

## Deletion of the *msdS*/*AfmsdC* gene induces abnormal polarity and septation in *Aspergillus fumigatus*

Yanjie Li, Lei Zhang, Depeng Wang, Hui Zhou, Haomiao Ouyang, Jia Ming and Cheng Jin

Correspondence  
Cheng Jin  
jinc@sun.im.ac.cn

State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, PR China

Received 14 February 2008  
Revised 25 March 2008  
Accepted 25 March 2008

$\alpha$ -Mannosidases play an important role in the processing of mannose-containing glycans in eukaryotes. A deficiency in  $\alpha$ -mannosidase is lethal in humans and cattle. In contrast to mammals, *Saccharomyces cerevisiae* does not require the endoplasmic reticulum  $\alpha$ -mannosidase gene for growth. However, little is known of the consequence of loss of function of class I  $\alpha$ -mannosidases in filamentous fungi. In this study, the *msdS*/*AfmsdC* gene was identified to encode 1,2- $\alpha$ -mannosidase MsdS in *Aspergillus fumigatus*. Soluble MsdS expressed in *Escherichia coli* was characterized as a typical class I  $\alpha$ -mannosidase. The *msdS* gene was deleted by replacement of the *msdS* gene with a *pyrG* gene. Although the mutant showed a defect in *N*-glycan processing, as well as a reduction of cell wall components and a reduced ability of conidiation, it appeared that the rate of hyphal growth was not affected. Morphology analysis revealed abnormal polarity and septation at the stages of germination, hyphal growth and conidiation. Although the mechanism by which the *N*-glycan processing affects polarity and septation is unclear, our results show that *msdS* is involved in polarity and septation in *A. fumigatus*.

### INTRODUCTION

*N*-Glycosylation starts with the transfer of Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> from dolichyl pyrophosphate to the nascent protein in the lumen of the endoplasmic reticulum (ER), and the product is converted into its final form by processing enzymes localized along the secretory pathway. *N*-Glycan processing is initiated by two ER  $\alpha$ -glucosidases, which remove the glucose residues, and it is followed by the action of various 1,2- $\alpha$ -mannosidases, which can remove one or more of the four 1,2- $\alpha$ -linked mannose residues. In mammalian cells, Man<sub>9</sub>GlcNAc<sub>2</sub> is converted to Man<sub>5</sub>GlcNAc<sub>2</sub> by the action of ER and Golgi  $\alpha$ -mannosidases, and Man<sub>5</sub>GlcNAc<sub>2</sub> is the precursor for complex hybrid and *N*-glycans with a high mannose content (Kornfeld & Kornfeld, 1985). In *Saccharomyces cerevisiae*, a specific ER 1,2- $\alpha$ -mannosidase converts Man<sub>9</sub>GlcNAc<sub>2</sub> into Man<sub>8</sub>GlcNAc<sub>2</sub>, which is elongated in the Golgi to form an outer chain containing up to 200 mannose residues (Kukuruzinska *et al.*, 1987; Tanner &

Lehle, 1987). *Aspergillus saitoi* and *Trichoderma reesei* have been found to produce *N*-glycan structures containing five mannose units (Man<sub>5</sub>GlcNAc<sub>2</sub>), suggesting further processing of the Man<sub>9</sub>GlcNAc<sub>2</sub> precursor (Chiba *et al.*, 1993; Maras *et al.*, 1997). Based on these observations, *N*-glycan synthesis in filamentous fungi seems to differ from that in yeast, and may be more similar to the processing in higher eukaryotes (Eades & Hintz, 2000).

The  $\alpha$ -mannosidases have been classified into two groups: class I and class II (Daniel *et al.*, 1994; Eades *et al.*, 1998; Moremen *et al.*, 1994). Class I  $\alpha$ -mannosidases include ER Man<sub>9</sub>-mannosidase, endomannosidase and Golgi mannosidase I. Class II  $\alpha$ -mannosidases contain the lysosomal mannosidases, Golgi mannosidase II, yeast vacuolar mannosidase (Yoshihisa & Anraku, 1989, 1990), and ER  $\alpha$ -mannosidase II (Weng & Spiro, 1993, 1996). Several Golgi  $\alpha$ -mannosidases have been cloned and characterized from *Penicillium citrinum* (Yoshida & Ichishima, 1995; Yoshida *et al.*, 1993), *A. saitoi* (Ichishima *et al.*, 1999), *Aspergillus oryzae* (Akao *et al.*, 2006), *T. reesei* (Maras *et al.*, 2000), and *Aspergillus nidulans* (Eades & Hintz, 2000). These enzymes are monomeric, with a molecular mass of 50–60 kDa, and they show maximal activity in the semi-acidic condition (pH 4–6).

*Aspergillus fumigatus* is known to cause fatal invasive aspergillosis in immunocompromised patients (Latgé,

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; HPAEC-PAD, high-performance anion-exchange chromatography pulsed ampere detector; LC-MS/MS, liquid chromatography/tandem MS; sMsdS, soluble MsdS.

The Genbank/EMBL/DDBJ accession number for the nucleotide sequence of *msdS*/*AfmsdC* is AY573554.

A table of LC-MS/MS data is available with the online version of this paper.

1999). The crude mortality rate for invasive aspergillosis is over 90%, and falls to around 50–70% if treatment is given (Steinbach *et al.*, 2003). The main reason for patient death is the low efficiency of the drug therapies available to treat invasive aspergillosis, and the lack of an assay that can detect the fungus early during the infection. The fungal cell wall is essential for fungal life, and therefore it is a unique specific target for antifungal drug development. Many glycoproteins are directly or indirectly involved in the synthesis and organization of the fungal cell wall, and thus it is of importance to assess the role of *N*-glycan processing in trafficking, localization and function of proteins in *A. fumigatus*.

To investigate *N*-glycan processing in *A. fumigatus*, a gene, which was previously designated *AfmsdC* and is now annotated as *msdS*, was identified to encode a class I 1,2- $\alpha$ -mannosidase. In this report, *MsdS*/*AfmsdC* was expressed and characterized. Also, the phenotypes associated with the deletion of the *msdS*/*AfmsdC* gene were analysed.

## METHODS

**Strains and growth conditions.** *A. fumigatus* strain YJ-407 (CGMCC0386; China General Microbiological Culture Collection Center, Beijing, PR China) was maintained on a potato glucose (2%) agar slant (Xia *et al.*, 2001). *A. fumigatus* strain CEA17 (Weidner *et al.*, 1998), a kind gift from C. d'Enfert, Institut Pasteur, Paris, France, was propagated at 37 °C on YGA (0.5% yeast extract, 2% glucose, and 1.5% Bacto-agar), complete medium (Cove, 1966), or minimal medium with 0.5 mM sodium glutamate as a nitrogen source (Cove, 1966). Uridine and uracil were added to the media at a concentration of 5 mM when CEA17 or a complemented strain was grown. Mycelium was harvested from strains grown in complete liquid medium at 37 °C, with shaking at 250 r.p.m. At the specified culture time point, mycelium was harvested, washed with distilled water, frozen in liquid N<sub>2</sub>, and then ground by hand. The powder was stored at -70 °C for DNA, RNA and protein extraction. Conidia were prepared by growing *A. fumigatus* strains on solid complete medium with uridine and uracil (CMU) for 48 h at 37 °C. The spores were collected, washed twice with 0.01% Tween-20 in physiological saline, and resuspended in 0.01% Tween-20 in saline. The concentration of spores was confirmed by haemocytometer counting and viable counting. Vectors and plasmids were propagated in *Escherichia coli* DH5 $\alpha$  (BRL).

**Computer analysis.** Sequence analysis of cDNA clones and multiple sequence alignments were performed using OMIGA v2.0, and a BLAST search was performed.

**Molecular cloning of *A. fumigatus* *msdS*.** The *AfmsdC/msdS* genomic sequence was identified in a search of the *A. fumigatus* genome database (<http://www.tigr.org/tdb/e2k1/afu1/>), using a tBLASTn program to search for sequences corresponding to the conserved amino acid sequence of *Penicillium citrinum* *msdC* that were homologous between *A. nidulans*, *S. cerevisiae* and human. A 1.6 kb genomic DNA fragment was found to contain the entire ORF. Based on the nucleotide sequence, the forward primer (5'-ATGCATTTACCCTCTTTGTCC-3') and the reverse primer (5'-TCACGTATGATGAATTCGGAC-3') were designed for cloning the cDNA of *AfmsdC* by PCR. The PCR products were subcloned into pGEM-T easy Vector (Promega), sequenced (T-*msdC*), and the

position of the intron was determined by comparing the cDNA with the genomic sequence.

**Expression and purification of *MsdS*.** The sequence encoding soluble *MsdS* (s*MsdS*) was amplified from T-*msdC* by truncation of the coding region for the transmembrane domain. Expression of s*MsdS* fused with a His-tag in *E. coli* was done by following Novagen protocols. The plasmid construct pET-*msdC* was introduced into *E. coli* BL21 (DE3). The recombinant strain was induced by the addition of 0.4 mM IPTG at 27 °C. After induction, cell extracts were prepared, and separated by metal chelation affinity chromatography (Novagen). Proteins were analysed by SDS-PAGE electrophoresis, and recombinant protein was identified by Western blotting with a His-tag-specific antibody.

**Construction of the *msdS* null mutant and the complemented strain.** To delete *msdS*, a deletion construct was designed to replace the entire coding region of *msdS* with a *pyrG* cassette by homologous recombination (d'Enfert, 1996). PCR primers were designed to amplify a 1.8 kb upstream non-coding region of the *msdS* before the ATG start codon (5' primer pair: 5'-ACGCGTCGAC GCGGCCGC-TCAGCTTGACTGAGAGAGGAG-3' and 5'-GGTGGTGATATC-GATACCGACCAACGAAAAGAAT-3'; the *SalI*, *NotI* and *EcoRV* restriction sites are underlined, respectively), and a 1.8 kb downstream non-coding region of the *msdS* after the stop codon (3' primer pair: 5'-GGTGGTGATATCTGTACATACC CTAGCTGGCT-3' and 5'-GCTCTAGAGCTGCACCACAAAAGCAC-3'; the *EcoRV* and *XbaI* restriction sites are underlined, respectively). These PCR fragments were cloned into the relevant sites of pBlueScript II SK. The *pyrG*-blaster cassette (8.6 kb), released by the digestion of pCDA14 (d'Enfert, 1996) with *HpaI*, was cloned into the site between the up- and downstream non-coding regions of the *msdS*, to yield the deletion construct pBK70. At a unique *NotI* site, the linearized pBK70 was transformed into strain CEA17 by protoplast transformation (Yelton *et al.*, 1984), and screened for mutants with uridine and uracil autotrophy. The deletions in the mutants were confirmed by PCR and Southern blotting.

