

# GDP-mannose pyrophosphorylase is essential for cell wall integrity, morphogenesis and viability of *Aspergillus fumigatus*

Hechun Jiang, Haomiao Ouyang, Hui Zhou and Cheng Jin

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

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

GDP-mannose pyrophosphorylase (GMPP) catalyses the synthesis of GDP-mannose, which is the precursor for the mannose residues in glycoconjugates, using mannose 1-phosphate and GTP as substrates. Repression of GMPP in yeast leads to phenotypes including cell lysis, defective cell wall, and failure of polarized growth and cell separation. Although several GMPPs have been isolated and characterized in filamentous fungi, the physiological consequences of their actions are not clear. In this study, *Afsrb1*, which is a homologue of yeast *SRB1/PSA1/VIG9*, was identified in the *Aspergillus fumigatus* genome. The *Afsrb1* gene was expressed in *Escherichia coli*, and recombinant AfSrb1 was functionally confirmed as a GMPP. By the replacement of the native *Afsrb1* promoter with an inducible *Aspergillus nidulans alca* promoter, the conditional inactivation mutant strain YJ-gmpp was constructed. The presence of 3% glucose completely blocked transcription of *P<sub>alca</sub>-Afsrb1*, and was lethal to strain YJ-gmpp. Repression of *Afsrb1* expression in strain YJ-gmpp led to phenotypes including hyphal lysis, defective cell wall, impaired polarity maintenance, and branching site selection. Also, rapid germination and reduced conidiation were documented. However, in contrast to yeast, strain YJ-gmpp retained the ability to direct polarity establishment and septation. Our results showed that the *Afsrb1* gene is essential for cell wall integrity, morphogenesis and viability of *Aspergillus fumigatus*.

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## INTRODUCTION

*Aspergillus fumigatus* is the most common opportunistic fungal pathogen of humans; it causes fatal invasive aspergillosis in immunocompromised patients (Latgé, 1999, 2001; Krappmann, 2006) and is the leading cause of death in patients with leukaemia and AIDS, and those that have had bone-marrow-transplants. The crude mortality from invasive aspergillosis is above 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. Therefore, there is an urgent need for a deep understanding of *A. fumigatus* at the molecular level.

The cell wall helps the fungus to battle against adverse environments, and it has a variety of biological functions, such as maintaining morphogenesis, and regulating the selective permeability (Yoda *et al.*, 2000; Agaphonov *et al.*,

2001). The *A. fumigatus* cell wall consists mainly of a covalently connected polysaccharide skeleton (glucans and chitin) that is interlaced and coated with glycoproteins, which contain mannose and galactose derived primarily from the process of glycosylation (Fontaine *et al.*, 2000; Latgé *et al.*, 2005; Upadhyay & Shaw, 2006). Some cell-surface proteins are further modified at their C terminus by the addition of a glycosylphosphatidylinositol (GPI) anchor, and they are transported to the plasma membrane and cell wall. These GPI proteins 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).

GDP-mannose, an activated form of mannose, is the precursor of mannose residues in galactomannan, glycoprotein and the GPI anchor. Activation of mannose requires three enzymes: phosphomannose isomerase, phosphomannomutase and GDP-mannose pyrophosphorylase (GMPP). To date, several GMPPs have been identified and characterized in different species (Griffin *et al.*, 1997; Ning & Elbein, 1999; Ohta *et al.*, 2000; Warit *et al.*, 1998; Sacchetti *et al.*, 2004). In *Saccharomyces cerevisiae* and *Candida albicans*, GMPP is essential (Hashimoto *et al.*, 1997; Warit *et al.*, 2000), while in *Leishmania mexicana* the GMPP is not required for

Abbreviations: DIC, differential interference contrast; GPI, glycosylphosphatidylinositol; GMPP, GDP-mannose pyrophosphorylase; PI, propidium iodide; Trx, thioredoxin.

The GenBank/EMBL/DBJ accession number for the nucleotide sequence of *Afsrb1* is DQ017035.

A table of primers used in this study is available with the online version of this paper.

viability (Garami & Ilg, 2001). Depletion of the GMPP in *S. cerevisiae* and *C. albicans* leads to pleiotropic phenotypes, including cell lysis, failure of cell separation, impaired budding and hyphal switching, clumping and flocculation, and defect of the cell wall (Warit *et al.*, 2000). The aforementioned reports imply that GMPPs are specifically crucial for synthesis and organization of the cell wall, and thus essential for species that have a cell wall.

To evaluate the impact of GMPP on the cell wall of *A. fumigatus*, we identified a homologue of yeast *SRB1/PSA1/VIG9* from a genomic database through bioinformatics analysis. In this report, the putative *Afsrb1* gene was expressed, and confirmed to encode a GMPP. In addition, the phenotypes associated with depletion of the *Afsrb1* gene were analysed.

## METHODS

**Strains and growth conditions.** *A. fumigatus* strain YJ-407 (CGMCC0386; China General Microbiological Culture Collection Center, Chinese Academy of Sciences, Beijing, China) was maintained on potato glucose (2%) agar slants (Xia *et al.*, 2001). *A. fumigatus* strain CEA17 (Weidner *et al.*, 1998), a gift from C. d'Enfert, Institut Pasteur, Paris, France, was propagated at 37 °C on YGA (0.5% yeast extract, 2% glucose, 1.5% Bacto-agar), complete medium (CM), or minimal medium with 0.5 mM sodium glutamate as a nitrogen source (MM) (Cove, 1966). Uridine and uracil were added at a concentration of 5 mM when strain CEA17 was grown.

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 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 CM with uridine and uracil for 48 h at 37 °C. The spores were collected, washed twice with 0.01% Tween 20 in physiological saline, resuspended in 0.01% 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, Bethesda, Maryland, USA).

