In vitro reconstitution of the yeast spore wall dityrosine layer discloses the mechanism of its assembly

Received for publication, March 14, 2017, and in revised form, July 31, 2017 Published, Papers in Press, August 9, 2017, DOI 10.1074/jbc.M117.786202

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Edited by Chris Whitfield

In response to nutrient starvation, diploid cells of the budding yeast *Saccharomyces cerevisiae* differentiate into a dormant form of haploid cell termed a spore. The dityrosine layer forms the outermost layer of the wall of *S. cerevisiae* spores and endows them with resistance to environmental stresses. LL-Bisformyl dityrosine is the main constituent of the dityrosine layer, but the mechanism of its assembly remains elusive. Here, we found that LL-bisformyl dityrosine, but not LL-dityrosine, stably associated *in vitro* with *dit1*Δ spores, which lack the dityrosine layer. No other soluble cytosolic materials were required for this incorporation. In several aspects, the dityrosine incorporated in *trans* resembled the dityrosine layer. For example, dityrosine incorporation obscured access of the dye calcofluor white to the underlying chitosan layer, and LL-bisformyl dityrosine molecules bound to *dit1*Δ spores were partly isomerized to the DL-form. Mutational analyses revealed several spore wall components required for this binding. One was the chitosan layer located immediately below the dityrosine layer in the spore wall. However, LL-bisformyl dityrosine did not stably bind to chitosan particles, indicating that chitosan is not sufficient for this association. Several lines of evidence demonstrated that spore-resident proteins are involved in the incorporation, including the Lds proteins, which are localized to lipid droplets attached to the developing spore wall. In conclusion, our results provide insight into the mechanism of dityrosine layer formation, and the *in vitro* assay described here may be used to investigate additional mechanisms in spore wall assembly.

In response to nutrient starvation, diploid cells of the budding yeast *Saccharomyces cerevisiae* undergo meiosis and differentiate into a dormant form of haploid cell termed a spore. Spore formation occurs inside of the mother cell where the four nuclei produced by meiosis are enclosed by newly synthesized spore plasma membranes and spore walls. The plasma membrane and cell wall of the mother cell become the ascal membrane and ascal wall, respectively. As such, the mother cell matures into the ascus harboring four spores (1, 2).

The spore plasma membrane is originally formed as a double membrane, termed the prospore membrane (3). This structure is formed *de novo* during sporulation, and the haploid nuclei are engulfed by the double membrane (2, 4). The outer membrane of the prospore membrane is broken down during the subsequent spore wall assembly process, whereas the inner membrane remains and becomes the spore plasma membrane (5). The mature spore wall consists of four layers composed of, from the inside to outside, mannan, glucan, chitosan, and dityrosine (6–9). These layers are synthesized from the inner layer in a sequential manner such that the glucan layer is deposited after mannan layer formation (10, 11). The mannan and glucan layers are constructed in the lumen of the prospore membrane. The outer membrane derived from the prospore membrane disappears, presumably before the completion of the glucan layer (5). Thus, the nascent spore wall is exposed to the ascal cytosol when the chitosan and dityrosine layers are constructed. The mannan and glucan layers of the spore wall are similar in composition to the vegetative cell wall, whereas the chitosan and dityrosine layers are unique to the spore wall (2). Compared with vegetative cells, spores are resistant to environmental stresses. This property depends largely on the chitosan and dityrosine layers (10, 12, 13).

In the spore wall, chitosan production involves two steps: synthesis of an *N*-acetylglucosamine polymer (chitin) and its deacetylation (10, 14). Yeast has three chitin synthases; among them, chitin synthase 3 (encoded by *CHS3*) is solely used in sporulation to produce chitin (10). Deacetylation is mediated by sporulation-specific chitin deacetylases (14–16). Deposition of the chitosan layer is prerequisite for dityrosine layer formation; therefore deletion of *CHS3* causes losses of both outer layers (10). The dityrosine layer is mainly composed of a cross-linked modified di-amino acid, LL-*N*,*N*′-bisformyl dityrosine (8). LL-*N*,*N*′-Bisformyl dityrosine is synthesized from L-tyrosine in the cytosol of nascent spores via two steps: *N*-formylation of L-tyrosine and cross-linking of L-formyl tyrosines through carbons in the aromatic rings. These reactions are mediated by the

This work was supported by National Natural Science Foundation of China Grant 21576118 and Fundamental Research Funds for the Central Universities Grant JUSRP51629B (to H. N.), Fundamental Research Funds for the Central Universities Grant JUSRP51319B (to X.-D. G.), and National Institutes of Health Grant R01 GM072540 (to A. M. N.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains supplemental Figs. S1–S3.

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Dit1 and Dit2 enzymes, respectively (13, 17). Subsequently, LL-*N*,*N*′-bisformyl dityrosine molecules are transported to the developing spore wall through transporters located at the spore plasma membrane (18). The mechanism of assembly of this precursor into the organized dityrosine layer remains elusive.