The complemented strain was constructed by the replacement of *pyrG* with a wild-type copy of *msdS* in the  $\Delta$ *msdS* mutant. *msdS*, with its 1.8 kb upstream and 1.8 kb downstream non-coding regions, was amplified by PCR (primer pair: 5'-TCAGCTTGACTGAGAGAGAG-3' and 5'-GAGCTGCACCACAAAAGCAC-3'), and the product was cloned into pGEM-T Easy Vector, and sequenced. The resulting plasmid T-long70 was then linearized with *XhoI*, before transformation into the  $\Delta$ *msdS* null mutant. The transformants were chosen by PCR, and then the transformation was confirmed by Southern blot analysis, using the upstream non-coding region as a probe. The probe was labelled by following the protocol of the DIG-labelled hybridization kit (catalogue no. 1093657; Roche Applied Science).

**Assay for 1,2- $\alpha$ -mannosidase activity.** The standard assay mixture containing 50  $\mu$ g Man( $\alpha$ 1,2)Man-OMe and the enzyme, in a total volume of 100  $\mu$ l with 10 mM sodium acetate (pH 5.5), was incubated at 37 °C. The reaction was terminated by heating, and the amount of reducing sugars was determined (Matta & Bahl, 1972). One unit of 1,2- $\alpha$ -mannosidase is defined as the amount of enzyme that releases 1  $\mu$ mol mannose per hour at 37 °C. A reaction mixture without enzyme was used as a control in each instance.

For the substrate specificity assay, 10  $\mu$ g purified s*MsdS* was incubated with 100 pmol Man<sub>9</sub>GlcNAc<sub>2</sub> (Sigma) or Man<sub>8</sub>GlcNAc<sub>2</sub> (Calbiochem) at 37 °C for 10–360 min. The reaction was terminated by heating at 100 °C for 5 min. Upon addition of 2 vols ethanol, proteins in the reaction mixture were precipitated, and removed by centrifugation. The supernatant was analysed with a CarboPac PA-100 column and a high-performance anion-exchange chromatography.

graphy pulsed ampere detector (HPAEC-PAD; Dionex). The products were eluted with 250 mM NaOH at a flow rate of 1.0 ml min<sup>-1</sup>. Man<sub>7</sub>GlcNAc<sub>2</sub>, Man<sub>6</sub>GlcNAc<sub>2</sub> and Man<sub>5</sub>GlcNAc<sub>2</sub> (Calbiochem) were used as standards.

The  $\alpha$ -mannosidase activity of the mutant was assayed as follows: the mutant was grown on a shaker (250 r.p.m.) in 100 ml liquid CMU medium at 37 °C for 48 h. The mycelium was filtered, washed extensively with water, and ground in liquid nitrogen. The powder was dissolved in 10 mM sodium acetate (pH 5.5). After removal of cell debris by centrifugation at 12 000 g at 4 °C for 10 min, the supernatant was used as a crude enzyme for the activity assay, under standard conditions.

**Western blotting of ChiB.** Chitinase ChiB, secreted by *A. fumigatus*, was induced and purified as previously described (Xia *et al.*, 2001). Anti-ChiB antibody developed in mouse was used in Western blotting. Proteins in the cell lysate or culture supernatant were run on a 12% SDS-PAGE gel, and transferred to PVDF (Bio-Rad) at 300 mA for 1.5 h. The anti-ChiB mouse serum was diluted at 1:5000. Protein was detected with the enhanced chemoluminescence substrate (Pierce) and autoradiography on film.

**Phenotypic analysis of the mutant.** Growth kinetics of *A. fumigatus* strains were assayed as follows. A 100  $\mu$ l slurry of spores ( $1 \times 10^9$  ml<sup>-1</sup>) was inoculated into 100 ml liquid CMU medium. After incubation at 37 °C with shaking (200 r.p.m.), three 1 ml aliquots of liquid were taken for each strain at set time intervals, and dried and weighed. The mean weight was used to plot the growth kinetics. The experiment was repeated three times.

To test the sensitivities of the mutant to antifungal reagents, conidiospores were collected from the wild-type, the mutant and the complemented strain, and, for each strain, similar numbers of conidiospores were spotted on CMU plates in the presence of 100  $\mu$ g Calcofluor white ml<sup>-1</sup>, 250  $\mu$ g Congo red ml<sup>-1</sup> or 60  $\mu$ g SDS ml<sup>-1</sup>. After incubation at 37 or 50 °C for 24–48 h, the plates were photographed.

For examination of conidial germination, 20 ml complete liquid medium was inoculated with 10<sup>7</sup> freshly harvested conidia, poured into a Petri dish containing a glass coverslip, and incubated at 37 °C for the time indicated in each experiment. At the specified times, the coverslips with adhering germlings were removed, and spore germination was observed and counted under differential interference contrast microscopy.

For examination of nuclei, septa and cell wall staining at the germination stage, the coverslips with adherent germlings were removed, and fixed in fixative solution (4% formaldehyde, 50 mM phosphate buffer, pH 7.0, and 0.2% Triton X-100) for 30 min. Coverslips were then washed with PBS, incubated for 15 min with 1  $\mu$ g 4',6-diamidino-2-phenylindole (DAPI) ml<sup>-1</sup> (Sigma), washed with PBS, and then incubated for 5 min with a 10 mg ml<sup>-1</sup> solution of fluorescent brightener 28 (Sigma), washed again, and germlings were photographed using a microscope.

For examination of nuclei, septa and cell wall staining at the conidiation stage, 100 ml complete liquid medium was inoculated with 10<sup>6</sup> conidia, and incubated with shaking (200 r.p.m.) at 37 °C for 17 h. The mycelia were taken, and placed on a glass coverslip. The glass coverslip was then put in a Petri dish containing two layers filter paper saturated with complete liquid medium. After incubation at 37 °C for 2–8 h, the coverslip was removed, and stained with DAPI and fluorescent brightener.

A 100 ml volume of complete liquid medium was inoculated with 10<sup>8</sup> freshly harvested conidia, and incubated at 37 or 50 °C with shaking (200 r.p.m.) for 12 h. Mycelia were harvested by filtering the culture

through two layers of Miracloth (Calbiochem), washed twice with distilled water, and streaked on minimal medium agar. After incubation at 37 or 50 °C for 2, 4, 6 and 8 h, mycelia were suspended in 5 ml distilled water, and conidial production was expressed as the mean number of conidia per volume (millilitre).

Conidia or mycelia produced on solid complete medium were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, and then examined with a Quanta 200 scanning electron microscope (FEI). Conidia were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, for 4 h, or overnight at 4 °C. Hyphal cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate, washed three times with 0.1 M phosphate, post-fixed in 1% osmium tetroxide, incubated for 2–4 h in 0.1 M phosphate, and the cells were then treated for 15–20 min in each of 30, 50, 70, 85, 95 and 100% methanol, and post-fixed in 2% uranyl acetate/30% methanol. Cells were rinsed, dehydrated, and embedded in Epon 812 by the floating sheet method. The sections were examined with an H-600 electron microscope (Hitachi).

**Chemical analysis of the cell wall.** Conidia were inoculated into 100 ml complete medium at a concentration of 10<sup>6</sup> conidia ml<sup>-1</sup> and incubated at 37 °C with shaking (200 r.p.m.) for 48 h. The mycelium was harvested by filtering the culture through two layers of Miracloth, washed twice with distilled water, and lyophilized. Three aliquots of 10 mg dry mycelium were used as independent samples for cell wall analysis, and the experiment was repeated twice. To remove unbound cell wall proteins and water-soluble sugar, each sample was boiled for 5 min in 2 ml 2% SDS in 50 mM Tris/HCl buffer supplemented with 100 mM Na-EDTA, 40 mM  $\beta$ -mercaptoethanol, and 1 mM PMSF (Elorza *et al.*, 1985; Hearn & Sietsma, 1994; Schoffemeer *et al.*, 1999). Mannoprotein was extracted with 3% NaOH at 75 °C for 1 h, and quantitatively determined by using the Lowry protein assay (Lowry *et al.*, 1951). Glucan and chitin were digested in 96% formic acid at 100 °C for 4 h. Formic acid was evaporated by lyophilization, and the residues were dissolved in 1 ml distilled water. Glucan and chitin were estimated by measuring the released glucose and *N*-acetylglucosamine after digestion. Glucose was measured by the phenol-sulfuric acid method (Dubois *et al.*, 1956). *N*-acetylglucosamine was measured by the method described by Lee *et al.* (2005).

**Glycan analysis by HPAEC-PAD.** The chitinase ChiB used for *N*-glycan analysis was induced from the wild-type, the mutant and the complemented strain of *A. fumigatus* as previously described (Xia *et al.*, 2001). The ChiB in the culture supernatant was precipitated with 30–60% ammonium sulfate. For comparison of *N*-glycans from different strains, the partially purified ChiB of the wild-type, the mutant and the complemented strain was run on SDS-PAGE, and then transferred to PVDF. The ChiB on PVDF was recovered, and treated with PNGase F (NEB) at 37 °C for 48 h. Upon the addition of 2 vols ethanol, the proteins were precipitated, and they were then removed by centrifugation. The supernatant was analysed with a CarboPac PA-100 column and HPAEC-PAD. The glycans were eluted with 250 mM NaOH at a flow rate of 1.0 ml min<sup>-1</sup>. Man<sub>8</sub>GlcNAc<sub>2</sub> and Man<sub>6</sub>GlcNAc<sub>2</sub> were used as standards.

**Analysis of virulence of the mutant.** The wild-type and mutant strains were used for experimental infections in white male BALB/c mice (18–20 g). Conidia were suspended in 0.01% Tween-20 in saline to give a challenge inoculum of  $3 \times 10^5$  c.f.u. (g body wt)<sup>-1</sup> in a 30  $\mu$ l volume. Mice were immunosuppressed by intraperitoneal injection of cyclophosphamide [150 mg (kg body wt)<sup>-1</sup>] on days -3 and -1, and one subcutaneous injection of hydrocortisone acetate [40 mg (kg body wt)<sup>-1</sup>] on day -1. On day 0, mice were anaesthetized by the inhalation of diethyl ether, and infected intranasally with 30  $\mu$ l spore suspension containing  $6 \times 10^6$  conidia. A concurrent control group consisted of mice that had been immunosuppressed, and then

inoculated with 30  $\mu$ l 0.01 % Tween 20 in saline. Immunosuppression was prolonged by cyclophosphamide injections [150 mg (kg body wt)<sup>-1</sup>] on days 3, 6 and 9. Mice were kept in sterile cages with filter tops, and they received sterile food and bedding. Tetracycline (1 mg ml<sup>-1</sup>) was added to the drinking water, which was changed twice daily. Four groups, each containing 20 mice, were inoculated, monitored twice daily for 30 days after inoculation, and mortality was recorded. Mice surviving the course of the experiment were killed humanely on day 30. The survival rate was analysed statistically by using the methods of Kaplan–Meier, with SPSS13 software. *P* values of <0.05 were considered significant in this analysis.

