

# Characterization of the *Aspergillus fumigatus* phosphomannose isomerase Pmi1 and its impact on cell wall synthesis and morphogenesis

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Phosphomannose isomerase (PMI) is an enzyme catalysing the interconversion of mannose 6-phosphate (Man-6-P) and fructose 6-phosphate (Fru-6-P). The reaction catalysed by PMI is the first committed step in the synthesis of mannose-containing sugar chains and provides a link between glucose metabolism and mannosylation. In this study, the *pmi1* gene was identified to encode PMI in the human fungal pathogen *Aspergillus fumigatus*. Characterization of *A. fumigatus* Pmi1 expressed in *Escherichia coli* revealed that this PMI mainly catalysed the conversion of Fru-6-P to Man-6-P and that its binding affinity for Man-6-P was similar to that of yeast PMIs, but different to those of PMIs from bacteria or animals. Loss of *pmi1* was lethal unless Man was provided in the growth medium. However, a  $\Delta pmi1$  mutant cell showed a significantly reduced growth rate at a high concentration of Man. Biochemical analysis revealed that both inadequate and replete Man led to an accumulation of intracellular Man-6-P and a reduction in the amount of  $\alpha$ -glucan in the cell wall. Uncoupling of the link between energy production and glycosylation by deletion of the *pmi1* gene led to phenotypes such as defects in cell wall integrity, abnormal morphology and reduced conidiation. Our results reveal that PMI activity is essential for viability and plays a central regulatory role in both cell wall synthesis and energy production in *A. fumigatus*.

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## INTRODUCTION

*Aspergillus fumigatus* is the predominant mould pathogen of humans, causing fatal invasive aspergillosis among the immunocompromised population (Latgé, 1999, 2001; Krappmann, 2006). The crude mortality is 60–90% and remains around 29–42% even when treatment is given (Zmeili & Soubani, 2007). A deep understanding of *A. fumigatus* at the molecular level will help in the development of efficient drug therapies to treat invasive aspergillosis. Since the fungal cell wall has a variety of biological functions, such as maintaining cell shape and providing osmotic and physical protection against an adverse environment (Yoda *et al.*, 2000; Agaphonov *et al.*, 2001), it has been recognized for a long time as essential and as a focus for the discovery of unique, specific drug targets. The *A. fumigatus* cell wall mainly consists of a

covalently connected polysaccharide skeleton interlaced and coated with glycoproteins and glycosylphosphatidylinositol (GPI) proteins, which contain *N*- and *O*-glycans derived primarily from the process of glycosylation (Latgé, 2007). Although these glycoproteins are involved in morphogenesis and cell wall organization (Mouyna *et al.*, 2000, 2005; Bruneau *et al.*, 2001; Chabane *et al.*, 2006; Romano *et al.*, 2006; De Groot *et al.*, 2005; Li *et al.*, 2007), it is poorly understood how glycosylation affects the cell wall organization.

Mannose (Man) is a major component of galactomannan and glycoprotein. Man activation first requires conversion to mannose 6-phosphate (Man-6-P), which occurs by one of two routes: direct phosphorylation of Man by hexokinase or interconversion from fructose 6-phosphate (Fru-6-P) via phosphomannose isomerase (PMI), the latter linking glycolysis to protein glycosylation. Man-6-P is converted to mannose 1-phosphate (Man-1-P) and then to GDP-mannose (GDP-Man), the central activated Man donor in glycosylation reactions. The interconversion of Man-6-P and Fru-6-P catalysed by PMI is the first committed step in the synthesis of Man-containing sugar chains and provides a link between glucose metabolism and mannosylation. In humans, PMI deficiency is the cause of carbohydrate-

**Abbreviations:** Fru-1,6-P, fructose 1,6-bisphosphate; Fru-6-P, fructose 6-phosphate; GDP-Man, GDP-mannose; Glc-6-P, glucose 6-phosphate; HPAEC-PAD, high-performance anion-exchange chromatography pulsed ampere detector; Man-1-P, mannose 1-phosphate; Man-6-P, mannose 6-phosphate; PMI, phosphomannose isomerase.

The GenBank/EMBL/DDBJ accession number for the *pmi1* gene sequence of *Aspergillus fumigatus* is AY700212.

deficient glycoprotein syndrome type Ib (CDG-Ib, OMIM 602579), but the clinical symptoms and aberrant glycosylation can be corrected with dietary Man supplements (Davis *et al.*, 2002). Although Man is beneficial for CDG-Ib patients, it is toxic to honeybees and becomes teratogenic to mid-stage rat embryos when given in high concentrations (Sols *et al.*, 1960; de la Fuente *et al.*, 1986; Freinkel *et al.*, 1984; Moore *et al.*, 1987; Buchanan *et al.*, 1985; DeRossi *et al.*, 2006). The toxicity appears to stem from an accumulation of Man-6-P which cannot efficiently enter glycolysis, instead becoming trapped in a cycle of dephosphorylation and rephosphorylation resulting in depletion of intracellular ATP.

To date, genes encoding PMIs have been isolated from several fungal species, including *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus nidulans* and *Cryptococcus neoformans* (Wells *et al.*, 1993; Coulin *et al.*, 1993; Smith & Payton, 1994; Wills *et al.*, 2001; Smith *et al.*, 1992). The *S. cerevisiae* PMI is encoded by the *PMI40* gene (Smith *et al.*, 1992). Loss of *pmi40* is lethal unless Man is provided in the growth medium (Payton *et al.*, 1991). However, the *pmi* mutant cells show a significantly reduced growth rate at a high concentration of Man. Biochemical and genome-wide analysis reveals that excess Man leads to an accumulation of intracellular Man-6-P, which mainly inhibits the activity of phosphoglucose isomerase and thus represses glycolysis, protein biosynthesis and cell wall biogenesis (Pitkänen *et al.*, 2004). Cells of an *A. nidulans manA1* mutant exhibited abnormal ballooning hyphal tips and eventually ceased to grow (Upadhyay & Shaw, 2006). A disrupted mutant of *Cry. neoformans* showed poor capsule formation, reduced polysaccharide secretion, morphological abnormalities and attenuated virulence (Wills *et al.*, 2001).

The aforementioned reports imply important roles for GDP-Man biosynthesis in fungi, insects and mammals; however, it seems that the significance of GDP-Man biosynthesis varies in different species. As PMI has been recognized as an antifungal drug target (Proudfoot *et al.*, 1994a; Bhandari *et al.*, 1998), investigation of the *A. fumigatus* PMI will provide us with a better understanding of fungal PMIs and should thus be helpful for the rational design of an inhibitor specific for fungal PMI without altering the activity of the mammalian enzyme. To this end, the *pmi1* gene in *A. fumigatus* was identified, expressed and characterized in this study. In addition, by construction of a null mutant through targeted gene disruption, the impact of Man metabolism on cell wall synthesis and morphogenesis was analysed.

## METHODS

**Strains and growth conditions.** *A. fumigatus* strain YJ-407 (CGMCC0386) was maintained on potato glucose (2%) agar slants (Xia *et al.*, 2001). *A. fumigatus* strain CEA17, a gift from C. d'Enfert, Institut Pasteur, Paris, France, was propagated at 37 °C on YGA with addition of 5 mM uridine and uracil (Weidner *et al.*, 1998). The PMI mutant strain was created from CEA17 and was maintained on CM

medium (Cove, 1966) supplemented with Man. *A. fumigatus* carrying the plasmid pPTR-II (Takara) was grown and maintained on Czapek-Dox (CD) medium supplemented with 0.1 mg pyrithiamine l<sup>-1</sup> and 0.8 M NaCl (Kubodera *et al.*, 2002). Strains were grown in liquid CM at 37 °C, with shaking at 250 r.p.m. At the specified culture time point, mycelia were harvested, washed with distilled water, frozen in liquid N<sub>2</sub>, and then ground using a mortar and pestle. The powder was stored at -70 °C for DNA, RNA and protein extraction. Conidia were prepared by growing *A. fumigatus* strains on solid CM with uridine and uracil for 48 h at 37 °C. The spores were collected, washed twice with 0.1% Tween 20 in physiological saline, resuspended in 0.1% Tween 20 in saline, and the concentration of spores was confirmed by haemocytometer counting and viable counting. Vectors and plasmids were propagated in *Escherichia coli* DH5 $\alpha$  (Bethesda Research Laboratories).