LL-*N*,*N*′-Bisformyl dityrosine molecules are probably cross-linked to produce a macromolecule that is covalently attached to the chitosan layer (19). In fact, they are tightly associated to the spore wall and are not liberated by any digestive enzymes. A strong acid hydrolysis can break down the dityrosine layer. However, dityrosine, rather than bisformyl dityrosine, is liberated by this treatment because the treatment results in the removal of the formyl groups (8, 9). Notably, LL-*N*,*N*′-bisformyl dityrosine molecules are partly isomerized during or after incorporation into the dityrosine layer by unknown mechanism (17). Thus, dityrosine molecules liberated from the spore wall by acid hydrolysis are a mixture of LL- and DL-forms.

Several genes involved in dityrosine layer assembly have been identified in previous studies (5, 20, 21). Intriguingly, many of them have functionally redundant paralogues (20). *LDS1*, *LDS2*, and *RRT8* are one such set of paralogues; their simultaneous deletion results in a severe loss of the dityrosine layer. What makes them distinctive from the other genes is that their gene products are localized to lipid droplets (20). The role of lipid droplets in dityrosine layer formation is unclear. However, cytological analyses showed that lipid droplets are attached to the ascal side of the prospore membrane or spore wall during the course of spore formation (20, 22). Although the function of Lds1, Lds2, and Rrt8 is not yet characterized, these proteins may link dityrosine layer formation and lipid droplets.

In the present study, we attempted to reconstitute the dityrosine layer assembly *in vitro*. Most cell wall macromolecules, such as β-glucan and chitosan, are secreted to the extracellular space as polymers. By contrast, the dityrosine layer is formed such that LL-*N*,*N*′-bisformyl dityrosine monomers assemble in the spore wall (18). Here, we report that LL-*N*,*N*′-bisformyl dityrosine provided in *trans* is a substrate for assembly onto the chitosan layer of intact spores *in vitro*. Furthermore, this assembly is blocked by mutations disrupting the wall of the acceptor spores, such as *chs3*Δ or *lds1*Δ*lds2*Δ*rrt8*Δ. This *in vitro* system will be of great use to investigate the assembly mechanism of LL-*N*,*N*′-bisformyl dityrosine in the spore wall.

Results

CFW staining in dit1Δ spores is inhibited by incubation with the ascal cytosolic lysate

*dit1*Δ spores lack the dityrosine layer because they cannot produce LL-*N*,*N*′-bisformyl dityrosine (hereafter referred to as bisformyl dityrosine or LL-bisformyl dityrosine if the configuration is important) (8, 13). We hypothesized that the dityrosine layer could be reconstituted by incubating *dit1*Δ spores with bisformyl dityrosine. To test this hypothesis, first we used ascal lysate of wild-type spores as a source of bisformyl dityrosine. The ascal lysate was prepared by rupturing the ascal membrane using sonication. It seems that the ascal lysate was primarily derived from the ascal cytosol because spores remained intact during the process. As shown in supplemental Fig. S1, viability of wild-type spores released from asci by sonication was comparable with that of the spores released by β-glucanase treatment. Similar results were obtained for *dit1*Δ and *chs3*Δ spores (supplemental Fig. S1).

Although we could not detect free bisformyl dityrosine in the lysate by high performance liquid chromatography (HPLC) (Fig. 1*A*), dityrosine was detected after hydrolysis of the lysate with strong acid (Fig. 1*B*). This result suggests that bisformyl dityrosine was conjugated with another molecule(s) in the lysate. Notably, the dityrosine peak observed in the ascal lysate exhibited a doublet. A similar pattern was observed for dityrosine molecules liberated from wild-type spores by acid hydrolysis (Fig. 1*B*). Previous work demonstrated that a fraction of the LL-dityrosine was isomerized to the DL-form in the spore wall, and this mixture was detected as a doublet peak by HPLC (19). Dityrosine peaks were not detected in the ascal lysate of *dit1*Δ spores (Fig. 1*B*). Thus, wild-type ascal lysate includes soluble dityrosine-containing molecules that include both the LL- and DL-dityrosine moieties. To identify dityrosine-containing molecules, HPLC chromatograms of wild-type and *dit1*Δ ascal lysates without the hydrolysis treatment were compared. However, major peaks detected in wild-type ascal lysate were also found in *dit1*Δ ascal lysates (data not shown). Thus, at present, the nature of the dityrosine-containing molecule is not clear.

The *dit1*Δ spores used in this experiment were first released from asci by breaking the ascal wall and membrane and then washed with high salt (0.6 M NaCl). They were incubated with wild-type or *dit1*Δ ascal lysate for 24 h and washed with the high salt. To assay whether the dityrosine layer is formed on the *dit1*Δ spores, we used calcofluor white (CFW)[3] staining. CFW can stain chitosan on *dit1*Δ spores (Fig. 1, *C* and *D*). However, wild-type spores are not stained by the dye because the dityrosine layer prevents the dye from binding the chitosan (Fig. 1, *C* and *D*). Strikingly, we found that the staining levels in *dit1*Δ spores were significantly decreased after incubation with wild-type ascal lysate (Fig. 1, *C* and *D*). *dit1*Δ spores incubated with *dit1*Δ ascal lysate were stained similarly to untreated spores (Fig. 1, *C* and *D*).

