The (1,3)β-D-glucan synthase subunit Bgs1p is responsible for the fission yeast primary septum formation.

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ABSTRACT

Cytokinesis is a crucial event in the cell cycle of all living cells. In fungal cells, it requires coordinated contraction of an actomyosin ring and synthesis of both plasmatic membrane and a septum structure that will constitute the new cell wall end. *Schizosaccharomyces pombe* contains four essential putative (1,3)β-D-glucan synthase catalytic subunits, Bgs1p to Bgs4p. Here we examined the function of Bgs1p in septation by studying the lethal phenotypes of *bgs1*$^+$ shut-off and *bgs1*Δ cells and demonstrated that Bgs1p is responsible and essential for linear (1,3)β-D-glucan and primary septum formation. *bgs1*$^+$ shut-off generates a more than 300-fold Bgs1p reduction, but the septa still present large amounts of disorganized linear (1,3)β-D-glucan and partial primary septa. Conversely, both structures are absent in *bgs1*Δ cells, where there is no Bgs1p. The septum analysis of *bgs1*$^+$-repressed cells indicates that linear (1,3)β-D-glucan is necessary but not sufficient for primary septum formation. Linear (1,3)β-D-glucan is the polysaccharide that specifically interacts with the fluorochrome Calcofluor white in fission yeast. We also show that in the absence of Bgs1p abnormal septa are formed, but the cells cannot separate and eventually die.

INTRODUCTION

Cytokinesis is the process of the cell cycle that generates a physical barrier in the cytoplasm to divide the cell into two new cells. This process is well conserved from fungi to mammals and requires coordinated contraction of an actomyosin ring and synthesis of plasma membrane (Balasubramanian *et al.*, 2004). Fungal cells contain a structure external to the plasma membrane, the cell wall, and therefore, cytokinesis requires coordinated synthesis during ring contraction of the cell wall structure termed division septum. The fission yeast *Schizosaccharomyces pombe* provides an excellent model for cytokinesis studies. The cells divide by medial fission and genetic studies have allowed a better understanding of the cytokinesis steps: medial ring positioning and assembly, recruitment of actin patches to the medial ring, and activation of ring contraction and septum formation by the septation initiation network (SIN) proteins (Krapp *et al.*, 2004; Wolfe and Gould, 2005). The septum is a three-layered structure composed of a middle disk named primary septum, flanked at both sides by the secondary septum (Johnson *et al.*, 1973). Finally, cell separation requires primary septum and adjacent cell wall degradation by the action of α and β-glucanases (Dekker *et al.*, 2004; Martín-Cuadrado *et al.*, 2003).

S. pombe cell wall consists of an outer layer rich in galactomannoproteins and an inner layer of (1,3)β, (1,6)β and (1,3)α-D-glucans (Durán and Pérez, 2004; Pérez and Ribas, 2004). The fluorochrome Calcofluor white (CW) binds to fibrillar (1,4)β polysaccharides, like chitin or cellulose. *S. pombe* has no chitin (Arellano *et al.*, 2000), but CW binds with high affinity to septum and with much less intensity to growing poles. However, the component that CW binds in fission yeast is still unknown. Immunoelectron microscopy studies localized the (1,3)β-D-branched (1,6)β-D-glucan in the cell wall and secondary septum; (1,6)β-D-branched (1,3)β-D-glucan in the cell wall and both primary and secondary septum; and a linear (1,3)β-D-glucan (L-BG) exclusively in the primary septum (Humbel *et al.*, 2001). In addition to (1,6)β-D-branched and linear (1,3)β-D-glucans, other polysaccharides, as the (1,3)α-D-glucan, could be present in the primary septum as well. The importance of each of these polysaccharides for the primary septum structure is still unknown. (1,3)β-D-glucan is the main

structural component of the fission yeast cell wall. The enzyme involved in its formation is the (1,3)β-D-glucan synthase complex (GS), composed by at least a regulatory and a catalytic subunit (Ribas *et al.*, 1991). The former is the GTPase Rho1p (Arellano *et al.*, 1996).

S. pombe contains four essential putative GS catalytic subunits, Bgs1p-4p. Bgs1p was the first subunit described. It was cloned from a mutant (*cps1-12*) hypersensitive to a spindle poison and named *cps1*$^+$ (later changed to *bgs1*$^+$). Bgs1p/Cps1p showed strong homology with the putative GS subunits Fks from *Saccharomyces cerevisiae*, but the *cps1-12* mutant presented no reduction in either cell wall β-glucan or GS activity. The *cps1-12* mutant displayed a multiseptated and branched phenotype, and it was suggested that Bgs1p could be a GS involved in cytokinesis, polarity and cell wall morphogenesis (Ishiguro *et al.*, 1997). Two other mutants, *swl1-N12* (*cps1-N12*) and *drc1-191* (*cps1-191*) were described as forming a stable actomyosin ring, but unable to assemble the division septum, suggesting that Bgs1p is involved in a septation checkpoint (Le Goff *et al.*, 1999; Liu *et al.*, 1999; Liu *et al.*, 2000b). The phenotype of these mutants led to the proposal that Bgs1p could be a GS essential for septum assembly. The finding that Bgs1p was localized at the medial ring and that it was essential for survival suggested that it was required for primary septum formation (Cortés *et al.*, 2002; Liu *et al.*, 2002). From that point on, however, new results complicated the situation and raised questions about Bgs1p function. First, Bgs1p was found to be localized not only at the contractile ring, but also at the septum, growing poles and sites of cell wall synthesis during sexual differentiation (Cortés *et al.*, 2002). Second, three new *bgs* genes were cloned, *bgs2*$^+$, *bgs3*$^+$ and *bgs4*$^+$. Although *bgs2*$^+$ was found to be expressed and essential only during sexual differentiation (Liu *et al.*, 2000a; Martín *et al.*, 2000), the other two homologues are required for survival and have been found to localize at the contractile ring (Cortés *et al.*, 2005; Martín *et al.*, 2003) in addition to the other sites where Bgs1p is found. Furthermore, the essential putative (1,3)α-D-glucan synthase Ags1p/Mok1p is needed for cell wall morphogenesis and was also located at the medial ring (Katayama *et al.*, 1999).

All these results prompted us to reexamine the function of Bgs1p. By shutting off the *bgs1*$^+$ expression more effectively than in the past and by examining the cells generated from *bgs1*Δ spores, we have been able to show that Bgs1p is required for the formation of L-BG and that the latter is essential,

although not sufficient, for the construction of the primary septum. Cells defective in Bgs1p are still able to use the machinery normally used for secondary septa to fashion a remedial septum. However, they are unable to separate and to maintain viability. These findings highlight remarkable similarities between septum formation in *S. pombe* and septa of plants and budding yeast.

RESULTS

Bgs1p is essential for vegetative cells and needed for correct septation and cell separation.

It has been suggested that Bgs1p is involved in septation, but its function in this process is unknown. Bgs1p is essential at least during spore germination. Therefore, the study of Bgs1p absence in vegetative cells could aid to uncover its specific essential function. *bgs1*$^+$ ORF was expressed under the control of the 81X version (very low expression) of the thiamine-repressible *nmt1*$^+$ promoter (Moreno *et al.*, 2000). *bgs1*$^+$ repression using a plasmid with a standard repression level (pJG23) maintained wild-type phenotype in a *bgs1*Δ strain (Table 1), as previously reported (Cortés *et al.*, 2002). Since it was possible that the residual *bgs1*$^+$ expression were still sufficient to maintain the cell wild type, it became necessary to find a procedure to increase *bgs1*$^+$ repression in an attempt to produce a lethal phenotype. It has been described that the gene expression level of the *nmt1*$^+$ promoter is extremely sensitive to the distance between promoter and start codon (Moreno *et al.*, 2000). For that purpose, *bgs1*$^+$ and *nmt1*$^+$-81X promoter were gradually separated and the corresponding shut-off phenotypes analyzed (Table 1). The phenotypes of altered morphology and lethality increased with the distance between promoter and ORF up to pJR102. The three most distant *bgs1*$^+$ insertions (pJR100, 101 and 102) showed the maximum lethal *bgs1*$^+$ shut-off phenotype that can be produced with the *nmt1*$^+$-81X promoter (Table 1) since the two most distant insertions (pJR100 and pJR101) already generated a morphological phenotype under *bgs1*$^+$-induced conditions. Thus, pJR102 was selected as the p81X-*bgs1*$^+$ plasmid for *bgs1*$^+$ shut-off studies in a *bgs1*Δ background. This indicates that the residual expression of an essential gene from a conventionally repressed promoter can still be high enough to maintain wild type phenotype.

bgs1$^+$ shut-off is ultimately lethal. After 12 hours, it produces a drastic cell growth reduction. Osmotic stabilization protects partially the cells, which maintain a slow cell growth (Fig. 1A). Surprisingly, septum synthesis is not impaired (Fig. 1B, C). Instead, after 15 hours of *bgs1*$^+$ shut-off most of the cells contain one septum, indistinguishable from wild-type septa by phase-contrast or CW staining (standard concentration of 50 μg/ml). At longer times, the cells are elongated, branched and contain multiple septa (mycelium form). The cells are viable for a long period (48-60 hours without sorbitol) and sorbitol helps to maintain cell shape and viability for at least three days (Fig. 1B, C), suggesting that Bgs1p plays an important role in cytokinesis, growth polarity and cell wall morphogenesis. In no case a defect in septum completion was observed; the cells present partial septa that eventually will be completed (Fig. 1B-D, Fig. S1 and data not shown), although, as it will be shown below, the septal structure is abnormal. Nuclear staining showed that mitosis proceeds normally, apparently coordinated with the slow growth of *bgs1*$^+$ shut-off cells (Fig. 1D).

In vitro GS activity decreases slightly (20%) after 15 hours of *bgs1*$^+$ shut-off and remains stable for at least 36 hours (Fig. 1E). Cell wall analysis revealed a considerable cell wall increase (38%), which is attenuated in the presence of sorbitol (Table S1). This increase is exclusively due to α-glucan (216%). The β-glucan does not decrease, probably due to a compensatory mechanism from other Bgs subunits or to Bgs1p contributing a minor (1,3)β-D-glucan essential for the septum and cell wall structures.

bgs1$^+$ shut-off causes Bgs1p reduction to undetectable level.

In order to analyze the Bgs1p decrease, GFP-Bgs1p localization and amount were examined during *bgs1*$^+$ shut-off. When the *nmt1*$^+$-81X promoter is induced, GFP-Bgs1p localizes to medial ring, septum and poles (Fig. 2A), indistinguishable from a strain expressing *GFP-bgs1*$^+$ from its own promoter (Cortés *et al.*, 2002), except for the less intense GFP fluorescence of P*nmt1*$^+$-81X-*GFP-bgs1*$^+$ strain (data not shown). After 15 hours of *bgs1*$^+$ repression, Bgs1p is either not detected (50% of the septa, n=141; data not shown) or is only faintly detected in the medial ring, where its localization is more stable (Fig. 2A). After 24 hours, the cells show multiple septa and Bgs1p is undetectable (Fig. 2A), although the cells maintain viability for several days.