**Molecular cloning of the *A. fumigatus pmi1* gene.** The *pmi1* gene 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 *Can. albicans PMI1* that are homologous between *A. nidulans*, *S. cerevisiae* and *Cry. neoformans*. A 1637 bp genomic DNA fragment was found to contain the entire ORF. Based on the nucleotide sequence, the forward primer P1 (5'-ATGCCTCCAGTACCACTACTTCGAC-3') and the reverse primer P2 (5'-CTAGT TTCCGTTTACCATGTCCTCC-3') were designed for cloning the cDNA of the *A. fumigatus pmi1* gene by PCR. The PCR products were subcloned into the pGEM-T easy vector (Promega) to obtain T-manA.

**Expression of the *pmi1* gene in *E. coli*.** The cDNA of the *A. fumigatus pmi1* gene was amplified from T-manA with primers P3 (5'-GGTGGTCCATGGCCATGATGCCTCCAGTACCACTAC-3') and P4 (5'-ACCACCGTCGACCTAGTTTCCGTTTACCATGTCCT-3') and subcloned into pET-30 $\alpha$  (Novagen). The resulting recombinant plasmid was designated pET30-PMI. The recombinant *E. coli* BL21(DE3) strain (Novagen) harbouring pET30-PMI was grown in 5 ml Luria-Bertani (LB) medium containing 50  $\mu$ g kanamycin ml<sup>-1</sup>, at 37 °C overnight. One millilitre of cell culture was inoculated into 100 ml LB containing 50  $\mu$ g kanamycin ml<sup>-1</sup>, and incubated at 37 °C. When the OD<sub>600</sub> value of the cell culture reached 0.6, the recombinant protein was induced by the addition of IPTG (Sigma-Aldrich) to a final concentration of 0.4 mM, followed by incubation at 25 °C for 8 h. The cells were harvested by centrifugation and resuspended in 50 ml 1  $\times$  binding buffer (0.02 M sodium phosphate, 0.5 M NaCl, 80 mM imidazole, pH 8.0). After sonication, the cell lysate was collected by centrifugation (17 500 g for 30 min at 4 °C), filtered through a 0.45  $\mu$ m membrane, and run on a HiTrap chelating HP column (Amersham Pharmacia Biotech). After washing with 20 column-volumes of binding buffer, the recombinant protein was eluted with a gradient of imidazole (80–500 mM) and dialysed against 50 mM Tris buffer, pH 7.6. The purity of the recombinant protein was judged by SDS-PAGE. The protein concentration was determined by the Bradford assay (Bradford, 1976).

**Activity assay.** PMI activity was assayed in the reverse direction by the method described by Slein (1950) using a modified assay mixture. A 0.5 ml reaction mix contained 50 mM Tris/HCl (pH 7.6), 1 mM EDTA, 2.5 mM ZnCl<sub>2</sub>, 0.5 mM Man-6-P, 0.5 mM NADP<sup>+</sup>, 1 unit phosphoglucose isomerase, 1 unit glucose 6-phosphate (Glc-6-P) dehydrogenase and 4 ng purified recombinant enzyme. The rate of NADP<sup>+</sup> reduction was monitored at 340 nm at 37 °C. One unit of enzyme activity is defined as 1  $\mu$ mol NADP<sup>+</sup> reduced per minute under the above-specified conditions.

**Kinetics and activity assay using HPAEC-PAD.** For measurement of the reversible isomerization between Fru-6-P and Man-6-P, the

product of the enzyme reaction was accurately analysed with a high-performance anion-exchange chromatography pulsed ampere detector (HPAEC-PAD, Dionex). Ten nanograms of purified recombinant Pmi1 was incubated with 0.297–11.88 mM Fru-6-P (Roche) or 0.25–12.5 mM Man-6-P, at 37 °C for 5 min. The reaction was terminated by heating to 100 °C for 15 min. Upon addition of 2 vols ethanol, proteins in the reaction mixture were precipitated and removed by centrifugation. The product in the supernatant, Man-6-P or Fru-6-P, was analysed with a CarboPac PA-1 anion-exchange column (Dionex). The products were eluted with 100 mM NaOH and 200 mM sodium acetate at a flow rate of 1.0 ml min<sup>-1</sup>.

**Generation of polyclonal antibodies against rPmi1 and Western blot analysis.** Purified recombinant Pmi1 was lyophilized, dissolved in PBS and injected subcutaneously into a mouse four times in 45 days. The collected serum was tested by Western blot analysis. Proteins in the cell lysate were run on a 12% SDS-PAGE gel and transferred to PVDF (Bio-Rad) at 300 mA for 1.5 h. The anti-rPMI mouse serum was diluted 1:500. Protein was detected with the enhanced chemiluminescence substrate (Pierce Biotechnology) and autoradiography film.

**Construction of the *pmi1* null mutant strain.** A deletion construct was designed to replace the entire coding region of *A. fumigatus pmi1* with a *pyrG* cassette by homologous recombination (d'Enfert, 1996). PCR primers were designed to amplify a 1.6 kb upstream flanking region of the *pmi1* gene before the ATG start codon (5' primer pair: 5'-GGTGGTTCGAC GCGGCCGCTCTGATACTTTCTGCCGTTTC-TTC-3' and 5'-ACCACCGAATTCCAATGACAAAACTACC-GCACTGGAG-3'; the *Sall*, *NotI* and *EcoRI* restriction sites are underlined), and a 1.6 kb downstream flanking region of the *pmi1* gene after the stop codon (3' primer pair: 5'-CCGGAATTC-CGATATCCCACTTCGACTACATTTGCTGCCG-3' and 5'-CCACT-CTAGAATCCACACAAACCCCTTACAGCCGCGC-3'; the *EcoRI*, *EcoRV* and *XbaI* restriction sites are underlined). 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 *pmi1* gene to yield the deletion construct pBKPMI. pBKPMI, linearized at two *NotI* sites, was transformed into strain CEA17 by protoplast transformation (Yelton *et al.*, 1984), and colonies were screened to detect mutants with mannose auxotrophy. The mutants were confirmed by PCR and then by Southern blot analysis. For PCR analysis, primers P5 (5'-TAACTCAGTCCGCTTGATTC-3') and P6 (5'-CAATCCGGTTCGCTTGCTG-3') were used to generate a fragment containing the upstream flanking sequence of the *pmi1* gene and a partial *neo* gene; primers P7 (5'-CAGGACATAGCGTTGGCTAC-3') and P8 (5'-ATACTCTAGGTCATCTCTCTT-3') were used to amplify a partial *neo* gene and the downstream non-coding sequence. For Southern blotting, genomic DNA was digested with *Bam*HI and the upstream non-coding region was used as a probe. The probe was labelled following the protocol of the DIG-labelled hybridization kit (Roche; cat. no. 1093657).

**Complementation of the  $\Delta$ *pmi1* mutant with the autonomously replicating shuttle vector pPTR II.** PCR was performed to amplify the entire *pmi1* gene and flanking region (from -279 to +2443) using primers P9 (5'-GGGGTACCATGACGGATGCGGTACGCAAAT-3') and P10 (5'-GGGGTACCCAGATCGGAGTGGCGGATGTTG-3'), pyrobst DNA polymerase and genomic DNA as template. The amplified 2.7 kb DNA fragment was digested with *KpnI* and inserted into the *KpnI* site of pPTR II (Takara) carrying the *ptrA* gene (pyrithiamine resistance gene from *Aspergillus oryzae*) as a selective marker, to yield pPTRII-*pmi1*. The *pmi1* mutant was transformed with pPTRII-*pmi1* and transformants were selected on CD medium supplemented with 0.1 mg pyrithiamine l<sup>-1</sup> and 0.8 M NaCl. Three

transformants were confirmed to carry pPTRII-*pmi1* by PCR analysis using primers P11 (5'-CAATTGATTACGGGATCCCAT-3') and P12 (5'-CTCTTGCACTTTGTTGTATT-3') to amplify the *ptrA* gene (Kubodera *et al.*, 2002).

