Interspore bridges: a new feature of the *Saccharomyces cerevisiae* spore wall

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The *Saccharomyces cerevisiae* spore wall is a multilaminar coat that surrounds individual spores and protects them from environmental insult. Scanning electron microscopy reveals that the four spores of an ascus are connected by interspore bridges. Transmission electron microscopy of spores indicates that these bridges are continuous with the outer layers of the spore wall. In *chs3* mutants, which lack the chitosan and dityrosine layers of the spore wall, bridges are absent. By contrast, in *dit1* mutants, which lack only the dityrosine layer, bridges are present, suggesting that the bridges may be composed of chitosan. Interspore bridges are shown to be necessary to hold spores together after release from the ascus. A function for these bridges in the maintenance of heterozygous markers in a homothallic yeast population is proposed.

INTRODUCTION

Sporulation in baker's yeast, *Saccharomyces cerevisiae*, is triggered in *MATa/MAT α* diploid cells by the absence of a nitrogen source and the presence of a non-fermentable carbon source (Kupiec *et al.*, 1997). Under these conditions, cells enter meiosis to produce four haploid nuclei. These nuclei are packaged into spores in two stages. In the first stage, secretory vesicles coalesce within the cytoplasm to form new double-membrane sheets, termed prospore membranes (Moens, 1971; Neiman, 1998). Each of the four haploid nuclei formed by the meiotic divisions becomes completely engulfed by a prospore membrane, resulting in the formation of four daughter cells, or prospores, within the cytoplasm of the mother cell. In the second stage, after capture of the nuclei within prospore membranes, formation of the spore wall proceeds by deposition of spore wall materials within the lumen of the prospore membrane (Lynn & Magee, 1970). The completed spore wall serves to protect the spore from environmental damage (Smits *et al.*, 2001).

The spore wall is a more elaborate structure than the vegetative wall of *S. cerevisiae*. The four major components of the spore wall are arranged in layers formed consecutively, beginning with the innermost layer and working outward (Tachikawa *et al.*, 2001). The two inner layers consist primarily of mannan and 1,3- β -glucan and are similar to the vegetative cell wall (Kreger-Van Rij, 1978). The two outer layers of the spore wall are unique to the spore and confer on the spore much of its resistance to environmental damage

(Smits *et al.*, 2001). Outside of the β -glucan layer is a layer of chitosan, a 1,4- β -glucosamine polymer (Briza *et al.*, 1988). This layer is formed by the combined action of the *CHS3*-encoded chitin synthase and the chitin deacetylases encoded by *CDA1* and *CDA2* (Christodoulidou *et al.*, 1996; Mishra *et al.*, 1997; Pammer *et al.*, 1992). Outside of the chitosan is a thin layer that consists predominantly of cross-linked molecules of dityrosine (Briza *et al.*, 1986, 1990, 1996). The dityrosine monomers are produced in the spore cytosol by the combined action of the Dit1p and Dit2p enzymes and then exported for assembly into the wall (Briza *et al.*, 1994; Felder *et al.*, 2002). After spores are fully formed, the remains of the mother cell then collapse around the four completed spores to form the ascus. As a result, the four spores of a tetrad are enclosed together inside an ascus membrane and ascus wall, which are derived from the mother cell plasma membrane and cell wall, respectively.

In an effort to develop new assays of spore wall assembly, we examined wild-type and mutant spores by scanning electron microscopy (SEM). This analysis revealed that the wild-type spore wall has a distinctive surface texture that is altered as the dityrosine and chitosan layers are removed. Moreover, this analysis has led to the identification of a previously undescribed feature of the spore wall, the interspore bridge. These bridges connect the outer spore wall layers of adjacent spores and serve to maintain the physical association of spores upon release from the ascus.

METHODS

Strains and media. Unless otherwise noted, standard media and methods were used (Rose & Fink, 1990). Strains used in this study are listed in Table 1. Strains were constructed as follows: *DIT1* and

Abbreviations: SEM, scanning electron microscopy; TEM, transmission electron microscopy.