Under physiological conditions (own promoter), Bgs1p amount is 8 and 24-fold lower than that of Bgs3p and Bgs4p, respectively (Fig. 2B). Therefore, the physiological level of each Bgs protein is very different. This is consistent with the repression level needed with each *bgs* gene to generate the corresponding lethal shut-off phenotype. Shut-off of *bgs4*$^+$ can be produced with either *nmt1*$^+$-41X (medium repression level) or *nmt1*$^+$-81X promoter (Cortés *et al.*, 2005), *bgs3*$^+$ with *nmt1*$^+$-81X (Martín *et al.*, 2003) and *bgs1*$^+$ only with very distant *nmt1*$^+$-81X. The Bgs1p amount with induced *nmt1*$^+$-81X promoter is 5-fold lower than physiological (Fig. 2C, lanes C and 0h+T). *bgs1*$^+$ shut-off produces a fast Bgs1p reduction of 6 and 75-fold (compared to the level with induced *nmt1*$^+$-81X) after 5 and 10 hours, respectively. After 15 hours, when the cells are still normal but starting to be defective in cell separation, Bgs1p is no longer detected (more than 300-fold reduction from the physiological amount; Fig. 2C, lanes C and 15h+T). This indicates that the Bgs1p level needed to synthesize apparently normal septa and maintain wild type phenotype is extremely low, and the multiseptated phenotype is only obtained when the Bgs1p amount is undetectable.

Formation of *bgs1*$^+$ shut-off septa exhibits the same requirements as those of wild type septa.

bgs1$^+$ shut-off septa are totally different in structure and synthesis from normal septa (see below). Therefore, it is possible that the septa formed with undetectable Bgs1p could be generated by a mechanism completely or partially independent from that of normal septation. To test this, different septation mutants defective in medial ring positioning (*mid1*), ring assembly (*cdc3*, *cdc15*) and activation of ring contraction and septation (SIN; *cdc11*, *cdc14*) (Gould and Simanis, 1997) were made in a *bgs1*Δ p81X-*bgs1*$^+$ background and analyzed for their ability to form *bgs1*$^+$ shut-off septa.

Induction of the septation mutant phenotypes in multiseptated *bgs1*$^+$-repressed cells (24 hours, +T, 28ºC, to induce formation of aberrant septa; then 15 hours, +T, 37ºC, to induce the septation mutant phenotype on the aberrant septa) showed the same phenotype of altered or absent septa as that of the corresponding single septation mutant (Fig. S1 and data not shown). Therefore, the synthesis of *bgs1*$^+$ shut-off septa depends, as the normal septa, on the medial ring positioning and assembly and on the SIN proteins. In addition, the disappearance of the previously formed *bgs1*$^+$ shut-off septa after inducing the septation mutant phenotype (new septum synthesis is blocked) indicates that the septa are

eventually degraded and the cell compartments separate. This is also consistent with the fact that *bgs1*$^+$ shut-off produces discrete multiseptated units but not continuous filamentous growth.

Bgs1p is at least necessary for correct primary septum and linear (1,3)β-D-glucan formation.

The analysis of *bgs1*$^+$ shut-off cells stained with an excess of CW (50 μg/ml) showed a non-uniform CW staining in some of the septa (Fig. 1B, C, 48h+T±S and data not shown). In order to find out whether the *bgs1*$^+$ shut-off septa have reduced the structure that specifically binds CW, the cells were stained with the lowest CW concentration that stains all the septa in wild-type cells (1.25 μg/ml; Fig. 3A, arrows in left panels). Under these conditions, many *bgs1*$^+$ shut-off septa, distinguishable by phase-contrast, were not stained or presented weak or uneven staining (Fig. 3A, arrows in right panels). Therefore, Bgs1p is at least necessary for correct synthesis of the septum constituent that binds CW. Due to its fibrillar structure, L-BG is likely the polymer that specifically binds CW in fission yeast. To determine if Bgs1p is important for L-BG synthesis, the cells were stained with an L-BG specific monoclonal antibody. Control cells showed staining in all the septa, even in some not detected by phase-contrast (Fig. 3B, arrows in left panels). However, many *bgs1*$^+$ shut-off septa exhibited weak or no staining at all (Fig. 3B, arrows in right panels). These data suggest that Bgs1p participates in L-BG synthesis.

In order to determine the Bgs1p role in septum synthesis, *bgs1*$^+$ shut-off cells were analyzed by transmission electron microscopy (TEM). Control cells present a clear three-layered septum structure (Fig. 4A). Septum synthesis starts forming the annular rudiments, small membrane invaginations where primary septum synthesis begins (Fig. 4C1, arrow). As the primary septum grows (arrow), the secondary septum is laid down at both sides (arrowhead). Finally, the complete septum presents a three-layer structure of primary and secondary septum (Fig. 4C). *bgs1*$^+$ shut-off cells are much larger and their cell wall and septa are thicker and multilayered (Fig. 4B). After 15 hours of *bgs1*$^+$ repression, when the cells still contain a septum apparently normal under phase-contrast, TEM revealed a defective primary septum and a considerably thicker secondary septum. In addition, the symmetry in annular rudiments formation and septum growth is lost (Fig. 4D). At longer *bgs1*$^+$ shut-off times the

septa present more dramatic defects. Cell wall and septa thickness increase by addition of multiple layers. The annular rudiments no longer appear. The septa initiate over a wide cell wall area and grow by successive depositions of secondary septum parallel to the cell wall. The primary septum is absent from the base, inner edge or entire septum, discontinuous, twisted or wrongly orientated, but in no case it is totally absent (Fig. 4E-K; Table 2 and data not shown). All these data demonstrate that Bgs1p is at least essential for correct primary septum formation. In addition, Bgs1p is also required for proper secondary septum and cell wall formation.

Next, the presence of L-BG in *bgs1*$^{+}$ shut-off cells was examined by immunoelectron microscopy (IEM). Control cells show normal distribution of L-BG along the primary septum (Fig. 5A). However, the *bgs1*$^{+}$ shut-off cells present multiple L-BG defects, similar to those observed in primary septum structure. After 15 hours of *bgs1*$^{+}$ repression, the signal is already reduced in some septa (Fig. 5B). At longer times, the defects increase showing no signal in base, growing edge or all the septum, or a reduced, discontinuous, wavy or diffuse signal along the septum (Fig. 5C-I; Table 2 and data not shown). As observed for the primary septum, in no case is L-BG altogether missing. Moreover, the amount of septa with no L-BG signal is much lower than that without primary septum (Table 2). Surprisingly, control cells also present a fainter signal along the cell wall, also detected in lower amount in *bgs1*$^{+}$ shut-off cells. These results show that Bgs1p is at least essential for correct L-BG synthesis and localization and that L-BG is necessary but not sufficient for primary septum formation.

Bgs1p is responsible for the assembly of the CW-stained septum material, but abnormal septa develop in its absence.

bgs1^{+} shut-off cells show that Bgs1p is required for correct primary septum formation, but not whether it is essential for its construction. A considerable amount of disorganized L-BG and primary septum remains in *bgs1*$^{+}$ shut-off cells, where Bgs1p is undetectable. In order to gain insight into the Bgs1p function, the phenotype produced by total absence of Bgs1p was analyzed in *bgs1*Δ cells.

*bgs1*Δ deletion is ultimately lethal. The *bgs1*Δ spores are able to germinate but unable to form colonies (Cortés *et al.*, 2002; Liu *et al.*, 1999). Germination of *bgs1*Δ spores results in slow growing large,

elongated, branched and multiseptated *bgs1*Δ cell units that eventually will die (Fig. 6A). In the absence of sorbitol, numerous germinated spores initially swell up, but later they develop growing projections, forming elongated and multiseptated cells. Like in *bgs1*$^{+}$ shut-off cells, sorbitol preserves *bgs1*Δ cell shape and viability for at least three days. In the presence of sorbitol, all the *bgs1*Δ cells are elongated and multiseptated (Fig. 6A and data not shown). Surprisingly, septation is not impaired in *bgs1*Δ cells, which are able to complete multiple septa (Fig. 6A, arrows). However, the *bgs1*Δ cells are unable to undergo cell separation, generating a single multiseptated unit per spore.

Growth of *bgs1*Δ cells is limited; so that further studies required the enrichment of *bgs1*Δ cells (see Experimental Procedures). Phase-contrast analysis confirmed that the *bgs1*Δ cells are able to complete multiple septa. Interestingly, no septum can be stained with CW (12.5 μg/ml, Fig. 6B). Higher CW concentrations did not show any septum staining (data not shown). This demonstrates that Bgs1p is responsible for the assembly of the CW-stained septum material, which is essential for cell separation. The *bgs1*Δ cells are able to perform several mitotic cycles. In the absence of sorbitol, many cells form syncytia, whereas others are elongated and with usually one or two nuclei per compartment (Fig. 6B; n=45 cells). In the presence of sorbitol, all the cells are elongated and most of the cell compartments contain one or two nuclei (Fig. 6B; n=95 cells), indicating that mitosis and septum synthesis remain at least partially coordinated during the slow growth of *bgs1*Δ cells. The presence of multiple binucleated compartments suggests that the septation checkpoint may be activated (Le Goff *et al.*, 1999), although in this case the cells are able to continue septum synthesis and cell growth. In addition, the presence of multiple partial septa (Fig. 6B, 7A), although they are ultimately completed (Fig. 7B), suggests that septum synthesis proceeds more slowly than the cell cycle.

Bgs1p is responsible for the linear (1,3)β-D-glucan and primary septum formation.

The *bgs1*Δ cells observed by TEM present extremely thick septum and cell wall structures. In addition, the electron density is significantly reduced (Fig. 7A, B), indicating a dramatic change in molecular binding and/or construction of *bgs1*Δ cell wall and septum. The annular rudiments are absent. The septa initiate over a wide cell wall area and present an altered growth pattern, resulting in

a multilayered structure due to successive secondary septum depositions parallel to the cell wall. Interestingly, in no case an electron-transparent layer resembling the primary septum structure is detected (Fig. 7B; n=80 septa). Instead, a discontinuous electron-dense line appears, resembling fused structures in each layer of the so-called "triangular dense material" (Johnson *et al.*, 1973). Similarly, IEM analysis of *bgs1*Δ cells revealed the total absence of L-BG in both septum and cell wall (Fig. 7C; n=155 septa). These data demonstrate that: a) Bgs1p is the subunit either directly or indirectly responsible for the L-BG and primary septum formation; b) the L-BG is responsible for the primary septum structure; c) the primary septum L-BG is the polysaccharide that specifically interacts with the fluorochrome CW; and d) although remedial septa form in the absence of Bgs1p and of a primary septum, cell separation does not take place under these conditions.

DISCUSSION

Yeast cytokinesis requires the strict coordination of simultaneous actomyosin ring contraction and centripetal septum synthesis (Balasubramanian *et al.*, 2004). *S. pombe* constitutes an attractive morphogenic model and, although appreciable progress has been made in understanding the cytokinesis steps prior to septum synthesis (Krapp *et al.*, 2004; Wolfe and Gould, 2005), little is known yet about how septum synthesis is performed, the synthases that are involved and how they cooperate to build up the different septum structures.

S. pombe cell wall and septum are especially interesting structures because they only contain glucans as structural polysaccharides. (1,6)β-D-branched (1,3)β-D-glucan is present in cell wall and both primary and secondary septum. L-BG had only been reported in primary septum (Humbel *et al.*, 2001), but now we have found also a small amount in the cell wall. To date Bgs4p is the only subunit shown to be involved in (1,3)β-D-glucan synthesis and GS activity (Cortés *et al.*, 2005), although its contribution to the cell wall or septum structure is still unknown.