**Chemical analysis of the cell wall.** Conidia were inoculated into 100 ml complete liquid 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 24 h. The mycelium was harvested, washed with deionized water and frozen at -80 °C. To isolate cell walls, 10 mg of dry mycelial pad was added to a tube containing 50 mM NH<sub>4</sub>HCO<sub>3</sub> at pH 8.0 and 0.2 g of glass beads (1 mm diameter). The mycelium was disrupted by successively shaking the tube with a Disruptor Genie (Scientific Industries) five times for 5 min each time. The cell homogenates were then centrifuged and washed several times. Three independent samples of lyophilized mycelial pad were used for cell wall analysis, and the experiment was repeated twice.

The cell walls were boiled for 5 min in 1 ml 2% SDS in 50 mM Tris/HCl buffer supplemented with 100 mM EDTA, 40 mM  $\beta$ -mercaptoethanol and 1 mM PMSF to remove noncovalently bound proteins and membrane fragments. Cell walls were collected by centrifugation, extracted for a second time and washed three times with deionized water. After washing, cell walls were treated with 1 M KOH and incubated at 70 °C for 30 min under N<sub>2</sub> to release glycoprotein and  $\alpha$ -glucan. The alkali-soluble materials were acidified with acetic acid to pH 5.0 and the precipitated  $\alpha$ -glucans were collected by centrifugation and washed with water. The glycoprotein in the supernatant was precipitated with 2 vols of ethanol, washed twice with 64% ethanol and dissolved in distilled water. The glycoprotein concentration was determined using the Bradford assay (Bradford, 1976). Monosaccharides were liberated from glycoprotein by acid hydrolysis (6 M HCl at 100 °C for 2 h) and separated on a CarboPac PA1 anion-exchange column, equipped with an Amino Trap guard column. Elution was performed at room temperature at a flow rate of 1 ml min<sup>-1</sup> with 18 mM NaOH.

The alkali-insoluble materials were washed with water several times and digested in 6 M HCl at 100 °C for 2 h to release monosaccharides from  $\beta$ -glucan and chitin. After digestion, HCl was evaporated and the residues were dissolved in 0.2 ml distilled water (Elorza *et al.*, 1985; Hearn & Sietsma, 1994; Schoffemeer *et al.*, 1999). The amounts of  $\alpha$ -glucan and  $\beta$ -glucan present were estimated by measuring released glucose using the phenol/sulfuric acid method (Dubois *et al.*, 1956). Chitin content was determined by measuring the *N*-acetylglucosamine released after digestion using the method described by Lee *et al.* (2005).

**Analysis of intracellular metabolites.** Conidia were inoculated into 100 ml liquid 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 24 h. The mycelium was harvested and quenched immediately in liquid nitrogen. The frozen suspension was lyophilized at -80 °C.

Intracellular sugars and sugar phosphates were extracted with chloroform/methanol buffer (Ruijter & Visser, 1996) with some modifications. Fifty milligrams of dry mycelial pad was resuspended in 0.5 ml cold chloroform (-40 °C), followed by 0.25 ml cold methanol (-40 °C). The sample was then incubated for 30 min to allow permeabilization of cells and denaturation of enzymes. Subsequently, 0.2 ml ice-cold buffer (3 mM HEPES, 3 mM EDTA, pH 7.0) was added to the mixture, followed by vigorous mixing and shaking at 200 r.p.m. for 30 min at <0 °C. The water/methanol and chloroform phases were separated by centrifugation at 5000 g for 5 min at <0 °C. The upper aqueous phase containing the polar metabolites was taken off and transferred to another tube. A second extraction was initiated by adding 0.2 ml methanol (-40 °C) and 0.2 ml ice-cold buffer to the chloroform phase, followed by vigorous mixing. After centrifugation at 5000 g for 5 min at less than 0 °C, the

upper aqueous phase was collected and incorporated with the previous extract and the solvents were removed by lyophilization.

Phosphosugars were analysed on a CarboPac PA-1 column (4 × 250 mm preceded by a PA-1 guard column) with HPAEC-PAD. Ten microlitres of metabolite extraction was injected onto the column and eluted with sodium acetate (in 5 mM NaOH). The sodium acetate gradient was increased from 0.1 to 0.2 M between 0 and 20 min, to 0.5 M between 20 and 30 min, to 0.8 M between 30 and 40 min, and then to 0.99 M between 40 and 45 min. The flow rate of the mobile phase was set at 1 ml min<sup>-1</sup>. Metabolites were quantified by comparison of calibration curves prepared with known quantities of Glc-6-P, Fru-6-P, fructose 1,6-bisphosphate (Fru-1,6-P), Man-6-P, Man-1-P or GDP-Man standards.

## RESULTS

### Expression and characterization of the *pmi1* gene

The putative gene encoding PMI was identified by a tBLASTn search of the *A. fumigatus* genome database with the *Can. albicans* *PMI1* gene (X82024). In the latest release of the *A. fumigatus* genome database, this gene is annotated as *pmi1* (AFUA\_1G13280) (<http://www.tigr.org/tdb/e2k1/afu1/>). The *pmi1* gene is 1637 bp in length and contains four introns and five exons. A 1374 bp region of the *pmi1* cDNA (AY700212) encodes a protein of 457 aa (AAU06585) that shares 81%, 47%, 45%, 41% and 39% identity with those from *A. nidulans*, *Can. albicans*, *S. cerevisiae*, *Cry. neoformans* and *Homo sapiens*, respectively.

As described in Methods, a 55 kDa recombinant PMI protein (rPmi1) was expressed and purified to homogeneity (data not shown). The purified rPmi1 exhibited a specific activity of 149.71 U mg<sup>-1</sup> toward Man-6-P. The highest activity was detected in a buffer of pH 8.0 at 40 °C. Its activity was completely inhibited by the addition of 5 mM EDTA and reactivated by cations in the following order: Mn<sup>2+</sup> > Ca<sup>2+</sup> > Zn<sup>2+</sup> > Co<sup>2+</sup> > Ni<sup>2+</sup> > Mg<sup>2+</sup>. Complete recovery was observed in the presence of 2.5 mM MnCl<sub>2</sub>. Incubation of the rPmi1 at 40 °C or 45 °C for 60 min caused a 25% or 70% loss of activity, respectively. When incubated at 50 °C for 5 min, the rPmi1 only retained 36% activity.

Using the method described by Slein (1950), the *K<sub>m</sub>* of the rPmi1 for Man-6-P was determined to be 1 mM, which is similar to the *K<sub>m</sub>* of the PMI proteins from *Can. albicans* (1.24 mM) or *S. cerevisiae* (0.65 mM), higher than those from pig (0.17 mM) and human (0.25 mM), and lower than those from *Xanthomonas campestris* (2 mM), *Pseudomonas aeruginosa* (3.03 mM) and *Burkholderia cepacia* (9.01 mM) (Proudfoot *et al.*, 1994a, b; Wells *et al.*, 1993; Coulin *et al.*, 1993; Darzins *et al.*, 1985; Papoutsopoulou & Kyriakidis, 1997; Shinabarger *et al.*, 1991; Gracy & Noltmann, 1968; Sousa *et al.*, 2007), suggesting that binding affinity of the *A. fumigatus* Pmi1 is similar to that of PMI proteins from *Can. albicans* or *S. cerevisiae* but different from those from bacteria or animals.

