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Deletion of *GEL2* encoding for a β(1–3)glucanosyltransferase affects morphogenesis and virulence in *Aspergillus fumigatus*

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Summary

The first fungal glycosylphosphatidylinositol anchored β(1–3)glucanosyltranferase (Gel1p) has been described in *Aspergillus fumigatus* and its encoding gene *GEL1* identified. Glycosylphosphatidylinositol-anchored glucanosyltransferases play an active role in the biosynthesis of the fungal cell wall. We characterize here *GEL2*, a homologue of *GEL1*. Both homologues share common characteristics: (i) *GEL1* and *GEL2* are constitutively expressed during over a range of growth conditions; (ii) Gel2p is also a putative GPI-anchored protein and shares the same β(1–3)glucanosyltransferase activity as Gel1p and (iii) *GEL2*, like *GEL1*, is able to complement the Δgas1 deletion in *Saccharomyces cerevisiae* confirming that Gelp and Gasp have the same enzymatic activity. However, disruption of *GEL1* did not result in a phenotype whereas a Δgel2 mutant and the double mutant Δgel1Δgel2 exhibit slower growth, abnormal conidiogenesis, and an altered cell wall composition. In addition, the Δgel2 and the Δgel1Δgel2 mutant have reduced virulence in a murine model of invasive aspergillosis. These data suggest for the first time that β(1–3)glucanosyltransferase activity is required for both morphogenesis and virulence in *A. fumigatus*.

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Introduction

Aspergillus fumigatus causes fatal invasive aspergillosis among immunocompromised patients (Latgé, 1999). The main reason for patient death is the low efficacy of the drug therapies available to treat aspergillosis and the lack of an assay that can detect the fungus early during the infection. The fungal cell wall is a rigid layer that protects the organism from its environment. Its composition and important functions make it an ideal target for the development of new therapeutic drugs. β(1–3)glucan is the main component of the cell wall of filamentous fungi and yeast (Latgé *et al*., 1991; Hearn and Sietsma, 1994). In fungal families, β(1–3)glucans are synthesized by a plasma membrane-bound glucan synthase complex. Following their synthesis, they are then extruded into the periplasmic space (Beauvais *et al*., 1993; 2001; Klis, 1994) where they become linked to other cell wall components resulting in the formation of a rigid structure. Of all filamentous fungi, both the cell wall composition and its organization are best understood in the human opportunistic pathogen *A. fumigatus* (Fontaine *et al*., 2000). We used this organism to search for enzymatic activities such as glucanases or transglycosidases which could modify β(1–3)glucans in the periplasmic space. During this search, a new β(1–3)glucanosyltransferase was identified biochemically in *A. fumigatus* (Hartland *et al*., 1996) and the encoding gene *GEL1* characterized (Mouyna *et al*., 2000a).

GEL1 is homologous to the *GAS*/*PHR*/*EPD* yeast gene families (Nuoffer *et al*., 1991; Vai *et al*., 1991; Popolo *et al*., 1993; Saporito-Irwin *et al*., 1995; Muhlschlegel and Fonzi, 1997; Nakazawa *et al*., 1998; 2000) which play a role in cell wall morphogenesis. We have shown that the yeast proteins Gas1p, Phr1p and Phr2p display the same β(1–3)glucanosyltransferase activity and share the same catalytic residues as Gel1p (Mouyna *et al*., 2000a,b).

In yeast, several genes of each family have been isolated. In this study, we looked for *GEL* homologues in *A.*

fumigatus. We report the identification and the characterization of *GEL2*, a homologue of *GEL1*. *GEL1* and *GEL2* are constitutively expressed. We showed that Gel2p displays the same β(1–3)glucanosyltransferase activity as Gel1p. However, unlike a gel1 deletion mutant which was without a phenotype, Δgel2 and Δgel2Δgel1 mutants showed reduced growth, morphological defects and a decrease of virulence.

Results

Sequence analysis of the homologue of GEL1*,* GEL2

Analysis of the sequences of the complementary and genomic DNA showed that the open reading frame (ORF) of *GEL2* was 1425 bp long and encoded a 475 amino acid polypeptide with a theoretical molecular weight of 52 kDa and a pI of 4.62. *GEL2* contained four introns, five putative N-glycosylation sites and has all the characteristics of GPI-anchored proteins: a hydrophobic amino-terminus (20 amino acids) according to the (–3, –1) rule (Von Heijne, 1986) and a hydrophobic C-terminus (25 amino acids) with a carboxy peptidase cleavage site consensus sequence found in GPI-proteins (Gerber *et al*., 1992). The carboxyl signal peptidase cleavage site is SAA for the attachment of the GPI anchor. The amino acid sequences of *GEL1* and *GEL2* showed a 38% identity. Reverse transcription polymerase chain reaction (RT-PCR) data analysis showed that *GEL1* and *GEL2* were constitutively expressed during active growth in a shake culture (1% glucose + 1% mycopeptone). Furthermore were detected by RT-PCR after 11 and 24 h of cultures at pH 4, 6 and 8, conditions that will span the entire development of the mycelial phase of both mutant and wild-type (WT) strains.

Gel2p shares the same enzymatic activity as Gel1p

The sequence homology observed between Gel2p and Gel1p and the conserved aspartic acid residues in the catalytic domains FF(A/S)GNE*V (for the catalytic acid-base) and F(F/L)SE*Y/GCN (for the nucleophilic residue) (Mouyna *et al*., 2000b) suggested that Gel2p displays the same glucanosyltransferase activity as Gel1p. As previous results showed that a recombinant Gel1p lacking the GPI signal sequence (Gel1p419) displayed the same β(1–3)glucanosyltransferase activity as the full length *A. fumigatus* protein (Mouyna *et al*., 2000a), the enzymatic activity of a recombinant protein Gel2p451, that was expressed without the GPI sequence and secreted in the culture medium, was investigated. SDS-PAGE analysis showed that Gel2p451 produced by *Pichia pastoris* migrated with an apparent Mr of 60–62 kDa. After N-deglycosylation, the protein had an apparent Mr of 50 kDa (data not shown). Analysis by High Performance Anion Exchange

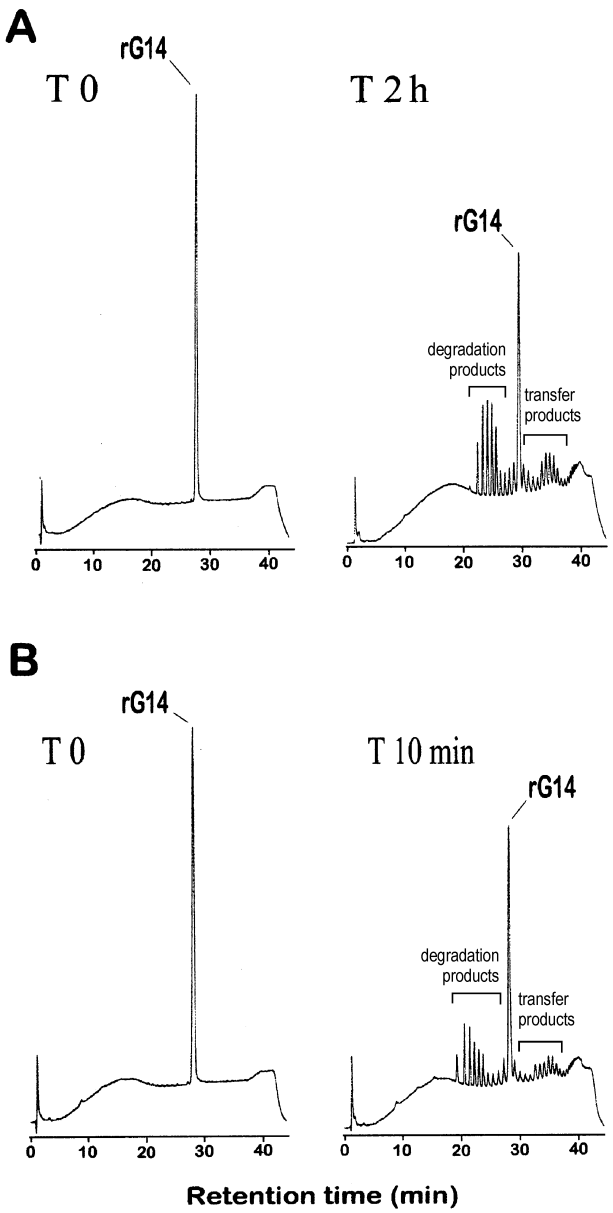

Fig. 1. HPAEC analysis of products from the incubation of the recombinant (A) Gel1p and (B) Gel2p with reduced laminarioligosaccharides.
A. Analysis of products after 0 and 2 h of incubation with Gel1p.
B. Analysis of products obtained with Gel2p after 0 and 10 min.

Chromatography (HPAEC) of the products resulting from the incubation of recombinant proteins Gel1p419 and Gel2p451 with reduced laminarioligosaccharides is shown in Fig. 1. After a 10–120 min incubation, a set of new β(1–3) glucan transfer products was seen. Comparison of the HPAEC pattern with control laminarioligosaccharides showed that all products contained only β(1–3)linkages, because the introduction of a linkage different from β(1–3) will result in a shift in the retention time of the branched oligosaccharide (Koizumi, 1989; Mouyna *et al*.,

1998). The pattern obtained for recombinant Gel2p451 was identical to the pattern obtained for Gel1p419. These results indicate that Gel2p displayed a β(1–3)glucanosyltransferase activity similar to the one characterized for Gel1p (Gel2p and Gel1p display the same activity after 10 and 120 min respectively).