**Computer analysis.** Sequence alignments were analysed by Vector NTI, and a BLAST search was performed.

**Isolation of the *Afsrb1* gene from *A. fumigatus*.** A homologue of yeast *SRB1/PSA1/VIG9* was identified by a tBLASTn search of the *A. fumigatus* genome database (<http://www.tigr.org/tdb/e2k1/afu1/>), and it was designated *Afsrb1*. cDNA of *Afsrb1* was isolated by RT-PCR. *A. fumigatus* total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instruction, and cDNA was synthesized with primer 1 (see supplementary Table S1, available with the online version of this paper) using M-MuLV (NEB). Thirty cycles (94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min) of PCR reaction were carried out using primers 2 and 3 (Table S1). The PCR products were subcloned into pGEM-T vector (Promega) to yield pGEM-T-GMPP, and then sequenced. The position of the intron was determined by comparing the cDNA with the genomic sequence.

**Expression of the *Afsrb1* gene in *E. coli*.** The cDNA of the *Afsrb1* gene was amplified from pGEM-T-GMPP with primers 4 and 5 (Table S1), and subcloned into pET-32a (Novagen). The recombinant expression vector containing the *Afsrb1* gene fused to the gene

encoding thioredoxin (Trx) was confirmed by PCR, restriction enzymes and sequencing. The resulting recombinant plasmid was designated pET-32GMPP.

The recombinant strain *E. coli* BL21(DE<sub>3</sub>) (Novagen), harbouring pET32-GMP, was grown in 5 ml Luria-Bertani (LB) medium containing 0.1 mg ampicillin ml<sup>-1</sup>, at 37 °C overnight. A 1 ml volume of cell culture was inoculated into 100 ml LB containing 0.1 mg ampicillin ml<sup>-1</sup>, and incubated at 30 °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 (final concn 0.4 mM; Sigma) at 30 °C for 4–6 h. The cells were harvested by centrifugation (12 000 r.p.m. for 15 min in an Avanti J-25 Centrifuge; Beckman), and resuspended in 100 ml 1× binding buffer (0.02 M sodium phosphate, 0.5 M NaCl, 40 mM imidazole, pH 7.4) containing 0.3 mg lysozyme ml<sup>-1</sup>. After incubation at 37 °C for 15 min, the cells were sonicated. The cell lysate was collected by centrifugation (12 000 r.p.m. for 15 min), filtered through a 0.45 µm membrane, and run on a HiTrap affinity column (Ni Sepharose 6 Fast Flow; GE Healthcare). After washing with 20 column vols binding buffer, the recombinant protein was eluted with a gradient of imidazole (40–500 mM), and dialysed against 50 mM Tris buffer, pH 7.5.

The purity of the recombinant protein was confirmed by SDS-PAGE. The protein concentration was determined by the Bradford assay (Bradford, 1976).

**Enzyme assay.** GMPP activity was determined as described by Ohta *et al.* (2000). Briefly, the reaction mixture contained 50 mM Tris (pH 7.5), 8 mM MgCl<sub>2</sub>, 100 µM GTP, 100 µM mannose 1-phosphate (Sigma), 1 mM DTT, 0.1 unit inorganic pyrophosphatase ml<sup>-1</sup> (Sigma), and 0.1 µg recombinant GMPP, in a total volume of 100 µl. The reaction was carried out at 30 °C for 30 min. A 40 µl volume of the reaction mixture was diluted with 120 µl water, mixed with 40 µl dye reagent (containing malachite green, sulfuric acid, ammonium molybdate and Tween 20), and incubated at 30 °C for 10 min. The amount of inorganic phosphate generated from the pyrophosphate was determined by measuring the absorbance at 630 nm. Trx was used as a negative control.

**Construction of the conditional inactivation mutant.** The conditional inactivation mutant was constructed by replacement of the native promoter (*P<sub>srb1</sub>*) of *Afsrb1* with the *alcA* promoter (*P<sub>alcA</sub>*). In order to achieve this, an 869 bp fragment from -14 to +855 of the *A. fumigatus Afsrb1* genomic sequence was amplified with primers 6 and 7 (see supplementary Table S1), and cloned into the *Bam*HI site of pAL3 (a gift from J. R. D. Lucas, Universidad de Alcalá, Madrid, Spain) to generate pALGMP. The upstream fragment of *P<sub>srb1</sub>* was amplified from *A. fumigatus* genomic DNA using primers 18 and 15 (94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min) (Table S1), and the downstream fragment of the *P<sub>srb1</sub>* was amplified from pALGMP using primers 14 and 7 (94 °C for 1 min, 56 °C for 1 min, and 72 °C for 3 min) (Table S1). The two PCR products were used as templates for the second-round PCR reaction, in which a fragment containing (in sequence) the upstream sequence of *P<sub>srb1</sub>*, the *pyr-4* gene, *P<sub>alcA</sub>*, and the downstream sequence of *P<sub>srb1</sub>*, was amplified with primers 7 and 18 (94 °C for 1 min, 56 °C for 1 min, and 72 °C for 4 min) (Table S1). The products from the second round of PCR were subcloned into pGEM-T to generate pGEM-P, and then sequenced.

The linearized pGEM-T was transformed into *A. fumigatus* strain CEA17 by PEG-mediated fusion of protoplasts (Langfelder *et al.*, 2002), and screened for cells with uridine/uracil autotrophy. The transformants were chosen by PCR, and the transformation was confirmed by Southern blot analysis. For PCR analysis, three pairs of primers (8 and 9, 10 and 11, and 12 and 13) (Table S1) were employed with the following cycling conditions: 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. The first pair of primers was

utilized as a positive control that could yield a 1268 bp *Afsrb1* fragment. The other two pairs of primers corresponded to a 1217 bp fragment of *pyr-4*, and a 1210 bp fragment of *P<sub>alca</sub>-Afsrb1*, respectively, in the transformant. For Southern blotting, genomic DNA was digested with *Bam*HI, separated by electrophoresis, and transferred to a nylon membrane (Zeta-probe<sup>+</sup>; Bio-Rad). A fragment of 876 bp, amplified from *Afsrb1* (+197 to +1072) with primers 16 and 17 (Table S1), was used as a probe. Labelling and visualization were performed using the DIG DNA labelling and detection kit (Roche Applied Science), according to the manufacturer's instructions.