Table 1. Strains

Strain	Genotype	Source
AN117-4B	<i>MATα ura3 leu2 trp1::hisG his3ΔSK lys2 arg4-NspI hoΔLYS2 rme1::LEU2</i>	Neiman <i>et al.</i> (2000)
AN117-16D	<i>MATa ura3 leu2 trp1::hisG his3ΔSK lys2 hoΔLYS2</i>	Neiman <i>et al.</i> (2000)
AN120	<i>MATa/MATα ura3/ura3 leu2/leu2 trp1::hisG/trp1::hisG his3ΔSK/his3ΔSK lys2/lys2 arg4-NspI/ARG4 hoΔLYS2/hoΔLYS2 rme1::LEU2/RME1</i>	Neiman <i>et al.</i> (2000)
AN262	<i>MATa/MATα ura3/ura3 leu2/leu2 trp1::hisG/trp1::hisG his3ΔSK/his3ΔSK lys2/lys2 arg4-NspI/ARG4 hoΔLYS2/hoΔLYS2 rme1::LEU2/RME1 chs3ΔHIS3MX6/chs3ΔHIS3MX6</i>	This work
AN264	<i>MATa/MATα ura3/ura3 leu2/leu2 trp1::hisG/trp1::hisG his3ΔSK/his3ΔSK lys2/lys2 arg4-NspI/ARG4 hoΔLYS2/hoΔLYS2 rme1::LEU2/RME1 dit1ΔHIS3MX6/dit1ΔHIS3MX6</i>	This work

CHS3 were individually deleted in the haploid strains AN117-4B and AN117-16D (Neiman *et al.*, 2000) by PCR-mediated knockout (Longtine *et al.*, 1998); the resulting haploids were mated to generate AN264 and AN262, respectively.

Sporulation. Intact cells were prepared for sporulation in 2% potassium acetate (KOAc) as described previously (Neiman, 1998). For sporulation of spheroplasts, cells were first grown to mid-exponential phase in YPAcetate medium (1% yeast extract, 2% Bacto-peptone, 2% KOAc). Cells were then pelleted, washed once in distilled water (dH₂O) and then resuspended in 0.7 M sorbitol. Zymolyase 100T (US Biological) was added to a final concentration of 0.1 mg ml⁻¹ and cells were incubated for 1 h at 30 °C. Cells were then washed once in SSM (0.7 M sorbitol, 1.4% KOAc), resuspended in SSM at a concentration of 2 × 10⁷ cells ml⁻¹ and incubated at 30 °C.

Scanning electron microscopy. For SEM analysis of spores, spheroplasts were first sporulated, washed once in 0.5% SDS to remove the ascal membrane, washed once in dH₂O, and then adhered to polylysine coated glass cover slips. The cells on the cover slips were fixed in 3% glutaraldehyde in cacodylate buffer (0.1 M sodium cacodylate pH 7.4, 5 mM CaCl₂) for 1 h at 23 °C, then stained for 1 h at 23 °C in 1% osmium tetroxide and 1% potassium ferricyanide in cacodylate buffer, washed with dH₂O and then dehydrated by 10 min incubations in a graded acetone series: two incubations each in 30%, 50%, 70% and 95% acetone, and four incubations in 100% acetone. The cover slips were then critical-point dried and sputter-coated with 4 nm of gold particles. Germinating cells, vegetative cells and asci were adhered to cover slips and prepared in the same way. Images were collected in a LEO1550 scanning electron microscope at 2.5 kV using an in-lens detector.

Transmission electron microscopy. Cells were prepared for transmission electron microscopy (TEM) using an osmium-thiocarbohydrazide staining protocol (Rieder *et al.*, 1996). Spores were prepared as for SEM, then fixed for 1 h in 3% glutaraldehyde in cacodylate buffer, washed once in cacodylate buffer, resuspended in 1% osmium tetroxide and 1% potassium ferricyanide in cacodylate buffer, and incubated for 30 min at 23 °C. Cells were then washed four times in dH₂O, resuspended in 1% thiocarbohydrazide in water, and incubated for 5 min at 23 °C. Cells were again washed in dH₂O, incubated in 1% osmium tetroxide and 1% potassium ferricyanide in cacodylate buffer for an additional 5 min, and washed again in dH₂O. The cells were then incubated in saturated uranyl acetate for 2 h and dehydrated through a graded series of acetone washes as for the SEM samples. The dehydrated samples were embedded in Epon 812, sectioned, and images were collected on a JEOL 1200EX microscope at 80 kV.

