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Evidence for the Essential Involvement of the Transglycosylase Domain of Ags1p in Cell Wall \( \alpha \)-Glucan Biosynthesis in Fission Yeast

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ABSTRACT

The fungal cell wall is important for morphology and structural integrity. For many fungi, α-glucan is a main polysaccharide of the cell wall. In the fission yeast *Schizosaccharomyces pombe*, cell wall α-glucan is an essential component, consisting mainly of (1,3)-α-glucan and some ten percent of α-(1,4)-linked glucose residues. Recently, we showed that the intracellular synthase (SYN) domain of the multidomain protein Ags1p is involved in the production of the (1,4)-α-glucan component of cell wall α-glucan. The other domain, the proposed extracellular transglycosylase (TGL) domain, shows strong similarity with glycosyltransferases. More specifically, it shares amino acid sequence motifs with the α-amylase superfamily, together with three highly conserved catalytic residues. Whether the TGL domain functions together with the SYN domain to produce the cell wall α-glucan has remained unclear. Here, we studied the role of the TGL domain of Ags1p by mutating its conserved putative catalytic residues by direct mutagenesis. We show that overexpression but also low expression of these mutant Ags1 proteins resulted in accumulation of (1,4)-α-glucan in *S. pombe* cells. Furthermore, the mutant Ags1 proteins failed to complement the cell lysis phenotype of the temperature-sensitive *ags1-1* mutant. Therefore, we propose that the TGL and SYN domains of Ags1p collaborate in the stepwise assembly process of cell wall α-glucan in *S. pombe* and that in this process the activity of the TGL domain appears to follow that of the SYN domain.
INTRODUCTION

Cell wall α-glucan is an essential component of the cell wall of the fission yeast *S. pombe* (Hochstenbach *et al*., 1998). This cell wall component is composed of two building blocks, each consisting of a main segment of (1,3)-α-glucan with some (1,4)-linked-α-glucose residues at its reducing end (Grün *et al*., 2005). The multidomain α-glucan synthase protein, Ags1p, is located at the plasma membrane and consists of two proposed catalytic domains on either side of the membrane and a multipass transmembrane domain, which might form a pore (Hochstenbach *et al*., 1998; Katayama *et al*., 1999). This domain structure is conserved in the *S. pombe* Ags1p homologs Mok11p–Mok14p and in Ags1p homologs in other cell wall α-glucan-containing fungi, like *Aspergillus fumigatus*, *Hisoplasma capsulatum* and *Cryptococcus neoformans* (Reese and Doering, 2003; Rappleye *et al*., 2004; Beauvais *et al*., 2005; Maubon *et al*., 2006). Recently, we showed that the proposed intracellular synthase (SYN) domain is involved in the biosynthesis of the (1,4)-α-glucan part of the cell wall α-glucan (Vos *et al*., 2007). Based on sequence similarity, the proposed extracellular domain was suggested to be a transglycosylase, referred to here as the TGL domain (Hochstenbach *et al*., 1998; Katayama *et al*., 1999).

The α-amylase superfamily consists of the glycoside hydrolase families GH-13, GH-70 and GH-77, based on the conserved catalytic mechanism (Henrissat, 1991; MacGregor *et al*., 2001; Coutinho *et al*., 2003). Because the fungal Ags1p TGL domains have been classified to family GH-13 (GH-13_22), we will focus on the α-amylases as the main member of family GH-13 (Stam *et al*., 2006; CAZy, http://www.cazy.org/). α-Amylases are (1,4)-α-D-glucan glucanohydrolases that catalyse hydrolysis or transglycosylation of α-glucans in which the anomeric configuration is retained (CAZy, http://www.cazy.org/). All members of this family contain a (β/α)₈ barrel, formed by eight parallel β-pleated strands in a cylindrical shape surrounded by eight α-helices, creating the catalytic domain A. The β-sheets and α-helices alternate in the peptide backbone, thereby forming loops from the β-sheets to the α-helices on which the catalytic residues are located (MacGregor *et al*., 2001). Many members have a long protruding loop from β-sheet 3, named domain B, which is probably involved in substrate binding or binding of Ca²⁺ (Jespersen *et al*., 1991; Janeček *et al*., 1997). Also present in a number of α-amylases is domain C, an antiparallel β-sandwich fold termed a Greek key motif. Its function is unknown, but, it influences the activity when mutated (Holm *et al*., 1990; Jespersen *et al*., 1991).