**Phenotypic analysis of the conditional inactivation mutant.** For the antifungal reagent sensitivity test, *A. fumigatus* strains were grown on solid repressive medium (RM) (MM containing 1 % glucose and 0.05 M threonine) containing 200 µg Calcofluor white ml<sup>-1</sup>, 20 µg hygromycin B ml<sup>-1</sup>, 150 µg Congo red ml<sup>-1</sup>, 40 µg G418 ml<sup>-1</sup> or 0.01 % SDS. After incubation at 37 °C for 2–3 days, the plates were taken out and photographed.

For growth characteristics, a 10 µl slurry of spores (1 × 10<sup>8</sup> ml<sup>-1</sup>) was spotted onto solid RM. The radius of each colony was measured at time intervals, and plotted. The experiment was repeated three times.

**Chemical analysis of the cell wall.** Conidia were inoculated into 100 ml liquid RM to a concentration of 1 × 10<sup>6</sup> ml<sup>-1</sup>, and the suspension was shaken (250 r.p.m.) at 37 °C for 36 h. The mycelia were harvested as described above, and lyophilized. Three aliquots of 10 mg dry mycelium were used as independent samples for analysis. Each sample was boiled for 5 min in 2 ml 50 mM Tris/HCl buffer containing 2 % SDS, 100 mM Na-EDTA, 40 mM β-mercaptoethanol and 1 mM PMSF (Elorza *et al.*, 1985; Hearn & Sietsma, 1994; Schoffemeer *et al.*, 1999) to remove unbound cell-wall proteins and water-soluble sugar. After treatment with 3 % NaOH at 75 °C for 1 h, proteins were released, and quantified 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 10 ml distilled water. Glucan and chitin were estimated by determining the amount of glucose and *N*-acetylglucosamine released after digestion, respectively. Glucose was measured by using the phenol sulphuric acid method (Dubois *et al.*, 1956). *N*-Acetylglucosamine was measured by using the method described by Lee *et al.* (2005). The experiment was repeated twice.

**Microscopic analysis.** A 10 ml volume of liquid RM was inoculated with 10<sup>7</sup> freshly harvested conidia, poured into a Petri dish containing glass coverslips, and incubated at 37 °C for the time indicated in each experiment. At the specified times, coverslips with adhering germlings were removed, and spore germination was observed and counted by using a differential interference contrast (DIC) microscope.

Propidium iodide (PI; Molecular Probes) staining was carried out by following the manufacturer's instruction. A 100 µl volume containing 1 × 10<sup>9</sup> ml<sup>-1</sup> conidia was inoculated into 100 ml liquid RM, and incubated at 37 °C with shaking (250 r.p.m.) for 25 h until the culture reached mid-exponential phase. The mycelia were collected, washed with PBS, and resuspended in 1 ml PBS. A 10 µl volume of 50 µg PI ml<sup>-1</sup> and 1 µl 1 % NP-40 were added to 10 µl mycelium suspension, followed by incubation at room temperature for 5 min. After centrifugation, mycelia were washed three times with PBS, and viewed with a fluorescence microscope.

Staining of the nuclei, septa and cell walls was performed as follows. Conidia were incubated as described above. The coverslips with adhering germlings were removed, and fixed in the fixative solution (8 % formaldehyde, 50 mM phosphate buffer, pH 7.0, and 0.2 % Triton X-100) for 30 min. Coverslips were then washed with water,

incubated for 5 min with 10 µg fluorescent brightener 28 ml<sup>-1</sup> (Sigma) and 1 mg 4',6-diamidino-2-phenylindole ml<sup>-1</sup> (Sigma) plus 0.1 % NP-40. After washing with water, germlings were photographed using a microscope.

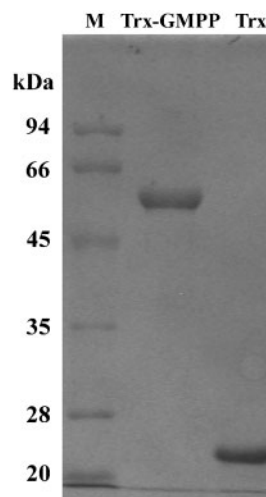
Conidia or mycelia were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, at room temperature for 4 h or at 4 °C overnight. After fixation, cells were washed three times in 0.1 M phosphate, post-fixed in 1 % osmium tetroxide and 0.1 M phosphate for 2–4 h, placed in increasing concentrations of methanol (30, 50, 70, 85, 95 and 100 %), and post-fixed in 2 % uranyl acetate/30 % methanol. Cells were rinsed, dehydrated, and embedded in Epon 812 for the floating sheet method. Sections were examined with an H-600 electron microscope (Hitachi).

## RESULTS

### Identification and expression of the *Afsrb1* gene

A tBLASTn search of the *A. fumigatus* genome database with the *S. cerevisiae* *SRB1/PSA1/VIG9* gene (GenBank accession no. NP010228) revealed the existence of a putative GMPP gene, which was designated *Afsrb1*. The *Afsrb1* gene is 1370 bp in length, and contains four introns and five exons. A 1095 bp length of *Afsrb1* cDNA (DQ017035) was isolated by RT-PCR. The *Afsrb1* gene encodes a protein of 364 aa (AAV40351) that has 71.2 % identity and 81.6 % similarity with *S. cerevisiae* *Srb1p* (NP010228).

From the expression of the *Afsrb1* gene in pET-32a/BL21(DE<sub>3</sub>), a 58 kDa fusion protein was expressed, and purified to homogeneity (Fig. 1). Enzyme activity was determined via a colorimetric assay coupled with inorganic pyrophosphatase, as described in Methods. Compared with the 0.03 ± 0.02 A<sub>630</sub> (mean ± SD) value of the Trx control,



**Fig. 1.** Expression of recombinant Trx-AfSrb1. SDS-PAGE analysis of purified Trx-AfSrb1. cDNA of the *Afsrb1* gene was cloned into the pET-32a/BL21(DE<sub>3</sub>) expression system. The recombinant protein was induced and purified as described in Methods.