Spore dispersal assay. Wild-type, *dit1* and *chs3* mutant cells were sporulated. Zymolyase was then added to a final concentration of

0.1 mg ml⁻¹ and the spores were incubated for 15 min at 37 °C. Spores were washed once in dH₂O, resuspended in dH₂O, and mounted on slides for examination by phase-contrast microscopy.

RESULTS

SEM reveals a distinct surface to the spore wall

The outermost layer of the *S. cerevisiae* spore wall is composed primarily of dityrosine (Briza *et al.*, 1994, 1986). By contrast, the exterior of the vegetative cell wall is primarily mannoproteins (Orlean, 1997). To determine if these two distinctive polymers could be distinguished by their surface appearance, vegetative cells, asci and isolated spores were examined by SEM. Vegetatively growing yeast cells had a smooth, velvety appearance (Fig. 1a). Intact asci had a similar surface texture to vegetative cells (Fig. 1b), consistent with the fact that the ascal wall is derived from the vegetative wall of the mother cell. By contrast, spore surfaces, visualized after removal of the ascus, had a distinctive ridged or scalloped appearance (Fig. 1c).

It was possible that the different surface features of the spore wall and the vegetative wall were in some way an artefact of the spore preparation process. To control for this possibility, isolated spores were transferred to rich medium for 3 h before fixation and preparation for SEM. Under these conditions, the germinating cells were seen to have buds, with the typical vegetative wall texture emerging from the scalloped spore coat (Fig. 1d). Thus, the spore wall and cell wall have distinct appearances even when present on different areas of the same cell.

Finally, it was possible that the appearance of the spores would be altered by the treatment necessary for removal of the ascus. In particular, removal of spores from the ascus requires digestion of the ascal wall with glucanases to release the spores. Although spores are resistant to glucanase digestion, it is nonetheless possible that the enzymes could cause changes in the spore wall. To avoid this problem, vegetative cells were spheroplasted before transfer to osmotically stabilized sporulation medium. After sporulation, the spores could then be removed from the ascus without enzymic digestion. When prepared in this way, spores appeared indistinguishable by SEM from spores

Fig. 1. The surface morphology of spores is distinct from that of vegetative cells. Vegetatively growing yeast cells (a), asci (b), isolated spores (c), and germinating spores (d) were examined by SEM. Representative cells from each type are shown. Over 100 cells of each class were examined. Arrowheads in (c) indicate interspore bridges. Arrows in (d) indicate germinating cells. Scale bars, 1 μ m.

isolated by direct digestion of asci with glucanases (data not shown). Thus, although we cannot exclude the possibility that the surface of the spore wall is affected by preparation for electron microscopy, the appearance of the wall is not an artefact of exposure to lytic enzymes.

Spores are connected to each other by bridges

The most striking finding of the SEM analysis was the presence of connections between spores (arrowheads, Fig. 1c). To explore the nature of these interspore bridges, spores were released from asci as for the SEM analysis and examined by TEM. Interspore bridges were readily visible in these preparations as darkly staining material connecting the outer layers of the walls of adjacent spores. In these preparations, the dityrosine and chitosan layers of the spore wall stain as a single dark layer (arrowheads, Fig. 2). This layer branches at the position of the bridges so that it both surrounds individual spores and connects adjacent spores. Thus, the bridges themselves appear to consist of chitosan and/or dityrosine.