Previously, Bgs1p was described as essential, but conventional *bgs1*$^+$ shut-off using the thiamine-repressible *nmt1*$^+$-81X promoter showed no apparent phenotype (Cortés *et al.*, 2002). It has been

described that the expression level of the β-galactosidase gene reporter varies considerably depending on the distance between *nmt1*$^+$ promoter and initiation codon (Moreno *et al.*, 2000). Using the same approach, we have been able to repress *bgs1*$^+$ to maximum lethal level by spacing out *bgs1*$^+$ ORF and *nmt1*$^+$-81X promoter. Under repressed conditions, the residual Bgs1p is still sufficient to produce partial primary septum and L-BG structures. Therefore, *bgs1*$^+$ shut-off can only prove that Bgs1p is required for correct L-BG and primary septum formation, but not whether it is completely responsible for one or both processes. It has been necessary to analyze *bgs1*Δ cells to observe total absence of both L-BG and primary septum and to demonstrate that Bgs1p is responsible and essential for the formation of both structures. Although our data demonstrate that the L-BG is required for the primary septum structure, the finding that in *bgs1*$^+$ shut-off cells many septa lack the primary septum but still contain L-BG (46% without primary septum vs. only 15% without L-BG after 60 hours of *bgs1*$^+$ repression) suggests that production of L-BG is necessary but not sufficient for primary septum formation. Defects in L-BG association with other polysaccharides or in L-BG localization, as in the observed septa with diffuse L-BG depositions, might be critical for primary septum assembly as well.

CW presents high affinity for fibrillar polysaccharides, like chitin or cellulose. *S. pombe* has no chitin (Arellano *et al.*, 2000), but CW binds with high affinity to the septum. We show for the first time a septum that is not stained with CW and demonstrate that L-BG is the polysaccharide that specifically interacts with CW in *S. pombe*. The same results were obtained with aniline blue (data not shown), a dye that binds specifically to (1,3)β-D-glucans and that has been shown to bind to the single-helix or partially opened triple-helix but not to the closed triple-helix conformation (Young and Jacobs, 1998; Young *et al.*, 2000). It has been described that the L-BG exhibits a gelation conformation of single-helix with a small proportion of triple-helix whereas the (1,6)β-D-branched (1,3)β-D-glucan presents the triple-helix form (Saito *et al.*, 1990), and that the (1,3)β-D-glucan with a less organized conformation is more susceptible to (1,3)β-D-glucanases (Pelosi *et al.*, 2003). According to this, the primary septum L-BG would present a loose conformation that would make it more susceptible to endo-(1,3)β-D-glucanases like Eng1p (Martín-Cuadrado *et al.*, 2003), helping to preserve the integrity of the rest of the cell wall during septum degradation.

S. pombe cells appear to need an extremely low Bgs1p amount and maintain wild type phenotype after

a more than 300-fold Bgs1p reduction, as observed after 15 hours of *bgs1*$^+$ shut-off. At later times, the shut-off phenotype with abnormal septa appears, but the cells still show a considerable amount of disorganized primary septa and L-BG. How is so little Bgs1p able to produce such a large amount of L-BG, albeit in a disorganized state? One possibility is that Bgs1p is a very efficient synthase. Alternatively, it is possible that Bgs1p is not the synthase, but a subunit of the catalytic complex essential to activate and direct L-BG synthesis. Very few Bgs1p molecules might be enough to activate and direct the synthase. Nonetheless, Bgs1p is essential for the L-BG (and primary septum) synthesis because the *bgs1*Δ cells do not contain any trace of L-BG (and primary septum). In addition, Bgs1p is responsible for correct L-BG localization because low Bgs1p generates disorganized L-BG (and primary septum) structures.

Bgs1p is predicted to contain a large number (15-16) of transmembrane domains and it is possible that it could be forming a pore in the plasma membrane with other Bgs1p subunits, or even with other Bgs3p or Bgs4p subunits. In this case, Bgs1p could activate and direct other Bgs subunits to synthesize and localize correctly the L-BG, and it could cooperate in that synthesis with a minor activity. Alternatively, the L-BG could be made by another unknown synthase that would form part of the catalytic complex with the Bgs subunits, which would extrude the nascent glucan chain trough the pore outside the cell. This is consistent with the fact that the Bgs, fungal Fks and plant CalS homologues are considered putative GS catalytic subunits, but their catalytic activity has never been proven and none contain the UDP-glucose binding consensus motif (R/K)XGG found in glycogen synthases, or the (Q/R)XXRW motif, considered the putative active site of β-glycosyltranferases like cellulose and chitin synthases. In addition, it has been suggested that the plant Bgs1p homologue callose synthase CalS1p forms a complex with UDP-glucose transferase Ugt1p, Rho1-like Rop1p and Phragmoplastin (Hong *et al.*, 2001a; Hong *et al.*, 2001b). The idea of a catalytic multiprotein complex is also consistent with the fact that *bgs1*$^+$ overexpression does not generate any increase but a considerable decrease in GS activity (14% residual activity; our unpublished results), as reported with *bgs4*$^+$ overexpression (Cortés *et al.*, 2005).

Our IEM studies show that Bgs1p is responsible for the L-BG present in primary septum and, as a minor component, in the cell wall. This is in agreement with Bgs1p localization to not only septum but

also poles (Cortés *et al.*, 2002) and with the fact that Bgs1p may be also involved in growth polarity and cell wall morphogenesis. Previously, the L-BG was exclusively localized in the primary septum (Humbel *et al.*, 2001). In that work, the cells were fixed by a high-pressure freezing method, whereas in our case we used a chemical method due to the cell fragility. It is possible that the former method only detects highly concentrated L-BG, skipping the small cell wall amount. We believe that the cell wall signal is real because decreasing antibody amounts result in proportional signal decrease in both primary septum and cell wall (data not shown) and because it is not detected in *bgs1*Δ cells.

Even in the absence of Bgs1p and primary septum, septa are made, but they have a different growth pattern. A normal septum grows by primary septum synthesis perpendicular to the cell wall and simultaneous secondary septum deposition parallel to the growing primary septum. The *bgs1*Δ septum lacks the primary septum backbone. As a result, the septum grows by successive depositions of secondary septum layers parallel to the cell wall, in a mechanism similar to that of *S. cerevisiae chs2*Δ cells, which lack the chitin primary septum made by Chs2p (Schmidt *et al.*, 2002).

Chitin-containing yeasts form a chitin primary septum. Then, how is *S. pombe* able to form a similar primary septum structure? Among all the different fission yeast glucans, one of them has the unusual feature of being linear. L-BG is absent in chitin-containing yeasts and like chitin, it can form fibrillar structures, it is almost exclusively present in the primary septum and we have found that it is essential for the primary septum structure. Although the primary septum contains other polysaccharides, the sole absence of L-BG is enough to prevent primary septum formation. This finding indicates that fission yeast has adopted a septation mechanism similar to that of budding yeast, by synthesizing an L-BG that mimics the chitin properties to form the primary septum. Interestingly, *S. pombe* presents also similarities with plant cytokinesis. Plant septum synthesis is different to that of fungal and animal cells because the septum is not made centripetally but by synthesis of a medial cell plate that will constitute the primary septum (Verma, 2001). However, like in *S. pombe*, the plant cell plate is made of callose (L-BG) and the protein involved is the Bgs homologue CalS1p (Verma and Hong, 2001). In this sense, fission yeast presents a mechanism of septum formation intermediate between chitin-containing yeasts and plants, similar to that of budding yeast in the strategy it has adopted and similar to that of plant cells in the subunit involved and in the primary septum structure.

S. pombe does not contain chitin but has evolved by making different crucial α- and β-glucans with four essential proteins. In contrast, *S. cerevisiae* presents a very distinct model of three Fks homologues (Inoue *et al.*, 1995; Mazur *et al.*, 1995) and three chitin synthases (Chs) (Cabib *et al.*, 2001; Roncero, 2002), none of them essential, to make the (1,3)β-D-glucan and chitin, respectively. Despite these differences, a distinct single subunit, Bgs1p (fission yeast) or Chs2p (budding yeast), has specialized in each species to make the primary septum. What are the differences that make Bgs1p essential and Chs2p dispensable? a) Chs2p is only involved in primary septum synthesis whereas Bgs1p seems to be also implicated in polarized growth (Cortés *et al.*, 2002). In fact, the L-BG is also detected in the cell wall and the *bgs1*$^+$ shut-off cells are altered in cell wall structure and polarized growth. b) The *bgs1*Δ septa do not contain chitin, which has been shown to be essential for *chs2*Δ cells viability (Cabib and Schmidt, 2003). c) Septation in *S. cerevisiae* only affects the narrow mother-bud neck region, whereas in *S. pombe* it concerns the entire cell diameter, probably making the cell more susceptible to integrity defects.

In spite of that, Bgs1p and Chs2p are functionally similar, presenting an evolutionarily convergent mechanism in yeasts by which a different and single subunit has specialized and is specifically responsible for the L-BG (fission yeast) or chitin (budding yeast) that forms in each case a primary septum structure distinct from the rest of septum and cell wall. By this means, the cell ensures that septum degradation during cell separation will only proceed through the primary septum and thus, the cell integrity will be preserved.

EXPERIMENTAL PROCEDURES

Strains and culture conditions.

The *S. pombe* strains used are listed in Table 3. *bgs1*Δ pJG25 strain 437 (*his3*$^+$ selection) (Cortés *et al.*, 2002) contains *bgs1*$^+$ expressed under the control of the 81X version (very low expression) of the thiamine-repressible *nmt1*$^+$ promoter (Moreno *et al.*, 2000). Other strains with different levels of *bgs1*$^+$ repression were made by genetic cross between strain 285 (Leu$^-$, Ura$^-$, His$^-$) transformed with the

corresponding version of p81X-*bgs1*$^+$ plasmid (*S. cerevisiae LEU2* selection) and strain 437 (*bgs1*Δ pJG25), and selection of a Leu$^+$ Ura$^+$ His$^-$ clone.

Diploid strain 857 (*bgs1*$^+$*::bgs1*$^+$*:leu1*$^+$/*bgs1*Δ*::ura4*$^+$) was made by transforming the diploid strain 387 (*bgs1*$^+$/*bgs1*Δ*::ura4*$^+$) with *Nco*I-cut pJK-*bgs1*$^+$, which directs its integration at position +4807 of *bgs1*$^+$ ORF. This strain was used for enrichment of *bgs1*Δ cells (see below).

GFP-bgs3$^+$ *bgs3*Δ strain 1217 contains *GFP-bgs3*$^+$ integrated (*Pac*I-cut pJG112) adjacent to *bgs3*Δ*::ura4*$^+$, at position -1857 of *bgs3*$^+$ promoter sequence. The *GFP-bgs3*$^+$ *bgs3*Δ strain displayed wild-type phenotype under all tested conditions and expressed *GFP-bgs3*$^+$ at physiological level, from a single integrated *GFP-bgs3*$^+$ gene under the control of its own promoter.

Standard complete yeast growth (YES), selective (EMM) and sporulation (SPA) media (Alfa *et al.*, 1993; Egel, 1984) have been described. Cell growth was monitored as described (Cortés *et al.*, 2005). General procedures for yeast and bacterial culture and genetic manipulations were carried out as described (Moreno *et al.*, 1991; Sambrook and Russell, 2001).