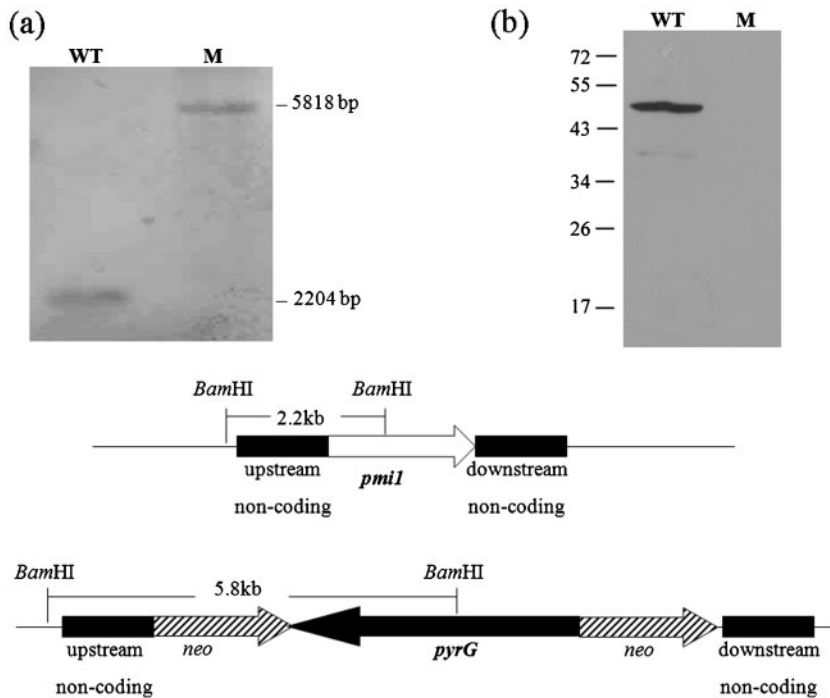
To accurately assess the conversion rate between Man-6-P and Fru-6-P, we also directly determined the amount of

Fru-6-P or Man-6-P when Man-6-P or Fru-6-P was used as a substrate separately. As a result, the *K<sub>m</sub>* values for Man-6-P and Fru-6-P were determined as 2.59 mM and 1.38 mM, respectively. The *k<sub>cat</sub>*/*K<sub>m</sub>* values for Man-6-P and Fru-6-P were calculated as 9.7 and 64.9 mM<sup>-1</sup> min<sup>-1</sup>, respectively. These results demonstrate that *A. fumigatus* Pmi1 mainly catalyses the conversion of Fru-6-P to Man-6-P.

### Deletion of the *pmi1* gene in *A. fumigatus*

To investigate the impact of the PMI activity in *A. fumigatus*, a null mutant was constructed by replacing a single copy of *pmi1* with *pyrG*. We hypothesized that this gene disruption would be lethal to the *A. fumigatus* cells if it was not rescued by the addition of exogenous Man to the growth medium. Thus, the transformants were grown on medium supplemented with D-Man. The Man auxotroph transformants were first screened by PCR amplification of the *pmi1* gene. Five transformants were identified as candidate mutant strains. Southern blotting analysis of *Bam*HI-digested genomic DNA of the mutants confirmed only one transformant to be the correct mutant, in which the wild-type 2.2 kb *Bam*HI fragment was converted into a 5.8 kb *Bam*HI fragment (Fig. 1a). Western blotting confirmed that the Pmi1 protein was not expressed in this mutant (Fig. 1b). No PMI activity was detected in the  $\Delta$ *pmi1* mutant. These results clearly showed that the *pmi1* gene was completely deleted.

To complement the *pmi1* gene in the  $\Delta$ *pmi1* mutant, the recombinant plasmid pPTR-*pmi1* was constructed by the insertion of the *pmi1* gene into the autonomously replicating vector pPTR II and this was transformed into the  $\Delta$ *pmi1* mutant. The transformants were screened on CD medium containing 0.1 µg pyrithiamine ml<sup>-1</sup> and three transformants were confirmed to carry pPTR-*pmi1* by PCR (data not shown). An activity assay revealed that PMI activity of the complemented strain grown in CD containing 0.1 µg pyrithiamine ml<sup>-1</sup> was 66% (0.11 ± 0.01 U mg<sup>-1</sup>) of that in the wild-type (0.17 ± 0.01 U mg<sup>-1</sup>) grown in CD medium, while no PMI activity was detected in the  $\Delta$ *pmi1* mutant in CD supplemented with 3 mM Man. Following incubation in CM medium, the PMI activity detected in the complemented strain was only 10% of that in the wild-type and no activity was detected in the mutant. The complemented strain grown on solid CM only showed a slight growth retardation compared to the wild-type, but its colony morphology was similar to that of the mutant grown on CM supplemented with 3 mM Man (Fig. 2a). These results indicate that pPTR-*pmi1* can apparently restore the growth phenotype of the mutant; however, it is not stably maintained without the addition of pyrithiamine in the medium. On the other hand, it should be pointed out that although the complemented strain grew well on CD containing 0.1 µg pyrithiamine ml<sup>-1</sup>, the wild-type strain was severely inhibited on the same medium, suggesting a toxicity of pyrithiamine to the wild-type *A. fumigatus*. We



**Fig. 1.** Confirmation of the  $\Delta pmi1$  mutants by Southern blotting (a) and Western blotting (b). (a) Genomic DNA digested with *Bam*HI was probed with a 1.6 kb upstream non-coding region of the *pmi1* gene. The electrophoretic positions and sizes of DNA are indicated. (b) The cytosolic fraction was detected by Western blotting with anti-rPmi1 serum. WT, wild-type; M,  $\Delta pmi1$  mutant.

therefore cultivated the complemented strain using CM or CD containing pyrithiamine in this study.

### Growth phenotypes of the $\Delta pmi1$ mutant

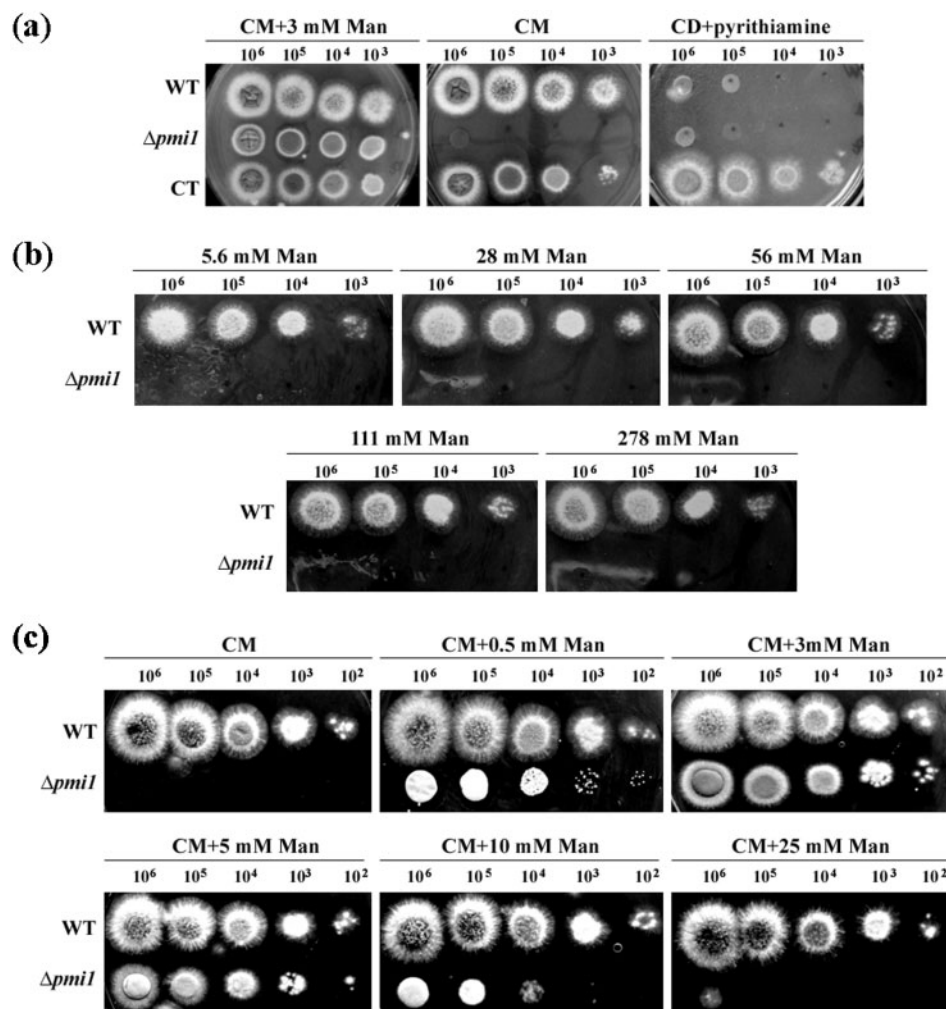
The wild-type *A. fumigatus* strain could use Man alone as a carbon source; however, the mutant was not able to grow on medium with Man as the sole carbon source (Fig. 2b). The growth of the mutant required both Glc and Man. When the mutant strain was exposed to increasing concentrations of Man (0.5, 3, 5, 10 or 25 mM), optimal growth was seen at 3 mM Man. The mutant showed a reduction in growth at concentrations higher than 3 mM, when compared to the wild-type grown on identical media. A slight reduction was seen at 5 mM, a more severe reduction at 0.5 mM and 10 mM, and complete retardation at 25 mM (Fig. 2c). These results demonstrate an essential role of Man for *A. fumigatus*; however, excess Man appears to cause toxicity in the  $\Delta pmi1$  mutant. Furthermore, although its growth rate was completely restored at 3 mM Man, the mutant formed smooth colonies, in contrast to the wild-type, suggesting a defect in conidia formation. Conidia counting revealed that the mean  $\pm$ SD conidia count of the  $\Delta pmi1$  mutant at 3 mM Man was  $9 \pm 1.1 \times 10^7$ , which was 41 % of that of the wild-type strain ( $22 \pm 2.7 \times 10^7$ ), and no conidia were produced by the  $\Delta pmi1$  mutant grown at 0.5 or 10 mM Man.