Complementation of Δgas1 with GEL2

Gel2p displays *in vitro* the same enzymatic activity as Gel1p suggesting a functional homology to Gas1p and in this respect it should be able to rescue *in vivo* the phenotype of the gas1 null mutant. The *GEL2* cDNA was cloned in a Yep24 vector under the control of the *GAS1* promoter and used to transform *gas1* cells. In transformed cells a unique transcript was amplified by RT-PCR whose expression level is comparable to *gas1* cells transformed with the Yep vector carrying the *GAS1* gene (data not shown). Total proteins from cells expressing the *GEL2* cDNA were analysed by immunoblot using antibodies against Gel2p. In these cells a band of about 60–62 kDa was detected that was absent in untransformed cells (Fig. 2A). Following treatment with tunicamycin (10 μg ml^{-1}) for 2 h, an immunoreactive form of about 50–52 kDa accumulated in cells (Fig. 2A, lane b) indicating that Gel2p was N-glycosylated in *Saccharomyces cerevisiae* as in *P. pastoris*. A null mutation in the *GAS1* gene caused several morphogenetic defects: cells had an abnormal morphology, became round and larger, were defective in bud maturation and cell separation, were more sensitive to calcofluor white (CF) and more resistant to Zymolyase (Popolo *et al*., 1993). In cells expressing the *GEL2* cDNA, the growth and the morphological defects, resulting from the deletion of *GAS1*, were reversed (data not shown). These data indicate that Gel2p significantly reduced the defects of the *gas1* mutant. For example, resistance to calcofluor white was increased compared with the control (Fig. 2B). Yet, in regards to the CW resistance, but *GEL2,* however, did not complement totally the *gas1* null mutant as well as *GEL1*.

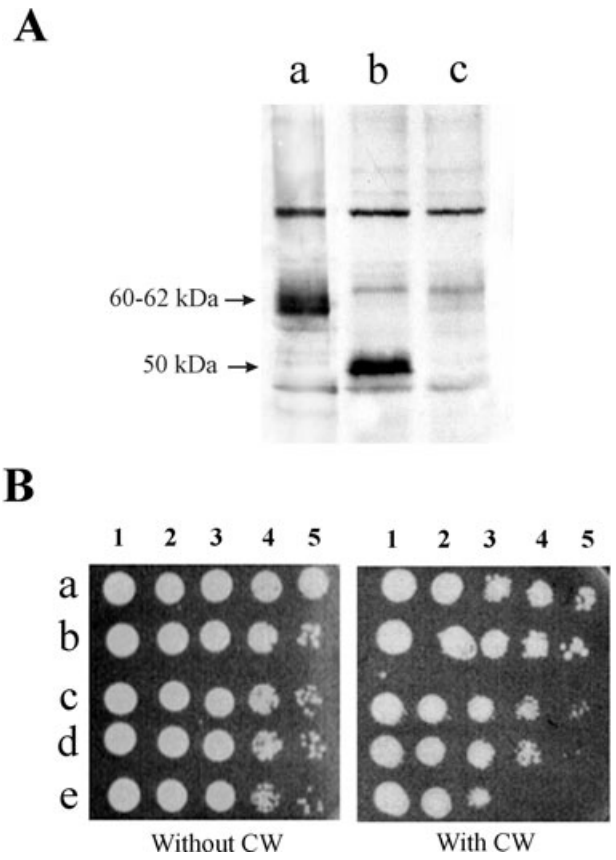

Fig. 2. A. Immunoblot analysis of *S. cerevisiae* extracts. Total extracts from exponentially growing cells of WB2d (YEp-Sc*GEL2*) strain (lane a), WB2d (YEp-Sc*GEL2*) after Tunicamycin treatment (2 h with 10 μg ml^{-1} at a cell density of 5 × 106 ml^{-1} (lane b), and WB2d (Yep) (lane c) were analysed by immunoblotting with anti-Gel2p antibodies. B. Sensitivity to calcofluor white (CW). (i) Not supplemented (CW) or (ii) supplemented (CW) with 50 μg ml^{-1} of calcofluor White. (a) Yep-ScGAS1; (b) Yep-ScGEL1; (c) Yep-ScGEL2 isolate a; (d) Yep-ScGEL2 isolate b (a and b are two independent isolates); (e) Yep.

Phenotypic analysis of Δgel1, Δgel2 and Δgel1Δgel2 mutants

Phenotypic analysis of Δgel1 mutant. The strategy described in Fig. 3A was used to produce an *A. fumigatus* strain with a non-functional copy of *GEL1*. Eighteen transformants resistant to hygromycin were analysed by restriction enzyme digestion. The results of a Southern blot analysis after *Eco*RV digestion of one of the Δgel1 null mutants are shown in Fig. 4A. The disappearance of three *Eco*RV fragments, 0.8 kb, 3 kb and 4 kb, from the parental strain and the appearance of a new fragment of 10 kb indicated the integration of the hph cassette at the *GEL1* locus for six transformants. No differences in hyphal and conidial morphology were found macroscopically or microscopically when the Δgel1 mutant was compared with the parental strain CBS 144.89. Minimal inhibitory concentration (MIC) values for SDS, Nikkomycin, Polyoxin D and Calcofluor white M2R were similar at 37°C in a 1% yeast extract medium for both parental and mutant strains (1.56, 0.019, >2, 0.3 mg ml^{-1} respectively). The kinetics of mycelial growth in liquid culture and conidial germination was not affected in the mutant, irrespective of the pH of the culture medium. The hexose and hexoseamine contents and composition of the alkali-insoluble (AI) fraction and the alkali-soluble (AS) fraction of the cell wall were similar in the mutant and parental strains (data not shown).

Phenotypic analysis of Δgel2 and Δgel1Δgel2 mutants. The strategy described in Fig. 3B was used to produce

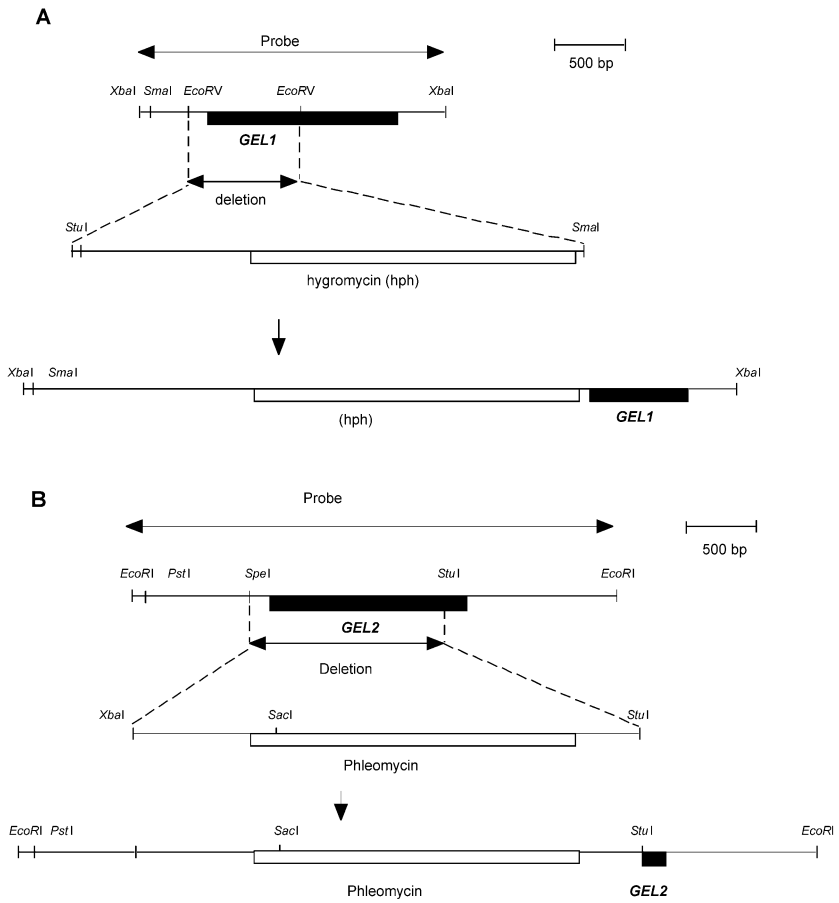

Fig. 3. A. Disruption of the *GEL1* gene. The internal *Eco*RV-*Eco*RV fragment was deleted from the *GEL1* ORF and replaced by the *Stu*I-*Sma*I fragment of pN4 plasmid carrying the hygromycin resistance gene.
B. Disruption of the *GEL2* gene. The internal *Spe*I-*Stu*I fragment containing most of the coding region was deleted and replaced by the *Xba*I-*Stu*I fragment of pAN8.1 plasmid carrying the phleomycin resistance gene.

either a Δgel2 mutant in CBS 144.89 strain or a Δgel1 strain with a non-functional copy of *GEL2*. Protoplasts of a WT strain and Δgel1 mutant of *A. fumigatus* were transformed using the fragment linearized by *Pst*I. The Southern blot analysis of the Δgel2 and Δgel2Δgel1mutants are shown in Fig. 4B. The 3.5 kb *Eco*RI/*Sac*I fragment has been replaced by two new *Eco*RI/*Sac*I fragments of 2 and 3 kb in the Δgel2 and Δgel1Δgel2 strains indicating the integration of the phl cassette at the *GEL2* locus. Germination of the Δgel2 and Δgel1Δgel2 mutant are the same as the WT, but in contrast to Δ gel1, the growth of Δgel2 mutant was severely impaired both in solid and liquid culture. The double disruption aggravated the phenotype. Reduction of growth, however, was higher on agar than in liquid cultures (Fig. 5A and B). In addition, the morphology of mycelial growth in liquid culture was different for the Δgel2Δgel1 mutant and for the Δgel2 mutant compared with the WT parental strain. After 16 h of growth in liquid medium, conidiophore vesicles and phialides were formed in the fermenter. Phialides produced conidia (Fig. 6A) or continued to grow (Fig. 6B–D). Phialides, which produced hyphal-like growth, and phialides with a normal morphology appeared at the same time in liquid culture on different mycelia. In addition, the number of growing phialides varied per conidiophore (Fig. 6C and D). When conidia were produced and released into the medium, they did not germinate but remained viable. These conidia were able to germinate when they were removed from the culture medium, washed with water and subsequently incubated in a fresh Sabouraud liquid medium. Expression of the sporulation genes *BRLA*, *ABAA*, *STUA*, *RODA* and *WETA*, was investigated by RT-PCR with RNA isolated from WT, Δgel2 and Δgel2Δgel1 mutants grown in liquid culture (Fig. 7). The results showed that the sporulation genes *BRLA*, *ABAA*, *STUA*, *RODA* and *WETA* were expressed in Δgel2 and Δgel2Δgel1 mutants and not in WT (as expected) after 24 h. This expression pattern correlated well with the production of conidiophores and conidia in liquid culture. Further, electron microscopy analysis showed that the conidia did not have the outer pigmented layer characteristic of conidia that are produced in solid media (Fig. 8). However, expression of *ALB1*, *ABR1, ABR2* (which are involved in melanin synthesis) was not detected in the mutants although it was detected in genomic DNA (data not shown). These results were in agreement with electron microscopy observations (Fig. 8) that showed the absence of melanin in the conidia of Δgel1Δgel2 and Δgel2 mutants compared with the WT.