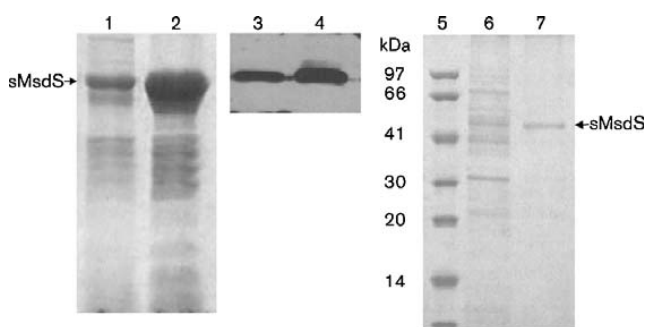
**Proteins secreted by the  $\Delta$ msdS mutant.** The *A. fumigatus* strains were incubated in the complete liquid medium at 37 °C at 250 r.p.m. for 60 h. The secreted proteins in the culture medium were precipitated from the culture filtrates by the addition of 4 vols ice-cold acetone, and the culture filtrates were kept at 4 °C overnight. The pellet was then recovered by centrifugation, resuspended in water, and separated by PAGE. After staining with Coomassie brilliant blue R-250, the protein bands of interest were cut, and analysed by liquid chromatography/tandem MS (LC-MS/MS).

## RESULTS

### Expression and characterization of sMsdS

A tBLASTn search (Altschul *et al.*, 1997) of the *A. fumigatus* genome database was performed with the protein sequence of the MsdC (P31723) of *P. citrinum*, and one gene, *AfmsdC/msdS*, was found. The *msdS* gene consists of a total of 1677 nt, and contains three introns. Its cDNA encodes a polypeptide of 504 aa with an estimated molecular mass of 55 kDa. The predicted sequence of MsdS exhibits a high score of amino acid identity with other class I  $\alpha$ -mannosidases: 78.1 % with *A. oryzae*  $\alpha$ -mannosidase, 73.5 % with *A. saitoi*  $\alpha$ -mannosidase, 72.3 % with *A. nidulans*  $\alpha$ -mannosidase IB, and 70.7 % with *P. citrinum* MsdC.

sMsdS was expressed and purified, as described in Methods. As shown in Fig. 1, a 55 kDa protein was



**Fig. 1.** Expression and purification of sMsdS expressed in *E. coli*. Lanes: 1 and 2, soluble and insoluble fractions of recombinant BL21(DE3)/pET-msdC; 3 and 4, soluble and insoluble fractions detected by Western blotting with a His-tag-specific antibody; 5, protein marker; 6, soluble protein of recombinant BL21(DE3)/pET-msdC; 7, recombinant sMsdS purified by metal chelation affinity chromatography.

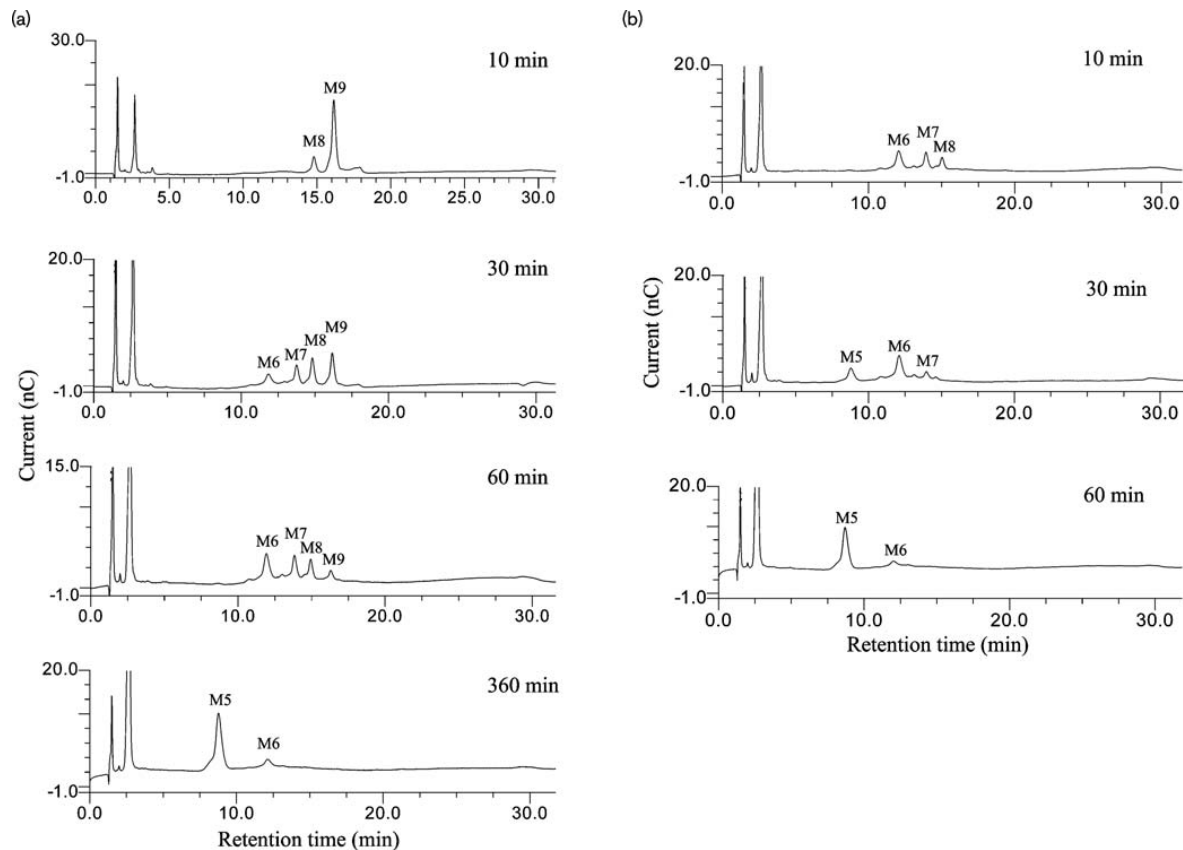
induced. After purification on a nickel column, sMsdS was purified to homogeneity, and exhibited a specific activity of 6.3 U mg<sup>-1</sup> toward Man( $\alpha$ 1,2)Man-OMe.

sMsdS did not act on pNP- $\alpha$ -mannoside. Using Man( $\alpha$ 1,2)Man-OMe as a substrate, the maximal activity (mean  $\pm$  SD, 9.15  $\pm$  0.26 units; assigned a relative activity of 100 %) of sMsdS occurred at 37–40 °C in 10 mM sodium acetate buffer (pH 5.5), and more than 80 % of the maximal activity was detected at 30–50 °C. Addition of 1 mM 1-deoxymannojirimycin to the standard assay mixture caused a complete loss of the  $\alpha$ -mannosidase activity of sMsdS, while swainsonine, a class II  $\alpha$ -mannosidase inhibitor, had no effect on its activity (9.02  $\pm$  0.15; 98.6 % relative activity); these results indicate that MsdS is a class I mannosidase. Addition of 1 mM EDTA resulted in a small decrease in activity (8.08  $\pm$  0.29 units; 88.3 % relative activity). sMsdS was able to degrade Man<sub>9</sub>GlcNAc<sub>2</sub> and Man<sub>8</sub>GlcNAc<sub>2</sub> to produce a final mixture of major Man<sub>5</sub>GlcNAc<sub>2</sub> and minor Man<sub>6</sub>GlcNAc<sub>2</sub>. It appeared that Man<sub>8</sub>GlcNAc<sub>2</sub> was the better substrate, since it was completely degraded within 30 min, while minor Man<sub>9</sub>GlcNAc<sub>2</sub> was detected in the reaction mixture after incubation for 60 min (Fig. 2).

### Deletion of the *msdS* gene in *A. fumigatus*

To investigate effects of loss of function of the *msdS* gene in *A. fumigatus*, we created the null mutant by replacing a single copy of *msdS* with *pyrG*. As a result, one mutant was obtained. PCR analysis demonstrated that the wild-type 1.67 kb fragment of *msdS* was converted into a 2.5 kb fragment containing an upstream non-coding region and *neo* in the mutant (Fig. 3a). Southern analysis of the *EcoRV*-digested genomic DNA of the mutant demonstrated that the 5.8 kb *EcoRV* fragment in the wild-type had been converted into a 6.5 kb *EcoRV* fragment (Fig. 3b). These results clearly demonstrated that the *msdS* gene was replaced by a *pyrG* gene in the  $\Delta$ msdS mutant. To ensure that all phenotypes noted for the  $\Delta$ msdS strain were the result of the specific deletion of *msdS*, the complemented strain was constructed by reintroduction of a wild-type copy of *msdS* directly into the mutated locus, under the control of its own promoter. The transformation of the complemented strain was also confirmed by PCR and Southern blot (Fig. 3).

$\alpha$ -Mannosidase activity of the  $\Delta$ msdS mutant was determined by measuring the mannose released from Man( $\alpha$ 1,2)Man-OMe (see Methods). The mean (SD)  $\alpha$ -mannosidase activity of the  $\Delta$ msdS mutant was 1.31  $\pm$  0.07 units mg<sup>-1</sup>, which was 44 % of the activity of the wild-type (2.98  $\pm$  0.23 units mg<sup>-1</sup>). The complemented strain showed an activity of 2.16  $\pm$  0.07 units mg<sup>-1</sup>. The MsdS that was secreted by the wild-type and the complemented strain was not found in the culture supernatant of the  $\Delta$ msdS mutant (Fig. 4a). Therefore, the activity detected in the mutant could be due to other  $\alpha$ -mannosidases able to cleave Man( $\alpha$ 1,2)Man-OMe. In



**Fig. 2.** Degradation of  $\text{Man}_9\text{GlcNAc}_2$  (a) and  $\text{Man}_8\text{GlcNAc}_2$  (b) by sMsdS. A 10  $\mu\text{g}$  quantity of purified sMsdS was incubated with 100 pmol  $\text{Man}_9\text{GlcNAc}_2$  or  $\text{Man}_8\text{GlcNAc}_2$  at 37  $^\circ\text{C}$  for 10–360 min. The products were analysed as described in Methods. M5,  $\text{Man}_5\text{GlcNAc}_2$ ; M6,  $\text{Man}_6\text{GlcNAc}_2$ ; M7,  $\text{Man}_7\text{GlcNAc}_2$ ; M8,  $\text{Man}_8\text{GlcNAc}_2$ ; M9,  $\text{Man}_9\text{GlcNAc}_2$ .  $\text{Man}_{5-9}\text{GlcNAc}_2$  standards were used to calibrate the retention time for each sugar chain.