The *chs3*Δ mutation causes a loss of the chitosan layer, so the dityrosine layer is not assembled on the mutant spores (10). As with the wild-type ascal lysate, free bisformyl dityrosine was not detected in ascal lysate of *chs3*Δ spores, but dityrosine was detected after hydrolysis (Fig. 1, *A* and *B*). The liberated dityrosine molecules are a mixture of LL- and DL-forms (Fig. 1*C*). *dit1*Δ spores incubated with *chs3*Δ ascal lysate were also resistant to CFW staining (Fig. 1, *C* and *D*).

Previous studies showed that dityrosine molecules detected in the spore cytosol are predominantly in the LL-form (8, 18). Accordingly, the dityrosine peak detected in *chs3*Δ spore lysate (lysate of *chs3*Δ spores released from asci) exhibited a single peak (supplemental Fig. S2). Thus, DL-dityrosine is formed in ascal cytosol even in *chs3*Δ mutant spores. As in the ascal lysate, soluble bisformyl dityrosine was not detected in the spore lysate (supplemental Fig. S2). These results suggest that dityrosine-containing molecules are an intermediate in the assembly of the

[3] The abbreviation used is: CFW, calcofluor white.

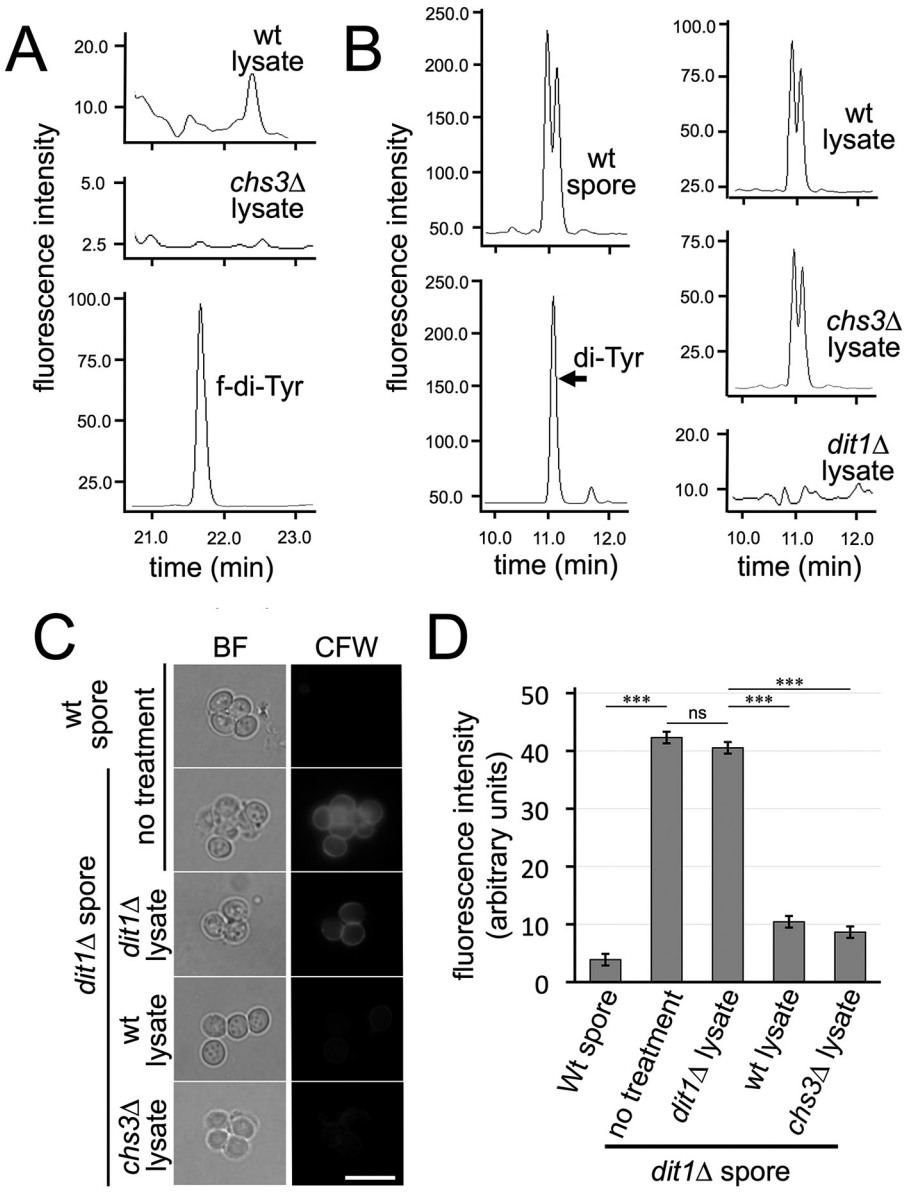

Figure 1. Detection of dityrosine in wild-type ascal lysate and CFW staining of *dit1*Δ spores incubated with the lysate. *A*, wild-type and *chs3*Δ ascal lysates and bisformyl dityrosine (*f-di-Tyr*) were subjected to HPLC, and fluorescence intensity characteristic of dityrosine (285-nm excitation and 425-nm emission) was monitored. *B*, wild-type spores or wild-type, *dit1*Δ, or *chs3*Δ ascal lysates were hydrolyzed with 6 N HCl and subjected to HPLC. LL-Dityrosine (*di-Tyr*; *arrow*) is shown as a control. *C*, wild-type spores, *dit1*Δ spores (no treatment), or *dit1*Δ spores incubated with *dit1*Δ, wild-type, or *chs3*Δ ascal lysate were washed with 0.6 M NaCl and stained with CFW. Images were obtained in fluorescence (*CFW*) or bright-field (*BF*) microscopy. All fluorescence microscopy images were obtained under the same imaging conditions. *Scale bar*, 5 μm. *D*, quantification of the fluorescence intensities of CFW of the spores described in *C*. Data presented are the mean of three independent samples. *Error bars* represent S.E. ***, $p < 0.001$; *ns*, not significant.