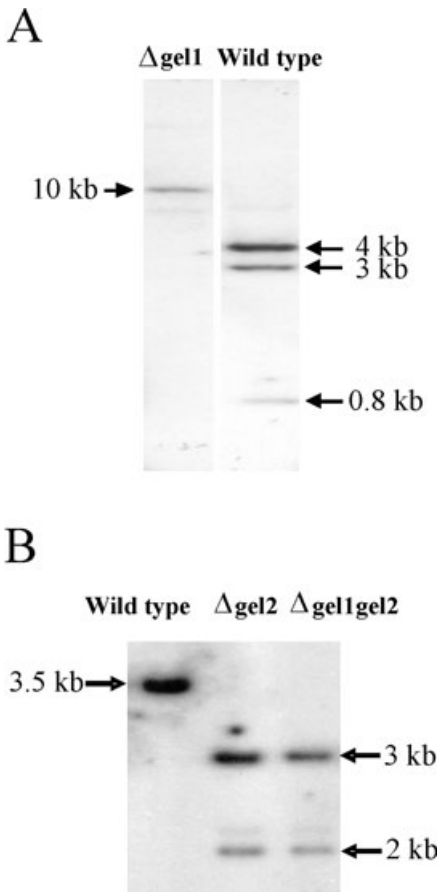

Fig. 4. A. Southern blot analysis of the Δgel1 mutant. Genomic DNA digested with *Eco*RV was probed with the *Xba*I fragment that included the entire *GEL1* ORF. The electrophoretic positions and sizes of DNA are indicated.
B. Southern blot analysis of the Δgel2 mutant. Genomic DNA digested with *Eco*RI/*Sac*I was probed with the *Eco*RI fragment including the entire *GEL2* ORF (see Fig. 3 for details).

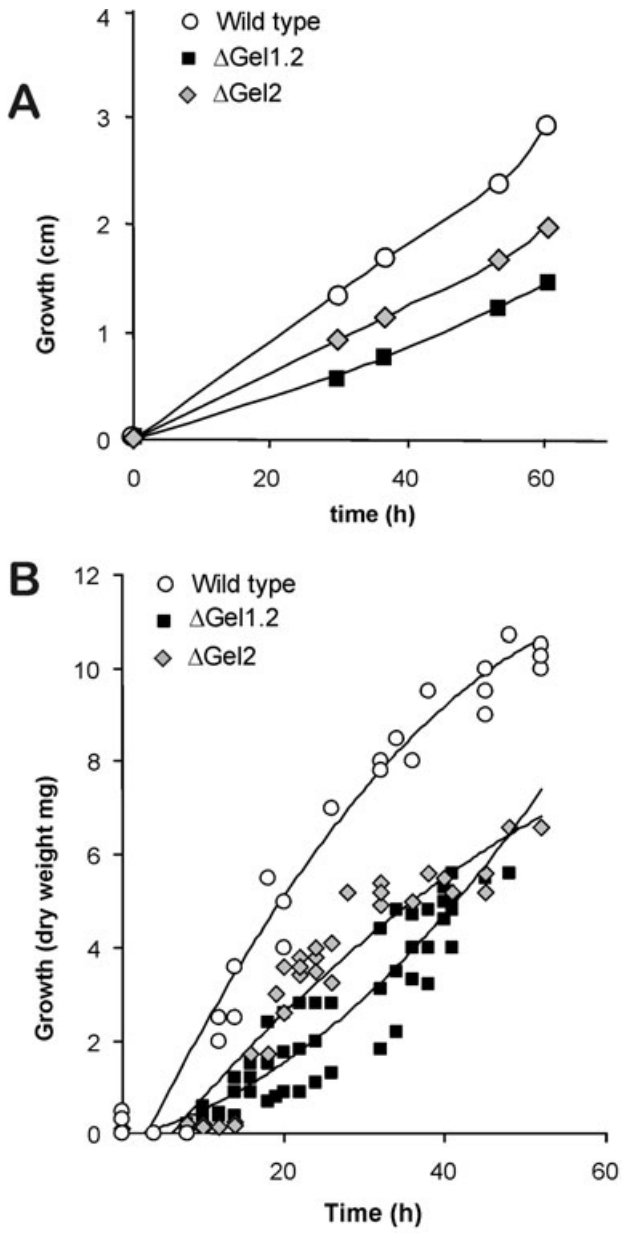

Fig. 5. Growth kinetics of Δgel2, Δgel2Δgel1 and wild type strains on (A) Sabouraud agar and (B) Sabouraud liquid medium. Each point represents one dry weight measurement (three experiments) and the tendency curves are indicated.

To compare precisely the virulence of Δgel2, Δgel2Δgel1 and WT strains, mixed infections followed by quantification of each DNA strain in the lung were undertaken. After mixed infections of Δgel2 and Δgel2Δgel1, there was no significant difference in the threshold cycle (Ct) values between the two strains (Table 1) (*t* paired test: prob > ltl 0.1695 for a d.f. of 7 for Δgel2Δgel1/Δgel2). This result indicated that equivalent amounts of DNA of both mutants were present in mouse and that accordingly Δgel2 and Δgel2Δgel1 showed the same virulence. The mixed infections of Δgel2 + WT and Δgel2Δgel1 + WT demonstrated that the two mutants were significantly less virulent than the WT parental strain. The amount of DNA estimated by the Ct values was significantly lower for the

Table 1. Average of Ct values obtained after mixed infections.

	WT + ΔGel2		WT + ΔGel1.2		ΔGel2 + ΔGel1.2	
	MDR4	Phleoquanti	MDR4	Gel1quanti	MDR4	Gel1quanti
Average of Ct values	32.26	33.91	29.5	33	30.61	31.03

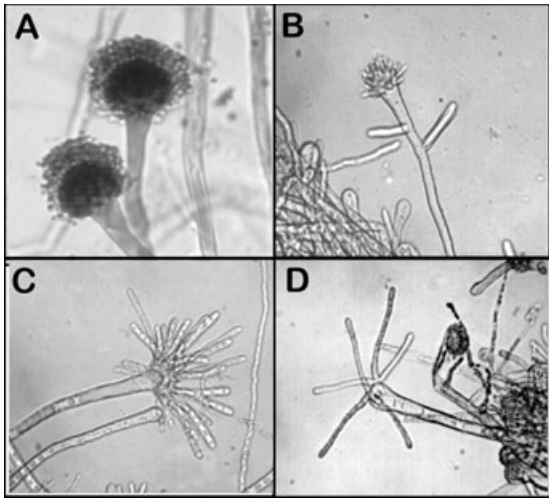

Fig. 6. Conidiophore morphology of the Δgel2 and Δgel1gel2 mutants grown on Sabouraud liquid medium. Two types of conidiophores are observed for the Δgel2Δgel1 and Δgel2 mutants. In (A) the conidophores of mutant Δgel2 and Δgel2Δgel1 are like those of wild type cells, but in B, C and D, the conidophores of the Δgel2 and Δgel2Δgel1 mutants are abnormal, either shorter (B) or resembling hyphae (C and D).

Δgel2 or Δgel2Δgel1 mutant than the WT parental strain (prob > |t| < 0.0001 for a d.f. of 4 and prob > |t| < 0.0001 for a d.f. of 7) (Table 1).

Δgel2 and Δgel2Δgel1 were slightly more sensitive to inhibitors of cell wall polysaccharide synthase such as Nikkomycin Z (20 μg ml^{-1} WT; 1 μg ml^{-1} Δgel2; 0.5 μg ml^{-1} Δgel2Δgel1), Mulundocandin (60 μg ml^{-1} WT, 10 μg ml^{-1} Δgel2, 5 μg ml^{-1} Δgel2Δgel1) and Lilly 303366 (30 μg ml^{-1} WT, 10 μg ml^{-1} Δgel2, 6 μg ml^{-1} Δgel2Δgel1). No difference was seen in the total amount of AS polysaccharides in the cell wall of the WT, Δgel2 and Δgel2Δgel1 mutant. This fraction accounts for 40% of the total cell wall. Although the AS fraction was mainly composed of glucan, a significant reduction in galactomannan was seen in the Δgel2 and Δgel2Δgel1 mutants, that represented 23%, 12% and 6% of the AS total carbohydrates respectively (for the WT, the Δgel2 and Δgel2Δgel1 mutants). No reducing sugar was released after a 3 day incubation of AS with the endo β1–3glucanase Quantazyme indicating that (i) no β(1–3)glucan was present in the AS of the WT and mutant and (ii) the AS glucan was α(1–3) linked, in agreement with the presence of 2,4,6 methylated glucose in this fraction (data not shown).