Trx-Afsrb1 had a corresponding value of  $0.55 \pm 0.02$ . The recombinant enzyme showed maximal activity toward mannose 1-phosphate and GTP at 35 °C and pH 8.5, and required 7.5 mM  $Mg^{2+}$ . It also exhibited 20% activity towards glucose 1-phosphate, and 40% activity towards UTP, as compared with mannose 1-phosphate and GTP, respectively. When ATP, CTP or ITP was used as the substrate, only 30% activity was detected, as compared with GTP. These results demonstrate that the *Afsrb1* gene encodes a GMPP in *A. fumigatus*.

### Construction of the conditional inactivation mutant

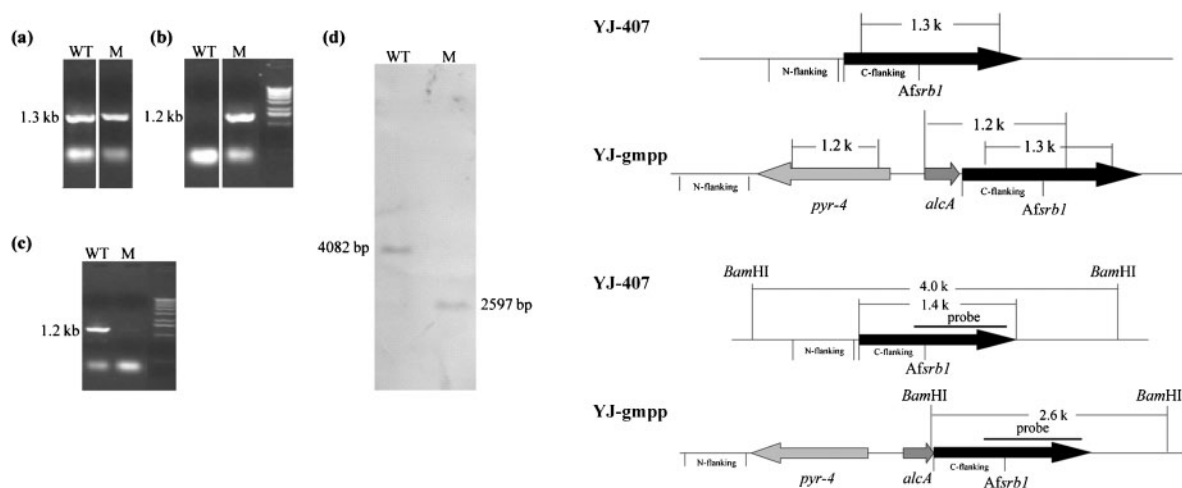
A conditional inactivation mutant was constructed by expressing the *Afsrb1* gene under the control of  $P_{alcA}$ , which is a strictly regulated promoter that can be triggered by ethanol or threonine, and is repressed completely by 3% glucose (Waring *et al.*, 1989; Romero *et al.*, 2003). A fragment in which the *pyr-4* gene and  $P_{alcA}$  were sandwiched with two flanking sequences of the  $P_{srb1}$  promoter was amplified via recombinant PCR (Zarrin *et al.*, 2005), and transformed into strain CEA17. One strain, namely YJ-gmpp, was obtained. PCR analysis showed that a 1217 bp fragment of *pyr-4* and a 1210 bp fragment of  $P_{alcA}$ -*Afsrb1* could be amplified from the genomic DNA of strain YJ-gmpp, while no such fragments were amplified from the wild-type strain YJ-407 (Fig. 2b, c). Southern blotting analysis of the *Bam*HI-digested genomic DNA of strain YJ-gmpp confirmed that wild-type 4082 bp *Bam*HI fragment was converted into a 2597 bp *Bam*HI fragment (Fig. 2d). These results demonstrated that native  $P_{srb1}$  was replaced by the *pyr-4* gene and  $P_{alcA}$  in strain YJ-gmpp.

Strain YJ-gmpp grew normally on MM medium containing 0.05 M threonine. When 3% glucose was added to MM medium, the conidia of strain YJ-gmpp were arrested at the stage of isotropic growth or early germination, while the wild-type strain grew normally (Fig. 3a, c). When an osmotic stabilizer (1 M sorbitol or 0.6 M KCl) was added to the MM medium containing 3% glucose, the growth of strain YJ-gmpp was compensated to some extent (Fig. 3b). These observations indicate that the *Afsrb1* gene is required for cell wall integrity, and that it is an essential gene in *A. fumigatus*.

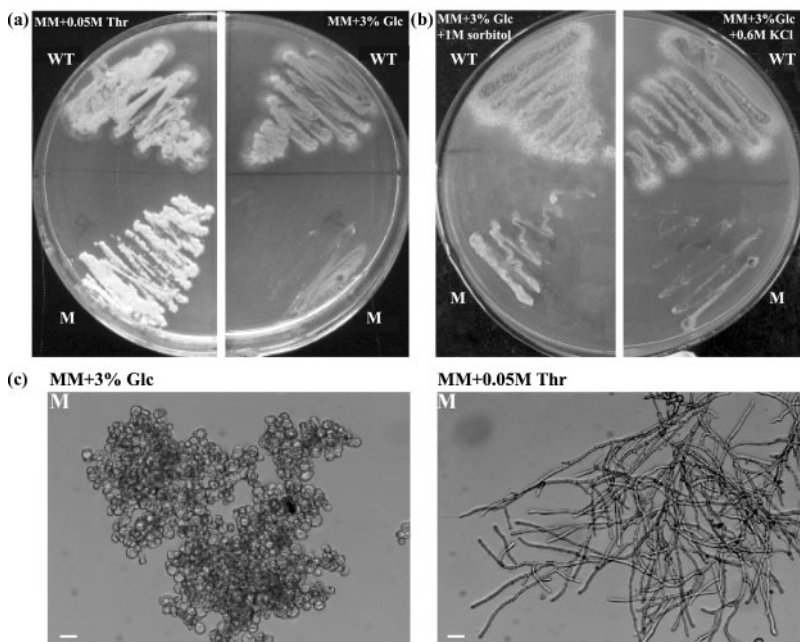
### Phenotypes associated with depletion of the *Afsrb1*

To determine the phenotypes associated with depletion of the *Afsrb1* gene, a series of culture conditions was tested to modulate the content and proportion of the inducer and repressor in the medium. Strain YJ-gmpp was able to grow on RM (Fig. 4a). Under this repressive condition, the expression of the *Afsrb1* gene was reduced to a minimal level that could not be detected by Northern blot (Fig. 4b). Thus, we cultivated strain YJ-gmpp in RM at 37 °C for phenotypic analyses.