In this study, we initiated an analysis into the role of the Ags1p TGL domain in cell wall α-glucan biosynthesis. The presence of both the TGL and SYN domains in Ags1p prompted us to investigate whether they function independently or whether they functionally interact. Our preliminary results show that the Ags1p TGL domain has a similar folding as other members of the α-amylase superfamily and shares the four
highly conserved amino acid regions in the putative catalytic centre. Furthermore, by using targeted mutagenesis and by isolating spontaneous mutants we generated Ags1 proteins with mutations in the TGL domain and showed that they accumulated (1,4)-α-glucan. Therefore, we suggest that the *S. pombe* Ags1p TGL domain has an essential function that follows (1,4)-α-glucan biosynthesis by the Ags1p SYN domain.

**RESULTS**

**Domain architecture and conserved regions of the α-amylase family**

Close examination of the putative extracellular domain of *S. pombe* Ags1p (residues 27–1068) revealed two main regions based on the predicted secondary structure (PSIPRED): a (β/α)₈ barrel structure (residues 27–585) and a β-sheet-rich region (residues 586–1068) (Figure 1). These regions are also present in α-amylases with which the extracellular domain shares amino acid sequence similarities, as reported previously (Hochstenbach et al., 1998; Katayama et al., 1999). The (β/α)₈ barrel consists

![Figure 1. Amino-acid sequence of the Ags1p TGL domain.](image-url)
of the catalytic domain A with a protruding loop between β-sheet 3 and α-helix 3 called domain B. These two domains are then followed by seven or eight β-sheets, domain C. Interestingly, the extracellular domain of Ags1p contains 35 predicted β-sheets following domains A and B. This indicates the presence of multiple domain C-like structures, only seen in certain cyclodextrin glucanotransferases (CGTases) where the additional β-sheet domains are named domain D, with unknown function.

Figure 2. Conserved regions of the α-amylase superfamily. (A) Schematic representation of S. pombe Ags1p indicating its putative catalytic TGL and SYN domains, as well as the predicted N-terminal signal peptide (SP), putative transmembrane region (TM), and multipass transmembrane domain (PORE), which might form a pore. The enlarged TGL domain shows the structural domains of this domain: domain A is the conserved (β/α)8 barrel, domain B is a large loop protruding out of domain A on loop 3, domain C consists of 7 β-sheets, and domain D/E is an additional β-sheet rich region. Relevant amino acid residues are indicated. (B) Seven conserved regions of the ClustalW alignment (Thompson et al., 1994) of fungal α-amylases (Aah1–4, AgtA–C, AMY1, Mal1, Mde5, Amy1, Amy2 and TAA), bacterial α-amylase (BLA) and fungal Ags1p TGL domains (Ags1 and Mok11–13). The best conserved regions of the (β/α)8 barrel are strands β3, β4, β5, and β7, indicated by the roman numbers I–IV. The three catalytic residues Asp315, Glu349, and Asp463 (in strands β4, β5, and β7, respectively) are highlighted with black shading. Dark-grey shading shows conserved histidines, Thr263 and His294 (in strands β3 and β7, respectively) important for subsite -1, and light-grey shading indicates a region between a conserved glycine (Gly122) and proline (Pro132), which length is enzyme specific. Dashes indicate gaps in the ClustalW alignment. The secondary structure above the alignment is based on the solved crystal structure of Taka-amylase A of Aspergillus oryzae (Protein DataBase accession number 6TAA). The species and included segments of the enzymes are: SpAgs1, S. pombe (SPCC1281.01); SpMok11, S. pombe (SPAC5272.01); SpMok12, S. pombe (SPBC226.09); SpMok13, S. pombe (SPBC163.15); CnaAgs1, C. neoformans Serotype A (CNAG_03120); SpAah1, S. pombe (SPCC757.12); SpAah2, S. pombe (SPAC2331.14); SpAah3, S. pombe (SPCC63.02c); SpAah4, S. pombe (SPBC1185.13); AbAgtA, Aspergillus niger (CAK40249); AbAgtB, A. oryzae (CAK41088); AbAgtC, A. niger (CAK42667); HcAMY1, H. capsulatum (ABK62854); SpMde5, S. pombe (SPAC25H1.09); SpAmy1, S. pombe (SPAC272E.02); SpAmy2, S. pombe (SPCC1180.09c); BiBLA, Bacillus licheniformis (P06278), and AoTAA, A. oryzae (P10529).
and domain E, for starch binding (reviewed by MacGregor et al., 2001). Here, we will refer to the α-amylase homology region of Ags1p (residues 27–672), comprised of domains A, B and C, as the TGL domain (Figure 2A).