Plasmids and DNA techniques.

pJK-*bgs1*$^+$ contains the 8.8 kb *Apa*I-*Spe*I *bgs1*$^+$ fragment cloned into the integrative plasmid pJK148 (*leu1*$^+$ selection). pJG23 is pJR1-81XL (*S. cerevisiae LEU2* selection and *nmt1*$^+$-81X promoter) (Moreno *et al.*, 2000) with the *bgs1*$^+$ ORF cloned into *Nru*I-*Apa*I sites. The resulting *bgs1*Δ pJG23 strain displayed wild type phenotype even in the presence of thiamine (Table 1), which represses the *nmt1*$^+$-81X promoter to a very low expression level (Moreno *et al.*, 2000). Other p81X-*bgs1*$^+$ plasmids with different *bgs1*$^+$ repression levels (Table 1) are as follows: pJR100 is pJR1-81XL with the *bgs1*$^+$ ORF cloned into the most distant sites from the *nmt1*$^+$-81X promoter, into *Bam*HI-*Sma*I of the 12 cloning sites. pJR101 to pJR107 are pJR100 with increasing deletions in the multiple cloning site (Table 1). pJR102 was the p81X-*bgs1*$^+$ plasmid selected for *bgs1*$^+$ shut-off studies in a *bgs1*Δ background (see Results).

pJK-*bgs3*$^+$ is pJK-148 with the 9.7 kb *Spe*I-*Spe*I *bgs3*$^+$ fragment. In order to obtain a functional GFP-Bgs3p fusion, the *GFP* fragment was inserted in-frame after the *bgs3*$^+$ start codon or in the three most

hydrophilic N-terminal regions, at the boundaries between predicted secondary structures, at bases 3, 198, 954 and 1038 (aminoacids 2, 66, 318 and 346), making pJG115, pJG112, pJG117 and pJG116, respectively. Only integrated pJG112 (pJK-*GFP-bgs3*$^+$) expressed a fully functional fusion protein in a *bgs3*Δ strain and was therefore selected for Bgs3p studies at physiological level.

bgs3$^+$ gene deletion was performed in a diploid strain removing the entire *bgs3*$^+$ coding sequence, as described for *bgs1*$^+$ and *bgs4*$^+$ gene deletions (Cortés *et al.*, 2002; Cortés *et al.*, 2005). Tetrad analysis of sporulated *bgs3*$^+$/*bgs3*Δ diploids showed Bgs3p to be essential, as reported (Martín *et al.*, 2003).

Enrichment of *bgs1*Δ cells from sporulated *bgs1*Δ/*bgs1*$^+$ diploids.

Germination of *bgs1*Δ spores in liquid medium extends for several days, whereas in solid medium most of the spores germinate in one day. The *bgs1*Δ cells are able to grow and divide, but are unable to separate, producing a single multiseptated unit per spore that after several days will die. On the other hand, germination of spores from *bgs1*Δ/*bgs1*$^+$ diploids results in a fast enrichment of wild-type cells due to the slow and limited growth of *bgs1*Δ cells. Therefore, a protocol for the enrichment of *bgs1*Δ cells from a mixture of germinated *bgs1*$^+$ and *bgs1*Δ spores was developed.

Diploid strain 857 *bgs1*$^+$*:leu1*$^+$/*bgs1*Δ contains pJK-*bgs1*$^+$ (*leu1*$^+$ selection) inserted into the *bgs1*$^+$ ORF, resulting in the *leu1*$^+$ gene integrated between two *bgs1*$^+$ copies. As a result, the *bgs1*$^+$ spores will be Leu$^+$ and the *bgs1*Δ will be Leu$^-$. Diploid cells were grown in EMM liquid medium at 28ºC for one week to induce complete sporulation during stationary phase, resulting in a mixture of *bgs1*$^+$*:leu1*$^+$ spores (Leu$^+$), *bgs1*Δ spores (Leu$^-$) and residual non-sporulated diploids (Leu$^+$). The spores/cells mixture was grown for 2 weeks at 28ºC in EMM containing aminoacids except leucine and 0.5% 2-deoxyglucose instead of glucose. This medium will only allow growth of diploid cells (Leu$^+$) and *bgs1*$^+$*:leu1*$^+$ spores (Leu$^+$), which will selectively lyse by incorporating the toxic analogous of glucose 2-deoxyglucose (Johnson, 1968). The *bgs1*Δ (Leu$^-$) spores will survive because their inability to germinate (and incorporate 2-deoxyglucose) in a medium devoid of leucine. This treatment did not produce any alteration in *bgs1*Δ spores viability or morphology during germination (data not shown). Other treatments (heat shock, addition of sodium azide, cycloheximide or nystatin, or

*bgs1*Δ*::KanMX6* spores selection with geneticin) were less lethal for Leu$^+$ cells or affected *bgs1*Δ spores viability and morphology during germination (data not shown). The surviving mixture of *bgs1*Δ and non-germinated *bgs1*$^+$ spores was washed overnight with EMM, spread on YES or YES + 1.2 M sorbitol plates and grown at 28ºC for 2-3 days. Cells were collected in YES + 1.2 M sorbitol liquid medium and the *bgs1*Δ cells were partially purified by their higher weight, by successive differential centrifugations in the same medium (20 sec at 1,500 g). Under these conditions, the *bgs1*Δ cells sediment while most of *bgs1*$^+$ cells remain in the supernatant.

(1,3)β-D-glucan synthase assay and labeling and fractionation of cell wall polysaccharides.

Cell extracts and GS assay, and ^{14}C-glucose labeling and fractionation of cell wall polysaccharides (3 μCi/ml control cells and 6 μCi/ml *bgs1*$^+$ shut-off cells) were carried out as described (Cortés *et al.*, 2005).

Immunoblot analysis.

Early log-phase cells were washed with 1 mM EDTA, suspended in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.1% Triton X-100 containing 1 mM phenylmethylsulfonylfluoride and 2 μg/ml aprotinin, leupeptin and pepstatin) and broken with glass beads (FastPrep FP120, 3x20-sec, speed of 6.0; Savant; BIO 101). Samples were diluted with lysis buffer and cell debris was removed by centrifugation (4,500 g, 30 sec, 4ºC). The supernatant was collected and the cell walls were eliminated by centrifugation (2,300 g, 5 min, 4ºC). Samples were diluted with 2x loading buffer (2x is 100 mM Tris-HCl pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 20% glycerol and 0.05% bromophenol blue) and boiled for 5 min. Proteins were subjected to 6.5% SDS-PAGE, blotted onto Immobilon-P (Millipore) and probed with monoclonal JL-8 anti-GFP (1:2,500 dilution; BD Biosciences) or monoclonal B-5-1-2 anti-α-tubulin (1:10,000 dilution; Sigma) antibodies. Immunodetection was with anti-mouse horseradish-conjugated antibody (1:7,500 dilution) and the ECL detection kit (Amersham). Protein amounts with the appropriated dilution were compared with the Quantity One software (V4.5.2, Bio-Rad).

Microscopy techniques.

Images of germinated spores were obtained from the plates as described (Cortés *et al.*, 2005). For cell wall staining, cells were visualized by adding a solution of CW (standard final concentration of 50 μg/ml or lower concentrations of 12.5 or 1.25 μg/ml) as described (Cortés *et al.*, 2002). Simultaneous nucleus and cell wall staining and direct GFP fluorescence visualization were as described (Cortés *et al.*, 2005)

Immunofluorescence for *in situ* labeling of L-BG was essentially as described (Hagan and Hyams, 1988). Primary anti-L-BG antibody was monoclonal 400-2 (1:100 dilution; Biosupplies), and secondary antibody was anti-mouse Alexa Fluor 594 (1:400 dilution; Molecular Probes). Cells were fixed (100% ethanol, 30 min, -20ºC) and incubated with 0.15-5.0 μg/ml Novozyme 234 (Novo Industries) for 45 min at 30ºC in PEMS buffer (100 mM Pipes pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$, 1.2 M sorbitol). For optimal L-BG labeling, the Novozyme 234 concentration was selected that preserved most the cell shape and allowed labeling of all (wild-type cells, 1 μg/ml) or the highest number of septa (*bgs1*$^+$ shut-off cells, 0.7 μg/ml).

Transmission electron microscopy.

Cells were fixed with 2% glutaraldehyde EM grade (GA; Electron Microscopy Science) in 50 mM phosphate buffer pH 7.2, 150 mM NaCl (PBS) for 2 h at 4ºC, postfixed with 1.2% potassium permanganate overnight at 4ºC and embedded in Quetol 653 as described (Konomi *et al.*, 2003). Ultrathin sections were stained in 4% uranyl acetate and 0.4% lead citrate, and viewed with a TEM H-800 (Hitachi) operating at 125 kV.

Immunoelectron microscopy.

The L-BG was detected *in situ* by immunoelectron microscopy (IEM) as described (Humbel *et al.*, 2001). Cells were fixed with 0.5% GA and 3% paraformaldehyde EM grade (TAAB) in PBS for 2 h at 4ºC, washed with PBS, treated with 1% sodium metaperiodate for 15 min and 50 mM ammonium

chloride for 30 min, and embedded in LR white resin (medium grade; London Resin) followed by polymerization for 24 h at 50ºC. Immunostaining with anti-L-BG specific monoclonal antibody (1:2,000 dilution; Biosupplies) (Humbel *et al.*, 2001; Meikle *et al.*, 1991) was performed on ultrathin sections, picked up on nickel grids after blocking with normal goat IgG (chromatographically purified; Zymed Laboratories) diluted 1:30 in 50 mM Tris-buffered saline (TBS) containing 0.1% BSA for 15 min as described (Kamasawa *et al.*, 1992). Ultrasmall colloidal gold-conjugated goat anti-mouse IgG (1:100 dilution; Aurion) was used as secondary antibody. After immunostaining, sections were fixed with 1% GA in 100 mM PBS, and the colloidal gold particles were enhanced by silver deposition, using high-efficiency silver enhancement reagents (R-GENT SE-EM; Aurion). The grids were stained in 4% uranyl acetate and viewed with a TEM H-800 (Hitachi) at 125 kV.

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FIGURE LEGENDS

Fig. 1. Bgs1p is essential for vegetative cells and required for correct septation and cell separation. **(A)** *bgs1*$^+$ shut-off promotes a considerable cell growth reduction and sorbitol partially rescues cell growth, although in both cases it is ultimately lethal (see below). *bgs1*Δ p81X-*bgs1*$^+$ (pJR102, *nmt1*$^+$-81X promoter) cells were grown on EMM±T (-thiamine, -T, *bgs1*$^+$ induced; +thiamine, +T, *bgs1*$^+$ repressed) either in the absence or in the presence of 1.2 M sorbitol at 28°C. Cell growth was monitored after 12 hours of growth with thiamine, in an interval where cell growth arrest or reduction is observed. **(B)** *bgs1*$^+$ shut-off produces multiseptated and branched cells that start to swell up and lose viability after 48 hours of *bgs1*$^+$ repression. Phase-contrast and Calcofluor white (CW, 50 μg/ml)-stained micrographs of *bgs1*Δ p81X-*bgs1*$^+$ (pJR102) cells grown on EMM+T at 28°C for the indicated times. **(C)** *bgs1*$^+$ shut-off in the presence of 1.2 M sorbitol (S) produces multiseptated, branched and uniformly shaped cells that are viable for at least three days. Cells were grown on EMM+T+S and visualized as in B. **(D)** *bgs1*$^+$ shut-off cells present normal mitosis, apparently coordinated with septum synthesis. Cells were grown for 36 hours as in C, ethanol-fixed and analyzed for CW (25 μg/ml)/Hoechst (10 μg/ml; nucleus) staining. Bars, 10 μm. **(E)** *bgs1*$^+$ shut-off induces only a slight decrease in (1,3)β-D-glucan synthase (GS) activity. Cells grown as in C were collected at the indicated times and assayed for GS activity. Values in parentheses are specific activity average, calculated from three independent extracts. Control wild-type cells grown in EMM+S±T showed similar GS activity to that of control *bgs1*Δ p81X-*bgs1*$^+$ (pJR102) cells grown in the absence of thiamine.