In contrast to the AS fraction, disruption of *GEL2* resulted in important modifications of the AI fraction (Table 2). The total amount of hexose in the AI fraction was significantly reduced from 39% of the total cell wall in the WT to 31% in the Δgel2 and Δgel2Δgel1 mutant. However, the hexose composition of the AI fraction remained similar in all strains (Table 2). A similar percentage of glucan (around 75%) was solubilized by incubation of AI with the endoβ(1–3)glucanase. MALDI-TOF analysis indicated that the material released by quantazyme was similar in both mutant and parental strains and consisted mainly of laminarioligosaccharides of dp5 with minor amounts of dp6 and dp7. Moreover, acetylation methylation analysis did not show any significant differences in the linkages between the different monosaccharides of the AI fraction (data not shown). These results suggested that the main structural organization of the gluco-galactomannan was conserved in the AI fraction of the WT and mutants. The 8% reduction of the hexose in the AI fraction of the mutant strains was compensated by an equivalent increase in the hexoseamine content. The hexoseamine concentration was 20, 27 and 30% of the WT, Δgel2 and Δgel2Δgel1 cell wall extract respectively. The hexoseamine composition was, however, similar in both WT and mutant strain with a glucosamine to galactosamine ratio equal to 1.

Complementation of the Δgel2 phenotype with GEL2 expression

Hygromycin-resistant transformants obtained after introduction of the pCBGla-*GEL2* into Δgel2 that restored the WT phenotype in the presence of maltose were analysed by Southern blots. One copy of the pCBGla-*GEL2* was integrated in all transformants. In the presence of xylose (that represses the glucoamylase promoter), the transformants that have an integrated pCBGla-*GEL2* retained the WT phenotype. Previously published data showed that a residual low expression level of Pgla was seen in *A. niger* in the presence of xylose (< 2%) (Verdoes *et al.*, 1994). Recent work in our laboratory (E. Mol, unpubl.) using glucuronidase as a reporter gene in *A. fumigatus* confirmed that this promoter Pgla was induced in presence of 2% maltose and repressed in presence of 2% xylose. Complementation of the phenotype was obtained with a

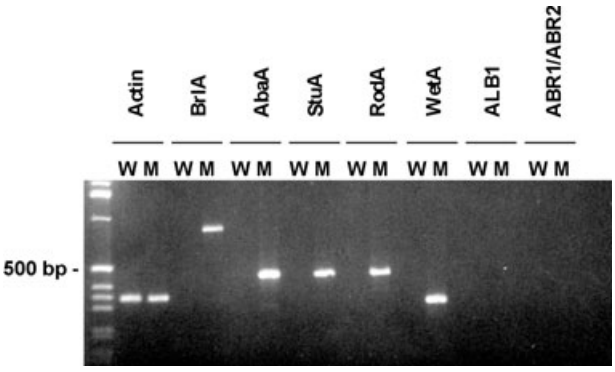

Fig. 7. RT-PCR of actin, *BRLA*, *ABAA*, *STUA*, *RODA*, *WETA*, *ALB1*, *ABR1* or *ABR2* genes in W: wild type and M: Δgel2 mutants grown for 24 h in Sabouraud medium (Δgel1Δgel2 mutants have exactly the same pattern as the Δgel2 mutants).

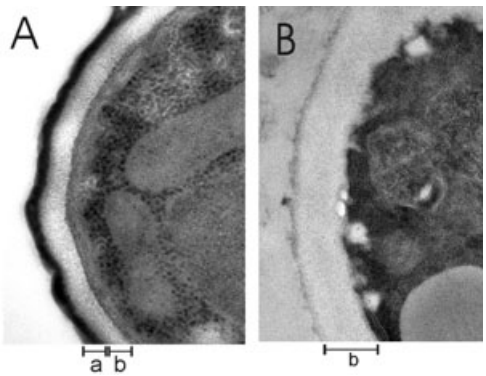

Fig. 8. Electron microscopy of wild type (A) and Δgel2 mutant (B) conidia obtained from fermentor cultures: (a) melanin; (b) cell wall.

very low amount of Gel2. These results also confirmed that the phenotype observed for the Δgel2 mutants was associated with the disruption of *GEL2*.

Discussion

We previously characterized *GEL1* of *A. fumigatus*, which encodes a β(1–3)glucanosyltransferase (Hartland *et al.*, 1996; Mouyna *et al.*, 2000a). We report here the molecular analysis of *GEL2, GEL1* and *GEL2* were expressed during growth in all culture media, and in spite of their homologous sequences and identical enzymatic activities of the encoded protein, disruption of these two genes resulted in very different phenotypes: the growth of Δgel2 was significantly reduced whereas Δgel1 grew as well as WT cells. It appears that members of several gene families that are involved in cell wall biosynthesis of *A. fumigatus* have different cellular functions, including chitin synthase (Mellado *et al.*, 1996; 2003), α(1–3)glucan synthase (Beauvais *et al.*, 2005). A similar observation has been made with the *FKS* gene family of *S. cerevisiae* that is 56–88% identical. *FKS1* and *FKS2* display the same glucan synthase activity (Mazur *et al.*, 1995) but are differentially regulated whereas *FKS3* is not involved in glucan synthesis but may act as a transporter (Lesage *et al.*, 2002). Investigations are presently underway to understand the discrepancies in the cellular function of Gel1p and Gel2p.

GEL1 was found to be homologous to the *GAS*/*PHR*/*EPD* genes that encode a family of GPI-anchored proteins required for correct morphogenesis in yeast. Homologues of *GAS*/*GEL* have been identified in other yeast species such as *Candida dubliniensis* (Kursai *et al.*, 1999), *Candida glabrata* (Weig *et al.*, 2001), or *Schizosaccharomyces pombe* http://broad.mit.edu/annotation/fungi/S.pombe) but also in filamentous species such as *Pneumocystis carinii* (Kottom *et al.*, 2001), *Neurospora crassa* (http://broad.mit.edu/annotation/fungi/neurospora/), *Coccidioides immitis* (http://www.tigr.org/tdb/ufmg), *Magnaporthe grisea* (http://www-genome.wi.mit.edu/annotation/fungi/magnaporthe/) *Aspergillus nidulans* and *A. oryzae* (http://www.broad.mit.edu/annotation/fungi/aspergillus). This study was undertaken before the completion of the genome sequencing of *A. fumigatus*. In the last release of the TIGR database (http://tigrblast.tigr.org/ufmg/), seven genes belonging to the *GEL* family have now been found in *A. fumigatus*. The percentage identity between members of the *GEL* families varies between 30 and 65%. They are clustered in two subfamilies: *GEL3*, *GEL4*, *GEL5* and *GEL7 (*from 53 to 65% of identity) and *GEL1*, *GEL2* and *GEL6* (from 36 to 38% of identity). Each of these genes belongs to the family 72 of the glycosylhydrolase classification of Henrissat and Coutinho (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html). For all proteins of different fungal species, the two catalytic residues identified in Gel1p are very conserved, suggesting that these proteins have the same β(1–3)glucanosyltransferase activity which has been demonstrated now for five members of this family, *GEL1*, *GEL2*, *GAS1*, *PHR1* and *PHR2* (Mouyna *et al.*, 2000b). Moreover, *GEL1, GEL2*, *PHR1* and *PHR2* can complement the *GAS1* mutation so that these proteins share the same enzymatic activity in spite of sequence variations. Variations in the sequences of these proteins occur mainly at the C- and N-terminus. At the C-terminus, some proteins like *GAS* display a lot of Serine

Table 2. Cell wall carbohydrate composition of the wild type and mutants Δgel2 and Δgel1Δgel2 (average of at least three experiments).

	Alkali-insoluble			Alkali-soluble		
	WT	Δgel2	Δgel1–2	WT	Δgel2	Δgel1–2
Hexose (total %CW)	39	31	31	39	41	37
Composition						
Glucose	68	78	70	77	88	94
Galactose	20	10	15	17	7	4
Mannose	12	12	15	6	5	2
Hexoseamine total	20	27	30			
Composition						
GlcNac	52	51	48			
GalNac	48	49	52			

(S) and Threonine (T). For example, *GAS1* of *S. cerevisiae* has 33 S + T, but in contrast, *GEL1* of *A. fumigatus* has only 23 S + T. In addition, Popolo and Vai (1999) have identified 13 conserved cysteine residues in *GAS1* whereas only six can be found in *GEL1* of *A. fumigatus*).