Quantification of radial growth on solid RM revealed a severely retarded growth of YJ-gmpp compared with that for the wild-type strain (YJ-407). As shown in Fig. 5(a), the growth rate of strain YJ-gmpp was approximately 30% of that of the wild-type. In liquid RM, hyphae of strain YJ-gmpp were swollen and hyperbranched, whereas the wild-type exhibited long and dispersed filaments (Fig. 5b). It was apparent that depletion of *Afsrb1* expression induced impaired polar maintenance in *A. fumigatus*.



**Fig. 2.** Construction of the conditional inactivation mutant YJ-gmpp. (a)–(c) Confirmation of transformation by PCR. Primers were utilized to amplify the 1268 bp *Afsrb1* fragment (a), the 1217 bp *pyr-4* fragment (b), and the 1210 bp *alcA*-*Afsrb1* fragment (c), using genomic DNA as a template, as described in Methods. (d) Southern blot analysis. Genomic DNA was digested with *Bam*HI, separated on agarose, and transferred to a nylon membrane. An 876 bp fragment of *Afsrb1* (+197 to +1072) was used as a probe. WT, YJ-407; M, YJ-gmpp.



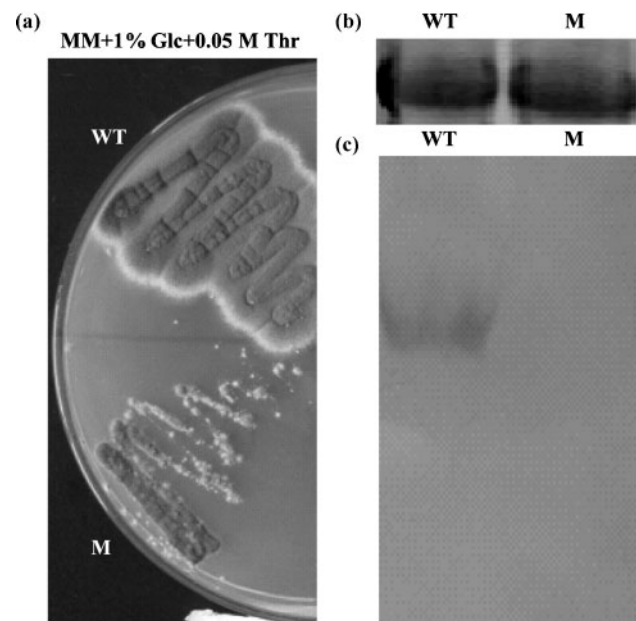
**Fig. 3.** Repression of the *Afsrb1* gene in strain YJ-gmpp. (a) YJ-407 (WT) and YJ-gmpp (M) were grown on medium that induced *Afsrb1* expression (left) and medium that repressed *Afsrb1* expression (right), at 37 °C for 3 days. (b) *A. fumigatus* strains were grown on medium that repressed *Afsrb1* expression, and contained 1 M sorbitol or 0.6 M KCl, at 37 °C for 3 days. (c) YJ-gmpp growth on medium that repressed *Afsrb1* expression (left) and medium that induced *Afsrb1* expression (right), at 37 °C for 36 h, was examined using DIC microscopy. Glc, glucose; Thr, threonine. Bars, 20 µm.

When the mycelia were stained PI, which is a dye commonly used for identifying apoptotic or dead cells, only the nuclei of strain YJ-gmpp were stained, even at exponential phase (37 °C for 25 h) (Fig. 6b); this suggested

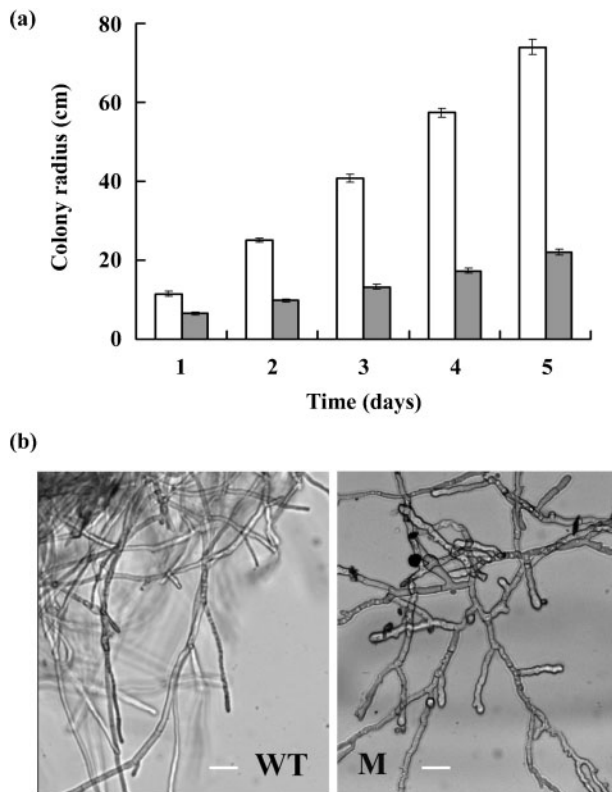
a significant increase in cell lysis, and is an observation similar to that seen in yeast *srb1* mutants (Warit *et al.*, 2000). However, in contrast to the yeast mutants (Warit *et al.*, 2000), YJ-gmpp formed septa normally, though its basal cells became shorter (Fig. 6d, e). Moreover, despite a small portion of the YJ-gmpp conidia being arrested at the stage of isotropic growth or early germination (Fig. 6d), most conidial cells retained the ability to establish polarized growth (Fig. 6e). Examination of the ultrastructure of the hyphal cell wall revealed that strain YJ-gmpp had a thinner cell wall under repressive conditions (Fig. 6f).