dityrosine layer rather than spore wall fragments solubilized when asci were broken down.

Dityrosine-containing molecules included in the ascal lysate can bind to dit1Δ spores

To verify that the dityrosine-containing molecules could bind to *dit1*Δ spores, the spores treated with wild-type ascal lysate were hydrolyzed, and liberated dityrosine molecules were detected by SDS-PAGE and HPLC. Dityrosine emits blue fluorescence under UV light (23). Using this property, we detected it by SDS-PAGE. As shown in Fig. 2*A*, synthesized dityrosine was observed as a single band running with the dye front, and a similar signal was observed in the hydrolytic lysate of wild-type spores. Notably, synthesized dityrosine solution includes tyrosine that was used as a raw material. However, tyrosine was not visualized by this method (Fig. 2*A*), showing that dityrosine is specifically detected by this method. The dityrosine band was detected in eluate of *dit1*Δ spores treated with wild-type ascal lysate but not in the hydrolytic lysate of *dit1*Δ spores or eluate of *dit1*Δ spores treated with *dit1*Δ ascal lysate (Fig. 2*A*). To further verify that the eluate of *dit1*Δ spores treated with wild-type ascal lysate includes dityrosine, we performed HPLC analysis. As shown Fig. 2*B*, the dityrosine peaks were detected in the hydrolytic lysate of *dit1*Δ spores treated with wild-type ascal lysate but not in those treated with *dit1*Δ ascal lysate. The hydrolytic lysate of wild-type spores was diluted to 20-fold

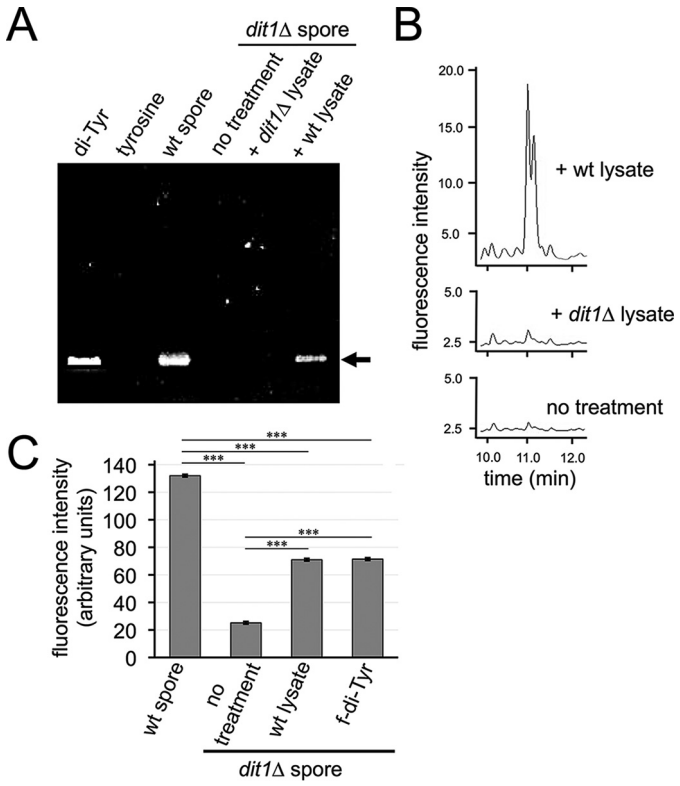

Figure 2. Detection of dityrosine-containing molecules bound to *dit1*Δ spores. *A*, wild-type spores, *dit1*Δ spores (no treatment), or *dit1*Δ spores incubated with *dit1*Δ or wild-type ascal lysate were washed with 0.6 M NaCl. The spores were hydrolyzed, and the lysates were subjected to SDS-PAGE. Hydrolysate of wild-type spores was diluted 20-fold. Dityrosine (*di-Tyr*) and tyrosine were also subjected to SDS-PAGE as controls. Dityrosine bands are indicated by an *arrow*. *B*, hydrolytic lysates of *dit1*Δ spores (no treatment) or *dit1*Δ spores treated with wild-type or *dit1*Δ ascal lysates were subjected to HPLC. *C*, quantification of the fluorescence of dityrosine on wild-type spores, *dit1*Δ spores (no treatment), or *dit1*Δ spores treated with wild-type or *dit1*Δ ascal lysate. Data presented are the mean of 30 spores. *Error bars* represent S.E. ***, $p < 0.001$.