One of the characteristic features of family 72 is that some members are pH-regulated; however, this characteristic is species-dependant. For example, *GEL1* and *GEL2* are constitutively expressed irrespective of pH of the culture medium (this study). *GAS1* in *S. cerevisiae*, *CgGAS1* and *CgGAS2* in *C. glabrata* (Popolo *et al*., 1993; Weig *et al*., 2001) are not pH regulated. In contrast, in *Candida albicans*, in *Candida maltosa* and in *P. carinii*, the pH of the culture medium plays a major role (Saporito-Irwin *et al*., 1995; Muhlschlegel and Fonzi, 1997; Nakazawa *et al*., 1998; Kottom *et al*., 2001). In *A. nidulans*, response to ambient pH and the pathway controlling pH responsive gene expression has been extensively studied. Central to the pH response is the activation of the zinc finger transcription factor pacC (Tilburn *et al*., 1995). In *C. albicans* or in *S. cerevisiae*, a homologue of pacC, designated, respectively, *PRR2* or *RIM101,* has been identified and cloned (Su and Mitchell, 1993; Ramon *et al*., 1999). The DNA motif recognized by pacC has been well characterized and contains the consensus sequence 5′-GCCAAG-3′ (Tilburn *et al*., 1995; Espeso *et al*., 1996; 1997). One copy of the pacC recognition site is found upstream of the alkaline induced gene *PHR1* (A.M. Ramon, unpubl. results). All homologues of the *A. nidulans* regulation cascade, including one homologue of pacC, are present in the *A. fumigatus* genome database (http://tigrblast.tigr.org/ufmg/), but in agreement with the lack of pH regulation of *GEL1* and *GEL2* expression, pH recognition sites have not been found upstream of any *GEL* genes. Based on the β(1–3)glucanosyltransferase activity of the *GEL* proteins, our working hypothesis for the biological function of Gelp is that it functions in the elongation of β(1–3)glucan side chains providing new non-reducing ends for anchoring of other polysaccharides of the cell wall network. Fonzi (1999) has also suggested that the Phrp homologues could process β(1–3)glucans and make available acceptor sites for the attachment of β(1–6) glucans. Based on this model, mutation in the *GEL* genes would induce perturbations in the cell wall composition. Modifications in cell wall composition have been indeed observed in the Δgel2 and Δgel2Δgel1 mutants. It was, however, impossible to correlate the modifications found in the cell wall of specific *GEL* deletions with the enzymatic activity of *GEL*. The only modifications of the cell wall that were in agreement with a β(1–3)glucan modifying activity is a decrease of the β(1–3)glucan content of the Δgel2 mutants associated to a three- to fourfold increase in the amount of β (1–3, 1–6) glucose linkages in the Quantazyme resistant cell wall pellet (data not shown). No release of β(1–3)glucan into the culture medium occurred, probably because of the inability of the mutant to anchor β(1–3)glucan to the polysaccharide core (data not shown). One of unexpected effects of the *GEL2* mutation is a significant reduction in the total cell wall galactomannan from 22% in the WT to 12% in the Δgel2 mutant (Table 2). In an analysis of the cell wall composition of phr mutants, Fonzi (1999) found an increase in linkages of β glucans to chitin. This modification is not apparent in the *A. fumigatus GEL* mutants. The difficulty in correlating cell wall modifications of the Δgel mutants with enzymatic activity may be partly resulting from the compensatory changes in the cell wall that occur as a consequence of the lack of *GEL* by *A. fumigatus*. The increase in levels of chitin in the Δgel2 mutants is a classical compensatory event in *S. cerevisiae* cell wall mutants (Valdivieso *et al*., 2000). An increase in glycoprotein secretion was found in Δgel2 mutants (data not shown), somewhat similar to gas mutants (Ram *et al*., 1998). Such a finding suggests that the cell wall mutant is more permeable. This is in agreement with the higher sensitivity of the mutant to echinochandin and Nikkomycin derivatives (such as Nikkomycin Z, Mulundocandin and Lilly 303366).

Experimental procedures

Strains and growth conditions

Aspergillus fumigatus strain CBS 144.89, was grown in Sabouraud liquid medium at 25°C (2% glucose + 1% mycopeptone, Biokar, Beauvais, France) for DNA and protein extraction as previously described (Hartland *et al*., 1996; Mouyna *et al*., 1998) or on malt slant to produce spores. For transformation experiments, a minimal medium (Cove, 1966) was used. The *S. cerevisiae* haploïd strain WB2d (gas1::LEU2), generated from the WT strain W303–1B (MATα ade2-1 his3-11,15 trp1-1 ura3-1 leu2-3112 can1-100) by one-step disruption (Vai *et al*., 1996), was used for complementation experiments. Yeast cells were grown at 30°C in Difco yeast nitrogen base (YNB) medium (6.7 g l^{-1}) containing 2% glucose and the required supplements.

Isolation of GEL2

An homologue of *GEL1* gene was identified (before the completion of the *A. fumigatus* genome sequence) and isolated by PCR amplification with the degenerate primers P1 5′-GSYTTCTTCKCYGGCAACGAGGTT-3′ and P2 5′-GTTG CAGCCGWATTCGGASAYGAA-3′. These primers encompass the AFFSGNEV and FLSEYGCN amino acid sequences that are conserved among the *A. fumigatus* Gel1p (Mouyna *et al*., 2000a), the *C. albicans* Phr1p (Saporito-Irwin *et al*., 1995) and *S. cerevisiae* Gas1p (Vai *et al*., 1991). PCR amplification was performed with template genomic DNA from *A. fumigatus* CBS 144.89. Thirty cycles of PCR were performed using the following steps: 1 min at 94°C, 1 min at

45°C, 1 min at 72°C. The reaction yielded specifically a fragment with the predicted size of 400 bp. Among the PCR products was identified one homologue of *GEL1*, called *GEL2*. The *GEL2* PCR product labelled with αP32CTP was used to screen approximately 5000 clones from a cosmid library of *A. fumigatus* (Borgia *et al*., 1994) immobilized on nylon membranes (Hybond N+, Amersham). Hybridization was performed in a solution containing 5× SSC, 5× Denhardt's solution and 0.5% SDS at 65°C for 24 h. The membranes were exposed to X-ray film after two 15 min washes at 65°C in 2× SSC, 0.1% SDS and two 15 min washes in 0.2× SSC; 0.1% SDS. Positive clones were purified and the cosmid DNA was isolated using the Qiagen Plasmid. Sequencing of *GEL2* was performed at Cybergene (Evry, France).

Cloning procedures and DNA manipulations

Agarose gel electrophoresis of restricted cosmid DNA, Southern blotting and subcloning of genomic DNA fragments into plasmids were performed according to standard protocols (Sambrook *et al*., 1989). *Escherichia coli* DH5α was used for plasmid propagation. Plasmid pUC19 was used in subcloning procedures. *A. fumigatus* DNA was isolated according to the procedure of Girardin *et al*. (1993). The position of introns was determined after amplification of cDNA by PCR using primers deduced from the genomic DNA sequence. The samples in a 100 µl reaction volume containing 200 µM of dNTPs, 50 pmol of each primer, 10 ng of cDNA and 1 U of *Taq* polymerase (Pharmacia) were subjected to 30 cycles of amplification consisting of the following steps: 1 min at 95°C, 1 min at 55°C and 1 min at 72°C. The PCR products were subcloned in pCR2.1 (TA Cloning kit, InVitrogen) and sequenced.

Expression of Gel2p in Pichia pastoris *and enzymatic analysis of recombinant Gel2p$_{451}$*

Pichia pastoris GS115 (Invitrogen) and the expression vector pKJ113 (Borg-von Zepelin *et al*., 1998) were used to express and secrete recombinant *A. fumigatus* Gel2p. A truncated form of *GEL2,* lacking 25 amino acid residues at the C-terminus, was generated by PCR amplification of the gene with the forward primer 5′-TGACTCGAGCCACTGCCA GCGCCGTCGTT-3′ and the reverse primer 5′-CGTGGATC CTTAACTCTCCTTGTCAGAGCTGGA-3′. The forward primer that was complementary to nucleotides +50 to +69, contained an *Xho*I site (underlined) at the 5′ end. The reverse primer was complementary to nucleotide 1333–1353 of the coding region, encompassing the codons from Ser 445 to Ser 451, and an in frame TAA stop codon adjacent to the Ser 451 codon, and a *Bam*HI site (underlined) at the 3′ end. Thirty cycles consisting of a 1 min 95°C melting step, a 1 min 60°C annealing step, and 1 min 70°C extension were performed. The resulting PCR product was digested with *Xho*I and *Bam*HI and cloned into the expression vector pKJ113 digested with the same enzymes, generating the plasmid pGel2p$_{451}$. *P. pastoris* spheroplasts were transformed with 10 µg pGel2p451 linearized by *Eco*RI. Transformants were selected and the recombinant Gel2p$_{451}$ expressed as previously described (Mouyna *et al*., 2000b). Deglycosylation of the Gel2p recombinant was carried out using a recombinant PNGaseF (Roche). After protein denaturation by boiling in 1% SDS for 5 min, the samples were adjusted to 0.1% SDS, 0.1% Triton X100 and 100 mM mercaptoethanol and incubated overnight at 37°C with PNGase F (5 U per 100 µl containing 1 to 2 µg protein). Purification of the recombinant Gel2p$_{451}$ and analysis of the β(1–3)glucanosyltransferase activity of the protein were performed as previously published (Mouyna *et al*., 2000b). Gel1p of 0.3 µg and Gel2p of 0.45 µg purified recombinant proteins were incubated with 3.75 mM reduced laminarioligosaccharide containing 14 glucose units (rG14) in 16 µl of 100 mM NaOAc pH 5.5 at 37°C. A 2.5 µl aliquot supplemented with 40 µl of 50 mM NaOH was analysed by HPLC with a CarboPAC PA-1 column (Dionex) and a pulsed electrochemical detector as previously described by Hartland *et al*. (1996).