Chitin synthase is required for construction of the bridges

To test the idea that the interspore bridges are constructed of outer spore wall materials, we examined the spore

surfaces of *dit1* and *chs3* mutants, which lack the dityrosine and chitosan layers of the spore wall, respectively (Briza *et al.*, 1994; Pammer *et al.*, 1992). Because both *dit1* and *chs3* spores are sensitive to Zymolyase digestion, cells were prepared for analysis by sporulation of spheroplasts, as described above. The surfaces of *dit1* mutant spores, which should consist primarily of chitosan without the overlying dityrosine, differed from that of wild-type spores in having a blistered, rather than scalloped appearance (Fig. 3a). Nonetheless, interspore bridges were readily visible in the *dit1* mutant (Fig. 3b). When the bridges in *dit1* cells were examined by TEM, the outer layer of the spore wall again appeared to branch and run between the spores (Fig. 3d). Because there is no dityrosine present in the *dit1* mutant, the remaining bridge material is probably chitosan.

To confirm that the bridges are indeed constructed of chitosan, spores were examined in a *chs3* mutant. *CHS3* encodes the chitin synthase required for synthesis of spore wall chitosan; in the *chs3* mutant both the chitosan and dityrosine layers of the spore wall are absent (Pammer *et al.*, 1992). In *chs3* spores, the surface of the underlying β -glucan layer is exposed and has a very smooth appearance by SEM (Fig. 4a, b). It was very difficult to identify *chs3* tetrads by SEM, as upon release from the ascus membrane the *chs3* spores dispersed. Figs 4(a) and 4(b) show two tetrads of *chs3* spores still held together by the remnants of

Fig. 2. Individual spores are connected by interspore bridges. (a) An interspore bridge as seen by SEM. (b) Higher magnification of spores in (a). (c, e) TEM images of interspore bridges. (d, f) Higher magnification of bridges in (c) and (e), respectively. Representative cells from over 200 cells examined are shown. Arrowheads in (d) and (f) mark the position of branches in the outer spore wall layers. Scale bars: (a) 1 μ m; (b–f) 200 nm.

the ascus membrane (arrowheads). These tetrads lack any structures characteristic of interspore bridges. Similar results were obtained by TEM analysis. Again, *chs3* spores were visualized within the ascus to prevent spores from dispersing. No connections between spores were visible at

positions where the β -glucan layers of adjacent spores came into contact (arrow, Fig. 4d). Thus, *CHS3* is required for the construction of interspore bridges, consistent with the inference that the bridges are themselves extensions of the chitosan layer of the spore wall.

Fig. 3. Interspore bridges are still formed in *dit1* mutant cells. (a) SEM image of *dit1* spores. (b) Higher magnification of spores in (a). (c) TEM image of *dit1* spores. (d) Higher magnification of spores in (c). Representative cells from over 200 cells examined are shown. Arrowheads in (d) mark the position of branches in the chitosan layer. Scale bars: (a) 1 μ m; (b) 200 nm; (c) 500 nm; (d) 100 nm.

Interspore bridges provide a physical connection between spores

The electron microscopic analysis of the *chs3* mutant suggests that the interspore bridges might hold spores together upon release from the ascus. To test this directly, wild-type, *dit1* and *chs3* mutant strains were sporulated and the spores were released from the ascus by digestion with Zymolyase. The released spores were then examined by light microscopy and scored as to whether they were in clumps of four, three, two, or isolated spores (Fig. 5). Before Zymolyase treatment, more than 90 % of the spores in all three cultures were grouped in tetrads. After digestion with Zymolyase, more than 80 % of the wild-type spores remained in groups of four. Consistent with the presence of bridges in *dit1* mutants, the *dit1* spores remained clustered together nearly as efficiently as wild-type. By contrast, less than 10 % of the *chs3* mutant spores maintained intact tetrads after digestion and nearly 80 % of the of the *chs3* mutant spores were dispersed as single, isolated spores. These results demonstrate that interspore bridges can

maintain a physical connection between spores after they are released from the ascus.

DISCUSSION

Our SEM studies of the *S. cerevisiae* spore wall have revealed that the surface of the spore wall is distinct from that of the vegetative cell wall. It is interesting in this regard that SEM images of spores from the fission yeast, *Schizosaccharomyces pombe*, have a similar surface appearance to *S. cerevisiae* spores, though the *Schiz. pombe* spore wall has not been reported to contain dityrosine (Nakamura *et al.*, 2004). The scalloped surface of the wall may therefore not be an inherent property of the constituents of the spore surface, but an architectural feature of the wall. When *dit1* and *chs3* mutations were used to expose different layers of the spore wall on the surface, these layers displayed distinct morphologies. Thus, SEM will be a useful technique in the characterization of other mutants with spore wall defects.