The *S. pombe* Ags1p TGL domain shares the four highly conserved regions of the catalytic site that are specific for the α-amylase family and contain conserved residues forming subsite −1, required for binding of α-glucose (MacGregor et al., 2001). These regions are found at the start of the loops following β-strands 3, 4, 5, and 7 of the (β/α)₈ barrel and contain five almost fully conserved residues (Figure 2B). The three residues essential for catalysis are conserved in the TGL domain: the first aspartic acid (Asp³¹⁵) is the nucleophile, the glutamic acid (Glu³⁴⁹) is the proton donor, and the second aspartic acid (Asp⁴⁶³) is required for stabilisation of the transition state. The two other residues are histidines. Although these are not invariant, their replacement has an effect on the reaction specificity (Leemhuis et al., 2004). In the TGL domain of *S. pombe* Ags1p, the second histidine (His⁴⁶⁵) is conserved whereas the first histidine is replaced by a threonine (Thr¹⁸³), which is specific for maltosyltransferases (MacGregor et al., 2001). In addition, three other short regions are conserved, namely those around β-strands 2 and 8 in domain A and a region within loop 3 that forms domain B. The variations in these regions may also indicate certain enzyme specificities. For example, in β-strand 2, a conserved glycine is followed by a conserved proline, separated by seven residues in α-amylases or eight residues in CGTases (MacGregor et al., 2001). Interestingly, the TGL domain has an interval of nine residues, which is unique for Ags1p and its homologs (Figure 2B). These results suggest that the Ags1p TGL domain may function as a transglycosylase rather than an α-amylase.

**Replacement of conserved Ags1p TGL domain residues results in (1,4)-α-glucan accumulation during overexpression**

To start an investigation into the function of the Ags1p TGL domain, we analysed the effect of the different proposed catalytic residues of this domain on Ags1p function. Previously we have shown that Ags1 proteins without the TGL domain (lacking residues 113–724) were still able to produce (1,4)-α-glucan upon overexpression in *S. pombe*, demonstrating that the TGL domain is not required for the (1,4)-α-glucan biosynthesis (Vos et al., 2007). We wondered whether similar effects are observed when the TGL domain is inactivated by directed mutagenesis. Therefore we introduced selected point mutations in the TGL domain of Ags1p, based on the conservation in α-amylases (Figure 1 and 2B). As a positive control, we used cells overexpressing wild-type Ags1p in our simple plate-based assay (Vos et al., 2007). These cells showed a yellowish staining under repression conditions (Figure 3, lane 2, LOW) similar to cells overexpressing the empty pREP4 vector (Figure 3,
lane 1), whereas overexpression of Ags1p resulted in a brown staining, indicating the accumulation of (1,4)-α-glucan (Figure 3, lane 2, HIGH). First, we checked the effect of the mutation of the proposed nucleophile Asp\textsuperscript{315} by replacing it with Asn to create Ags1p-D315N. Overexpression of this mutant Ags1p resulted in brown staining (Figure 3, lane 3, HIGH). Surprisingly, under low expression conditions an even more intense brown staining was observed (Figure 3, lane 3, LOW). To confirm the accumulation of (1,4)-α-glucan in these cells, we used our amylase assay to measure the amount of glucose residues released from insoluble cell fractions after treatment with α-amylase and glucoamylase (Vos et al., 2007). We detected an increase in the cellular amount of (1,4)-α-glucan after overexpression of pAgs1-D315N (Figure 4, lane 6), comparable to the amount observed after overexpression of Ags1p (Figure 4, lane 4). However, for the low Ags1p-D315N expression samples we detected no pronounced increase in (1,4)-α-glucan levels, even though the plate-based assay showed a more intense brown staining for cells containing pAgs1-D315N than for cells containing wild-type pAgs1 (Figure 4, compare lanes 3 and 5).