### Cell wall defect of the $\Delta pmi1$ mutant

To evaluate the impact of the initial concentration of Man on the *A. fumigatus* strain lacking PMI, we analysed the

phenotypes of the  $\Delta pmi1$  mutant grown under both Man-starvation (0.5 mM Man) and Man-repletion (3 mM Man) conditions. We first tested the sensitivity of *A. fumigatus* strains to various chemical reagents. As compared with the wild-type, the  $\Delta pmi1$  mutant showed an increased sensitivity to Congo red, Calcofluor white and SDS under both Man-repletion and Man-starvation conditions (Fig. 3a). These results reveal that exogenous Man can not complement the defect in cell wall integrity of the mutant. The  $\Delta pmi1$  mutant did not show any temperature-sensitive phenotype (Fig. 3b).

We further analysed the mycelial cell wall contents of the mutant. As summarized in Table 1, when the mutant was grown at 37 °C under Man-starvation conditions, the  $\alpha$ -glucan content decreased by 33 % as compared with the wild-type, while the mannoprotein and chitin content increased by 42 % and 43 %, respectively. Under Man-repletion conditions, the  $\alpha$ -glucan content decreased by 20 %, whereas the mannoprotein and  $\beta$ -glucan content increased by 51 % and 22 %, respectively. Although an increase in mannoprotein was observed under both conditions, the Man residues released from mannoproteins decreased by 43 % under Man-starvation conditions and increased by 97 % under Man-repletion conditions, as compared with those released from the wild-type mannoproteins. Similarly, the amount of GlcNAc and Gal residues released from the mutant mannoproteins was decreased under Man-starvation conditions and increased under Man-repletion conditions. The wild-type was also found to be sensitive to the high Man concentration; although the content of mannoprotein remained constant, the GlcNAc, Gal and Man residues released from mannoproteins were significantly increased at the higher Man concentration,



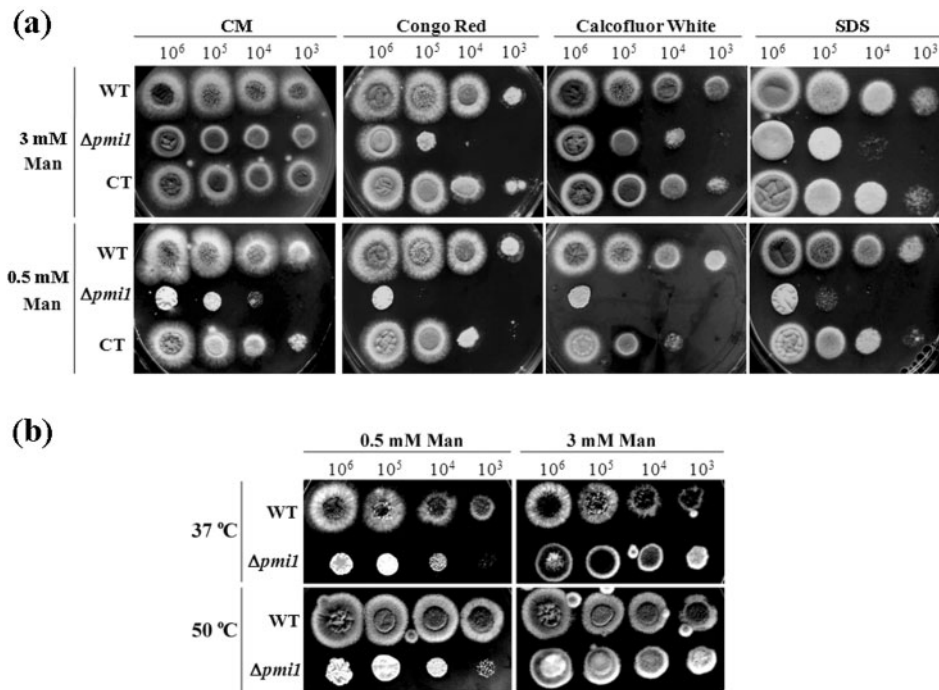
**Fig. 2.** Growth of *A. fumigatus* strains on various media. (a) *A. fumigatus* strains were grown on CM with 3 mM Man, on CM or on CD with  $0.1 \mu\text{g}$  pyrithiamine  $\text{ml}^{-1}$  at  $37^\circ\text{C}$  for 24 h. (b) *A. fumigatus* strains were grown on CM with 5.6, 28, 56, 111 or 278 mM Man as the sole carbon source at  $37^\circ\text{C}$  for 24 h. (c) *A. fumigatus* strains were grown on CM supplemented with 0, 0.5, 3, 5, 10 or 25 mM Man at  $37^\circ\text{C}$  for 24 h. WT, wild-type strain; CT, complemented strain.

and a decrease (21%) of  $\alpha$ -glucan in the cell wall was also observed. These results suggest that PMI is a key enzyme in the regulation of cell wall synthesis in *A. fumigatus*. Although an inappropriate extracellular Man concentration leads to an under- or overglycosylation of mannoproteins, the increase of these proteins might compensate for the decrease of  $\alpha$ -glucan synthesis. It should be pointed out that, in addition to the increase of mannoproteins, it appeared that the mechanisms triggered to compensate for the loss of  $\alpha$ -glucan in the mutant were different from those in the wild-type and dose-dependent on Man; Man-starvation led to an increase of chitin, and Man-repletion led to an increase of  $\beta$ -glucan.

### Morphogenesis of the $\Delta pmi1$ mutant

To study the effect of PMI activity on hyphal development in *A. fumigatus*, the mutant conidia were allowed to adhere

to glass coverslips during incubation in medium with or without Man at  $37^\circ\text{C}$ . Coverslips with adhering germlings were examined under a microscope. As shown in Fig. 4, when conidia were incubated in CM, both wild-type and  $\Delta pmi1$  conidia appeared to begin the normal germination process by swelling slightly. After the first one or two rounds of mitotic division, the wild-type conidia began to develop the first germ tubes (6 h). The second germ tubes and the first septation occurred after four rounds of mitosis (8 h). In contrast to the wild-type, the mutant conidia ceased to grow after two rounds of mitotic division and became enlarged, misshapen and vacuolated, with irregular distribution of chitin in their cell walls. When the mutant conidia were incubated under Man-starvation conditions, 41% (7 h) and 66% (8 h) of the mutant conidia germinated the second germ tube at a random angle after the third mitotic division. After four rounds of mitotic



**Fig. 3.** Sensitivity of the mutant to chemical reagents (a) and temperature (b). (a) Serially diluted conidia ( $10^6$ – $10^3$  cells) were spotted and incubated at 37 °C for 24 h on CM plates containing 50  $\mu\text{g}$  Calcofluor white  $\text{ml}^{-1}$ , 50  $\mu\text{g}$  Congo red  $\text{ml}^{-1}$ , or 75  $\mu\text{g}$  SDS  $\text{ml}^{-1}$ . (b) A series of 10-fold dilutions ( $10^6$ – $10^3$  cells) of the wild-type and  $\Delta pmi1$  strains were cultivated on solid CM medium with 0.5 or 3 mM Man at 37 °C or 50 °C for 36 h. WT, wild-type strain; CT, complemented strain.