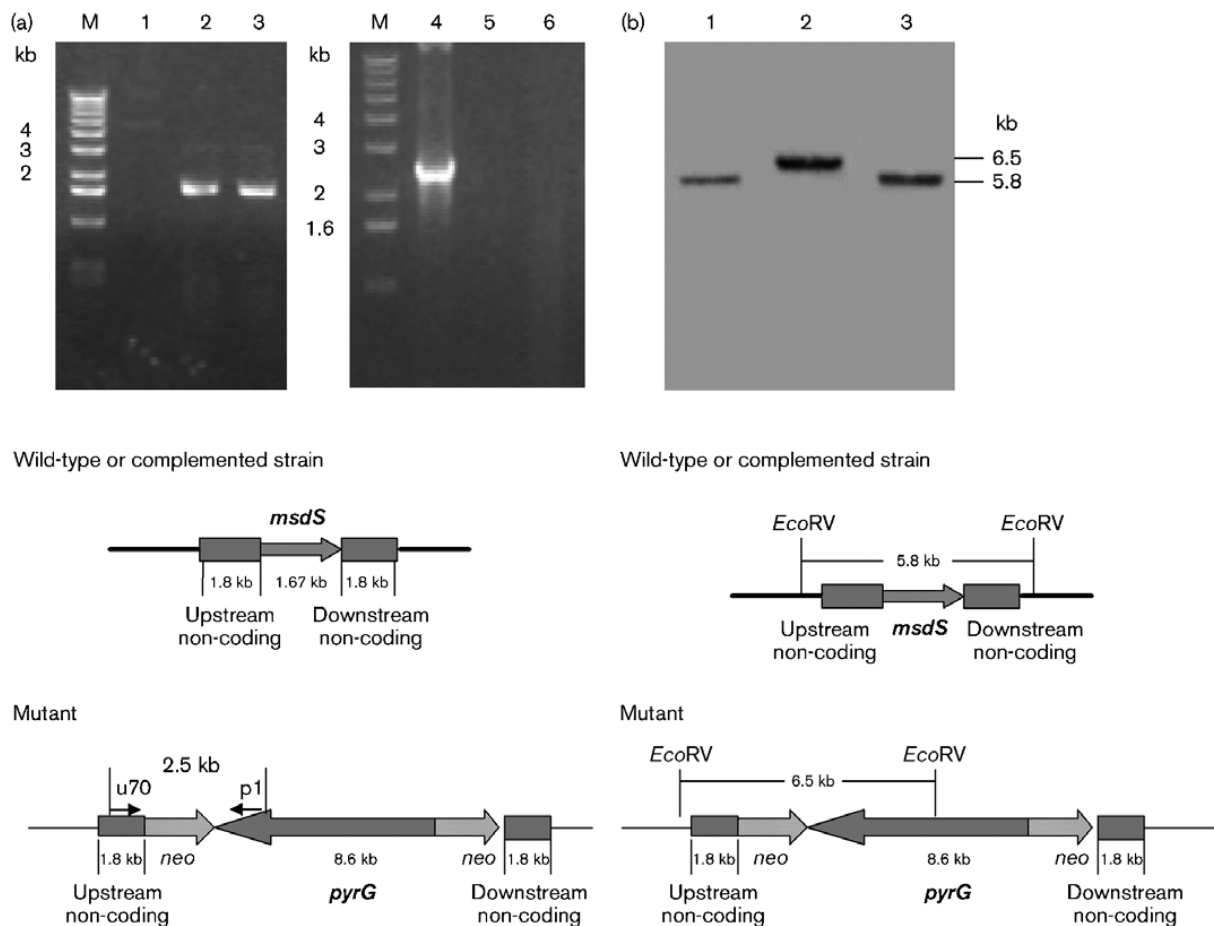
addition, the proteins, such as ChiB and Alg1, secreted by the  $\Delta\text{msdS}$  mutant were identified to be larger than their counterparts secreted by the wild-type or the complemented strain (see supplementary Table S1, available with the online version of this paper). Western blotting revealed that only one ChiB band was detected in the intracellular and secreted preparations of the mutant, and this band was slightly larger than the those from the wild-type and the complemented strain (Fig. 4b). Moreover, the *N*-glycan released from the ChiB secreted by the mutant was determined to be  $\text{Man}_8\text{GlcNAc}_2$ , whereas the *N*-glycan from the wild-type and the complemented strain was  $\text{Man}_6\text{GlcNAc}_2$  (Fig. 5). These results indicate that deletion of *msdS* in *A. fumigatus* leads to a complete loss of MsdS activity, and a defect in *N*-glycan processing. However, it appeared that *msdS* was not required for protein secretion, at least for secretion of ChiB.

### Phenotypes and virulence of the $\Delta\text{msdS}$ mutant

When the growth kinetics were determined, as described in Methods, the mutant mycelia did not show any significant difference in growth rate as compared with that of the

wild-type or the complemented strain (data not shown). We also tested the growth rate of the mutant at both 37 and 50  $^\circ\text{C}$  on solid complete medium. As shown in Fig. 6, the hyphal growth of the  $\Delta\text{msdS}$  mutant was similar to that of the wild-type or the complemented strain. When the mutant was grown in the presence of Calcofluor white or Congo red, hyphal growth was not affected at 37  $^\circ\text{C}$ . However, the mutant showed a slightly increased sensitivity to Calcofluor white and Congo red at 50  $^\circ\text{C}$ . Also, the mutant was not sensitive to SDS, as compared with the wild-type strain. These observations demonstrated that the cell wall integrity of the mutant was slightly affected at a higher temperature.

Considering that glycoproteins are involved in the synthesis and organization of the fungal cell wall, we also analysed the cell wall contents, including mannoproteins, glucans and chitin. The content of  $\alpha$ -glucan, mannoprotein,  $\beta$ -glucan and chitin in the mycelial cell wall of the mutant grown at 37 or 50  $^\circ\text{C}$  was reduced to some extent (10–27%) compared with the wild-type and the complemented strain (Table 1). Interestingly, when the temperature was elevated to 50  $^\circ\text{C}$ , the wild-type showed increases



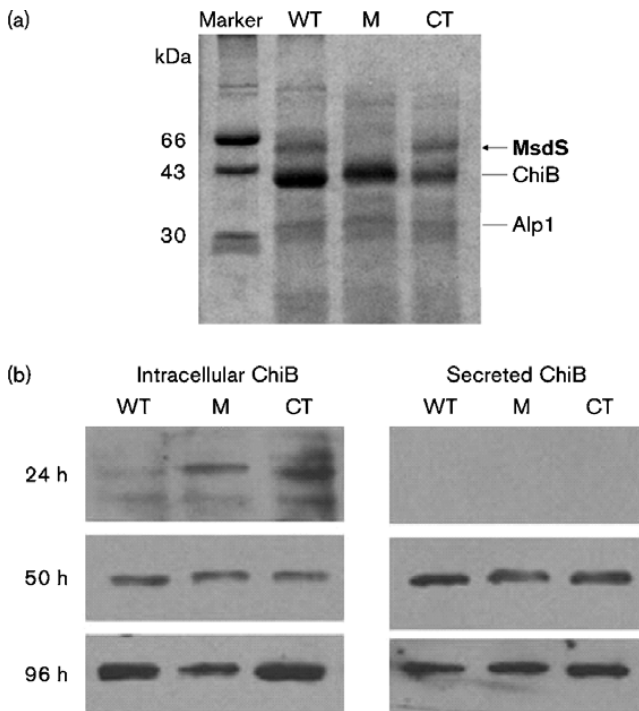
**Fig. 3.** Confirmation of the identities of the  $\Delta msdS$  mutant and the complemented strain by PCR (a) and Southern blotting (b). (a) PCR analysis was carried out as described in Methods. Lanes: 1–3, primers (forward, 5'-ATGCATTACCTCTTTGTC C-3'; and reverse, 5'-TCACGTATGATGAATTCGGAC-3') were used to amplify the 1.67 kb coding region of *msdS*; 4–6: primers (*u70* and *p1*) were used to generate a 2.5 kb DNA fragment containing an upstream non-coding region of *msdS* and partial *neo* in the  $\Delta msdS$  mutant; 1 and 4,  $\Delta msdS$  mutant; 2 and 5, wild-type; 3 and 6, complemented strain. (b) Genomic DNA digested with *EcoRV* was probed with a 2.0 kb upstream non-coding region of *msdS*. The electrophoretic positions and sizes of DNA are indicated in the panel. Lanes: 1, wild-type; 2,  $\Delta msdS$  mutant; 3, complemented strain.

of 15, 47 and 34% for  $\alpha$ -glucan,  $\beta$ -glucan and chitin, respectively, and mannoprotein was decreased by 17%, as compared with cultivation at 37 °C. Also at 50 °C, the mutant exhibited increases of 25, 33 and 55% in  $\alpha$ -glucan,  $\beta$ -glucan and chitin, respectively, and a decrease of 13% in mannoprotein, as compared with growth at 37 °C. These data suggest that an elevated temperature could induce an increase of  $\alpha$ -glucan and chitin in the cell wall of the mutant. This is probably the reason that the mutant showed a minor temperature-sensitive defect in cell wall integrity. Electron microscopic analysis revealed that the mycelial cell wall of the mutant grown at 37 °C was normal, while the cell wall of the mutant at 50 °C was less dense, as judged by electron density, but there was no change in thickness (Fig. 7a).

Although the ultrastructure of the conidium of the mutant appeared normal (Fig. 7b), a severe defect in metulae

formation of the mutant was observed at 50 °C (Fig. 7c). Conidia counting confirmed that the mean  $\pm$  SD ( $\times 10^7$ ) conidia count of the mutant at 37 °C was  $105 \pm 13$ , and this was one-third of that of the wild-type strain ( $326 \pm 19$ ). The number of conidia produced by the mutant at 50 °C was  $0.4 \pm 0.1$ , which was only 3% of that produced by the wild-type ( $12 \pm 1$ ). These results suggest a severe defect in conidiation of the mutant, especially at a higher temperature. Conidia counts for the complemented strain were  $247 \pm 22$  and  $11 \pm 0.9$ , and for CEA17 they were  $273 \pm 13$  and  $10.6 \pm 0.7$ , at 37 and 50 °C, respectively.

Moreover, in the immunocompromised mouse model, the difference in virulence between the wild-type and the  $\Delta msdS$  mutant was not statistically significant ( $P > 0.05$ ). Taking these results together, we concluded that the *msdS* gene was not essential for the growth and virulence of *A. fumigatus*, and that deletion of this gene led to a defect in



**Fig. 4.** Detection of secreted proteins (a) and ChiB (b) of the  $\Delta msdS$  mutant. (a) The protein bands were visualized by Coomassie brilliant blue R-250, and analysed by LC-MS/MS (Table S1). (b) The strains were cultivated at 37 °C for 24, 50 and 96 h, as described in Methods. Proteins in the cell lysate or culture supernatant were run on a 12% SDS-PAGE gel, and transferred to PVDF at 300 mA for 1.5 h. The anti-ChiB mouse serum was diluted to 1:5000. Protein was detected with the enhanced chemoluminescence substrate and autoradiography on film. The mycelia and culture supernatant were collected. WT, Wild-type; M,  $\Delta msdS$  mutant; CT, complemented strain.

conidia formation, and a minor defect in cell wall integrity at 50 °C.