Second, we replaced the second proposed catalytic residue, the proton donor Glu\textsuperscript{349}, with Gln, creating Ags1p-E349Q. This mutant Ags1p shows similar results to those for wild-type Ags1p. In the plate-based assay, a yellowish staining was observed during low expression, whereas a brown staining was observed during overexpression (Figure 3, lane 4). And these results were confirmed in the amylase assay, showing an increase in amount of (1,4)-α-glucan during overexpression (Figure 4, compare lanes 7 and 8). Third, we analysed the highly conserved Tyr\textsuperscript{143}, which was shown to

Figure 3. Replacement of conserved residues in the TGL domain results in increased iodine staining upon overexpression. Cells (10\textsuperscript{5}) were spotted onto EMMA plates without (high expression; –T) or with (low expression; +T) the addition of thiamine. After 3 days of growth at 28°C, plates were exposed to iodine vapour to stain for (1,4)-α-glucan. The following cells were analysed: cells containing pREP4 (strain AV027, lane 1), pAgs1 (strain AV028, lane 2), pAgs1-D315N (strain BS012C, lane 3), pAgs1-E349Q (strain BS013B, lane 4), pAgs1-Y143A (strain AV146, lane 5), or pAgs1-G696S (strain AV149, lane 6). (For a full colour version, see page 207)

Figure 4. Replacement of conserved residues in the TGL domain results in (1,4)-α-glucan accumulation upon overexpression. Cells were grown in EMMA medium without (high expression; –T) or with (low expression; +T) the addition of thiamine for 20 h. (1,4)-α-Glucan levels of the insoluble cell fraction were measured in an assay using α-amylase and glucoamylase. The following cells were analysed: cells containing pREP4 (strain AV027, lanes 1 and 2), pAgs1 (strain AV028, lanes 3 and 4), pAgs1-D315N (strain BS012C, lanes 5 and 6), or pAgs1-E349Q (strain BS013B, lanes 7 and 8).
be important in the active site for subsite –1 (Robert et al., 2005). A mutant Ags1p in which Tyr143 was substituted by Ala resulted in a similar phenotype in the plate-based assay as that obtained with Ags1p-D315N, brown staining upon overexpression of Ags1p-Y143A, but an even more intense brown staining at low expression (Figure 3, lane 5). Finally, we introduced the temperature-sensitive mutation of the ags1-1ts mutant in pAgs1, creating pAgs1-G696S (Hochstenbach et al., 1998). Overexpression of this mutant Ags1p resulted in the typical brown staining that was not observed during low expression (Figure 3, lane 6). In conclusion, these results confirm that the TGL domain of Ags1p is not involved in the production of (1,4)-α-glucan. Importantly, they demonstrate that replacement of conserved residues in the Ags1p TGL domain may result in an accumulation of (1,4)-α-glucan, suggesting that its activity influences the activity of the SYN domain.

The Ags1p TGL domain is important for the in vivo function of Ags1p
To determine whether the activity of the TGL domain is important for Ags1p function in vivo, we tested the ability of the mutant Ags1 proteins to complement the cell lysis phenotype of the temperature-sensitive ags1-1 strain. As shown previously, this mutant strain grows well at the permissive temperature of 28°C, but is unable to grow at the restrictive temperature of 36°C (Hochstenbach et al., 1998). Low expression of Ags1p recovers the growth defect at 36°C (Vos et al., 2007). We show that one of the catalytic TGL mutant proteins, Ags1p-E349Q, completely failed to complement the ags1-1ts strain (Figure 5, row 4). By contrast, Ags1p-D315N and Ags1p-Y143A partially complemented the cell lysis phenotype of this strain (Figure 5, rows 3 and 5), suggesting that these mutant Ags1 proteins were partially active. In contrast to previous results, we observed very weak growth for Ags1p-G696S, the temperature-sensitive Ags1p, but these results slightly differed between experiments, perhaps because of small differences in temperature or slight variations in amount of plasmids present in these cells (Vos et al., 2007). From these results, we conclude that the activity of the TGL domain is important for the Ags1p function in vivo.