More strikingly, this analysis revealed that *S. cerevisiae*

Fig. 4. Interspore bridges are absent in *chs3* mutant cells. (a, b) SEM images of *chs3* asci. Examples of intact tetrads of *chs3* were rare due to dispersal of the spores (see text). These spores are held together by remnants of the ascus membrane (arrowheads). (c) TEM image of *chs3* spores within an ascus. (d) Higher magnification of spores in (c); the β -glucan layers are adjacent but not connected (arrow). Scale bars: (a, b) 1 μ m; (c) 500 nm; (d) 200 nm.

spores isolated from the ascus are connected by structures we have termed interspore bridges. TEM analysis of isolated spores suggested that the bridges are formed by a branching of the outer layers of the spore wall so that these layers both surround individual spores and connect adjacent spores. This inference was confirmed by analysis of *chs3* mutant cells. In *chs3* mutants, the interspore bridges were absent, indicating that chitin or chitosan is required for bridge construction. These results demonstrate that chitin or chitosan is essential for construction of the bridges. Whether there are additional constituents of the bridges is unclear. For instance, the bridges appear somewhat less robust in a *dit1* mutants (Fig. 3) suggesting that, although bridges are still functional in *dit1* mutants (Fig. 5), dityrosine may normally be present in the bridges. Additionally, the composition of the grey-staining material in the central region of the bridges is unknown.

The existence of these bridges raises several interesting issues. For example, how are the bridges constructed? One possibility is that bridges may be formed at points of contact during spore wall construction. Perhaps contact triggers branching and interconnection of the chitosan layers of adjacent spores. Understanding bridge assembly will require the identification of the enzymes responsible for this process. It may be possible to use the spore dispersal assay (Fig. 5) to identify genes involved in bridge synthesis.

Another important question raised by the presence of these bridges is what functional role these structures play in the yeast life cycle. One possibility is suggested by the homothallic nature of *S. cerevisiae*. Outside of the laboratory, *S. cerevisiae* is found primarily in a diploid (or higher ploidy) state (Mortimer & Hawthorne, 1969). Starvation induces sporulation and the generation of haploids, but *HO*-mediated mating-type switching guarantees that isolated haploid spores will be able to mate and return to diploidy upon germination (Herskowitz & Jensen, 1991). However, if haploid spores self-mate to restore diploidy, then any heterozygosities that may have been present in the parental population will be lost. A large body of literature suggests that maintenance of heterozygosity can be selectively advantageous to a population (Milton, 1997). Therefore, one possible role for bridges is to promote, by physically connecting spores, the mating of sister spores to produce diploids and thereby restore the heterozygous state present in the parental cell. The fact that mating-type switching does not begin until the second division after germination (Strathern & Herskowitz, 1979) might, in combination with the bridges, help promote mating between sister spores.

While this is a speculative hypothesis, some indirect support for it can be found by comparison to *Schiz. pombe*. *Schiz. pombe* is also a homothallic yeast, but unlike *S. cerevisiae* it exists primarily as a haploid, only mating to form diploids

Fig. 5. Spore dispersal assay. Spores of the wild-type (a), a *dit1* mutant strain (b), and a *chs3* mutant strain (c) were examined by light microscopy and scored as clustered in groups of 1, 2, 3 or 4, both before (black bars) and after (grey bars) Zymolyase digestion. Over 500 spore clusters were scored in each culture.

immediately prior to sporulation (Mortimer, 1969). Because *Schiz. pombe* haploids do not mate upon germination, our model would predict no role for interspore bridges in this yeast. Indeed, in *Schiz. pombe*, no bridges are evident by SEM and wild-type spores disperse upon release from the ascus (Gutz *et al.*, 1974; Nakamura *et al.*, 2004). It will be of interest to determine whether, in other homothallic yeasts, the presence of bridges between ascospores correlates with the tendency to mate immediately upon germination.

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