Table 1

Amount of dityrosine released from wild-type spores and *dit1*Δ spores incubated with wild-type ascal lysate (per unit weight of spores)

The amount of dityrosine released from wild-type spores and *dit1*Δ spores incubated with wild-type ascal lysate by acid hydrolysis was measured based on HPLC data. Data presented are the mean ± S.E. of three independent samples.

	Wild-type spores	*dit1*Δ spores incubated with wild-type ascal lysate
Amount of dityrosine (ng/mg of spores)	21.03 ± 2.43	0.118 ± 0.017

Table 2

Amount of dityrosine detected in wild-type ascal lysate before and after incubation with *dit1*Δ spores

200 μl of wild-type ascal lysate was incubated with 52 mg of *dit1*Δ spores. The amounts of dityrosine detected in the lysate before and after the incubation were measured based on HPLC data. Based on these results, the amount of dityrosine bound to *dit1*Δ spores was calculated. Data presented are the mean ± S.E. of three independent samples (amount of dityrosine per unit weight of spores is shown in parentheses).

	Before incubation	After incubation	Bound to *dit1*Δ spores
Amount of dityrosine (ng)	9.66 ± 0.67	4.41 ± 0.23	5.25 ± 0.31 (0.10 ± 0.31 ng/mg of spores)

before the SDS-PAGE and HPLC analyses. Furthermore, to directly detect dityrosine on the spore wall, its fluorescence was measured in the fluorescence microscope. As shown in Fig. 2*C*, levels of dityrosine fluorescence were increased by incubation with wild-type ascal lysate. Similarly, the fluorescence levels in *dit1*Δ spores were increased by incubation with synthesized bisformyl dityrosine (Fig. 2*C*). As described below, bisformyl dityrosine can also bind to *dit1*Δ spores. HPLC was used to quantify the amount of dityrosine liberated from wild-type spores and *dit1*Δ spores incubated with ascal lysate. The result showed that the amount of dityrosine bound to *dit1*Δ spores was ~180 times less than that in wild type (Table 1). We also measured the amount of dityrosine detected in the ascal lysate before and after incubation with *dit1*Δ spores (Table 2). Based on these results, we calculated the amount of dityrosine bound to *dit1*Δ spores (Table 2). The value was in agreement with that liberated from *dit1*Δ spores incubated with ascal lysate (Tables 1 and 2). Thus, the amount of dityrosine bound to *dit1*Δ spores was much less than the dityrosine levels in the wild-type spore wall. Perhaps for this reason no obvious difference was seen when spores treated with or without ascal lysate were examined by scanning electron microscopy analysis (data not shown). Nevertheless, our results demonstrate that the dityrosine-containing molecules can bind to *dit1*Δ spores, and their incorporation makes the spores resistant to CFW staining.

In the fluorescence microscopy assay, the fluorescence signals in *dit1*Δ spores treated with wild-type ascal lysate or bisformyl dityrosine were about half of that in wild-type spores (Fig. 2*C*). Thus, it seems that HPLC and SDS-PAGE analyses are more sensitive than the fluorescent microscopy analysis. For this reason, we used HPLC and SDS-PAGE analyses to detect dityrosine bound to spores in this study.

Dityrosine molecules bound to dit1Δ spores are divided into two fractions

In wild-type spores, dityrosine molecules bind tightly to the chitosan layer, so they are not liberated either by high-salt or detergent wash (19) (Fig. 3*A*). As described above, dityrosine molecules bound to *dit1*Δ spores were not liberated by high-salt wash. However, we found that the *dit1*Δ spores incubated with wild-type ascal lysate became susceptible to CFW staining after detergent wash (0.5% Triton X-100) (Fig. 3, *A* and *B*). Dityrosine was detected in the supernatant after *dit1*Δ spores treated with wild-type ascal lysate were washed with high salt and detergent (Fig. 4, *A* and *B*). 82 and 60% of *dit1*Δ spores were viable after high-salt and detergent washes, respectively (supplemental Fig. S3). Thus, it is not likely that the gain of CFW staining in *dit1*Δ spores washed with detergent was attributable to cell lysis.

Although *dit1*Δ spores were stained by CFW after detergent wash, the SDS-PAGE and HPLC analyses revealed that dityrosine-containing molecules remained in the spore wall even after washing with high salt and detergent (Fig. 4, *A* and *B*). These results indicate that dityrosine-containing molecules bound to *dit1*Δ spores were divided into two fractions. One fraction is those liberated by detergent, and thus they may bind to the spore wall via hydrophobic interactions. Notably, this type of dityrosine-containing molecule can make *dit1*Δ spores resistant to CFW staining. The other fraction of dityrosine-containing molecules is tightly incorporated into *dit1*Δ spores, probably via a covalent association. After the wash with deter-