Figure 5. Activity of the TGL domain is important for the in vivo function of Ags1p. Cells were spotted onto EMMA plates supplemented with thiamine (low expression) in 10-fold serial dilutions as indicated. After 3 days of incubation at 28°C (left panel) or 36°C (right panel), plates were exposed to iodine vapour to stain for (1,4)-α-glucan. The following cells were analysed: ags1-1ts cells containing pREP4 (strain AV059, row 1), pAgs1 (strain AV060, row 2), pAgs1-D315N (strain BS018, row 3), pAgs1-E349Q (strain BS019, row 4), pAgs1-Y143A (strain AV150, row 5), or pAgs1-G696S (strain AV153, row 6). (For a full colour version, see page 207)
Spontaneous mutations in the Ags1p TGL domain allow overexpression of Ags1p and accumulation of (1,4)-α-glucan

To investigate the involvement of the TGL and SYN domains in the (1,4)-α-glucan biosynthesis, we set up a simple screen to isolate mutant Ags1 proteins that allow Ags1p overexpression. *ags1-1* cells containing pAgs1 were plated on EMMA culture plates lacking thiamine to induce overexpression of wild-type Ags1p, at a density of 10⁴ cells per plate. After three days of growth at 28°C, these plates showed a thin layer of tiny single colonies, an effect caused by the growth inhibition induced Ags1p overexpression. Importantly, several larger colonies were observed (data not shown). Preliminary analysis of some mutant strains showed that these cells did not display the typical growth inhibition observed upon overexpression of wild-type Ags1p (Figure 6A, lane 3, compare HIGH and LOW). In addition, these mutant strains showed a positive iodine staining upon overexpression (Figure 6A, lane 3, HIGH), and were able to complement the temperature-sensitive ags1-1 cells at 36°C (Figure 6B). Sequence analysis of mutant pAgs1 plasmids from several independently isolated mutants identified single point mutations located predominantly in the TGL domain, Ags1p-I359M, -G529D, -G796E, or -W2370M (Figure 1). In conclusion, this screen allowed the identification of mutant Ags1 proteins that showed a normal in vivo Ags1p function, both during low expression and during overexpression. Importantly, these mutant proteins showed an intense iodine staining, indicating that they accumulated (1,4)-α-glucan.

DISCUSSION

In this study, we initiated an investigation into the function of the TGL domain, one of the two probable catalytic domains of *S. pombe* Ags1p. First, we showed that the TGL domain shares the four highly conserved amino acid regions of the catalytic site of the α-amylase superfamily, including the three proposed catalytic residues.
(Figure 2). Second, we observed that direct mutagenesis of these proposed catalytic residues and an important residue of the glucose-binding subsite −1 resulted in (1,4)-α-glucan accumulation upon Ags1p overexpression, as indicated by the specific brown staining with iodine vapour and by a quantitative amylase assay (Figures 3 and 4). Third, we showed that these conserved residues of the TGL domain are important for the *in vivo* function of Ags1p, because their replacement reduced the ability to complement the cell lysis phenotype of the *ags1-l*ts strain, compared to wild-type Ags1p (Figure 5). Finally, in a preliminary screen for mutant Ags1 proteins that support normal cellular growth rates upon Ags1p overexpression, we identified single missense mutations in the TGL domain. Cells overexpressing these mutant Ags1 proteins were found to accumulate (1,4)-α-glucan (Figure 6). We conclude that impairment of the function of the TGL domain may affect the synthase activity of the SYN domain.