### Morphogenesis of the $\Delta msdS$ mutant

In general, a filamentous fungus initiates its life cycle from conidial germination, and terminates it with conidiation. When the conidia break dormancy, nuclear division is accompanied by a series of ordered morphological events, including the switch from isotropic to polar growth, the emergence of second germ tubes from the conidia, and septation. In *A. fumigatus*, it has been shown that the switch from isotropic to polar growth precedes the first mitosis during the early stage of germination. The earliest emergence of second germ tubes from the conidia occurs after the third mitotic division, and the first septation usually occurs in germlings that have undergone four rounds of mitosis (Momany & Taylor, 2000).

As shown in Fig. 8, when incubated at 37 °C in rich medium containing glucose as the carbon source, the wild-

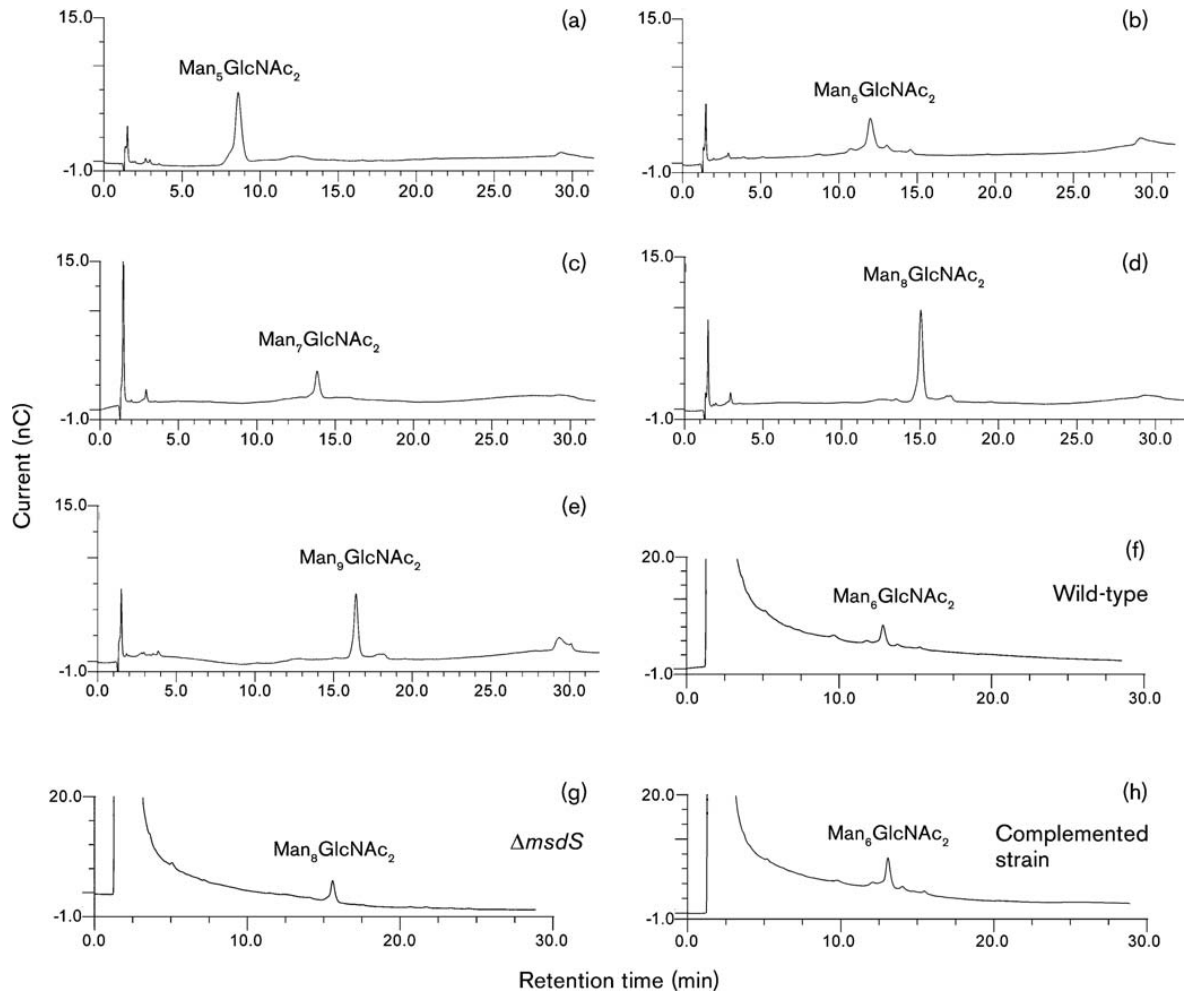
type conidium germinated in a typical bi-polar pattern at an angle of 180°, and the second germ tube and the first septation occurred after four rounds mitosis (7–8 h). The septum formed at the ‘neck’ site near the region of what was once the conidium. In comparison with the wild-type, the earliest emergence of the second germ tube occurred in the  $\Delta msdS$  mutant after the second mitotic division (5 h) at an angle of 120°, and the third germ tube, or branching of the germling, was found after the third or fourth nuclear division (6–7 h). After four rounds of mitotic division, some of germinated conidia of the mutant were not able to form a septum, while for those conidia that could form a septum, it was usually formed at the neck site of the newly emerged germ tube or germling, instead of the neck site of the first germling, as in the wild-type strain. Moreover, the germling of the mutant was more swollen than that of the wild-type. As summarized in Table 2, after two rounds of mitotic division (5 h), over 15% of the conidia of the mutant formed a second germ tube, while about 30% were found with a third germ tube after four rounds of mitosis (6 h). About 40% of conidia of the mutant formed a fourth germ tube or branching after four rounds of mitosis. These results clearly demonstrated that deletion of the *msdS* gene led to random budding and septation at an early stage of germination.

Although the growth rate of the mutant was similar to that of the wild-type, the hyphae of the mutant were found to be swollen, and contain more nuclei in each basal cell, and no septum was formed in hyphal tip after four rounds of mitotic division (Fig. 9a), suggesting reduced polar growth and septation during hyphal growth.

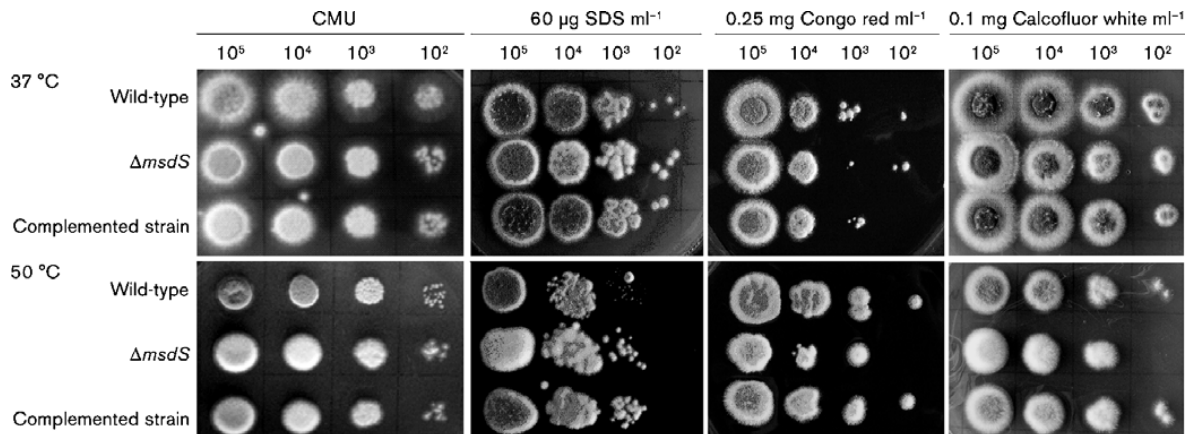
Since conidiation is the preparation for the beginning of the next life cycle, and is thus also an important event of development, we also examined the morphogenesis of the mutant at an early stage of conidiation. As shown in Fig. 9(b), the mutant was able to form a conidiophore vesicle; however, this vesicle contained more nuclei and formed less metulae than the vesicle of the wild-type, and this again suggested aberrant polarity and septation at an early stage of conidiation.

### DISCUSSION

$\alpha$ -Mannosidase I is known to play an important role in the processing of mannose-containing glycans. In humans (Rose, 1967) and cattle (Burditt *et al.*, 1978; Norden *et al.*, 1973; Phillips *et al.*, 1974), a deficiency in  $\alpha$ -mannosidase can result in the lethal disease mannosidosis. In *Drosophila melanogaster*, deletion of the Golgi mannosidase I (MAS-1) is viable, and the null organisms synthesize the same range of oligosaccharides as the wild-type, albeit with different ratios (Roberts *et al.*, 1998). In *S. cerevisiae*, disruption of the ER  $\alpha$ -mannosidase gene does not prevent outer chain synthesis (Puccia *et al.*, 1993). These observations suggest that the consequences of  $\alpha$ -mannosidase I activity vary in different species.



**Fig. 5.** Determination of *N*-glycan released from ChiB secreted by the mutant strain. The *N*-glycan was released from ChiB, as described in Methods. The released *N*-glycan was applied onto a CarboPac PA-100 column, and eluted with 250 mM NaOH at a flow rate of 1.0 ml min<sup>-1</sup>. (a–e) Standard *N*-glycan, (f) *N*-glycan from the wild-type, (g) *N*-glycan from the mutant, and (h) *N*-glycan from the complemented strain.



**Fig. 6.** Hyphal growth and sensitivity to antifungal reagents of the mutant at 37 and 50 °C. A series of 10-fold dilutions (10<sup>5</sup>–10<sup>2</sup> cells) of the wild-type, *ΔmsdS* and the complemented strain were spotted on CMU agar, with and without the antifungal reagent, and cultivated at 37 or 50 °C for 24–48 h.