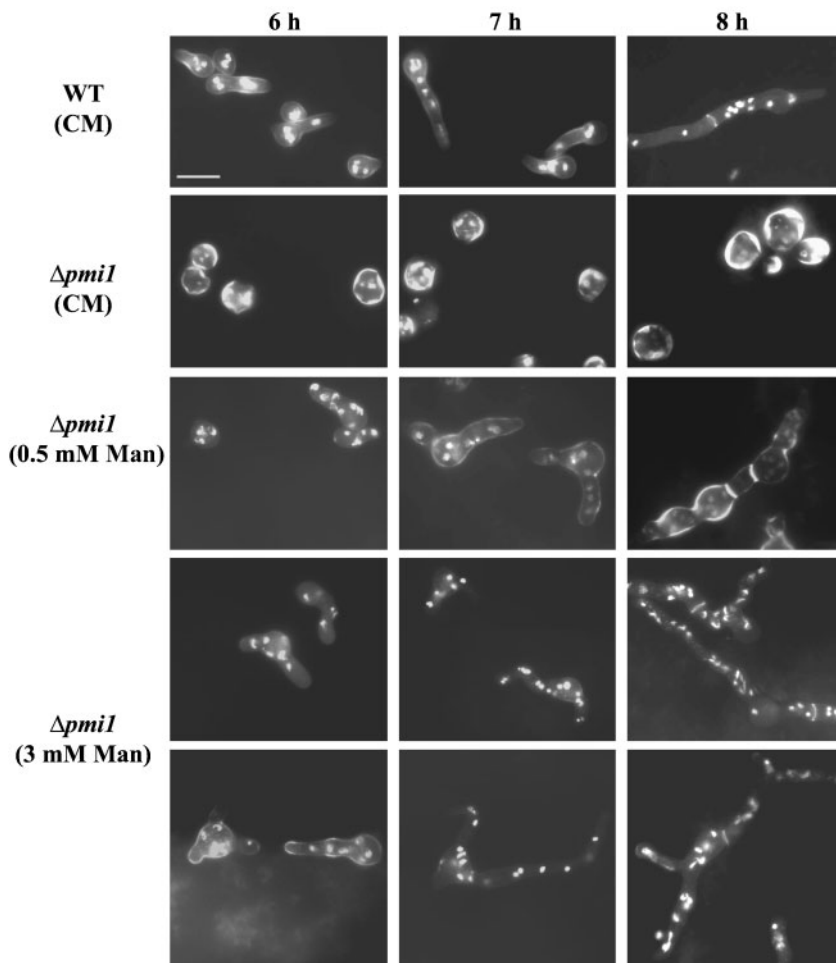
division, some basal cells were ballooned, with a thickened chitin-enriched cell wall. In particular, a prolonged incubation up to 12 h led to ballooned hyphal tips and some ballooned basal cells in the mutant (Fig. 5). Propidium iodide staining revealed that these ballooned cells were dead (Fig. 5b). Under Man-repletion conditions, 51 % of the mutant conidia germinated the second germ tube at a random angle after the third mitotic division and 36 % of the mutant conidia germinated the third germ tube (Fig. 4, Table 2). In addition, an increase in mitotic division was observed in the mutant. After 8 h

incubation, 4–5 rounds of mitotic division occurred in 76 % of the mutant conidia, while only 3–4 rounds of mitotic division were seen in 72 % of the wild-type conidia, suggesting an alteration of the cell cycle in the mutant. Although the growth rate of the  $\Delta pmi1$  mutant cells could be completely restored by the addition of 3 mM Man and partially restored by the addition of 0.5 mM Man, it was apparent that morphogenesis of the  $\Delta pmi1$  mutant was abnormal under both conditions, indicating an essential role of the *pmi1* gene in growth and morphogenesis of *A. fumigatus*.

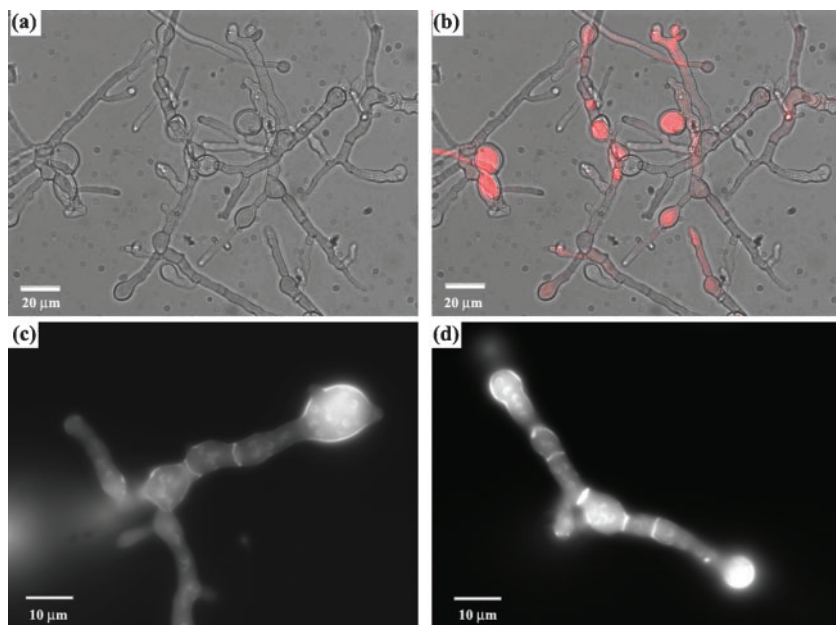
**Table 1.** Chemical analysis of the  $\Delta pmi1$  mutant cell wall

Conidia were inoculated into 100 ml medium at a concentration of  $10^6$  conidia  $\text{ml}^{-1}$ , and incubated at 37 °C with shaking (200 r.p.m.) for 24 h. The mycelium was then harvested for isolating cell walls. Three aliquots of 10 mg lyophilized cell walls were used as independent samples for cell wall analysis, and the experiment was repeated twice. The values shown are  $\mu\text{g}$  cell wall component per 10 mg dry cell walls ( $\pm$ SD).

Culture condition	Strain	Alkali soluble				Alkali insoluble		
		Mannoprotein				$\alpha$ -Glucan ( $\mu\text{g}$ )	Chitin ( $\mu\text{g}$ )	$\beta$ -Glucan ( $\mu\text{g}$ )
		Protein ( $\mu\text{g}$ )	GlcNAc ( $\mu\text{g}$ )	Gal ( $\mu\text{g}$ )	Man ( $\mu\text{g}$ )			
0.5 mM Man	YJ-407	57 $\pm$ 5	0.43 $\pm$ 0.04	1.78 $\pm$ 0.05	0.65 $\pm$ 0.04	434 $\pm$ 22	181 $\pm$ 9	831 $\pm$ 49
	$\Delta pmi1$	81 $\pm$ 8	0.35 $\pm$ 0.03	1.33 $\pm$ 0.09	0.37 $\pm$ 0.03	291 $\pm$ 29	259 $\pm$ 18	821 $\pm$ 31
3 mM Man	YJ-407	59 $\pm$ 5	0.36 $\pm$ 0.01	2.48 $\pm$ 0.28	1.09 $\pm$ 0.12	342 $\pm$ 29	186 $\pm$ 12	848 $\pm$ 66
	$\Delta pmi1$	89 $\pm$ 9	0.73 $\pm$ 0.06	3.20 $\pm$ 0.3	2.15 $\pm$ 0.2	277 $\pm$ 16	188 $\pm$ 16	1037 $\pm$ 65



**Fig. 4.** Germination and septation of the  $\Delta pmi1$  mutant. Freshly harvested conidia ( $10^7$ ) were poured into a Petri dish containing glass coverslips, and incubated in 10 ml medium at 37 °C for the time indicated. The coverslips with adherent germlings were removed and fixed in 3.7% formaldehyde in PBS for 30 min. Coverslips were then washed with PBS, incubated for 20 min with 1  $\mu\text{g ml}^{-1}$  4',6-diamidino-2-phenylindole (DAPI) (Sigma), washed with PBS, and then incubated for 10 min with a 10  $\mu\text{g ml}^{-1}$  solution of fluorescent brightener 28 (Sigma), washed again, and germlings were photographed using a fluorescence microscope (Carl Zeiss). Typical photographs are shown. Bar, 10  $\mu\text{m}$ .