Many fungal mutants with defective cell walls are sensitive to antifungal reagents such as Calcofluor White and Congo Red (Bai *et al.*, 2006). To test whether there is a similar phenotype associated with the conditional depletion of the *Afsrb1*, we inoculated strain YJ-gmpp and the wild-type onto RM containing 150 µg Congo Red ml<sup>-1</sup> or 200 µg Calcofluor white ml<sup>-1</sup>. As expected, strain YJ-gmpp was hypersensitive to these two reagents (Fig. 7), suggesting a defect in the cell wall. In addition, depletion of *Afsrb1* also led to hypersensitivity to 40 µg G418 ml<sup>-1</sup>, 20 µg hygromycin B ml<sup>-1</sup>, and 0.01 % SDS (Fig. 7).

To further explore the effect of GMPP activity on the cell wall of *A. fumigatus*, the cell wall components were analysed after sequential alkali and acid treatment. As summarized in Table 1, the alkali-soluble  $\alpha$ -glucan and alkali-insoluble  $\beta$ -glucan contents remained unchanged in strain YJ-gmpp depleted of *Afsrb1*, while the alkali-soluble protein and alkali-insoluble chitin contents were 1.3- and 2.0-fold higher, respectively, than those of the wild-type. These results suggested that repression of *Afsrb1* gene expression in *A. fumigatus* induced an increased content of chitin and protein in the cell wall.



**Fig. 4.** Northern blot analysis of strain YJ-gmpp depleted of *Afsrb1*. (a) Strains YJ-407 (WT) and YJ-gmpp (M) were grown on RM at 37 °C for 3 days. Total RNAs were extracted (b) and hybridized with the Dig-labelled *Afsrb1* fragment (+197 to +1072) (c). Ethidium-bromide-stained rRNAs are shown to indicate RNA loading.



**Fig. 5.** Growth characteristics of strain YJ-gmpp depleted of *Afsrb1*. (a) Conidia ( $1 \times 10^6$ ) were inoculated onto RM agar, and incubated at 37 °C. The colony radius was measured every 24 h for 5 days. White bars, YJ-407; grey bars, YJ-gmpp (b) Liquid RM was inoculated with freshly harvested conidia, poured into a Petri dish containing a glass coverslip, and incubated at 37 °C. At intervals, coverslips with adhering germlings were removed, and spore germination was observed under a DIC microscope. WT, YJ-407; M, YJ-gmpp. Bars, 20 μm.

As shown in Fig. 8, the conidia of strain YJ-gmpp germinated earlier and more rapidly than those of the wild-type. The earliest emergence of the first germ tube occurred after incubation for 7 h; this is about 1 h earlier than the wild-type. Statistical analysis revealed that the emergence of the first germ tube in strain YJ-gmpp occurred at rates of 29, 42, 60 and 65% after incubation at 37 °C for 7, 8, 9 and 10 h, respectively. Meanwhile, the corresponding rates for the wild-type strain were 0, 20, 52 and 65% at 7, 8, 9 and 10 h, respectively. About 6–14% of strain YJ-gmpp conidia formed the second germ tube after 8–9 h, while only 5% of the wild-type cells formed a second germ tube after 10 h. These results demonstrated that depletion of the *Afsrb1* gene led to an earlier and more rapid germination in *A. fumigatus*.

Reduced conidiation was also associated with depletion of the *Afsrb1* gene in strain YJ-gmpp. After cultivation at 37 °C for 5 days, strain YJ-gmpp produced about  $2.02 \times 10^8$  spores, while the wild-type produced  $14 \times 10^8$

spores. However, the conidia produced by strain YJ-gmpp appeared morphologically normal (data not shown).