Complementation of the S. cerevisiae *Δgas1 by A. fumigatus GEL2*

The coding region of the *GEL2* cDNA lacking the NH2-terminal was PCR amplified by the forward primer 5′-AGCGCCCGGGTTCCCATCGAG-3′ and the reverse primer 5′-GTAACTAGTTTACAGCATGAAGAACGC-3′ with a *Spe*I site (underlined) after the stop codon, generating a fragment of about 1.3 kb with a *Sma*I site (underlined) introduced at nucleotide + 64 (corresponding to Valine 22) to facilitate the fusion with the 5′ region of the *GAS1* gene. The PCR fragment of *GEL2* cDNA was digested by *Sma*I and *Spe*I and cloned into plasmid pHSP containing the promoter, the start codon and the signal sequence of *GAS1*, digested by *Sma*I and *Xba*I *(*compatible site with *Spe*I) (Mouyna *et al*., 2000a). DNA sequencing confirmed the desired in frame-fusion. The *GAS1*/*GEL2* fusion was then cloned in the high copy number vector YEp24 and the resulting plasmid was used to transform the WB2d strain. Transformation of *S. cerevisiae* cells was carried out by the lithium acetate procedure (Hill *et al*., 1991) and transformants were selected on YNB w/o aminoacids, 2% glucose and auxotrophic supplements. Transformed strains are indicated throughout the text as WB2d (YEp-*GAS1*)(Vai *et al*., 1996), WB2d (Yep-Sc*GEL1*) (Mouyna *et al*., 2000a), WB2d (YEp-Sc*GEL2*) and WB2d (YEp) the latter obtained after transformation of WB2d with the YEp24 plasmid without insert. The sensitivity to Calcofluor White was tested at a cell density of 5×10^6 ml^{-1}, 5 µl of a concentrated suspension of cells, and of 1:10 serial dilutions, was spotted on standard minimal agar plates. Protein extracts, prepared as described by Vai *et al*. (1986), were resolved by SDS-PAGE on 8% polyacrylamide slab gels. Immunolabelling of Western blots was carried out as previously described (Vai *et al*., 1996) using anti-Gel2p antibodies at 1:1000 dilution. Binding was visualized with the ECL Western Blotting Detection Reagent (Amersham). To obtain antisera labelling Gel2p in Western blots, rabbits were immunized with a *GEL2* peptide NH2- SGFTKDKDPLSDPDAC- CONH2 designed on the basis of sequence data. Coupling of the peptide through cysteine residue to the m-Maleimido-Benzoyl-N-hydroxy-Succinimide Ester and Keyhole limpet haemocyanin (KLH) protein immunization of the animal, and titre determinations

of the antiserum were performed by Eurogentec (Seraing, Belgium).

Aspergillus fumigatus *transformation and construction of the Δgel1, Δgel2 and Δgel1Δgel2 mutants*

Plasmid pN4 carrying the *E. coli* hygromycin B phosphotransferase gene (hph) flanked by the trpC terminator and gpd promoter of *Aspergillus nidulans* was used for transformation of *A. fumigatus* to obtain the Δgel1 mutant (Paris *et al*., 1993; Mouyna *et al*., 1998). pN4 was derived from the pAN7.1 plasmid (Punt *et al*., 1987) in which *Hin*dIII was replaced by *Sma*I. To construct a Δgel1 mutant, a 800 bp *Eco*RV fragment of *GEL1* containing more than 50% of the coding region, was replaced by the 3.65 kb *Stu*1-*Sma*I fragment of pN4 containing the hph gene flanked by the trpC terminator and gpd promoter of *A. nidulans* (Fig. 3A). A linear fragment obtained by *Sma*I digestion was used for transformation of protoplasts of *A. fumigatus* CBS 144.89. After overnight expression of hph, transformants were selected on minimal medium containing 200 μg ml^{-1} of hygromycin B for 7 days at 25°C.

Plasmid pAN8.1 (Mattern *et al*., 1988) was used for transformation of *A. fumigatus* to obtain a Δgel2 mutant. pAN8.1 carries the *E. coli* phleomycin B phosphotransferase gene (Phl) flanked by the trpC terminator and gpd promoter of *A. nidulans*. To construct a Δgel2 mutant, a 1.6 kb *Spe*I-*Stu*I fragment containing most of the coding region was replaced by a *Xba*I-*Stu*I fragment of pAN8.1 (Fig. 3B). The construct was linearized by *Pst*I and used for transformation of protoplasts of *A. fumigatus* CBS 144.89 or a Δgel1 mutant to obtain, respectively, a Δgel2 and a Δgel1Δgel2 mutant. After overnight expression of Phl, transformants were selected on minimal medium containing 20 μg ml^{-1} of phleomycin for 7 days at 25°C.

To complement the Δgel2 mutant, a pGla-*GEL2* plasmid was constructed in which the pGla glucoamylase promoter of *A. niger* (a *Sal*I-*Nco*I fragment purified from pGUS64 vector [kindly provided by P. Punt (the Netherlands)] (Verdoes *et al*., 1994) was fused to the cDNA of *GEL2* (a *GEL2* PCR fragment flanked by two introduced *Nco*I restriction enzyme sites) and cloned in SK+ (Bluescript, Stratagene). After confirming the desired in frame fusion by DNA sequence at ESGS (Evry), the complementation cassette Gla-*GEL2* was inserted at the polylinker site of pCB1004 vector (kind gift of MH Lebrun (Lyon) containing the hph marker gene outside of the polylinker to produce pCBgla-*GEL2*). pCBgla-*GEL2* was used to complement Δgel2 mutants by electroporation using swollen conidia (Sanchez and Aguirre, 1996). Hygromycin positive transformants were obtained, grown in presence of Maltose (pGla on) or xylose (pGla off) (Verdoes *et al*., 1994) and analysed by Southern blots.

Phenotypic analysis of mutants

Growth kinetics of *A. fumigatus* Δgel1, Δgel2, Δgel1Δgel2 and parental strains were followed in a 15 l fermenter containing Sabouraud medium as previously described (Mouyna *et al*., 1998). For estimating MIC values, *A. fumigatus* was grown in a 1% Yeast extract medium containing various concentrations of cell wall inhibitors, including, Calcofluor White (Sigma) (0–100 μg), SDS (0–250 μg), Nikkomycin Z (Sigma) (0–125 μg), Polyoxin D (Calbiochem) 1–125 μg, Mulundocandin (0–20 μg) (kind gift from Aventis) and Lilly (0–100 μg) (303366, Madrid). End growth points were determined visually in 96-well microtitre plates after 48 h of growth at 37°C (Mouyna *et al*., 1998). For mycelial growth on plates, diameter of the colonies was measured with a rule. For the conidial germination, number of germinated spores were counted every hours after deposition of a conidial suspension (2 μl of a suspension of 10^6 conidia ml^{-1}) on 2% malt agar incubated at 37°C.

Electron microscopy

Conidia produced in agar media or in liquid media were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7 overnight at 4°C. Cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate, washed three times in 0.1 M cacodylate, postfixed in 1% osmium tetroxyde, 90 min in 0.1 M cacodylate, then 30 min in methanol 30%, postfixed in 2% Uranyl acetate-methanol 30%. Cells were rinsed, dehydrated and embedded in either epon or propylene oxide for the floating sheet method. The sections were examined with a JEOL 1200 Ex electron microscope X20000.

RNA extraction and RT-PCR

RNA was isolated using the QIAGEN RNA/DNA kit. Strains CBS 144.89, Δgel1, Δgel2 and Δgel1Δgel2 were grown in Sabouraud liquid medium buffered with 150 mM HEPES at 37°C in shake culture (200 r.p.m.) for 11 and 24 h at pH 4, pH 6, or pH 8. Reverse transcription was carried out with the Promega Reverse Transcription System kit following the instructions of the manufacturer. A tube containing all the reaction components and heat-inactivated (10 min at 94°C) AMV-RT (Avian myeloblastosis virus) was always included as a negative control to check for the presence of contaminating DNA. The cDNA products were then used as target DNAs for amplifications as described above. The expression of the two *GEL* genes (*GEL1*, *GEL2*) and eight genes involved in conidiation of *Aspergillus* was investigated. Primers deduced from the sequence of these genes are listed in Table 3. *A. fumigatus* homologues of *BRLA*, *ABAA*, *STUA*, *WETA* of *A. nidulans* (Adams *et al*., 1998) were found in the TIGR database (http://tigrblast.tigr.org/ufmg/), TIGR release of November, 2004). Sequences of other genes associated with a conidiation function, *RODA*, *ALB1*, *ABR1*, *ABR2* in *A. fumigatus* were previously reported (Thau *et al*., 1994; Langfelder *et al*., 1998; Tsai *et al*., 1999). The actin gene used as a control was deduced from the *A. nidulans* gene (Fidel *et al*., 1988). The primers used for amplification of *GEL1*, *GEL2* were deduced from their genomic sequence. The RT-PCR products were resolved by electrophoresis in 1.4% agarose gels and stained with ethidium bromide for photography.

Carbohydrate analysis of cell wall extracts

Cell wall extracts were prepared from 24 h old mycelial cultures grown at 25°C in Sabouraud liquid medium. The fungal material was filtered, extensively washed with water, and

Table 3. Primers used in this study.

