, sialidases were only detected in a few fungi from diverse lineages suggesting that these genes were acquired horizontally or have been lost from, or changed significantly in, all but a few ascomycete fungi. A possible bacterial origin of the fungal sialidases is suggested by our analyses; while the majority of *A. fumigatus* genes (77%) contain introns with an average of 1.8 per gene [18], the *A. fumigatus* sialidase does not contain any introns; the mean difference in codon usage between the fungal sialidase and other *A. fumigatus* genes was greater than the difference between the sialidase codon usage and all *S. avermitilis* genes (24.3% *versus* 10.4%, respectively). The % G + C value of the *A. fumigatus* sialidase (64.7%) was intermediate between the average % G + C for *A. fumigatus* exons (54%) and the average for streptomycetes (approximately 70%). Finally, the Actinobacteria and the fungi that contain sialidase share similar habitats, which

increase the possibility of gene transfer; all are filamentous, soil-dwelling saprobes that secrete hydrolytic enzymes to break down naturally-occurring polymers as a source of carbon and energy. Nevertheless, these data do not rule out the possibility that the gene was acquired by a common ancestor of the Pezizomycotina (which includes all of the fungal species with putative sialidases) [16] but then retained in only a few species.

Sialidase expression in *A. fumigatus* was upregulated during germination in MEM containing 10% human serum. Using microarray analysis, we have found that serum exposure induces stress response genes (unpublished data) and mediators of the stress response may activate *A. fumigatus* gene expression through the STRE motif or via other uncharacterized mechanisms. Alternatively, sialidase expression may be developmentally regulated in *A. fumigatus*.

The role of the *A. fumigatus* sialidase in the life cycle of the fungus or in pathogenesis is currently unclear. Recently, we have shown that α2-6-linked unsubstituted sialic acids are present on the surface of *A. fumigatus* conidia and that these sugars play important roles in mediating the interaction between conidia and mouse macrophages and human type II epithelial cells, *in vitro* [60]. Despite their presence on the conidial surface, the mechanism of their biosynthesis and transport to the surface are unknown. In other microorganisms, several pathways have been elucidated for the biosynthesis of sialic acid and its display on the cell surface. For example, some bacteria have a complete biosynthetic pathway while others have only a few enzymes and rely on environmental sources for sialic acid or its precursors [58]. Even though our results indicate that sialic acid is synthesized *de novo* in *A. fumigatus*, work in our laboratory and by others [5, 55] has failed to identify orthologues of known sialic acid biosynthetic genes in the genomes of *A. fumigatus* and other fungi. The *A. fumigatus* genome does contain genes encoding several nucleotide-sugar transporters and glycosyltransferases, which together may function to sialylate the surface of *A. fumigatus* conidia. Furthermore, [43] have partially purified a sialyltransferase from another fungal pathogen, *Cryptococcus neoformans* although the gene has not yet been identified. Nevertheless, supplementation of growth media with sialic acid or *N*-acetylmannosamine (a sialic acid precursor in other organisms) did not enhance sialic acid levels in *A. fumigatus* [55]. Alpha-2,3-linked sialic acids are undetectable in *A. fumigatus* [60, 62] and the preference for this substrate suggests that the *A. fumigatus* sialidase is likely not involved in glycan remodelling.

Thus, sialic acid metabolism in fungi has many intriguing findings but the full picture of sialobiology in this Kingdom has yet to emerge.

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