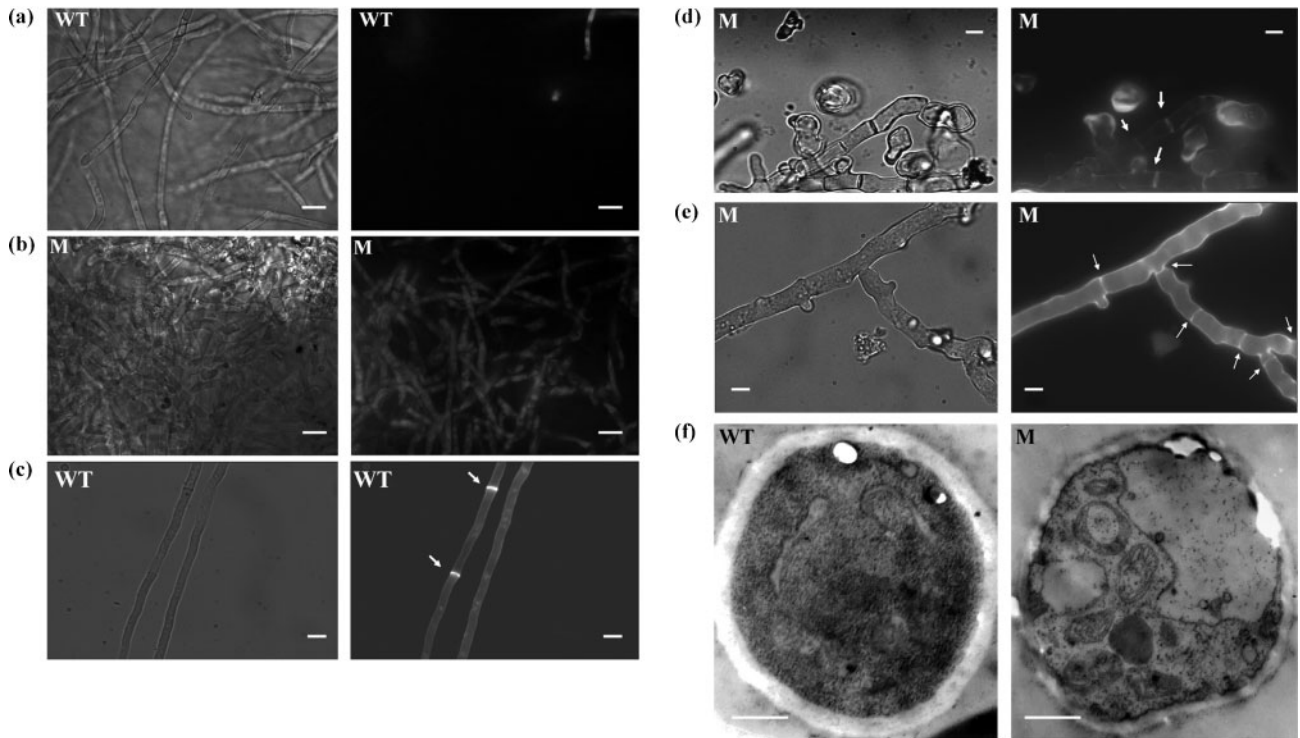
## DISCUSSION

GMPP is the final enzyme in GDP-mannose biosynthesis pathway to create GDP-mannose, which is the precursor for the mannose residues in glycan and the GPI anchor. Thus GMPP is the final component required for cell-wall organization (Ohta *et al.*, 2000; Bruneau *et al.*, 2001; De Groot *et al.*, 2005; Upadhyay & Shaw, 2006). In *S. cerevisiae* and *C. albicans*, GMPP is indispensable, and depletion leads to pleiotropic phenotypes, including lysis, failure of cell separation, impaired budding, clumping and flocculation, as well as defects in the cell wall, and impaired hyphal switching ability (Warit *et al.*, 2000). In contrast to yeasts, little is known about the physiological consequences of the action of GMPP in filamentous fungi.

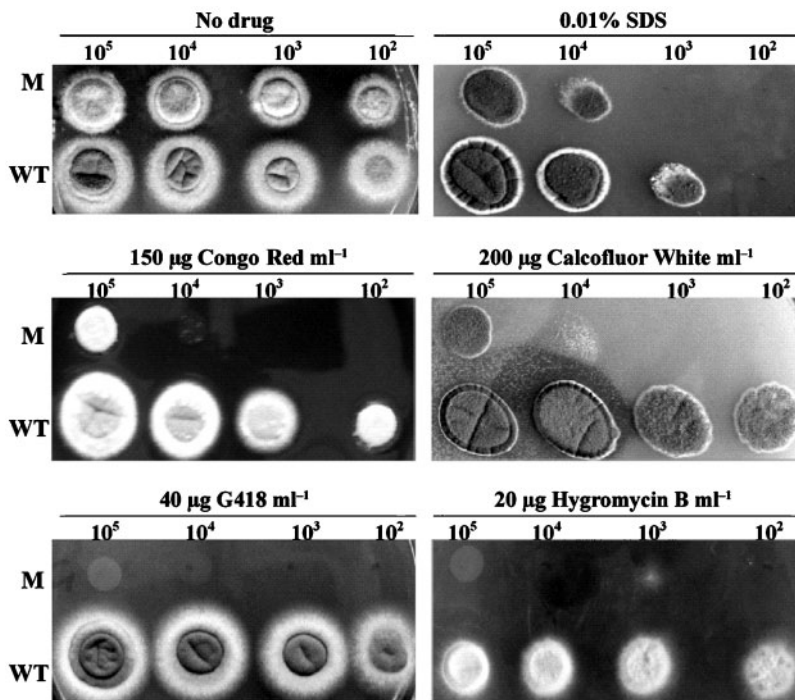
In this study, the *Afsrb1* gene was identified to encode the GMPP in *A. fumigatus*. To evaluate its physiological consequences in *A. fumigatus*, a conditional inactivation mutant, strain YJ-gmpp, was constructed by replacing the *Afsrb1* promoter with an inducible promoter,  $P_{alcA}$ . Three promoters have been successfully used in *A. fumigatus*, including the *A. nidulans alcA* promoter, the *E. coli* tetracycline-regulated promoter, and the *A. fumigatus NiiA* nitrogen-regulated promoter (Romero *et al.*, 2003; Hu *et al.*, 2007). The *alcA* promoter is induced, through the positive transcriptional regulator *alcR*, by various substrates such as ethanol or threonine, and repressed by glucose in the presence of the negative regulator *CreA* (Panozzo *et al.*, 1998). In strain YJ-gmpp, the *Afsrb1* gene was expressed under the control of the promoter of *alcA*. The presence of 3% glucose in the medium effectively blocked transcription of the  $P_{alcA}$ -*Afsrb1* expression cassette. Glucose-mediated repression of the *Afsrb1* gene caused a lethality of strain YJ-gmpp, suggesting that, as documented in *S. cerevisiae* and *C. albicans*, the *Afsrb1* gene is essential for viability of *A. fumigatus*.

Under repressive conditions, strain YJ-gmpp displayed some phenotypes similar to the yeast mutants, such as increased cell lysis and a defective cell wall (Warit *et al.*, 2000). On the other hand, in contrast to the yeast mutants, strain YJ-gmpp retained the ability to direct polarity establishment and cell separation, despite the fact that its mycelia were shorter, swollen and hyperbranched. Clearly, these phenotypes are different from those observed in the yeast mutants.

Recently, we have shown that the GPI anchor is not required for the viability of *A. fumigatus*. Blocking of GPI-anchor synthesis causes increased death or cell lysis (Li *et al.*, 2007). In the present study, a similar, but more severe, sensitivity to PI staining was associated with the repression of the *Afsrb1* gene. This significant increase in death might be attributed to the depletion of both GPI anchoring and protein glycosylation.