Fig. 2. *bgs1*$^+$ shut-off results in undetectable GFP-Bgs1p before the appearance of phenotype. **(A)** Bgs1p is only detected in some cells as a faint signal in the medial ring after 15 hours, and is absent after 24 hours of *bgs1*$^+$ shut-off. *bgs1*Δ P*nmt1*$^+$-81X-*GFP-bgs1*$^+$ (integrated p81X-*GFP-bgs1*$^+$) cells were grown as in Fig. 1C and visualized for GFP fluorescence and CW staining at the indicated times. The exposure time was increased to detect the low level of GFP fluorescence. Bar, 2 μm. **(B)** The physiological level of each Bgs protein is very different. Proteins from total cell extracts (100 μg,

left panel; 25 μg, right panel) were separated in 6.5% SDS-PAGE, blotted and probed with monoclonal anti-GFP or anti-α-tubulin antibody. A control without GFP tag (-) is shown in the fourth lane. Numbers are relative amount of each Bgs protein compared to tubulin, giving a value of 1.0 to Bgs1p. **(C)** Bgs1p amount is 5-fold reduced in *bgs1*Δ P*nmt1*$^+$-81X-*GFP-bgs1*$^+$ strain and decreases drastically during *GFP-bgs1*$^+$ shut-off. Cells expressing *GFP-bgs1*$^+$ from its own promoter (control, C) or from the *nmt1*$^+$-81X promoter were grown as in Fig. 1C and collected at the indicated times. GFP-Bgs1p (100 μg total protein) was analyzed as in B. The film was overexposed to increase Bgs1p detection during *bgs1*$^+$ shut-off.

Fig. 3. *bgs1*$^+$ shut-off generates apparently normal septa, but not detected with specific septum stainings.

(A) Many *bgs1*$^+$ shut-off septa show defective staining with limiting CW concentration. Cells were examined as in Fig. 1C using a lower CW concentration (1.25 μg/ml) that stains all the septa in control cells (arrows, left panels) but not in *bgs1*$^+$ shut-off cells (arrows, right panels). **(B)** Numerous *bgs1*$^+$ shut-off septa are not detected with L-BG antibody. All the septa of control cells are stained (arrows, left panels), whereas many *bgs1*$^+$ shut-off septa are not (arrows, right panels). Bar, 10 μm.

Fig. 4. Bgs1p is at least required for correct primary septum assembly.

*bgs1*Δ p81X-*bgs1*$^+$ cells were grown as in Fig. 1C and processed for TEM analysis. **(A)** Normal morphology of *bgs1*Δ p81X-*bgs1*$^+$ strain expressing *bgs1*$^+$. **(B)** Altered morphology after 48 hours of *bgs1*$^+$ repression. **(C)** Detail of septum formation steps in control cells. **(D)** Septum formation steps after 15 hours of *bgs1*$^+$ shut-off. The symmetry in septum growth is lost, the primary septum is discontinuous and the secondary septum is remarkably thicker. **(E-K)** Increasing septum and cell wall defects after 24 (E), 36 (F, G), 48 (H, I) and 60 (J, K) hours of *bgs1*$^+$ shut-off. Cell wall and secondary septum thickness increase and the primary septum presents multiple defects: absent, reduced, discontinuous, twisted, delocalized, etc. Septa representative of the different defects are shown. Arrow, primary septum; arrowhead, secondary septum; N, nucleus; S, septum.

Fig. 5. Bgs1p is at least necessary for correct linear (1,3)β-D-glucan formation.

Cells as in Fig. 4 were processed for IEM analysis of L-BG. **(A)** L-BG distribution during septum formation in control cells. **(B-I)** L-BG defects after 15 (B), 24 (C), 36 (D, E), 48 (F) and 60 (G-I) hours of *bgs1*$^{+}$ shut-off. L-BG is absent, reduced or with altered distribution. Septa representative of the different defects are shown.

Fig. 6. Bgs1p is dispensable for septum synthesis but is responsible for the CW-stained septum formation.

(A) Germinated *bgs1*Δ spores generate multiseptated *bgs1*Δ cells that cannot perform cell separation and eventually will die. Micrographs of *bgs1*Δ cells obtained from *bgs1*Δ spores incubated on YES±S plates at 28ºC for 2-3 days. Arrows indicate the multiple septa of *bgs1*Δ cells. **(B)** The *bgs1*Δ septa are not stained with CW (left). Mitosis is not impaired in *bgs1*Δ cells (right). *bgs1*Δ cells grown as in A were enriched (see Experimental Procedures) and analyzed for CW (12.5 μg/ml) or Hoechst (nucleus) staining. Bars, 10 μm.

Fig. 7. Bgs1p is responsible for the linear (1,3)β-D-glucan and primary septum formation.

*bgs1*Δ cells grown on YES+S plates were purified as in Fig. 6B and processed for TEM (A, B) or IEM (C) analysis. **(A)** Altered morphology, septa and cell wall of *bgs1*Δ strain. **(B)** Detail of septum formation steps in *bgs1*Δ cells generating thick multilayered septum and cell wall structures, in which the electron density is severely reduced. No septum presents a primary septum structure. **(C)** L-BG labeling of *bgs1*Δ cells. No septum or cell wall shows L-BG labeling. Control cells processed in parallel with *bgs1*Δ cells showed the corresponding primary septum and L-BG labeling.

Table 1. Increasing morphological and lethal *bgs1*$^+$ shut-off phenotypes of *S. pombe bgs1*Δ p81X-*bgs1*$^+$ strains containing *bgs1*$^+$ ORF cloned at different distances from the *nmt1*$^+$-81X promoter.

Plasmids[a]	Cloning sites[b]	Spacer basepairs[c]	Shut-off phenotype
pJG23	**P*nmt1*$^+$- 81X -*Xho*I-*Sal*I-(*Nru*I/*Hpa*I)- *bgs1*$^+$-ORF**	**22**	±
pJR107	**P*nmt1*$^+$- 81X -(*Xho*I/*Not*I)-*Bam*HI- *bgs1*$^+$-ORF**	**22**	±
pJR106	**P*nmt1*$^+$- 81X -*Xho*I-(*Sal*I/*Not*I)-*Bam*HI- *bgs1*$^+$-ORF**	**28**	±
pJR105	**P*nmt1*$^+$- 81X -*Xho*I-*Sal*I-(*Nru*I/*Not*I)-*Bam*HI- *bgs1*$^+$-ORF**	**32**	+ +
pJR104	**P*nmt1*$^+$- 81X -*Xho*I-*Sal*I-*Nru*I-(AscI/*Bam*HI)- *bgs1*$^+$-ORF**	**33**	+ +
pJR103	**P*nmt1*$^+$- 81X -*Xho*I-*Sal*I-*Nru*I-(*Asc*I/*Not*I)-*Bam*HI- *bgs1*$^+$-ORF**	**41**	+ + +
pJR102	**P*nmt1*$^+$- 81X -*Xho*I-*Sal*I-*Nru*I-*Asc*I-*Nsi*I-(*Apa*I/*Not*I)-*Bam*HI- *bgs1*$^+$-ORF**	**52**	+ + + +
pJR101	**P*nmt1*$^+$- 81X -*Xho*I-*Sal*I-*Nru*I-*Asc*I-*Nsi*I-*Apa*I-*Sac*II-*Nco*I-(*Spe*I/*Not*I)-*Bam*HI- *bgs1*$^+$-ORF**	**74**	+ + + +
pJR100	**P*nmt1*$^+$- 81X -*Xho*I-*Sal*I-*Nru*I-*Asc*I-*Nsi*I-*Apa*I-*Sac*II-*Nco*I-*Spe*I-*Not*I-*Bam*HI- *bgs1*$^+$-ORF**	**77**	+ + + +

- : No *bgs1*$^+$ shut-off phenotype; + + + + : Maximum *bgs1*$^+$ shut-off phenotype (see Results).

[a] Initial plasmid pJG23 showed no *bgs1*$^+$ shut-off phenotype.

[b] *bgs1*$^+$ ORF was cloned into the most distant sites of pJR1-81XL, *Bam*HI-*Sma*I, making pJR100, and a series of deletions were made (shown in parenthesis) to gradually approach *bgs1*$^+$ ORF to *nmt1*$^+$-81X promoter. *bgs1*$^+$ shut-off phenotype increased up to pJR102, with no difference in further constructs. pJR102 was selected as the p81X-*bgs1*$^+$ plasmid used for *bgs1*$^+$ shut-off studied.

[c] Number of bases between *nmt1*$^+$-81X promoter and *bgs1*$^+$ ORF.

Table 2. Increasing primary septum and linear (1,3)β-D-glucan defects during *bgs1*$^+$ shut-off of *S. pombe bgs1*Δ p81X-*bgs1*$^+$ strain.

Time of *bgs1*$^+$ shut-off (hours)			15h	24h	36h	48h	60h
Primary septum[a]	No. of septa		28	26	175	66	80
	% Defective[b]	Absent from base	43	50	38	44	43
		Absent from inner edge[c]	18	15	24	29	26
		Discontinuous	29	35	31	29	35
		Twisted or delocalized	36	38	44	36	43
	% Absent		0	8	36	44	46
Linear (1,3)β-D-glucan[a]	No. of septa		81	146	159	327	138
	% Defective[b]	Absent from base	22	19	30	31	38
		Absent from inner edge[c]	20	26	29	31	36
		Discontinuous	7	23	29	32	38
		Twisted or delocalized	12	27	31	32	40
		Diffuse[d]	12	31	37	47	49
	% Absent		0	2	8	12	15

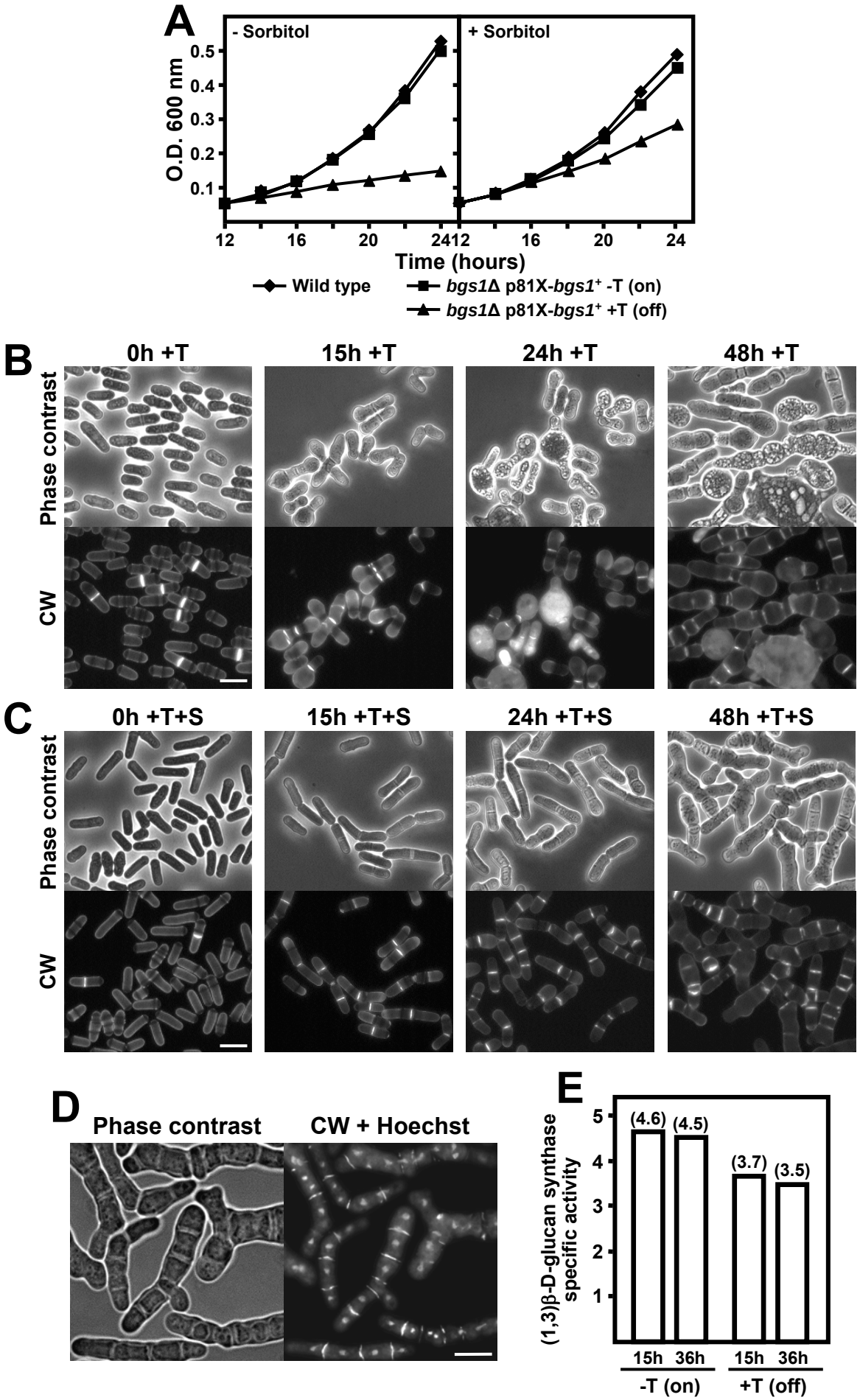
[a] Septa from cells as in Fig. 4 and 5 were analyzed for defects or absence of primary septum or linear (1,3)β-D-glucan.
[b] The septa can present different defects simultaneously.
[c] The absence from the growing edge can only be analyzed in incomplete septa.
[d] The signal appears dispersed in part of or all the septum.