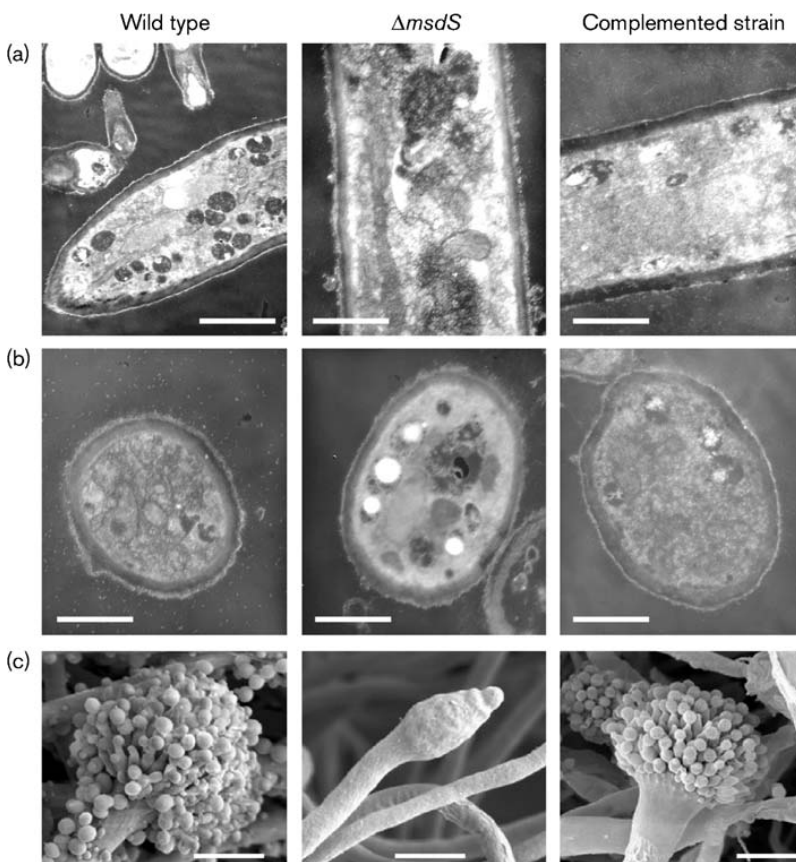
**Table 1.** Cell wall components of the mutant

Conidia were inoculated into 100 ml YPD liquid medium at a concentration of  $10^6$  conidia  $\text{ml}^{-1}$ , and incubated at 37 or 50 °C with shaking (200 r.p.m.) for 48 h. The mycelium was then harvested and lyophilized, and three aliquots of 10 mg dry mycelium were used as independent samples for the analyses of unbound cell wall proteins and water-soluble sugars, as described in Methods. The experiment was repeated three times. The values shown are mg cell wall component per 10 mg dry mycelium ( $\pm$ SD). Values in parentheses are percentages relative to the those for the wild-type.

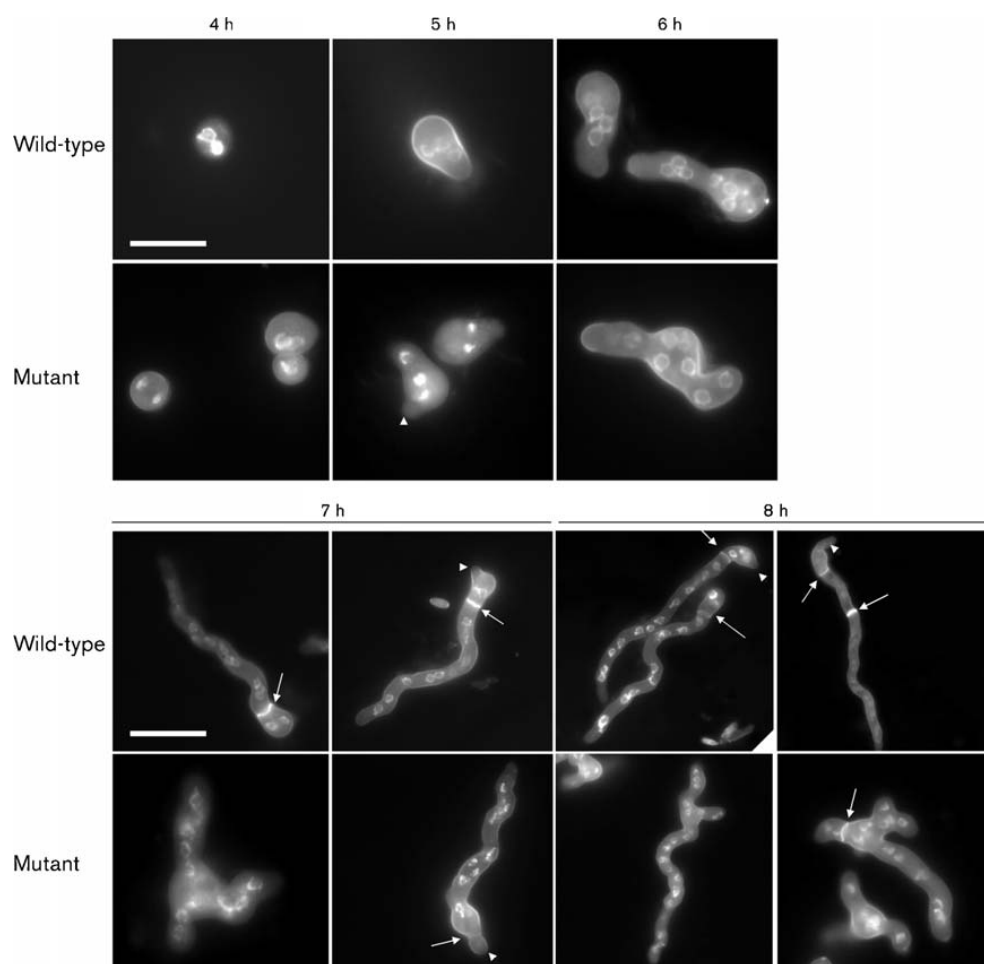
Temperature (°C)	Strain	Alkali-soluble		Alkali-insoluble	
		$\alpha$ -Glucan	Mannoprotein	$\beta$ -Glucan	Chitin
37	Wild-type	5465 $\pm$ 111 (100)	286 $\pm$ 7 (100)	8610 $\pm$ 284 (100)	471 $\pm$ 13 (100)
	$\Delta$ msdS	4520 $\pm$ 160 (83)	198 $\pm$ 7 (70)	7480 $\pm$ 237 (87)	341 $\pm$ 25 (72)
	Complemented	5129 $\pm$ 205 (94)	246 $\pm$ 14 (86)	8108 $\pm$ 222 (94)	423 $\pm$ 12 (90)
50	Wild-type	6267 $\pm$ 260 (100)	238 $\pm$ 5 (100)	12663 $\pm$ 394 (100)	629 $\pm$ 40 (100)
	$\Delta$ msdS	5663 $\pm$ 241 (90)	173 $\pm$ 2 (73)	9909 $\pm$ 255 (78)	529 $\pm$ 11 (84)
	Complemented	6354 $\pm$ 259 (101)	200 $\pm$ 12 (84)	11534 $\pm$ 465 (91)	578 $\pm$ 17 (92)

In filamentous fungi, investigations into  $\alpha$ -mannosidases have mostly concentrated on characterization, and their role in processing *N*-glycans. Little is known about their physiological importance in filamentous fungi. Our investigation of  $\alpha$ -mannosidase in *A. fumigatus* was initiated before the completion of the genome sequencing of *A. fumigatus*, and three genes were identified (including AY573554, AY852252 and AY852253). Since we originally

identified the putative class I  $\alpha$ -mannosidase gene (AY573554) by a tBLASTn search with the MsdC (P31723) of *P. citrinum*, we named the gene *AfmsdC*. In the latest release of TIGR database ([www.tigr.org/tdb/e2k1/afu1/](http://www.tigr.org/tdb/e2k1/afu1/)) (Galagan *et al.*, 2005), nine genes are annotated to encode  $\alpha$ -mannosidases, and these include: XP\_749038.1, XP\_754794.1, XP\_751252.1, XP\_751819.1, XP\_752444.1, XP\_752825.1, XP\_753592.1, XP\_751114.1 and



**Fig. 7.** Electron microscopy of the conidia and mycelia produced by the  $\Delta$ msdS mutant at 50 °C. The wild-type, the  $\Delta$ msdS mutant and the complemented strain were cultivated on solid complete medium at 50 °C. (a) After incubation for 24 h, the mycelia were fixed, and examined with a H-600 electron microscope. Bars, 0.7  $\mu\text{m}$ . (b) After incubation for 40 h, the conidia were fixed and examined with the same microscope. Bars, 0.8  $\mu\text{m}$ . (c) After incubation for 40h, the conidia were fixed, and examined with a Quanta 200 Scanning Electron Microscope. Bars, 10  $\mu\text{m}$ . Representative electron micrographs are shown.



**Fig. 8.** Germination and septation of the  $\Delta msdS$  mutant. Freshly harvested conidia ( $10^7$ ) were poured into a Petri dish containing a glass coverslip, and incubated in 10 ml complete liquid medium at 37 °C. The coverslips with adherent germlings were removed, fixed in fixative solution, and stained with Calcofluor white and DAPI, as described in Methods. Typical photographs are shown. Bars, 125  $\mu$ m. The second germ tube and the septum are marked with an arrowhead and an arrow, respectively.

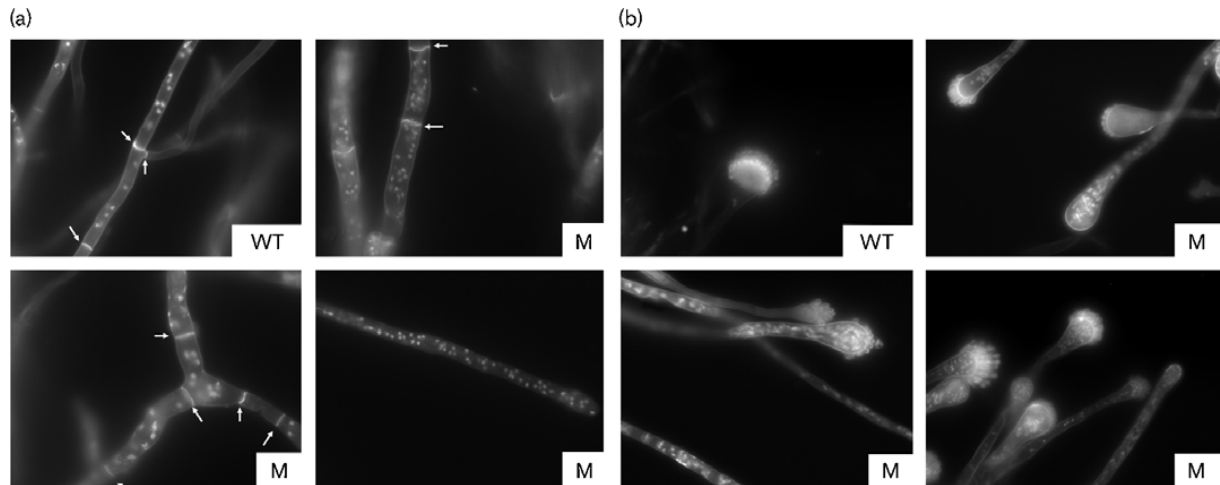
XP\_750572.1. Among these mannosidases, MsdS (XP\_752825.1) has an overall protein sequence identity

of 98 % with AfMsdC. Therefore, the gene reported here is referred to as AfmsdC/msdS.