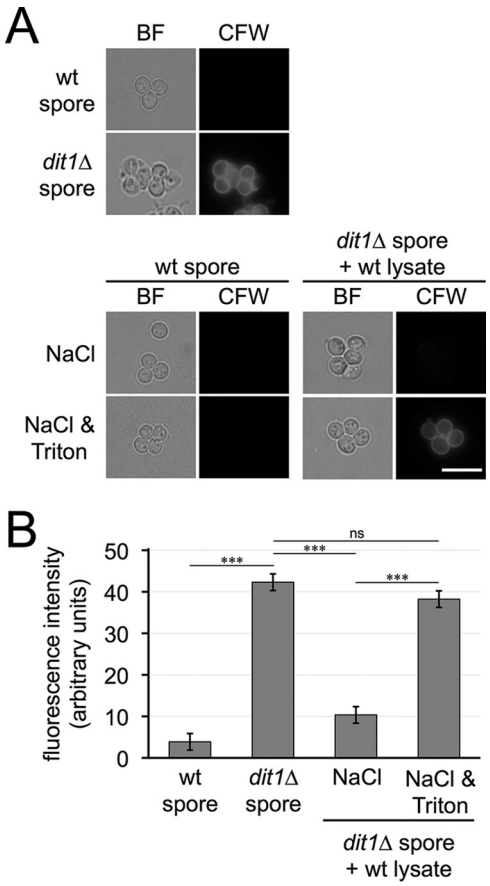

Figure 3. Effect of high-salt and detergent wash on CFW staining of *dit1*Δ spores incubated with wild-type ascal lysate. *A*, *upper panel*, wild-type or *dit1*Δ spores were stained with CFW, and images were obtained by fluorescence (*CFW*) or bright-field (*BF*) microscopy. *Lower panel*, wild-type spores or *dit1*Δ spores incubated with wild-type ascal lysate were washed with 0.6 M NaCl or 0.6 M NaCl and 0.5% Triton X-100. The spores were stained with CFW, and images were obtained under by fluorescence (*CFW*) or bright-field (*BF*) microscopy. *Scale bar*, 5 μm. *B*, quantification of the fluorescence intensities of CFW of wild-type spores, *dit1*Δ spores, or *dit1*Δ spores incubated with wild-type ascal lysate washed with 0.6 M NaCl or 0.6 M NaCl and 0.5% Triton X-100. Data presented are the mean of three independent samples. *Error bars* represent S.E. ***, $p < 0.001$; *ns*, not significant.

gent, the ratio of DL-dityrosine was largely decreased both in the spores and supernatant (Fig. 4*B*). This phenomenon was observed reproducibly, although the basis for this change is not clear.

The chitosan layer and spore wall resident protein(s) are required for the binding of the dityrosine-containing molecules to the spore wall

The chitosan layer is prerequisite for dityrosine layer formation (10, 11). To examine whether the chitosan layer is required for the interaction of the dityrosine-containing molecules, *dit1*Δ*chs3*Δ spores, which lack both the chitosan and dityrosine layers, were incubated with wild-type ascal lysate. After washing with high salt, the spores were hydrolyzed, and the eluate was subjected to SDS-PAGE and HPLC. As shown in Fig. 5, *A* and *B*, dityrosine was not detected in the hydrolytic lysate, suggesting that the dityrosine-containing molecules did not bind to *dit1*Δ*chs3*Δ spores. Next, to examine whether chitosan is sufficient for the interaction, chitosan particles were incubated with wild-type ascal lysate. After the incubation, chitosan particles were washed with either high salt or detergent and subjected to hydrolysis with HCl. As shown in Fig. 5, *C* and *D*, the binding of the dityrosine-containing molecules to chitosan par-

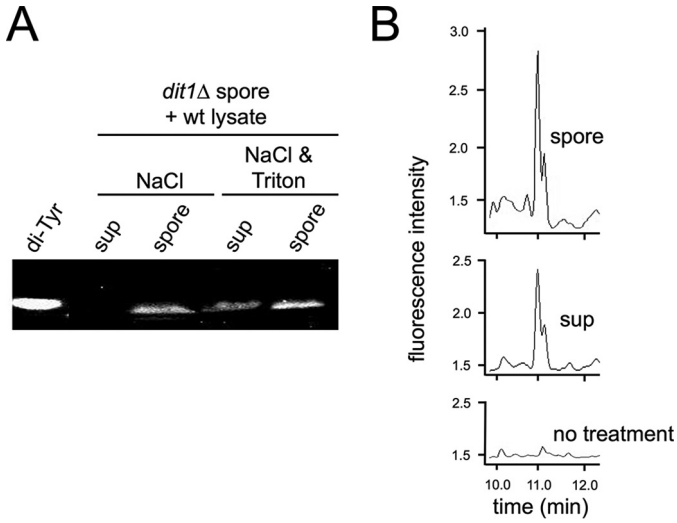

Figure 4. Effect of high-salt and detergent wash on the dityrosine-containing molecules bound on *dit1*Δ spores. *A*, *dit1*Δ spores were first incubated with wild-type ascal lysate. After washing with 0.6 M NaCl or 0.6 M NaCl and 0.5% Triton X-100, the spores or supernatants (*sup*) were hydrolyzed with 6 N HCl and subjected to SDS-PAGE. Dityrosine (*di-Tyr*) is shown as a control. *B*, *dit1*Δ spores were incubated with wild-type ascal lysate. After washing with 0.6 M NaCl and 0.5% Triton X-100, the spores or supernatant (*sup*) were hydrolyzed with 6 N HCl and subjected to HPLC. Hydrolytic lysate of *dit1*Δ spores (no treatment) is shown as a control.