	Primers 5′	Primers 3′	Genomic size (bp)	CDNA size (bp)
GEL1	GACGACGTTACTCCCATCACT	GTTGGTGTTGCAGCCGTATTC	780	720
GEL2	GAGCAGAGTGTCAAGAAC	AGAGGCGTCGGCAAAGTC	380	330
BRLA	ATGCACCAATATCCGCCAATG	CATTCCATACTGATCTCGCG	840	840
ABAA	AGTATCGGGCGCGGCCTC	TGGACCGACTGGCTGATG	450	450
RODA	GCGCTGCTGTCCTCGCTTTC	AGGATAGAACCAAGGGCAATG	550	450
WETA	TGCTGCGCAAACAGAGCTTC	GGCTGACGGTGATGGCATAG	250	250
STUA	CGATGAGCAGCCAGGTTCC	GTGCACGAACAAGGGGTAG	600	430
ALB1	TGAGTTGCACCGCAGTCAGC	GAGACCAGTGGAACCCGTGGT	510	510
ABR1	CGCGTACCCTGCGCGAGAC	CCTCGCCGTATTGGACGAC	500	500
ABR2	CTGGACAAGGAACCAGCC	TGGCCGGTTCCCAGGAGG	490	490
Actin	GGTGATGAGCCACAGTCCAAG	GGGGACGACGTGGGTAACAAC	260	260
MDR4	TTCTACGATCCCGATTCAGG	GACGACACTAAGCCATATGC	150	158
*GEL1*quanti	TTCGCTACCGTTGATGCTTTCG	TGCGGCTACGGATGTACTGAC	150	147
Phleoquanti	GGCGAAGTCGTCCTCCAC	AGTTGACCAGTGCCGTTCC	150	109

disrupted for 3 min with 1 mm diameter glass beads in a Dyno-Myll cell breaker in water (WAB, Basel, Switzerland). The cell wall homogenates were centrifuged for 10 min at 4000 *g* to isolate the cell wall debris and the pellet (CWP), washed three times with 0.5 M NaCl, four times with water, and freeze dried. Colorimetric determination of total hexose and hexosamine was done by the phenol sulphuric acid method of Dubois *et al*. (1956) using glucose as a standard and the dimethylaminobenzaldehyde method of Johnson, (1971) using glucosamine as standard. Treatment of cell wall with NaOH 1N 65°C for 1 h two times resulted in the separation of AS and AI fractions. Concentrations of total hexose and hexoseamine were expressed as percentage of total cell wall material (AI + AS) estimated by the phenol and diaminobenzaldehyde methods on the AI and AS fractions. Monosaccharides were analysed by GLC as alditol acetates obtained after 4 h hydrolysis at 100°C in ATFA 4N for hexoses and HCl 8N for hexosamines, reduction and peracetylation (Sawardeker *et al*., 1967). The monosaccharide composition was expressed in percentage of hexose or percentage of hexoseamine determined in each fraction. Analysis of the glucan linkages before or after Quantazyme [recombinant endo β(1–3)glucanase, Quantum-Bioprobe] degradation of the AI and the AS was done by methylation according to the procedure described by Paz Parente *et al*. (1985) and modified by Fontaine *et al*. (1991). Partially methylated alditol acetates were prepared from permethylated samples for GC-MS linkage analysis as described (Albersheim *et al*., 1967). Quantazyme degradation of the AS and AI fractions (4 mg ml^{-1}) was carried out using the following conditions: 100 U in 1.5 ml of 100 mM imidazole buffer, pH 7.4, for up to 4 days at 37°C. Release of reducing sugars was measured at various times during the 4 days of incubation from 50 µl of sample that was mixed with 950 µl of p-Hydroxybenzoic acid hydrazide (PAHBAH) buffer (0.2 M CaCl2, 5 M NaOH, 0.5 M Na Citrate, 2.5 mM $NaSO_3$, 3.2 mM PAHBAH. Material solubilized by quantazyme was analysed by MALDI-TOF MS for size estimation.

Analysis of mutant virulence

Mutants and WT strain were used for experimental infections in female Swiss mice (16–18 g). The mice were infected intranasally with an equal mixture of mutant and WT (2×10^6 conidia in total in 25 µl of PBS-Tween 20 0.01%) intranasally after immunosuppression treatment with cortisone acetate (5 mg per mouse on days −3, 0, +2) (Beauvais *et al*., 2005). The DNA of each strain was quantified *in vivo* in lungs by the quantitative real-time PCR (QPCR). The lungs from euthanasied mice were disrupted in a mortar under liquid nitrogen and the total DNA was prepared (Girardin *et al*., 1993). The DNA extracts were further diluted 5× in sterile DDH2O for real-time PCR. SYBr green was performed for detection and quantification of the different strains in each mouse. Primers were designed to amplify and detect each Δgel mutant and WT strain. All primers were designed using the Beacon Designer 2 software (Table 3). The primer set MDR4 was specific for total fungi DNA (WT and Δgel2 and Δgel1Δgel2), GEL1quanti was specific for WT and Δgel2, Pheoquanti was specific for Δgel2. To quantify the Δgel1Δgel2 DNA in the mixture of WT + Δgel1Δgel2, MDR4 will quantify total DNA and GEL1quanti will quantify only WT. To quantify the Δgel2 DNA, in the mixture WT + Δgel2, MDR4 will quantify total DNA and Pheoquanti will quantify the Δgel2 mutant only. To quantify the Δgel1Δgel2 DNA, in the mixture Δgel1Δgel2 + Δgel2, MDR4 will quantify total DNA and GEL1quanti will quantify the Δgel2 mutant only. Differences between Ct indicated the amount of WT compared with the Δgel mutants. Each 25 µl of QPCR mixture included 5 µl of DNA, 0.08 µl of primers (300 nM), 12.5 µl of IQ supermix (2×) (ABgene) qsp H2O. QPCR amplification was performed using an iCycler thermal cycler (Bio-Rad) with the following parameters, 15 min at 95°C and 40 cycles of two steps consisting of 30 s at 95°C, 30 s at 55°C. Ct values were analysed by the paired *t*-test using JMP5 software (SAS, Cary, NC, USA).

Nucleotide accession number

The nucleotide sequence data of *GEL1* and *GEL2* has been submitted to GenBank under the accession number AF072700 for *GEL1* and AF208039 for *GEL2*.

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References

Adams, T.H., Wieser, J.K., and Yu, J.H. (1998) Asexual sporulation in *Aspergillus nidulans*. *Microbiol Mol Biol Rev* **62:** 35–54.

Albersheim, P., Nevins, D.J., English, P.D., and Karr, A. (1967) A method for the analysis of sugars in plant cell wall polysaccharides by gas-liquid chromatography. *Carbohydr Res* **5:** 340–345.

Beauvais, A., Drake, R., Ng, K., Diaquin, M., and Latgé, J.P. (1993) Characterization of the 1,3 β glucan synthase of *A. fumigatus*. *J Gen Microbiol* **139:** 3071–3078.

Beauvais, A., Bruneau, J.M., Mol, P.C., Buitrago, M.J., Legrand, R., and Latgé, J.P. (2001) Glucan synthase complex of *Aspergillus fumigatus*. *J Bacteriol* **183:** 2273–2279.

Beauvais, A., Maubon, D., Park, S., Morelle, W., Tanguy, M., Huerre, M., *et al.* (2005) Two α(1–3) glucan synthases with different function in *A. fumigatus*. *Appl Env Microbiol* **71:** 1531–1538.

Borgia, P.T., Dodge, C.L., Eagleton, L.E., and Adams, T.H. (1994) Bidirectional gene transfer between *Aspergillus fumigatus* and *Aspergillus nidulans*. *FEMS Microbiol Lett* **122:** 227–232.

Borg-von Zepelin, M., Beggah, S., Boggian, K., Sanglard, D., and Monod, M. (1998) The expression of the secreted aspartyl proteinases Sap4 to Sap6 from *Candida albicans* in murine macrophages. *Mol Microbiol* **28:** 543–554.

Cove, D.J. (1966) The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochim Biophys Acta* **13:** 51–56.

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

Espeso, E.A., and Penalva, M.A. (1996) Three binding sites for the *Aspergillus nidulans* PacC Zinc-finger transcription factor are necessary and sufficient for regulation by ambient pH of the isopenicillin N synthase gene promoter. *J Biol Chem* **271:** 28825–28830.

Espeso, E.A., Tilburn, J., Sanchez-Pulido, L., Brown, C.V., Valencia, A., Arst, H.N., *et al.* (1997) Specific DNA recognition by the *Aspergillus nidulans* three zinc finger transcription factor PacC. *J Mol Biol* **274:** 466–480.

Fidel, S., Doonan, J.H., and Morris, N.R. (1988) *Aspergillus nidulans* contains a single actin gene which has a unique intron locations and encodes a gamma-actin. *Gene* **70:** 283–293.

Fontaine, T., Talmont, F., Dutton, G.G.S., and Fournet, B. (1991) Analysis of pyruvic acid acetate containing polysaccharides by methanolysis and reductive cleavage methods. *Anal Biochem* **199:** 154–161.

Fontaine, T., Simenel, C., Dubreucq, G., Adam, O., Delepierre, M., Lemoine, J., *et al.* (2000) Molecular organization of the alkali-insoluble fraction of *Aspergillus fumigatus* cell wall. *J Biol Chem* **275:** 27594–27607.

Fonzi, W.A. (1999) *PHR1* and *PHR2* of *Candida albicans* encode putative glycosidases required for proper cross-linking of β(1–3) and β(1–6)glucans. *J Bacteriol* **181:** 7070–7079.

Gerber, L.D., Kodukula, K., and Udenfriend, S. (1992) Phosphatidylinositol glycan (PI-G) anchored membrane proteins. *J Biol Chem* **267:** 12168–12173.

Girardin, H., Latgé, J.P., Srikantha, T., Morrow, B., and Soll, D.R. (1993) Development of DNA probes for fingerprinting *Aspergillus fumigatus*. *J Clin Microbiol* **31:** 1547–1554.

Hartland, R.P., Fontaine, T., Debeaupuis, J.P., Simenel, C., Delepierre, M., and Latgé, J.P. (1996) A novel β(1–3)glucanosyltransferase from the cell wall of *Aspergillus fumigatus*. *J Biol Chem* **271:** 26843–26849.

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

Hill, J., Jan, K.A., Donald, G., and Griffiths, E. (1991) DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Res* **19:** 5791.

Johnson, A.R. (1971) Improved method of hexosamine determination. *Anal Biochem* **44:** 628–635.

Klis, F.M. (1994) Cell wall assembly in yeast. *Yeast* **10:** 851–869.

Koizumi, K., Kubota, Y., Tanimoto, T., and Okada, Y. (1989) High-performance anion-exchange chromatography of homogeneous D-gluco-oligosaccharides and -polysaccharides (polymerization degree greater than or equal to 50) with pulsed amperometric detection. *J Chromatogr* **464:** 365–373.