**Table 2.** Statistics of germination of the  $\Delta msdS$  mutant

Freshly harvested conidia ( $10^7$ ) were poured into a Petri dish containing a glass coverslip and incubated in 10 ml complete liquid medium at 37 °C. The coverslips with adherent germlings were removed, and the number of germ tubes was counted under a microscope. For each independent experiment, 100 conidia were counted, and three independent experiments were carried out. The values shown are means  $\pm$  SD.

Time (h)	No. of germ tubes							
	Wild-type				$\Delta msdS$			
	0	1	2	3 or more	0	1	2	3 or more
5	33 $\pm$ 2	65 $\pm$ 4	0	0	29 $\pm$ 2	56 $\pm$ 3	15 $\pm$ 1	0
6	10 $\pm$ 1	90 $\pm$ 2	0	0	2 $\pm$ 0	68 $\pm$ 4	27 $\pm$ 2	3 $\pm$ 1
7	2 $\pm$ 1	87 $\pm$ 3	7 $\pm$ 1	4 $\pm$ 0	3 $\pm$ 1	36 $\pm$ 4	38 $\pm$ 2	23 $\pm$ 2
8	1 $\pm$ 0	77 $\pm$ 5	20 $\pm$ 2	2	1 $\pm$ 0	31 $\pm$ 2	31 $\pm$ 3	37 $\pm$ 1



**Fig. 9.** Hyphal growth (a) and conidia formation (b) of the mutant (M). A 100 ml volume of complete liquid medium was inoculated with  $10^6$  conidia, and incubated with shaking (200 r.p.m.) at 37 °C for 17 h. The mycelium was removed, and placed on a glass coverslip, which was then put in a Petri dish containing two layers filter paper saturated complete liquid medium. After incubation at 37 °C for 2–8 h, the coverslip was removed and stained, as described in Methods. The arrow indicates the septum. Representative micrographs of the mycelium (a) ( $\times 100$ ) and conidiophore vesicle (b) ( $\times 63$ ) are shown. WT, wild-type.

In this study, we showed that 1-deoxymannojirimycin was able to completely inhibit MsdS, while swainsonine had no effect. We found that sMsdS could act on all  $\alpha 1,2$  linkages in  $\text{Man}_9\text{GlcNAc}_2$  and  $\text{Man}_8\text{GlcNAc}_2$ , to produce a final product of  $\text{Man}_5\text{GlcNAc}_2$ , and that deletion of the *msdS* gene resulted in conversion of the *N*-glycan on mature ChiB from  $\text{Man}_8\text{GlcNAc}_2$  to  $\text{Man}_6\text{GlcNAc}_2$ , suggesting that MsdS hydrolyses  $\text{Man}_8\text{GlcNAc}_2$  to yield  $\text{Man}_6\text{GlcNAc}_2$ .

Although 44 % of the  $\alpha$ -mannosidase activity of the wild-type was detected in the mutant, there were several lines of evidence to show that the mutant was completely devoid of the MsdS: (i) PCR and Southern blotting analysis confirmed that the *msdS* gene was deleted in the mutant; (ii) the substrate we used for activity assay was  $\text{Man}(\alpha 1,2)\text{Man-OMe}$ , which is not a specific substrate for class I  $\alpha$ -mannosidase, and it can be cleaved by other  $\alpha$ -mannosidases found in *A. fumigatus*; (iii) the MsdS detected in the culture supernatant of the wild-type was missing in the supernatant of the mutant; and (iv) the proteins secreted by the mutant were larger than their counterparts secreted by the wild-type or the complemented strain. Indeed, the *N*-glycan on mature ChiB secreted by the mutant was  $\text{Man}_8\text{GlcNAc}_2$ , instead of  $\text{Man}_6\text{GlcNAc}_2$ , as in the wild-type and the complemented strain.

Although we observed a reduction in a number of cell wall components, and a reduced ability of conidia formation, it was apparent that deletion of *msdS* had no effect on the growth and virulence.

*O*-Mannosylation has been shown to be involved in polarized growth of *A. nidulans*. The *swaA* mutant has been identified as a single-locus temperature-sensitive

mutant that fails to switch from isotropic to polar growth. At 42 °C, each cell is swollen, and contains 64 or more nuclei after a 14 h period of growth. During growth at a restrictive temperature, multiple points of polarity are established, but polar growth can not be maintained (Momany *et al.*, 1999). The *swaA* mutant can be complemented by *pmtA*, which is the gene encoding the Pmt2 subfamily *O*-mannosyltransferase. Disruption of *pmtA* leads to a phenotype identical to that of the *swaA* mutant (Shaw & Momany, 2002). In addition, a hypersensitivity to Congo red and a decrease in conidia formation are observed (Oka *et al.*, 2004). Similarly, screening of the polarity-defective mutants in *Neurospora crassa* has led to the identification of two genes in the mannosylation pathway: *alg-1* and *sec-53*, which encode 1,4- $\beta$ -mannosyltransferase (ALG1) and phosphomannomutase (SEC53), respectively (Seiler & Plamann, 2003). Recently, a polarity defective phenotype has also been observed by disruption of the *AapmtA* gene in *Aspergillus awamori* (Oka *et al.*, 2005). These observations clearly demonstrate the involvement of the *O*-mannosylation in cell wall integrity and polarized growth in filamentous fungi. More recently, we have shown that the *Afpmt1*, a gene encoding the Pmt1 subfamily *O*-mannosyltransferase 1, is crucial for cell wall integrity and conidia morphology. However, disruption of the *Afpmt1* gene does not affect polarized growth of *A. fumigatus* (Zhou *et al.*, 2007), suggesting that proteins involved in the polarity of filamentous fungi might be *O*-mannosylated by Pmt2 subfamily *O*-mannosyltransferase.

For what is believed to be the first time, we showed that the *N*-glycan processing was involved in polarity of *A. fumigatus*. However, deletion of the *msdS* gene did not

give a phenotype identical to *A. nidulans* mutants with defective *O*-mannosylation. The  $\Delta$ *msdS* mutant showed a slight increase in sensitivity to Congo red and Calcofluor white at a higher temperature. Analysis of morphogenesis revealed that the mutant displayed a random emergence of germ tube and septum formation at an early stage of germination, swollen and multinucleate basal cells and hyphal tips during hyphal growth, and multinucleate conidiospore vesicles and reduced metulae formation, suggesting abnormalities of polarity establishment and septation during development of the mutant. Since *N*-glycosylation of proteins is known to play a variety of roles, such as protein folding, trafficking, localization and function, the phenotypes associated with the  $\Delta$ *msdS* mutant could be explained as: (i) the proteins that are required for polar growth and septation are substrates of *MsdS*, and require *N*-glycosylation for their correct localization and function; (ii) for the proteins that are involved in cell wall synthesis and organization, their localization and function are less dependent on *N*-glycosylation, as compared with *O*-mannosylation. Obviously, an understanding of the role of the *msdS* in polar growth and septation will depend on identification of the substrates of *MsdS*, and their roles in the cell.

It is not surprising that both the complemented strain and CEA17 displayed phenotypes similar to the mutant when they were used as controls. Both the complemented strain and CEA17 are devoid of the *pyrG* gene that encodes an orotidine-5'-phosphate decarboxylase, which is an enzyme that catalyses the last step of *de novo* UMP biosynthesis, which is a precursor for synthesis of glycoproteins. It has been shown that uridine/uracil deprivation in *A. fumigatus* CEA17 results in a low rate of conidium swelling, and in the inability of the conidia of the mutant to produce germ tubes (d'Enfert, 1996). Therefore, we attributed the abnormal polarity of the complemented strain to the depletion of glycoprotein synthesis, which then led to abnormal polarity.

In conclusion, we have shown that the *msdS* gene is not essential for growth and virulence of *A. fumigatus*. Although the mechanism remains unclear, our results clearly show that  $\alpha$ -mannosidase I activity of *MsdS* is involved in polarity and septation in *A. fumigatus*.

## ACKNOWLEDGEMENTS

This project was supported by the State '863' High-tech Project (2007AA02Z164), the National Natural Science Foundation of China (30621005 and 30470023), and the Chinese Academy of Sciences of China (KSCX2-3-02-01), who funded C.J. We thank C. d'Enfert for his kind supply of *A. fumigatus* strain CEA17 and plasmid pCDA14.

## REFERENCES

Akao, T., Yamaguchi, M., Yahara, A., Yoshiuchi, K., Fujita, H., Yamada, O., Akita, O., Ohmachi, T., Asada, Y. & Yoshida, T. (2006). Cloning and expression of 1,2- $\alpha$ -mannosidase gene (*fmanIB*)

from filamentous fungus *Aspergillus oryzae*: *in vivo* visualization of the FmanIBp-GFP fusion protein. *Biosci Biotechnol Biochem* **70**, 471–479.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Burditt, L. J., Phillips, N. C., Robinson, D., Winchester, B. G., Van-de-Water, N. S. & Jolly, R. D. (1978). Characterization of the mutant  $\alpha$ -mannosidase in bovine mannosidosis. *Biochem J* **175**, 1013–1022.

Chiba, Y., Yamagata, Y., Iijima, S., Nakajima, T. & Ichishima, E. (1993). The carbohydrate moiety of the acid carboxypeptidase from *Aspergillus saitoi*. *Curr Microbiol* **27**, 281–288.

Cove, D. J. (1966). The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochim Biophys Acta* **113**, 51–56.

d'Enfert, C. (1996). Selection of multiple disruption events in *Aspergillus fumigatus* using the orotidine-5'-decarboxylase gene, *pyrG*, as a unique transformation marker. *Curr Genet* **30**, 76–82.

Daniel, P. F., Winchester, B. & Warren, C. D. (1994). Mammalian  $\alpha$ -mannosidases-multiple forms but a common purpose? *Glycobiology* **4**, 551–566.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**, 350–356.

Eades, C. J. & Hintz, W. E. (2000). Characterization of the class I  $\alpha$ -mannosidase gene family in the filamentous fungus *Aspergillus nidulans*. *Gene* **255**, 25–34.

Eades, C. J., Gilbert, A. M., Goodman, C. D. & Hintz, W. E. (1998). Identification and analysis of a class 2  $\alpha$ -mannosidase from *Aspergillus nidulans*. *Glycobiology* **8**, 17–33.

Elorza, M. V., Murgui, A. & Sentandreu, R. (1985). Dimorphism in *Candida albicans*: contribution of mannoproteins to the architecture of yeast and mycelial cell walls. *J Gen Microbiol* **131**, 2209–2216.

Galagan, J. E., Calvo, S. E., Cuomo, C., Ma, L. J., Wortman, J. R., Batzoglou, S., Lee, S. I., Bastürkmen, M., Spevak, C. C. & other authors (2005). Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **438**, 1105–1115.