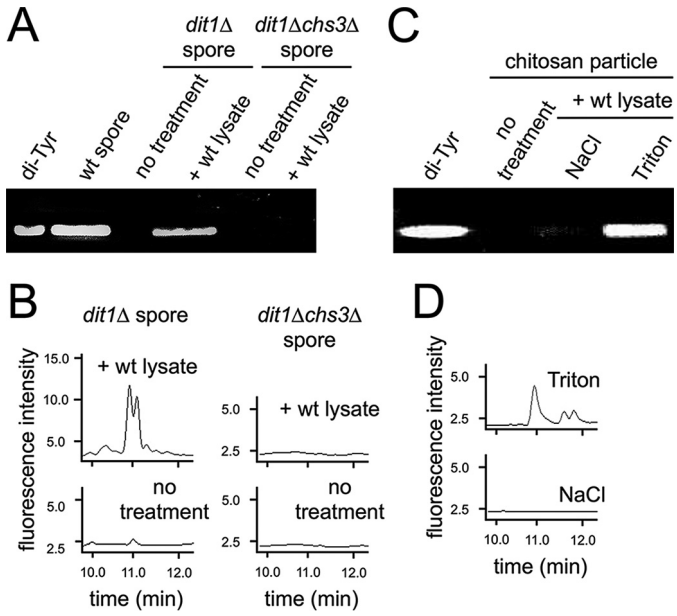

Figure 5. Binding assay of dityrosine-containing molecules to *dit1*Δ*chs3*Δ spores or chitosan particles. *A*, wild-type spores, *dit1*Δ spores (no treatment), *dit1*Δ*chs3*Δ spores (no treatment), or *dit1*Δ or *dit1*Δ*chs3*Δ spores incubated with wild-type ascal lysate were washed with 0.6 M NaCl and hydrolyzed with 6 N HCl. The hydrolytic lysates were subjected to SDS-PAGE. *B*, *dit1*Δ spores (no treatment), *dit1*Δ*chs3*Δ spores (no treatment), or *dit1*Δ or *dit1*Δ*chs3*Δ spores incubated with wild-type ascal lysate were washed with 0.6 M NaCl and hydrolyzed with 6 N HCl. The hydrolytic lysates were subjected to HPLC. *C*, chitosan particles (no treatment) or chitosan particles incubated with wild-type ascal lysate washed with 0.6 M NaCl or 0.5% Triton X-100 were hydrolyzed with 6 N HCl. The hydrolytic lysates were subjected to SDS-PAGE. Dityrosine (*di-Tyr*) is shown as a control. *D*, chitosan particles incubated with wild-type ascal lysate washed with 0.6 M NaCl or 0.5% Triton X-100 were hydrolyzed with 6 N HCl. The hydrolytic lysates were subjected to HPLC.

ticles was not like that to *dit1*Δ spores; dityrosine was detected in the lysate of the chitosan particles after washing with detergent but not in those washed with high salt. Thus, the dityrosine-containing molecules probably bound to chitosan particles via electrostatic interaction. These results demonstrate that the chitosan layer is required for binding of the dityrosine-containing molecules, but chitosan is not sufficient for their binding.

These results raise the possibility that the interaction between the dityrosine-containing molecules and the chitosan layer is mediated by proteins residing in the spore wall. To test this hypothesis, *dit1*Δ spores and wild-type ascal lysate were treated with proteinase K prior to the binding assay. *dit1*Δ spores incubated with ascal lysate treated with proteinase K became resistant to CFW staining, indicating that no proteins in the lysate are required for this reaction (Fig. 6, *A* and *B*). However, *dit1*Δ spores treated with proteinase K were stained by CFW even after incubating with wild-type ascal lysate (Fig. 6, *A* and *B*). 45% of *dit1*Δ spores remained viable after proteinase K treatment (supplemental Fig. S3), indicating that the gain of CFW staining is not attributed to cell lysis. The SDS-PAGE assay provided further evidence that spore wall-resident proteins are involved in the incorporation of the dityrosine-containing molecule. As shown in Fig. 6, *C* and *D*, binding of the dityrosine-containing molecules to *dit1*Δ spores was decreased with the increase of the incubation period of the spores with proteinase K. After the 5-h incubation, dityrosine detected from the spores was 7% of that bound to spores incubated in the reaction buffer without proteinase K for 5 h (Fig. 6*D*). *dit1*Δ spores treated with proteinase K for 5 h were stained by CFW similarly to untreated spores, ruling out the possibility that the treatment causes a loss of the chitosan layer (Fig. 6*E*). Thus, the stable incorporation of dityrosine into the spore wall requires proteins present in the wall, suggesting that the process may be enzymatic.