**Fig. 5.** Germination of the  $\Delta pmi1$  mutants under Man-starvation conditions at 37 °C for 12 h. Coverslips with adhering germlings were removed, fixed and washed with PBS three times, incubated for 5 min with 50  $\mu\text{g ml}^{-1}$  propidium iodide (Molecular Probes), washed again, and germlings were photographed using a differential interference contrast (DIC) microscope (a) or a fluorescence microscope (b) (Carl Zeiss). The mycelium was stained with Calcofluor white and DAPI and examined under a fluorescence microscope (c, d). Representative photographs are shown. Bars, 20  $\mu\text{m}$  (a, b) or 10  $\mu\text{m}$  (c, d).

**Table 2.** Statistics of germination of the  $\Delta pmi1$  mutant

Coverslips with adhering germlings were removed from incubation in 10 ml medium at 37 °C and the numbers of germ tubes per conidium were counted under a microscope. Three independent experiments were carried out, with 100 conidia counted in each.

Time (h)	Number of germlings in each germ tube class											
	Wild-type				$\Delta pmi1$ (Man starvation)				$\Delta pmi1$ (Man repletion)			
	Germ tubes:	0	1	2	≥3	0	1	2	3	0	1	2
6	8 ± 2	92 ± 3	0	0	16 ± 3	66 ± 3	18 ± 1	0	12 ± 2	39 ± 3	43 ± 5	6 ± 1
7	5 ± 1	86 ± 5	8 ± 2	1 ± 0	5 ± 1	38 ± 2	41 ± 4	16 ± 2	4 ± 2	30 ± 4	52 ± 6	14 ± 1
8	0	72 ± 8	25 ± 3	3 ± 0	0	23 ± 3	66 ± 5	11 ± 2	0	13 ± 1	51 ± 3	36 ± 3

**Intracellular metabolites of the  $\Delta pmi1$  mutant**

To monitor the sugar metabolism in the  $\Delta pmi1$  mutant, we analysed intracellular sugar phosphates under both Man-starvation and Man-repletion conditions (Table 3). After 24 h cultivation at 37 °C, Man-starvation led to a twofold increase in Man-6-P as compared with the wild-type. However, this increase of Man-6-P was not sufficient to drive GDP-Man synthesis. Indeed, significant decreases in Glc-6-P, Fru-6-P, Man-1-P and GDP-Man were observed in the mutant. Thus, it is reasonable to conclude that the decrease of GDP-Man was due to the insufficient supply of Man and extremely low concentration of Fru-6-P, which forced the conversation of Man-6-P into Fru-6-P instead of GDP-Man. In addition, Fru-1,6-P was significantly decreased in the mutant. Considering that Fru-1,6-P is the precursor of UDP-GlcNAc, a substrate of chitin synthesis, the reduction in Fru-1,6-P, and thus Fru-6-P, might be caused by an increased synthesis of chitin in the mutant to compensate for the cell wall defect.

When the  $\Delta pmi1$  mutant was grown under Man-repletion conditions, a 20-fold increase in Man-6-P and an increase in Man-1-P were observed, which is consistent with the overglycosylation observed in the cell walls of the mutant. On the other hand, levels of Fru-6-P and GDP-Man were found to be similar to the wild-type, whereas those of Glc-6-P and Fru-1,6-P were reduced by 77% and 66%, respectively. These observations demonstrate that a high

concentration of extracellular Man can force GDP-Man synthesis in the  $\Delta pmi1$  mutant to a level similar to that in the wild-type; however, it also leads to a significant accumulation of Man-6-P and a significant decrease of the flux through glycolysis, as Glc-6-P is the precursor of UDP-Glc, a substrate for glucan synthesis. Therefore, the decrease in  $\alpha$ -glucan in the cell wall of the mutant cultivated under both Man-starvation and Man-repletion conditions can be explained by the decrease in Glc-6-P. Although details of the mechanism are not clear yet, it appears that the  $\Delta pmi1$  mutant possesses a mechanism to ensure  $\beta$ -glucan synthesis.

Moreover, the sugar phosphates in the wild-type *A. fumigatus* grown under Man-repletion were similar to those in this strain grown under Man-starvation, which is consistent with tolerance of the wild-type to high concentrations of Man. Indeed, we found that the wild-type grew well on CM supplemented with a high concentration of Man, up to 300 mM, or on medium with 1110 mM Man as the sole carbon source (data not shown).

**DISCUSSION**

The cell wall helps fungi to resist adverse environments and has a variety of biological functions, such as maintaining morphogenesis and regulating selective permeability (Yoda

**Table 3.** Intracellular sugar phosphates in the  $\Delta pmi1$  mutant

Conidia were inoculated into 100 ml 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 24 h. The mycelium was then harvested for sugar phosphate extraction. Three aliquots of 50 mg lyophilized mycelia were used as independent samples for sugar phosphate analysis, and the experiment was repeated twice. The values shown are nmol sugar phosphate per 50 mg dry mycelium (±SD).

Culture condition	Strain	Sugar phosphate (nmol)					
		Glc-6-P	Fru-6-P	Fru-1,6-P	Man-6-P	Man-1-P	GDP-Man
0.5 mM Man	YJ-407	46 ± 3	25 ± 5	95 ± 15	47 ± 6	64 ± 11	13 ± 2
	$\Delta pmi1$	29 ± 2	6 ± 1	46 ± 5	99 ± 18	10 ± 1	1 ± 0.2
3 mM Man	YJ-407	73 ± 11	24 ± 5	89 ± 8	56 ± 14	75 ± 16	13 ± 2
	$\Delta pmi1$	17 ± 3	23 ± 3	30 ± 4	1151 ± 111	135 ± 18	15 ± 2

*et al.*, 2000; Agaphonov *et al.*, 2001). The *A. fumigatus* cell wall comprises four major components:  $\alpha$ -glucan,  $\beta$ -glucan, chitin and mannoprotein. Although the molecular structure of the *A. fumigatus* cell wall has been studied in detail (Fontaine *et al.*, 2000; Latgé *et al.*, 2005; Upadhyay & Shaw, 2006), very little work has been undertaken to identify the biochemical pathways involved in biosynthesis of cell wall polysaccharides and mannoprotein. We hypothesized that the GDP-mannose biosynthesis pathway in this filamentous fungus is linked to cell wall biosynthesis and is biochemically similar to that found in *S. cerevisiae*, *Can. albicans* and *A. nidulans*.