Hearn, V. M. & Sietsma, J. H. (1994). Chemical and immunological analysis of the *Aspergillus fumigatus* cell wall. *Microbiology* **140**, 789–795.

Ichishima, E., Taya, N., Ikeguchi, M., Chiba, Y., Nakamura, M., Kawabata, C., Inoue, T., Takahashi, K., Minetoki, T. & other authors (1999). Molecular and enzymic properties of recombinant 1,2- $\alpha$ -mannosidase from *Aspergillus saitoi* overexpressed in *Aspergillus oryzae* cells. *Biochem J* **339**, 589–597.

Kornfeld, R. & Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem* **54**, 631–664.

Kukuruzinska, M. A., Bergh, M. L. & Jackson, B. J. (1987). Protein glycosylation in yeast. *Annu Rev Biochem* **56**, 915–944.

Latgé, J. P. (1999). *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* **12**, 310–350.

Lee, J. I., Yu, Y. M., Rho, Y. M., Park, B. C., Choi, J. H., Park, H. M. & Maeng, P. J. (2005). Differential expression of the *chsE* gene encoding a chitin synthase of *Aspergillus nidulans* in response to developmental status and growth conditions. *FEMS Microbiol Lett* **249**, 121–129.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.

Maras, M., De Bruyn, A., Schraml, J., Herdewijn, P., Claeysens, M., Fiers, W. & Contreras, R. (1997). Structural characterization of *N*-linked oligosaccharides from cellobiohydrolase I secreted by the

filamentous fungus *Trichoderma reesei* RUTC 30. *Eur J Biochem* **245**, 617–625.

Maras, M., Callewaert, N., Piens, K., Claeysens, M., Martinet, W., Dewaele, S., Contreras, H., Dewerte, I., Penttila, M. & Contreras, R. (2000). Molecular cloning and enzymatic characterization of a *Trichoderma reesei* 1,2- $\alpha$ -D-mannosidase. *J Biotechnol* **77**, 255–263.

Matta, K. L. & Bahl, O. P. (1972). Glycosidases of *Aspergillus niger*. IV. Purification and characterization of  $\alpha$ -mannosidase. *J Biol Chem* **247**, 1780–1787.

Momany, M. & Taylor, I. (2000). Landmarks in the early duplication cycles of *Aspergillus fumigatus* and *Aspergillus nidulans*: polarity, germ tube emergence and septation. *Microbiology* **146**, 3279–3284.

Momany, M., Westfall, P. J. & Abramowsky, G. (1999). *Aspergillus nidulans* *swo* mutants show defects in polarity establishment, polarity maintenance and hyphal morphogenesis. *Genetics* **151**, 557–567.

Moremen, K. W., Trimble, R. B. & Herscovics, A. (1994). Glycosidases of the asparagine-linked oligosaccharide processing pathway. *Glycobiology* **4**, 113–125.

Norden, N. E., Lundblad, A., Ockerman, P. A. & Jolly, R. D. (1973). Mannosidosis in Angus cattle: partial characterization of two mannose containing oligosaccharides. *FEBS Lett* **35**, 209–212.

Oka, T., Hamaguchi, T., Sameshima, Y., Goto, M. & Furukawa, K. (2004). Molecular characterization of protein O-mannosyltransferase and its involvement in cell-wall synthesis in *Aspergillus nidulans*. *Microbiology* **150**, 1973–1982.

Oka, T., Sameshima, Y., Koga, T., Kim, H., Goto, M. & Furukawa, K. (2005). Protein O-mannosyltransferase A of *Aspergillus awamori* is involved in O-mannosylation of glucoamylase I. *Microbiology* **151**, 3657–3667.

Phillips, N. C., Robinson, D., Winchester, B. G. & Jolly, R. D. (1974). Mannosidosis in Angus cattle. The enzymic defect. *Biochem J* **137**, 363–371.

Puccia, R., Grondin, B. & Herscovics, A. (1993). Disruption of the processing  $\alpha$ -mannosidase gene does not prevent outer chain synthesis in *Saccharomyces cerevisiae*. *Biochem J* **290**, 21–26.

Roberts, D. B., Mulvany, W. J., Dwek, R. A. & Rudd, P. M. (1998). Mutant analysis reveals an alternative pathway for N-linked glycosylation in *Drosophila melanogaster*. *Eur J Biochem* **253**, 494–498.

Rose, D. P. (1967). Tryptophan metabolism in carcinoma of the breast. *Lancet* **1**, 239–241.

Schoffelmeer, E. A., Klis, F. M., Sietsma, J. H. & Cornelissen, B. J. (1999). The cell wall of *Fusarium oxysporum*. *Fungal Genet Biol* **27**, 275–282.

**1** Seiler, S. & Plamann, M. (2003). The genetic basis of cellular morphogenesis in the filamentous fungus *Neurospora crassa*. *Mol Biol Cell* **14**, 4352–4364.

Shaw, B. D. & Momany, M. (2002). *Aspergillus nidulans* polarity mutant *swoA* is complemented by protein O-mannosyltransferase *pmtA*. *Fungal Genet Biol* **37**, 263–270.

Steinbach, W. J., Stevens, D. A. & Denning, D. W. (2003). Combination and sequential antifungal therapy for invasive aspergillosis: review of published *in vitro* and *in vivo* interactions and 6281 clinical cases from 1966 to 2001. *Clin Infect Dis* **37**, S188–S224.

Tanner, W. & Lehle, L. (1987). Protein glycosylation in yeast. *Biochim Biophys Acta* **906**, 81–99.

Weidner, G., D'Enfert, C., Koch, A., Mol, P. C. & Brakhage, A. A. (1998). Development of a homologous transformation system for the human pathogenic fungus *Aspergillus fumigatus* based on the *pyrG* gene encoding orotidine 5'-monophosphate decarboxylase. *Curr Genet* **33**, 378–385.

Weng, S. & Spiro, R. G. (1993). Demonstration that a kifunensine-resistant  $\alpha$ -mannosidase with a unique processing action on N-linked oligosaccharides occurs in rat liver endoplasmic reticulum and various cultured cells. *J Biol Chem* **268**, 25656–25663.

Weng, S. & Spiro, R. G. (1996). Endoplasmic reticulum kifunensine-resistant  $\alpha$ -mannosidase is enzymatically and immunologically related to the cytosolic  $\alpha$ -mannosidase. *Arch Biochem Biophys* **325**, 113–123.

Xia, G., Jin, C., Zhou, J., Yang, S. & Zhang, S. (2001). A novel chitinase having a unique mode of action from *Aspergillus fumigatus* YJ-407. *Eur J Biochem* **268**, 4079–4085.

Yelton, M. M., Hamer, J. E. & Timberlake, W. E. (1984). Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. *Proc Natl Acad Sci U S A* **81**, 1470–1474.

Yoshida, T. & Ichishima, E. (1995). Molecular cloning and nucleotide sequence of the genomic DNA for 1,2- $\alpha$ -D-mannosidase gene, *msdC* from *Penicillium citrinum*. *Biochim Biophys Acta* **1263**, 159–162.

Yoshida, T., Inoue, T. & Ichishima, E. (1993). 1,2- $\alpha$ -D-mannosidase from *Penicillium citrinum*: molecular and enzymic properties of two isoenzymes. *Biochem J* **290**, 349–354.

Yoshihisa, T. & Anraku, Y. (1989). Nucleotide sequence of AMS1, the structure gene of vacuolar  $\alpha$ -mannosidase of *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun* **163**, 908–915.

Yoshihisa, T. & Anraku, Y. (1990). A novel pathway of import of  $\alpha$ -mannosidase, a marker enzyme of vacuolar membrane, in *Saccharomyces cerevisiae*. *J Biol Chem* **265**, 22418–22425.

Zhou, H., Hu, H., Zhang, L., Li, R., Ouyang, H., Ming, J. & Jin, C. (2007). Protein O-mannosyltransferase I (AfPmt1p) in *Aspergillus fumigatus* is crucial for cell wall integrity and conidia morphology especially at an elevated temperature. *Eukaryot Cell* **6**, 2260–2268.

Edited by: S. D. Harris

Dear Authors,

Please find enclosed a proof of your article for checking.

When reading through your proof, please check carefully authors' names, scientific data, data in tables, any mathematics and the accuracy of references. Please do not make any unnecessary changes at this stage. All necessary corrections should be marked on the proof at the place where the correction is to be made; please write the correction clearly in the margin (if in the text they may be overlooked).

Any queries that have arisen during preparation of your paper for publication are listed below and indicated on the proof. Please provide your answers when returning your proof.

Please return your proof by Fax (+44 (0)118 988 1834) within 2 days of receipt.

Query no.	Query
1	

PAPER mic2008/017525

Please quote this number in any correspondence

Authors Y. Li and others

Date \_\_\_\_\_

I would like 25 free offprints, plus  additional offprints, giving a total of  offprints

Dispatch address for offprints (BLOCK CAPITALS please)

\_\_\_\_\_  
 \_\_\_\_\_  
 \_\_\_\_\_

Please complete this form **even if you do not want extra offprints**. Do not delay returning your proofs by waiting for a purchase order for your offprints: the offprint order form can be sent separately.

Please pay by credit card or cheque with your order if possible. Alternatively, we can invoice you. All remittances should be made payable to 'Society for General Microbiology' and crossed 'A/C Payee only'.

Tick one

- Charge my credit card account (give card details below)
- I enclose a cheque/draft payable to Society for General Microbiology
- Purchase order enclosed

Return this form to: *Microbiology* Editorial Office, Marlborough House, Basingstoke Road, Spencers Wood, Reading RG7 1AG, UK.

### CHARGES FOR ADDITIONAL OFFPRINTS

Copies	25	50	75	100	125	150	175	200	Per 25 extra
No. of pages									
1-2	£23	£40	£58	£76	£92	£110	£128	£145	£23
3-4	£35	£58	£81	£104	£128	£150	£173	£191	£29
5-8	£46	£76	£104	£133	£162	£191	£219	£249	£35
9-16	£58	£92	£128	£162	£196	£231	£267	£301	£40
17-24	£70	£110	£151	£191	£231	£272	£312	£353	£46
each 8pp extra	£18	£23	£29	£35	£40	£46	£53	£58	

### OFFICE USE ONLY

Issue:
Vol/part:
Page nos:
Extent:
Price:
Invoice: IR/

### PAYMENT BY CREDIT CARD (Note: we cannot accept American Express)

Please charge the sum of £\_\_\_\_\_ to my credit card account.

My Mastercard/Visa number is (circle appropriate card; no others acceptable):

<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
----------------------	----------------------	----------------------	----------------------

Expiry date

<input type="text"/>	<input type="text"/>
----------------------	----------------------

Security Number

<input type="text"/>	<input type="text"/>
----------------------	----------------------

Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Cardholder's name and address\*: \_\_\_\_\_

\*Address to which your credit card statement is sent. Your offprints will be sent to the address shown at the top of the form.