Table 3. Fission yeast strains.

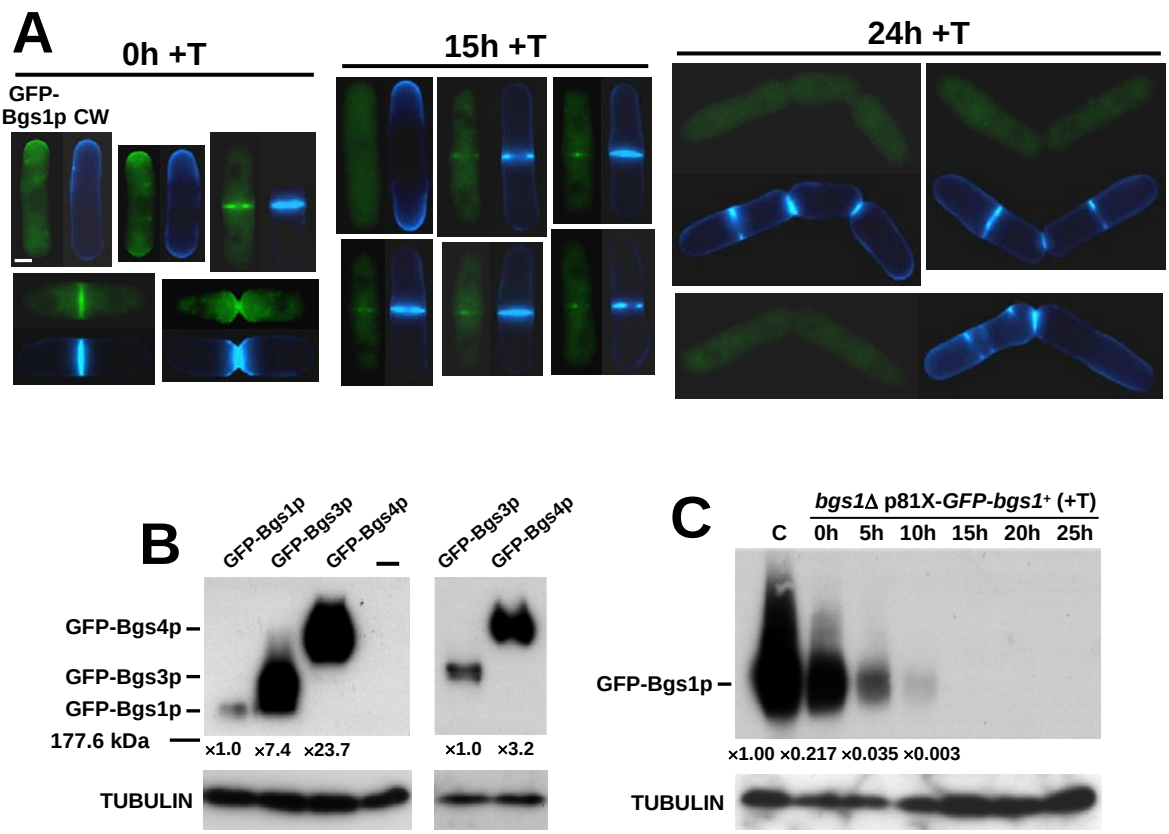
Strain	Genotype	Source
33	972 h^-	P. Munz[a]
284	*leu1-32 ura4-Δ18 his3-Δ1* h^-	J. C. Ribas
285	*leu1-32 ura4-Δ18 his3-Δ1* h^+	J. C. Ribas
419	*leu1-32 ura4-Δ18* h^-	This study
420	*leu1-32 ura4-Δ18* h^+	This study
635	*mid1-366 leu1-32* h^+	P. Nurse[b]
439	*cdc3-6 leu1-32* h^+	J. C. Ribas
574	*cdc15-140 leu1-32* h^+	J. C. Ribas
581	*cdc16-116 ura4-Δ18* h^-	J. C. Ribas
901	*cdc11-119 leu1-32 ura4-Δ18 his3-Δ1* h^+	This study
572	*cdc14-118 leu1-32* h^+	J. C. Ribas
811	*leu1-32 ura4-Δ18 his3-Δ1 ade6-M216 bgs3Δ::ura4*$^+$ pIM51 (p81XH-*bgs3*$^+$) h^-	This study
1217	*leu1-32 ura4-Δ18 his3-Δ1 bgs3Δ::ura4*$^+$ P*bgs3*$^+$*::GFP-bgs3*$^+$*:leu1*$^+$ h^+	This study
562	*leu1-32 ura4-Δ18 his3-Δ1 bgs4Δ::ura4*$^+$ P*bgs4*$^+$*::GFP-bgs4*$^+$*:leu1*$^+$ h^+	J. C: Ribas
317	*leu1-32*/*leu1-32 ura4-Δ18*/*ura4-Δ18 his3-Δ1*/*his3-Δ1 ade6-M210*/*ade6-M216* h^-/h^+	J. C. Ribas
387	*leu1-32*/*leu1-32 ura4-Δ18*/*ura4-Δ18 his3-Δ1*/*his3-Δ1 ade6-M210*/*ade6-M216* *bgs1*$^+$/*bgs1Δ::ura4*$^+$ h^-/h^+	J. C. Ribas
857	*leu1-32*/*leu1-32 ura4-Δ18*/*ura4-Δ18 his3-Δ1*/*his3-Δ1 ade6-M210*/*ade6-M216* *bgs1*$^+$*::bgs1*$^+$*:leu1*$^+$/*bgs1Δ::ura4*$^+$ h^-/h^+	This study
1180	*leu1-32*/*leu1-32 ura4-Δ18*/*ura4-Δ18 his3-Δ1*/*his3-Δ1 ade6-M210*/*ade6-M216* *bgs3*$^+$/*bgs3Δ::ura4*$^+$ h^-/h^+	This study
437	*leu1-32 ura4-Δ18 his3-Δ1 bgs1Δ::ura4*$^+$ h^- pJG25 (p81XH-*bgs1*$^+$)	J. C. Ribas
410	*leu1-32 ura4-Δ18 his3-Δ1 ade6-M216 bgs1Δ::ura4*$^+$ h^+ pJG23 (p81X-*bgs1*$^+$)	This study
526	*leu1-32 ura4-Δ18 his3-Δ1 bgs1Δ::ura4*$^+$ h^- pJR102 (p81X-*bgs1*$^+$)	This study
727	*leu1-32 ura4-Δ18 his3-Δ1 bgs1Δ::ura4*$^+$ P*nmt1*$^+$-81X-*GFP-bgs1*$^+$*:his3*$^+$ h^-	This study
969	*mid1-366 leu1-32 ura4-Δ18 bgs1Δ::ura4*$^+$ h^- pJR102 (p81X-*bgs1*$^+$)	This study
964	*cdc3-6 leu1-32 ura4-Δ18 bgs1Δ::ura4*$^+$ h^- pJR102 (p81X-*bgs1*$^+$)	This study
967	*cdc15-140 leu1-32 ura4-Δ18 bgs1Δ::ura4*$^+$ h^- pJR102 (p81X-*bgs1*$^+$)	This study
905	*cdc11-119 leu1-32 ura4-Δ18 his3-Δ1 bgs1Δ::ura4*$^+$ h^- pJR102 (p81X-*bgs1*$^+$)	This study
966	*cdc14-118 leu1-32 ura4-Δ18 bgs1Δ::ura4*$^+$ h^- pJR102 (p81X-*bgs1*$^+$)	This study

[a] Institute of General Microbiology, University of Bern, Switzerland.
[b] Laboratory of Yeast Genetics and Cell Biology, The Rockefeller University, New York, USA.