Kottom, T.J., Thomas, C.F., and Limper, A.H. (2001) Characterization of *Pneumocystis carinii PHR1*, a pH regulated gene important for cell wall integrity. *J Bacteriol* **183:** 6740–6745.

Kursai, O., Heinz, W.J., Sullivan, D.J., Coleman, D.C., Frosch, M., and Muhschlegel, F.A. (1999) Rapid PCR test discriminating between *Candida albicans* and *Candida dubliniensis* isolates using primers derived from the pH-regulated *PHR1* and *PHR2* genes of *C. albicans*. *J Clin Microbiol* **37:** 1587–1590.

Langfelder, K., Gehringer, H., Schmidt, A., Wanner, G., and Brakhage, A.A. (1998) Identification of a polyketide synthase gene (pksP) of *Aspergillus fumigatus* involved in conidial pigment biosynthesis and virulence. *Med Mol Immunol* **187:** 79–89.

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

Latgé, J.P., Debeaupuis, J.P., Moutaouakil, M., Diaquin, M., Sarfati, J., *et al.* (1991) Galactomannan and the circulating Antigens of *Aspergillus Fumigatus*. In *Fungal Cell Wall and Immune response.* latgé J. P. and Drion B. (eds). NATO ASI series. Heidelberg: Springer-Verlag, pp. 143–155.

Lesage, G., Li, H., Sdicu, A.M., Menard, P., and Bussey, H. (2002) *Beta-glucan synthesis in* S. Cerevisiae*: a genome-wide synthetic lethal analysis of participating gene families.* IMC7 Congress, Oslo, 11–17 August 2002.

Mattern, I.E., Punt, P.J., and Van den Hondel, C.A.M.J.J. (1988) A vector of *Aspergillus* transformation conferring

phleomycin resistance. *Fungal Genet Newsletter* **35:** 25.

Mazur, P., Morin, N., Baginsky, W., El-Sherbeini, M., Clemas, J.A., Nielsen, J.B., and Foor, F. (1995) Differential expression and function of two homologous subunits of yeast 1,3 β-D-glucan synthase. *Mol Cell Biol* **15:** 5671–5681.

Mellado, E., Aufauvre-Brown, A., Gow, N.A.R., and Holden, D.W. (1996) The *Aspergillus fumigatus chsC* and *chsG* genes encode class III chitin synthases with different functions. *Mol Microbiol* **20:** 667–679.

Mellado, E., Dubreucq, G., Mol, P., Sarfati, J., Paris, S., Diaquin, M., *et al.* (2003) Cell wall biogenesis in a double chitin synthase mutant (*chsG-/chsE-*) of *Aspergillus fumigatus*. *Fungal Genet Biol* **38:** 98–109.

Mouyna, I., Hartland, R.P., Fontaine, T., Diaquin, M., Simenel, C., Delepierre, M., *et al.* (1998) A β(1–3)glucanosyltransferase isolated from the cell wall of *Aspergillus fumigatus* is an homolog of the yeast Bgl2p. *Microbiology* **144:** 3171–3180.

Mouyna, I., Fontaine, T., Vai, M., Monod, M., Fonzi, W., Diaquin, M., *et al.* (2000a) Glycosylphosphatidylinositol-anchored glucanosyltransferases play an active role in the biosynthesis of the fungal cell wall. *J Biol Chem* **275:** 14882–14889.

Mouyna, I., Monod, M., Fontaine, T., Henrissat, B., Léchenne, B., and Latgé, J.P. (2000b) Identification of the catalytic residues of the first family of β(1–3)glucanosyltransferases identified in fungi. *Biochem J* **347:** 741–747.

Muhlschlegel, F.A., and Fonzi, W.A. (1997) *PHR2* of *Candida albicans* encodes a functional homolog of the pH-regulated *PHR1* with an inverted pattern of pH-dependent expression. *Mol Cell Biol* **17:** 5960–5967.

Nakazawa, T., Horiuchi, H., Ohta, A., and Takagi, M. (1998) Isolation and characterization of *EPD1*, an essential gene for pseudohyphal growth of a dimorphic yeast, *Candida maltosa*. *J Bacteriol* **180:** 2079–2086.

Nakasawa, T., Takahashi, M., Horiuchi, H., Ohta, A., and Takagi, M. (2000) Cloning and characterization of *EPD2*, a gene required for efficient pseudohyphal formation of a dimorphic yeast, *Candida maltosa*. *Biosci Biotechnol Biochem* **64:** 369–377.

Nuoffer, C., Jeno, P., Conzelmann, A., Horvath, A., and Riezman, H. (1991) Determinants for glycophospholipid anchoring of the *Saccharomyces cerevisiae GAS1* protein to the plasma membrane. *Mol Cell Biol* **11:** 27–37.

Paris, S., Monod, M., Diaquin, M., Lamy, B., Arruda, L.K., Punt, P.J., *et al.* (1993) A transformant of *Aspergillus fumigatus* deficient in the antigenic cytotoxin *ASPFI*. *FEMS Microbiol Lett* **111:** 31–36.

Paz Parente, J., Cardon, P., Leroy, Y., Montreuil, J., Fournet, B., and Ricart, G. (1985) A convenient method for methylation of glycoproteinglycans in small amount by using lithium methyl sulfinyl carbanion. *Carbohydr Res* **141:** 41–47.

Popolo, L., and Vai, M. (1999) The Gas1 glycoprotein, a putative wall polymer cross-linker. *Bioch et Bioph Acta* **1426:** 385–400.

Popolo, L., Vai, M., Gatti, E., Porello, S., Bonfante, P., Balestrini, R., *et al.* (1993) Physiological analysis of mutants indicates involvement of the *S. cerevisiae* GPI-anchored protein gp115 in morphogenesis and cell separation. *J Bacteriol* **175:** 1879–1885.

Punt, P.J., Oliver, R.P., Dingemanse, M.A., Pouwels, P.H., and Van den Hondel, C.A.M.J.J. (1987) Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene* **56:** 117–124.

Ram, A.F.J., Kapteyn, J.C., Montijn, R.C., Caro, H.P., Douwes, J.E., Baginsky, W., *et al.* (1998) Loss of the plasma membrane bound protein Gas1p in *Saccharomyces cerevisiae* results in the release of β(1–3)glucan into the medium and induces a compensation mechanism to ensure cell wall integrity. *J Bacteriol* **180:** 1418–1424.

Ramon, A.M., Porta, A., and Fonzi, W.A. (1999) Effect of environmental pH on morphological development of *Candida albicans* is mediated via the PacC-related transcription factor encoded by *Prr2*. *J Bacteriol* **181:** 7524–7530.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning, a Laboratory Manual*, 2nd edn. New York, NY: Cold Spring Harbor Laboratory Press.

Sanchez, O., and Aguirre, J. (1996) Efficient transformation of *Aspergillus nidulans* by electroporation of germinated conidia. *Fungal General News* **43:** 48–51.

Saporito-Irwin, S.M., Birse, C.E., Sypherd, P.S., and Fonzi, W.A. (1995) *PHR1*, a pH regulated gene of *Candida albicans* is required for morphogenesis. *Mol Cell Biol* **15:** 601–613.

Sawardeker, J.S., Sloneker, J.H., and Jeanes, A. (1967) Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. *Anal Chem* **37:** 1602–1604.

Su, S.S.Y., and Mitchell, A.P. (1993) Molecular characterization of the yeast meiotic regulatory gene *Rim1*. *Nucleic Acid Res* **21:** 3789–3797.

Thau, N., Monod, M., Crestani, B., Rolland, C., Tronchin, G., and Latgé, J.P. (1994) Rodletless mutants of *Aspergillus fumigatus*. *Infect Immun* **62:** 4380–4388.

Tilburn, J., Sarkar, S., Widdick, D.A., Espeso, E.A., Orejas, M., Mungroo, J., *et al.* (1995) The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid and alkaline expressed genes by ambient pH. *EMBO J* **14:** 779–790.

Tsai, H.F., Wheeler, M.H., Chang, Y.C., and Kwon-Chung, K.J. (1999) The developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. *J Bacteriol* **181:** 6469–6477.

Vai, M., Popolo, L., and Alberghina, L. (1986) Immunological cross reactivity of fungal and yeast plasma membrane H^+-ATPase. *FEBS Lett* **206:** 135–141.

Vai, M., Gatti, E., Lacana, E., Popolo, L., and Alberghina, L. (1991) Isolation and Deduced Amino Acid Sequence of the gene encoding gp115, a yeast glycophospholipid-anchored protein containing a serine-rich region. *J Biol Chem* **266:** 12242–12248.

Vai, M., Orlandi, I., Cavadini, P., Alberghina, L., and Popolo, L. (1996) *Candida albicans* homologue of *GGP1/GAS1* gene is functional in *Saccharomyces cerevisiae* and contains the determinants for glycosylphosphatidylinositol attachment. *Yeast* **12:** 361–368.

Valdivieso, M.H., Ferrario, L., Vai, M., Duran, A., and Popolo, L. (2000) Chitin synthesis in a gas1 mutant of *Saccharomyces cerevisiae*. *J Bacteriol* **182:** 4752–4757.

Verdoes, J.C., Punt, P.J., Stouthamer, A.H., and Van den Hondel, C.A.M.J.J. (1994) The effect of multiple copies of

the upstream region on expression of the *Aspergillus niger* glucoamylase-encoding gene. *Gene* **145:** 179–187.

Von Heijne, G. (1986) A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res* **14:** 4683–4690.

Weig, M., Haynes, K., Rogers, T.R., Kurzai, O., Frosch, M., and Muhlschlegel, F.A. (2001) A *GAS*-like gene family in the pathogenic fungus *Candida glabrata*. *Microbiology* **147:** 2007–2019.