The four highly conserved amino acid regions of the α-amylase superfamily, forming the catalytic site, are also conserved in the proposed extracellular domain of Ags1p in *S. pombe* (Figure 2). Replacement of the equivalent residues of the nucleophile and proton donor, Asp315 and Glu349, and another important residue in the catalytic site, Tyr143, demonstrated that these residues are important for TGL function. Overexpression of these mutant Ags1 proteins resulted in accumulation of (1,4)-α-glucan (Figure 3), similar to the accumulation shown previously for overexpression of wild-type Ags1p (Vos *et al.*, 2007). Surprisingly, under low expression conditions we observed a dramatic increase in iodine-staining material for Ags1p-D315N and Ags1p-Y143A, compared to wild-type Ags1p (Figure 3). At present, we do not know whether this increase in iodine-staining material represents an increase in polymer length of (1,4)-α-glucan accumulating in these cells or whether it represents an increase in (1,4)-α-glucan levels. The latter possibility appears more likely, because (1,4)-α-glucan molecules with an increased length of 60 glucose residues or more would stain purple or blue when forming a complex with iodine, rather than the observed red-brown staining, which indicates the presence of (1,4)-α-glucan oligosaccharides (Bailey and Whelan, 1961). A preliminary analysis of this material in the amylase assay, however, did not confirm the presence of abundant amounts of (1,4)-α-glucan in the insoluble fraction of cells expressing Ags1p-D315N (Figure 4). Further studies are required to study the soluble fraction of these cells. Previously, we demonstrated that the (1,4)-α-glucan biosynthesis is not a function of the TGL domain (Vos *et al.*, 2007). Consequently, we speculate that a TGL domain rendered dysfunctional by mutagenesis may be unable to process the (1,4)-α-glucan produced by the SYN domain. This may result in the accumulation of (1,4)-α-glucan segments of approximately 12 (1,4)-linked α-glucose residues as found in cell wall α-glucan (Grün *et al.*, 2005).
Based on our close examination of the regions in the TGL domain and new observations with the Ags1p TGL mutants (Figures 1, 3 and 6), we propose a refinement of our speculative model for cell wall α-glucan biosynthesis with respect to the function of the TGL domain and the presence of different regions in the TGL and SYN domains (Hochstenbach et al., 1998; Grün et al., 2005; Vos et al., 2007). The intracellular SYN domain synthesises small (1,4)-α-glucan oligosaccharides, which may be directed towards the putative pore domain along the Ser-rich region. Before crossing the plasma membrane, the (1,4)-α-glucan segments may be elongated by a hypothesised (1,3)-α-glucan synthase (Vos et al., 2007) resulting in a single building block. After crossing the membrane, this building block may be directed to the TGL domain, via the extracellular β-sheet-rich region, where it is linked to another building block, forming mature cell wall α-glucan (Figure 7). This model would explain the phenotypes of the Ags1p TGL mutants. Dysfunctional TGL domains, like that found in the ags1-1" mutant expressing Ags1p-G696S, may be unable to link two building blocks (Grün et al., 2005). As a consequence, the route of the growing building block might become physically obstructed and the synthesised (1,4)-α-glucan segments may subsequently accumulate intracellularly. Further studies will be required to determine the precise contribution of the Ags1p TGL domain to cell wall α-glucan biosynthesis in S. pombe.

The bifunctional protein Ags1p is an integral part of the protein machine for cell wall α-glucan biosynthesis. Like activation of Bgs1p by Rho1p, Ags1p is activated by Rho2p via Pck2p (Arellano et al., 1999; Calonge et al., 2000). In addition, very recently it was suggested that cell wall α-glucan biosynthesis may also involve GPI-anchored transglycosylases. In Aspergillus niger, two GPI-anchored transglycosylases, AgtAp and AgtBp, have been identified which are conserved in cell wall α-glucan-containing fungi. These agt genes are often localised next to ags genes and their gene products can perform a linkage reaction between a (1,4)-α-glucan donor and a (1,3)-

![Figure 7. Schematic model for the cell wall α-glucan biosynthesis in S. pombe.](image)

Schematic representation of the hypothetical model for cell wall α-glucan biosynthesis by Ags1p and additional proteins in S. pombe. The Ags1p SYN domain (2) synthesises (1,4)-α-glucan, whereas an unknown (1,3)-α-glucan synthase (3) might synthesise the (1,3)-α-glucan component of cell wall α-glucan, together synthesising a single cell wall α-glucan building block (A). This building block is transported across the plasma membrane through the putative pore domain of Ags1p to the extracellular space. Then, two building blocks are linked to form mature cell wall α-glucan (B) by the Ags1p TGL domain (1) or by the transglycosylase Aah3p (4).
α-glucan acceptor (van der Kaaij et al., 2007). Moreover, in S. pombe, four homologs were identified, Aah1p, Aah2p, Aah3p, and Aah4p, the genes of which are often located in the neighbourhood of the mok genes (Morita et al., 2006). Although the catalytic activity of these homologs in S. pombe was not investigated, deletion of the aah3 gene resulted in a cell wall defect without changing the content of α-glucan in the cell wall (Morita et al., 2006). However, these results do not exclude the possibility that the cell wall α-glucan structure may have changed in these mutants. Further research will be required to investigate this.