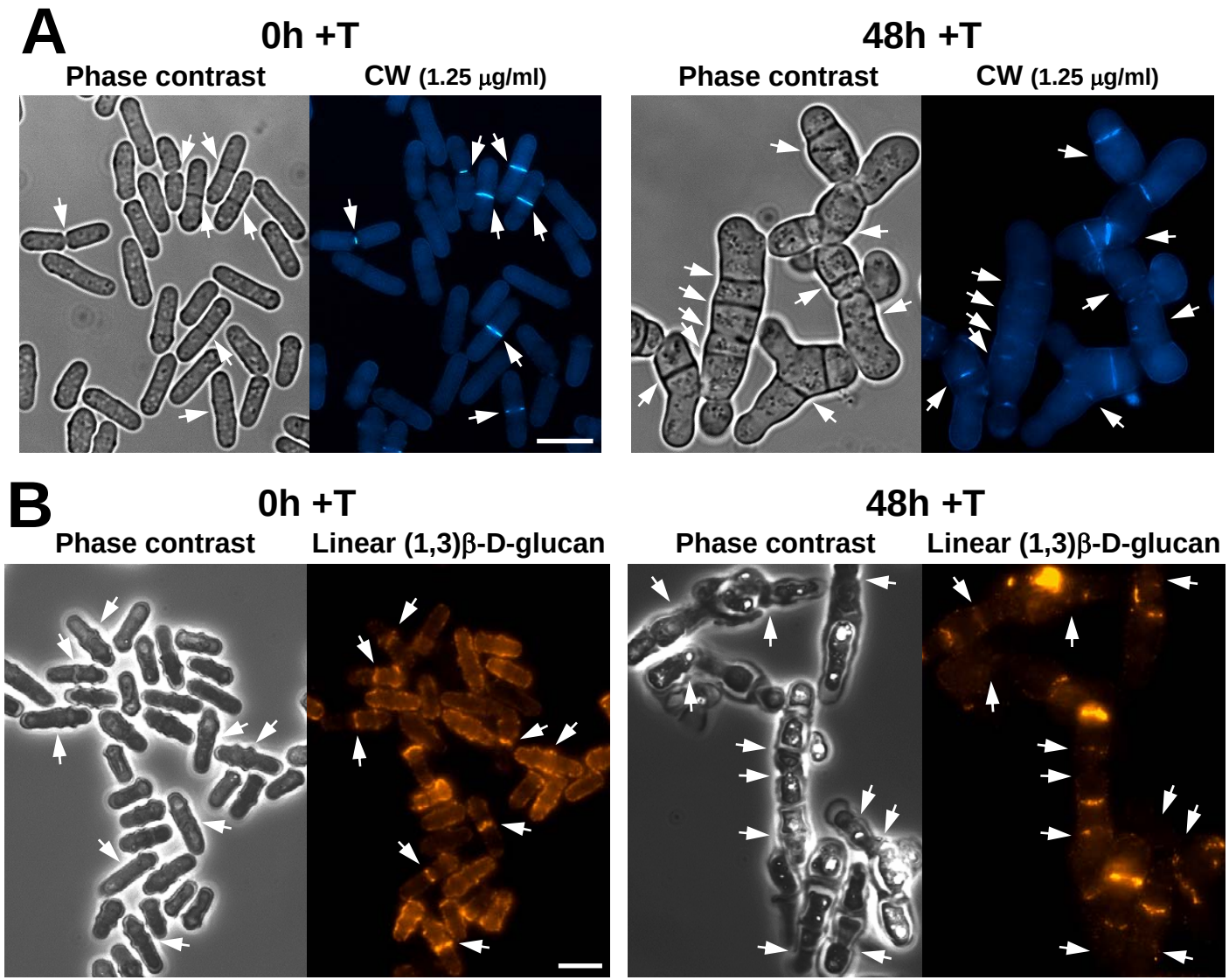
Cortés *et al.* Fig. 1



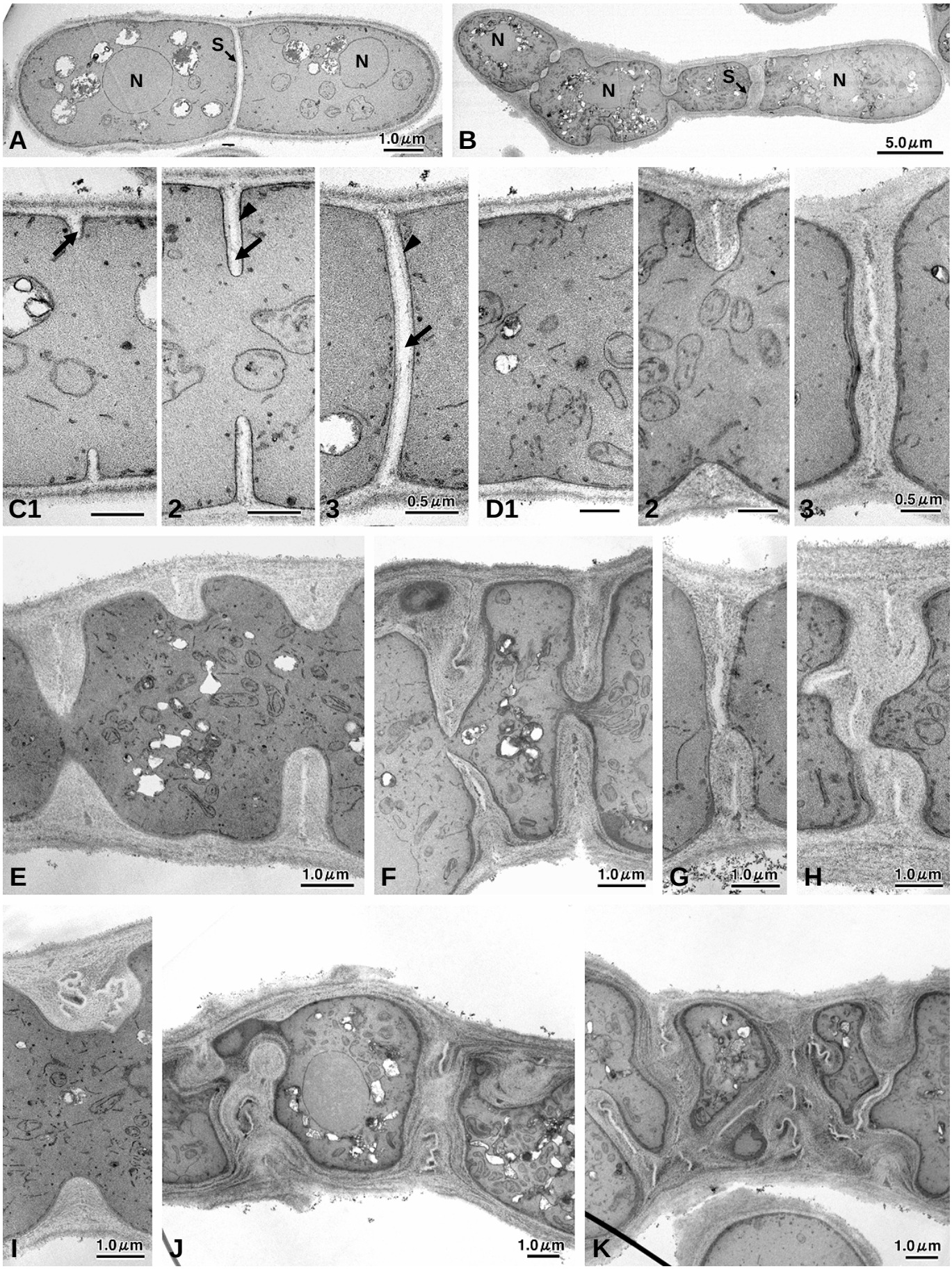
Cortés *et al.* Fig. 2



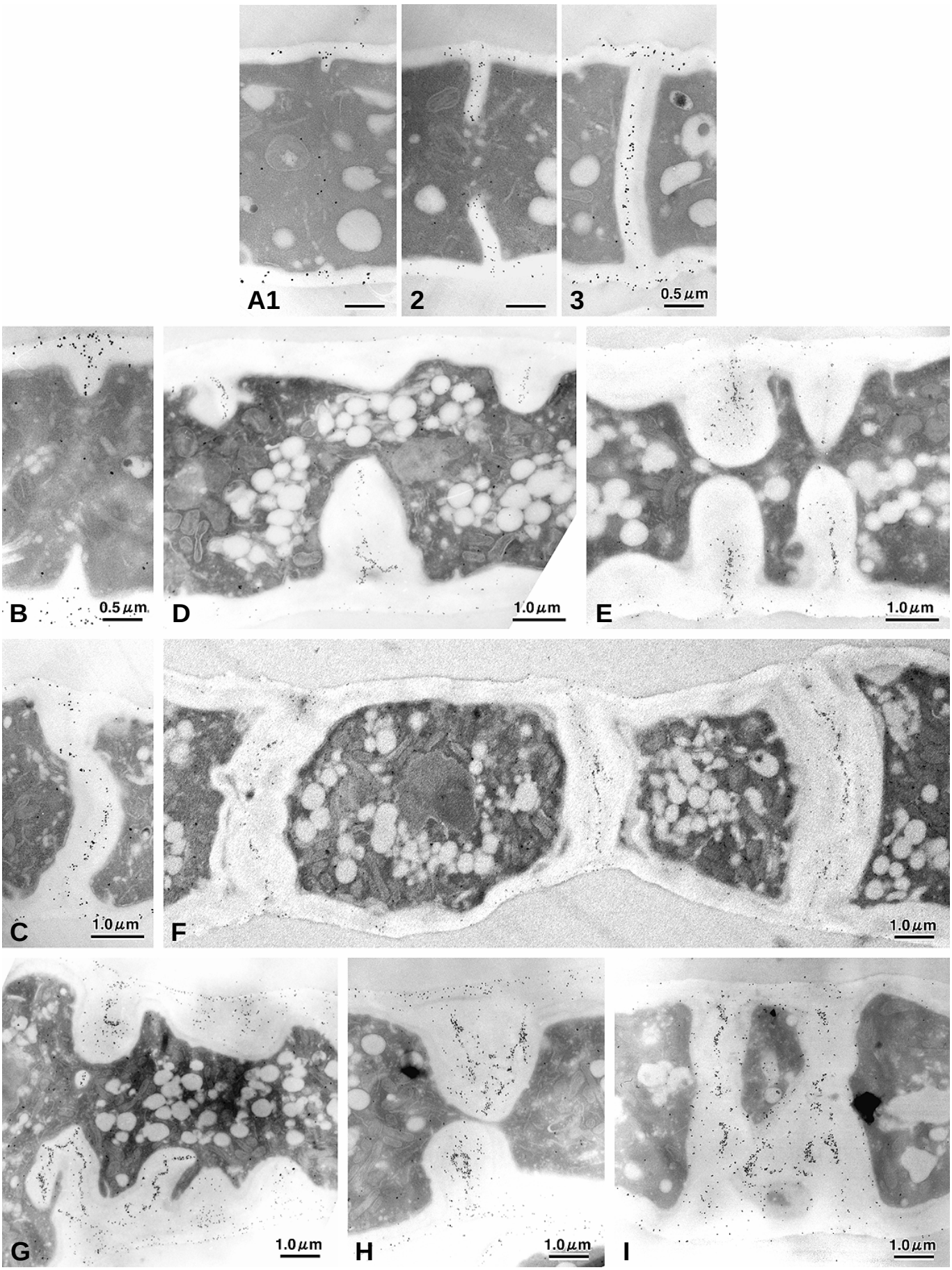
Cortés *et al.* Fig. 3

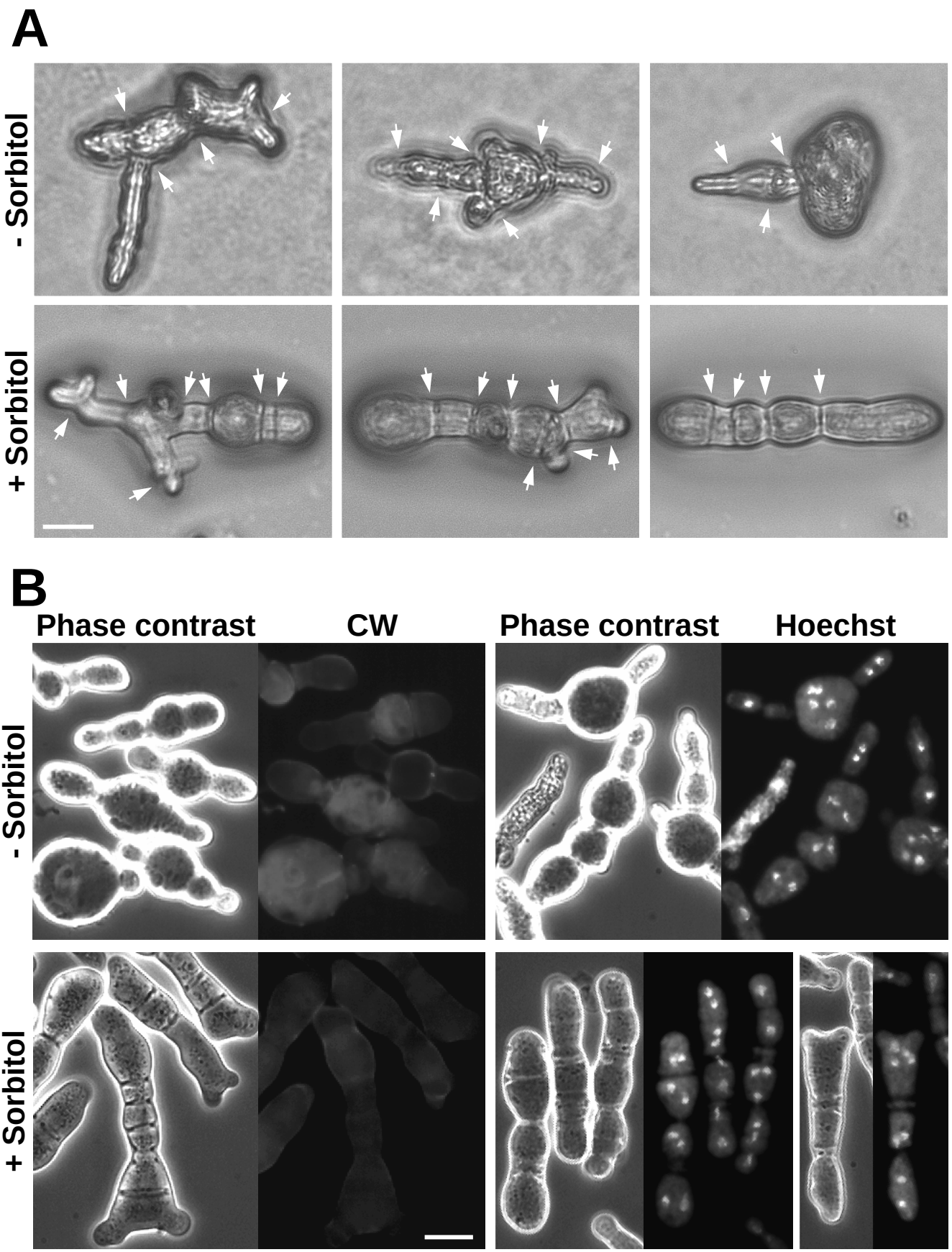


Cortés *et al.* Fig. 4



Cortés *et al.* Fig. 5




A
- Sorbitol
+ Sorbitol
B
Phase contrast
CW
Phase contrast
Hoechst
- Sorbitol
+ Sorbitol