In our study, the *pmi1* gene encoding PMI was identified in *A. fumigatus* and we expressed this gene in *E. coli*. The recombinant Pmi1 protein was shown to be a member of the type I PMIs and required bivalent metals for its activity; however,  $Mn^{2+}$  was required instead of the  $Zn^{2+}$  required by PMIs from other sources (Proudfoot *et al.*, 1994b; Roux *et al.*, 2007). Another interesting finding was that the  $K_m$  value of the *A. fumigatus* PMI for Man-6-P was close to that of the PMIs from *Can. albicans* and *S. cerevisiae* but different from that of the PMIs from bacteria or mammals, indicating a different substrate-binding affinity. Computer modelling of the human PMI has putatively identified several amino acid residues as zinc-binding (Gln<sup>110</sup>, His<sup>112</sup>, Glu<sup>137</sup> and His<sup>276</sup>) and substrate-binding sites (Arg<sup>295</sup>, Lys<sup>301</sup>, Ser<sup>108</sup> and Tyr<sup>278</sup> for Man-6-P) (Xiao *et al.*, 2006). However, alignment of the *A. fumigatus* PMI with human PMI reveals that metal-binding (Gln<sup>108</sup>, His<sup>110</sup>, Glu<sup>135</sup> and His<sup>292</sup>) and substrate-binding sites (Arg<sup>311</sup>, Lys<sup>317</sup>, Ser<sup>106</sup> and Tyr<sup>294</sup>) are conserved in the *A. fumigatus* PMI. It is not understood why the AfPmi1 exhibits a different binding affinity from the human PMI. It is also not clear whether  $Mn^{2+}$  makes a contribution to this difference. In addition, the  $K_m$  and  $V_{max}$  values of the *A. fumigatus* PMI were also determined in both directions. Surprisingly, the catalytic efficiency ( $K_{cat}/K_m$ ) for Fru-6-P was 6.5 times that for Man-6-P, indicating that the conversion from Fru-6-P to Man-6-P was more efficient in *A. fumigatus*.

PMI allows the essential interconversion of the sugars Man-6-P and Fru-6-P and provides a link into the GDP-mannose biosynthesis pathway and the glucose metabolism pathway, respectively. PMI mutants in other species have typically shown secretion defects and abnormal cell morphologies, such as clumping, cell wall 'ballooning' and an inability to separate from the mother cell (Payton *et al.*, 1991; Smith *et al.*, 1995; Wills *et al.*, 2001). These defects were completely restored in some species by the addition of exogenous Man and Glc to the growth medium (Payton *et al.*, 1991), which suggests that the utilization of exogenous Man by the cell can be accomplished without the involvement of the PMI enzyme. However, high Man concentration also has a toxic effect on growth in yeast lacking the homologue of PMI (Pitkänen *et al.*, 2004), as well as in honeybees with a non-functional PMI (de la Fuente *et al.*, 1986), which is called 'honeybee effect' and defined as a depletion of intracellular ATP through a futile

cycle of Man-6-P de- and rephosphorylation (de la Fuente & Hernanz, 1988).

In our study, the addition of Glc and Man could rescue the mycelial growth of the  $\Delta pmi1$  mutant but the conidiation defect that occurred in the absence of PMI could not be restored. This observation suggests that, although mannose is essential for survival of *A. fumigatus*, the expression of PMI activity is also needed for complete restoration of wild-type morphogenesis, such as conidia formation. Our observations demonstrate that in *A. fumigatus* exogenous Man is not handled in the same way as interconverted Man and that uptake of exogenous Man is inefficient.

We further assessed the impact of the PMI enzyme on cell wall synthesis by incubating the  $\Delta pmi1$  mutant under Man-starvation or Man-repletion conditions. Under both conditions, the mutant displayed a decrease in  $\alpha$ -glucan content in its cell wall. The wild-type was also affected by a high concentration of exogenous Man and showed a decrease in  $\alpha$ -glucan. However, it is apparent that inadequate Man and replete Man trigger different responses. To compensate for the loss of  $\alpha$ -glucan, the mutant showed an increase in chitin under Man-starvation and an increase in  $\beta$ -glucan under Man-repletion. Although the mechanism is not clear, these observations emphasize that a suitable Man supply and mannosylation are necessary for normal cell wall biogenesis in *A. fumigatus*. Furthermore, high Man in the environment appears to cause toxicity in the *A. fumigatus*  $\Delta pmi1$  cell, such that the cell seems unable to grow at levels above 25 mM Man.

In addition to the defects in the cell wall, the morphological changes observed in the *A. fumigatus*  $\Delta pmi1$  mutant were dose-dependent on Man. Given the involvement of glycoproteins in the synthesis and organization of the fungal cell wall, it is not surprising to observe a severe phenotype featuring an irregular cell wall and ballooned dead cells under Man-starvation conditions. Further analysis of the intracellular sugar phosphates revealed that inadequate Man caused a twofold increase in Man-6-P and significant decreases in Glc-6-P, Fru-6-P, Man-1-P and GDP-Man in the mutant, indicating that Man-6-P can accumulate even at lower Man concentrations. Previously, it has been demonstrated that an accumulation of Man-6-P limits the flux through glycolysis in a yeast *PMI40* deletion mutant (Pitkänen *et al.*, 2004), while in a mouse mutant lacking PMI, Man-6-P accumulation has been shown to limit glycolysis and deplete intracellular ATP (DeRossi *et al.*, 2006). Therefore, we conclude that the severe phenotypes associated with the *A. fumigatus*  $\Delta pmi1$  mutant under Man starvation are due to an insufficient supply of GDP-Man for cell wall synthesis, limitation of energy production and depletion of intracellular ATP. Moreover, Fru-1,6-P was also significantly decreased in the mutant grown under Man-starvation. Considering that Fru-1,6-P is the precursor of UDP-GlcNAc, a substrate of chitin synthesis, the reduction of Fru-1,6-P might be caused by an increased synthesis of chitin in the mutant to compensate for the cell wall defect.

Although the mutant apparently grew well under Man-repletion conditions, an abnormal morphology and a change in cell cycle were observed. Further analysis showed that, although replete Man allowed the mutant cell to produce GDP-Man to a level similar to that of the wild-type, it also caused a marked accumulation of Man-6-P and a reduction in Fru-1,6-P and Glc-6-P. Thus, the abnormal morphology associated with the  $\Delta pmil$  mutant under Man-repletion conditions can be ascribed to limitation of energy production and depletion of intracellular ATP. In addition, considering the remarkably high level of Man-6-P detected in the mutant, it is reasonable to conclude that a futile cycle of Man-6-P de- and rephosphorylation may contribute to the phenotypes observed under Man-repletion conditions.

In our study, both inadequate and replete Man supply caused a reduction in  $\alpha$ -glucan synthesis in the mutant, which can be explained by the decrease in Glc-6-P, a precursor of UDP-Glc that is required for glucan synthesis. It is not clear yet whether the decrease in  $\alpha$ -glucan is the cause of the defect in conidiation associated with the mutant. However, in contrast to  $\alpha$ -glucan,  $\beta$ -glucan levels in the mutant remained constant under Man-starvation and were increased under Man-repletion, as compared with the wild-type. Although Glc-6-P is also the precursor for  $\beta$ -glucan synthesis, it is likely that the mutant cell possesses a mechanism to ensure the synthesis of  $\beta$ -glucan prior to  $\alpha$ -glucan, probably through an upregulation of 1,3- $\beta$ -glucan synthase to compete for a limited substrate supply and ensure  $\beta$ -glucan synthesis in the mutant.

Recently, we have shown that GDP-mannose pyrophosphorylase is essential in *A. fumigatus* and that repression of this enzyme leads to a similar cell wall defect as seen in the  $\Delta pmil$  mutant under Man-starvation conditions; however, in comparison to a complete loss of the ability to produce conidia and the death of swollen cells in the  $\Delta pmil$  mutant under Man-starvation conditions, a severe hyphal lysis and reduced conidiation were seen (Jiang *et al.*, 2008). These observations suggest a different mechanism. It is likely that repression of the GDP-mannose pyrophosphorylase only leads to a limitation of GDP-Man synthesis and that no significant accumulation of Man-6-P occurs, which mainly affects the biogenesis of the cell wall and therefore leads to phenotypes different from those associated with Man-6-P toxicity.

In conclusion, our functional studies showed that PMI activity is essential for viability and plays a central regulatory role in both glycosylation and energy production in *A. fumigatus*. Although exogenous Man can rescue the growth of the mutant deficient in PMI, it is not handled in the same way as interconverted Man. Deletion of the *pmil* gene leads to uncoupling of the link between energy production and glycosylation and accumulation of Man-6-P, which then results in phenotypes such as defects in cell wall integrity, conidiation and morphology.

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