EXPERIMENTAL PROCEDURES

Strains and culture media

Escherichia coli strain DH5α (Invitrogen) was used for all plasmid isolations. S. pombe plasmid transformations were performed using strains with genotype h− ura4-D18 or h− ags1-1ts ura4-D18 using a lithium acetate method at pH 4.9 (Ito et al., 1983). Cells were grown in YEA medium (Hochstenbach et al., 1998) or EMM2 medium (Moreno et al., 1991) supplemented with 250 mg/l adenine sulphate (EMMA). Following plasmid transformation, expression of cloned genes was repressed by growing the cells on EMMA plates supplemented with 10 μM thiamine at 28°C for 3–5 days. For overexpression experiments, cells were grown overnight at 28°C in EMMA medium containing 10 μM thiamine, washed twice with EMMA medium (which lacks thiamine), and grown at 28°C in EMMA medium for 20 h. The S. pombe strains used in this study are listed in Table 1.

Sequence alignments and secondary structure predictions

The sequencing project used to obtain protein sequences of C. neoformans was the Fungal Genome Initiative, Broad Institute of Harvard and MIT (www.broad.mit.edu). The selected amino acid sequences were aligned using the algorithm ClustalW

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(version 2.0) (Thompson et al., 1994; Larkin et al., 2007). Secondary structure predictions resulted from the program PSIPRED (version 2.5) (McGuffin et al., 2000; Bryson et al., 2005).

Construction of plasmids
The primers used in this study are listed in Table 2. All PCR amplifications were performed using Phusion™ polymerase (Finnzymes). To introduce defined mutations into the gene fragment encoding the TGL domain, the Xhol-EcoRI fragment was cloned into the pUC19-based vector pAdH101 (Chapter 5), and site-directed mutagenesis was performed using primers BS001 and BS002 (Y143A), BS005 and BS006 (D315N), or BS007 and BS008 (E349Q). The Xhol-SpeI fragment of pAV011 was replaced with the mutated Xhol-SpeI fragments, generating pAV134, pBS025, or pBS026, respectively. All constructs used in this study were sequenced in both directions using a series of overlapping PCR amplification products (BigDye Terminator sequencing kit, Applied Biosystems). The plasmids used in this study are listed in Table 3.

Plate-based assay for (1,4)-α-glucan accumulation
Cells were grown in EMMA medium containing 10 μM thiamine at 28°C to an optical density at 595-nm wavelength (OD$_{595}$) of 1.0–1.5, washed and taken up in MQ-$H_2$O to a final concentration of 2.5 × 10$^7$ cells/ml. Four microliters of cell suspension (1 × 10$^5$ cells) were spotted onto EMMA plates supplemented with or without 10 μM thiamine. Plates were incubated at 28°C for 3 days and exposed to iodine vapour for 4 min.

(1,4)-α-Glucan determination
Cells (10$^9$) were taken up in 5 mM sodium azide and 20 mM Tris•HCl (pH 7.6), broken with acid-washed glass beads using Fastprep 120 (Bio 101, Inc) at speed 6 for five intervals of 15 sec, and centrifuged at 16,000 × g at 4°C for 10 min. Cell pellets were resuspended in 2% (w/v) SDS, 100 mM EDTA, 20 mM DTT, and 50 mM Tris (pH 7.6), boiled for 10 min, and centrifuged again. The resulting pellets were washed twice with MQ-$H_2$O and once with 10 mM sodium acetate (pH 5.6) and were divided