**Fig. 6.** Morphology of strain YJ-gmpp depleted of *Afsrb1*. Mycelia grown in liquid RM at 37 °C for 36 h were collected for PI staining (a and b, right panel), DAPI and Calcofluor White staining (c, d and e, right panel) and electron microscope analysis (f). Photographs taken under a DIC microscope are shown in the left panels of (a) ( $\times 630$ ), (b) ( $\times 630$ ), (c), (d) and (e). Septa are indicated by arrows. WT, YJ-407; M, YJ-gmpp. Bars, (a, b), 10  $\mu\text{m}$ ; (c, d, e), 5  $\mu\text{m}$ ; (f) 1  $\mu\text{m}$ .



**Fig. 7.** Sensitivity of strain YJ-gmpp to chemical reagents. Conidia were serially diluted and dotted onto RM plates containing G418, hygromycin B, Congo Red, Calcofluor White or SDS. After incubation at 37 °C for 3 days, photographs of the plates were taken. WT, YJ-407; M, YJ-gmpp.

**Table 1.** Cell-wall component analysis

Conidia ( $1 \times 10^8$ ) were added to 100 ml RM, and incubated at 37 °C with shaking (250 r.p.m.) for 36 h. The mycelia were harvested and lyophilized. Three aliquots of 10 mg dry mycelium were used as independent samples for the analysis of cell-wall proteins and water-soluble sugar, as described in Methods. The experiment was repeated twice. Values are means  $\pm$  SD.

Strain	Cell-wall component ( $\mu\text{g}$ per 10 mg dry mycelium)			
	Alkali-soluble protein	$\alpha$ -Glucan	Alkali-insoluble chitin	$\beta$ -Glucan
Wild-type	156 $\pm$ 4	47 $\pm$ 2	326 $\pm$ 25	1190 $\pm$ 17
YJ-gmpp	209 $\pm$ 10	42 $\pm$ 8	677 $\pm$ 37	1185 $\pm$ 52

In yeast, a defect in the cell wall requires the cells to induce the cell-wall integrity pathway to survive, and the compensatory mechanism featured with increased chitin content is triggered (Carotti *et al.*, 2002). In the present study, we observed an increased content of chitin in the cell wall of strain YJ-gmpp depleted of *Afsrb1*. Although the mechanism by which the increased content of chitin is triggered remains unclear, it is likely that *A. fumigatus* compensates for its cell-wall defect by synthesizing more chitin. In addition, an increased content of protein in the cell wall was also observed, and we hypothesize that this is also a compensatory mechanism. There are two lines of evidence to indirectly support our hypothesis: (i) the hypersensitivity of strain YJ-gmpp to G418 and hygromycin B, which are known to inhibit protein synthesis, may suggest a compensated role of protein in the cell wall; (ii) the hypersensitivity of strain YJ-gmpp to SDS may imply that the increased numbers of proteins are loosely attached to the cell wall, and this may be due to lack of GDP-mannose under repressive conditions.

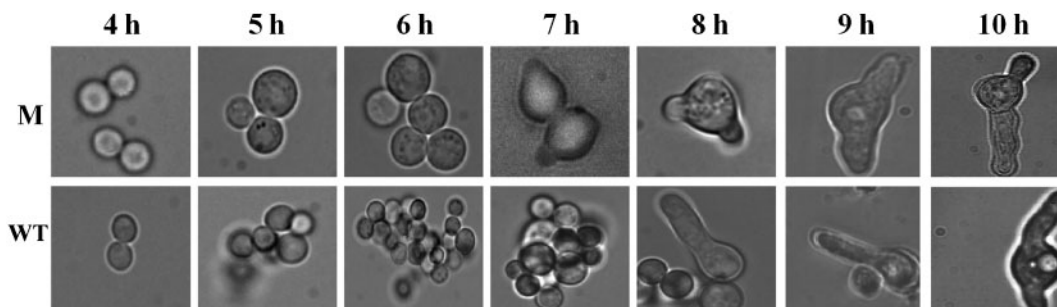
Germination is characterized by a series of ordered morphological events, including the switch from isotropic to polar growth, the emergence of second germ tube from the conidium, and septation. In *A. fumigatus*, depletion of *Afsrb1* resulted in an earlier emergence of the first and second germ tubes. A similar phenotype has also been observed in *A. fumigatus* mutants with defective *ECM33* or *afpig-a* (Romano *et al.*, 2006; Li *et al.*, 2007); both mutants

are defective in the cell wall. Since ECM33 is a GPI protein, it is reasonable to conclude that the rapid germination induced by repression of the *Afsrb1* might be due to a decrease or loss of some GPI proteins in the cell wall of *A. fumigatus*.

Some *A. fumigatus* mutants with reduced conidiation have been identified previously. One example is the *A. fumigatus*  $\Delta$ *Afpmt1* mutant, which exhibits a severe reduction of conidiation, especially at high temperatures, and this suggests that *O*-mannosylation is required for the conidiation of *A. fumigatus* (Zhou *et al.*, 2007). We also found a reduction in the amount of conidiation in strain YJ-gmpp depleted of *Afsrb1*. Thus, we postulate that depletion of *O*-mannosylation in *A. fumigatus* might be one of the causes for the reduced conidiation, since GDP-mannose is the precursor of *O*-glycan. Interestingly, unlike the case of the  $\Delta$ *Afpmt1* mutant, strain YJ-gmpp was able to form normal conidia under repressive conditions, suggesting that *A. fumigatus* may possess a mechanism to ensure its survival in conditions of GDP-mannose starvation, by which fewer, but normal, conidia are formed.

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**Fig. 8.** Conidial germination of strain YJ-gmpp depleted of *Afsrb1*. Freshly harvested conidia ( $1 \times 10^7$ ) of YJ-407 (WT) and YJ-gmpp (M) were poured into a Petri dish containing glass coverslips, and incubated at 37 °C for 4–10 h. At the specified times, coverslips with adhering germlings were removed, and spore germination was examined under a DIC microscope ( $\times 1000$ ).

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