Cortés *et al.* Fig. 7

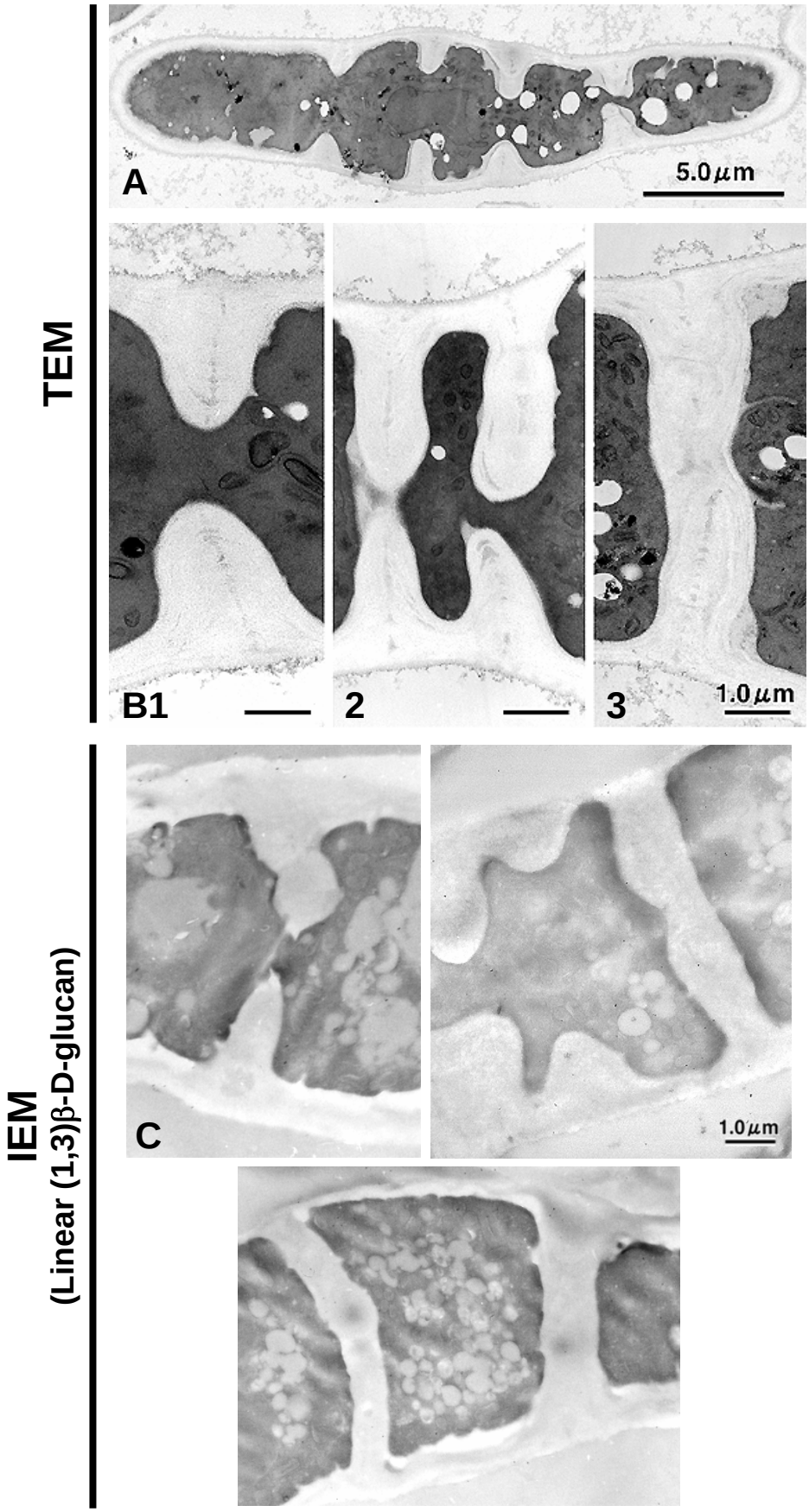


Table S1. Incorporation of radioactivity from [^{14}C]glucose into cell wall polysaccharides during *bgs1*$^{+}$ shut-off of *S. pombe bgs1*Δ p81X-*bgs1*$^{+}$ strain grown either in the absence or in the presence of 1.2 M sorbitol.

			% Incorporation of [^{14}C]glucose (mean ± s.d.)[b]			
Growth	Strain	Thiamine[a]	Cell wall	α-Glucan	β-Glucan	Galactomannan
EMM	*bgs1*Δ p81X-*bgs1*$^{+}$	- (on, wt)	32.2 ± 1.3 (100)[c]	10.8 ± 0.2 (33.5)	18.3 ± 0.5 (56.9)	3.1 ± 0.1 (9.6)
	*bgs1*Δ p81X-*bgs1*$^{+}$	+ (off)	44.4 ± 3.0 (100)	23.4 ± 2.5 (52.6)	18.7 ± 0.5 (42.2)	2.3 ± 0.1 (5.2)
EMM+S	*bgs1*Δ p81X-*bgs1*$^{+}$	- (on, wt)	29.1 ± 0.9 (100)	9.5 ± 0.4 (36.6)	16.7 ± 0.5 (53.4)	2.9 ± 0.1 (10.0)
	*bgs1*Δ p81X-*bgs1*$^{+}$	+ (off)	33.5 ± 0.3 (100)	15.1 ± 0.2 (47.3)	16.3 ± 0.6 (46.7)	2.1 ± 0.1 (6.0)

[a] Induced p81X-*bgs1*$^{+}$ maintains the *bgs1*Δ cells with wild-type morphology and GS activity. *bgs1*$^{+}$-repressed cell cultures were grown for 48 hours in the presence of thiamine (T) and [^{14}C]glucose was added 5 hours (control cells, -thiamine) or 24 hours (*bgs1*$^{+}$-repressed cells, +thiamine) before harvesting. Control wild type cells grown in EMM±S±T showed similar cell wall amount and composition to that of control *bgs1*Δ p81X-*bgs1*$^{+}$ cells grown in the absence of thiamine.

[b] Percentage incorporation of [^{14}C]glucose = cpm incorporated per fraction x 100/total cpm incorporated. Values are the means and standard deviations (s.d.) calculated from three independent experiments.

[c] Values in parentheses are percentages of the corresponding polysaccharide in the cell wall.

S: 1.2 M sorbitol.

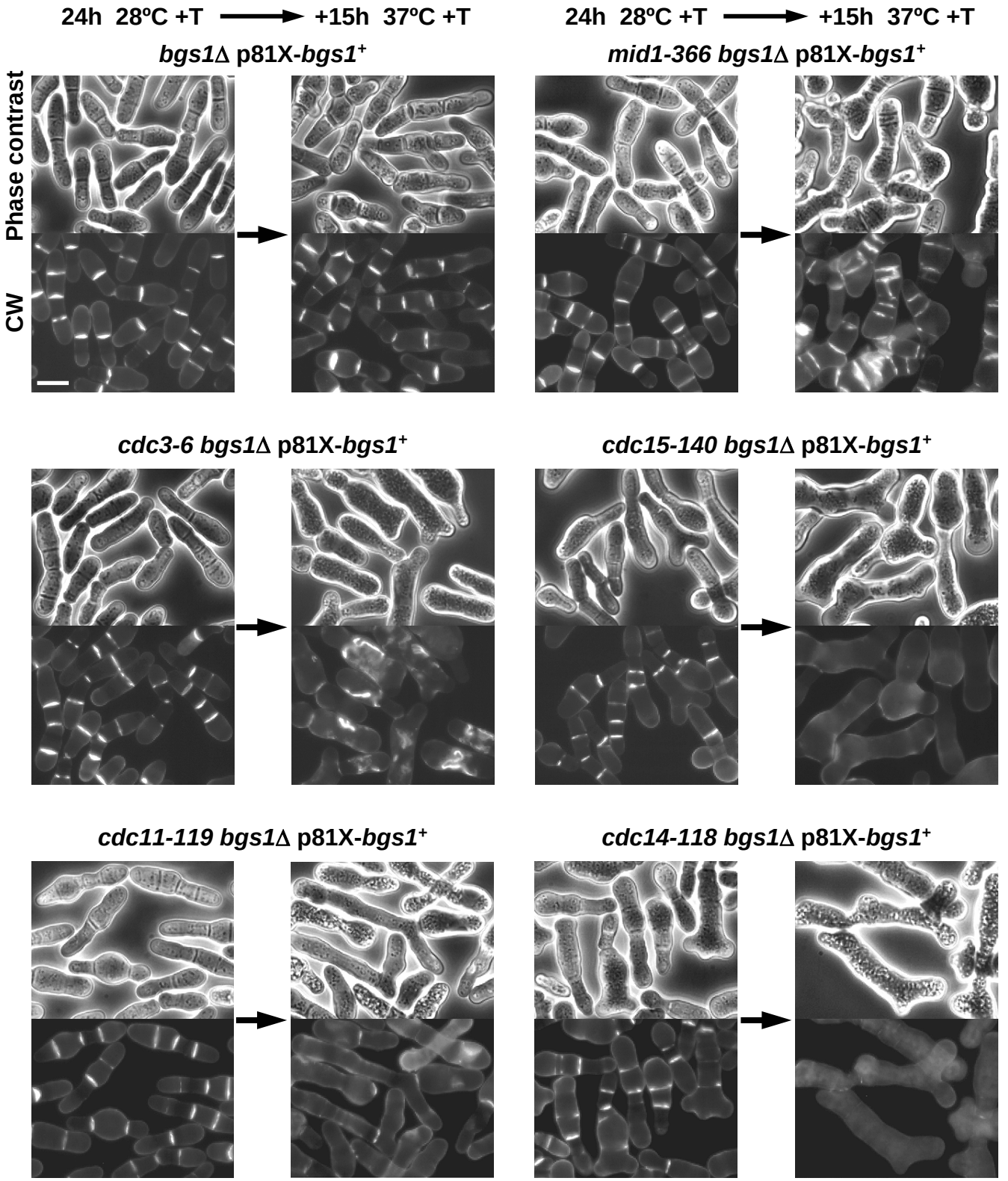


Figure S1. Synthesis of *bgs1*$^+$ shut-off septa depends on the medial ring positioning and assembly and on the SIN proteins.

*bgs1*Δ p81X-*bgs1*$^+$ cells harboring the indicated septation mutations were grown on EMM+T+S at 28°C for 24 hours to induce the multiseptated *bgs1*$^+$ shut-off phenotype. Then, the cell cultures were transferred to 37ºC for 15 hours to induce the corresponding septation mutation phenotype while maintaining *bgs1*$^+$ repressed. Cells were grown and visualized as in Fig. 1C. Bar, 10 μm.