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS001</td>
<td>5’–CCCTGGGGAGCTGATCAAGCTTCTCTCTTTGGATTAC</td>
<td>To mutate ags1, Y143A; FW</td>
</tr>
<tr>
<td>BS002</td>
<td>5’–GTAATCCAAAGGAGAAGCTTGATCAGCTCCCCAGGG</td>
<td>To mutate ags1, Y143A; REV</td>
</tr>
<tr>
<td>BS005</td>
<td>5’–GTCGATGGATTCCGTATTAACAAGGCTACCCAGATGAC</td>
<td>To mutate ags1, D315N; FW</td>
</tr>
<tr>
<td>BS006</td>
<td>5’–GTCATCTGGGTAGCCTTGTTAATACGGAATCCATCGAC</td>
<td>To mutate ags1, D315N; REV</td>
</tr>
<tr>
<td>BS007</td>
<td>5’–GATCGTTTGGTCAAGTCTGGCTCAGCTCATCTAGC</td>
<td>To mutate ags1, E349Q; FW</td>
</tr>
<tr>
<td>BS008</td>
<td>5’–GCTAGATGAGCCAGTAACTTGACCAAACGATC</td>
<td>To mutate ags1, E349Q; REV</td>
</tr>
</tbody>
</table>
equally over four fractions. Fractions were resuspended in 200 μl 10 mM sodium acetate (pH 5.6) and incubated at 37°C for 2.5 h in the presence of 1.8 units of α-amylase (Roche Applied Science; 102814) and 6.0 milliunits of glucoamylase (Roche Applied Science; 1202332), 0.1 unit of Zymolyase-100T (Seikagaku Corp.; 120493), all three enzymes, or buffer only. After digestion, supernatants were collected and the amount of liberated reducing ends was measured using a colorimetric assay (Lever, 1972). The reducing ends were converted to glucose equivalents, the background (buffer-only fraction) was subtracted, and the glucose equivalents/10^9 cells were corrected for the β-glucan amounts (Zymolyase-100T digestion). Digestion of (1,4)-α-glucan was checked visually for completion by resuspending the remaining pellets in 100 μl of MQ-H_2O and adding 900 μl of a potassium triiodide reagent prepared from 0.01% (w/v) iodine and 0.1% (w/v) potassium iodide in 5 M calcium chloride.

**Complementation assay**

Cells were grown in EMMA medium containing 100 μM thiamine at 21°C to OD_595~1.0, washed, and taken up in MQ-H_2O to a final concentration of 2.5 × 10^7 cells/ml. Four microliters of cell suspension were spotted in 10-fold serial dilutions onto EMMA plates containing 100 μM of thiamine. Plates were incubated at 28°C or 36°C for 3–5 days and exposed to iodine vapour for 4 min.

**(1,4)-α-glucan accumulation screen**

ags1-1ts cells containing pAgs1 were grown at 28°C to OD_595~1.0 and plated on 20 EMMA plates, which lack thiamine to induce the expression of pAgs1, in a density of 10^4 cells/plate. After three days of growth at 28°C, plates showed a thin layer of tiny single colonies with some large and medium sized colonies. These bigger colonies were taken and regrown on EMMA plates supplemented with 10 μM of thiamine. Plates were incubated at 28°C for 3–5 days. Cells were then checked for growth and iodine staining upon overexpression and for complementation at 36°C. Mutant cells that showed no growth inhibition, a positive iodine staining upon overexpression, and that were able to complement the temperature-sensitive ags1-1 cells at 36°C were chosen for further characterisation by sequencing.

**Table 3. Plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Name</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pREP4</td>
<td>pAgs1</td>
<td><em>S. pombe</em> expression plasmid</td>
<td>Maundrell, 1993</td>
</tr>
<tr>
<td>pAV011</td>
<td>pAgs1-Y143A</td>
<td>pREP4 with ags1</td>
<td>(Vos et al., 2007)</td>
</tr>
<tr>
<td>pAV134</td>
<td>pAgs1-G696S</td>
<td>pAV011 with ags1-T427G, A428C</td>
<td>This study</td>
</tr>
<tr>
<td>pAV137</td>
<td>pAgs1-D315N</td>
<td>pAV011 with ags1-G2086A</td>
<td>(Vos et al., 2007)</td>
</tr>
<tr>
<td>pBS025</td>
<td>pAgs1-E349Q</td>
<td>pAV011 with ags1-C943A, T945C</td>
<td>This study</td>
</tr>
<tr>
<td>pBS026</td>
<td>pAgs1-G1045C</td>
<td>pAV011 with ags1-G1045C</td>
<td>This study</td>
</tr>
</tbody>
</table>

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REFERENCES


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