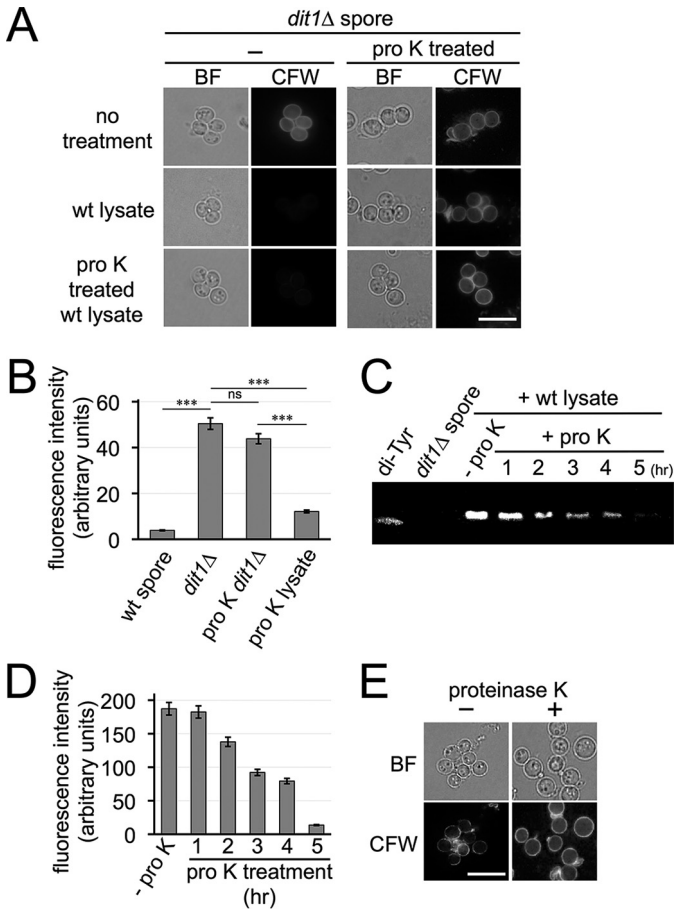

Figure 6. Effect of proteinase K treatment on the binding of dityrosine-containing molecules to *dit1*Δ spores. *A*, *dit1*Δ spores were first treated with or without proteinase K (*pro K*) for 2 h, and then they were incubated with wild-type ascal lysate treated with or without proteinase K for 2 h. The spores washed with 0.6 M NaCl were stained with CFW, and images were obtained by fluorescence (*CFW*) or bright-field (*BF*) microscopy. CFW staining was also performed on *dit1*Δ spores without incubation with wild-type ascal lysate (no treatment) after treatment with or without proteinase K. *Scale bar*, 5 μm. *B*, quantification of the fluorescence intensities of CFW of wild-type spores, *dit1*Δ spores, proteinase K-treated *dit1*Δ spores incubated with wild-type ascal lysate (*pro K dit1*Δ), or *dit1*Δ spores incubated with proteinase K-treated wild-type ascal lysate (*pro K lysate*). Data presented are the mean of three independent samples. *Error bars* represent S.E. ***, $p < 0.001$; *ns*, not significant. *C*, *dit1*Δ spores were treated with proteinase K for the indicated times (hours) or incubated in the reaction buffer without proteinase K for 5 h (−*pro K*). These spores were then incubated with wild-type ascal lysate. After washing with 0.6 M NaCl, the spores were hydrolyzed, and the lysates were subjected to SDS-PAGE. Hydrolytic lysate of *dit1*Δ spores and dityrosine (*di-Tyr*) are shown as controls. *D*, quantification of fluorescence intensities of dityrosine signals as shown in *C*. Data presented are the mean of three independent samples. *Error bars* represent S.E. *E*, *dit1*Δ spores were stained with CFW before (−) or after (+) treatment with proteinase K for 5 h, and images were obtained by fluorescence (*CFW*) or bright-field (*BF*) microscopy. *Scale bar*, 5 μm.

Synthesized bisformyl dityrosine, but not dityrosine, can bind to dit1Δ spores

Because no proteins are required in the ascal lysate, simple addition of bisformyl dityrosine may be sufficient for incorporation into the spore wall. When *in vitro* synthesized bisformyl dityrosine was added to *dit1*Δ spores and then analyzed by SDS-PAGE assay, the bisformyl dityrosine was found to bind tightly to *dit1*Δ spores, and it was not released by either high-salt or detergent wash, suggesting that it had been incorporated similarly to the dityrosine-containing molecule in the ascal lysates (Fig. 7*A*). However, bisformyl dityrosine did not make *dit1*Δ spores resistant to CFW (Fig. 7, *B* and *C*). The synthesized bisformyl dityrosine solution used in this study was a mixture of bisformyl dityrosine and formyl tyrosine, but formyl tyrosine was not detected by the SDS-PAGE assay (data not shown). In contrast to bisformyl dityrosine, dityrosine could not bind tightly to *dit1*Δ spores and was extracted by 0.6 M NaCl (Fig. 7*A*). Thus, in our experimental condition, the formyl group is necessary for bisformyl dityrosine to bind tightly to the spore wall.

Bisformyl dityrosine bound to *dit1*Δ spores was further analyzed by HPLC. Interestingly, we found that dityrosine molecules hydrolytically released from the spores exhibited a double peak (Fig. 7*D*). The bisformyl dityrosine used in this experiment was that LL-form, and its hydrolytic product, LL-dityrosine, exhibited a single peak in HPLC (Fig. 7*D*). This result suggests that LL-bisformyl dityrosine molecules are partly isomerized to the DL-form when they bind to *dit1*Δ spores. This is consistent with the possibility of enzymatic incorporation into the wall that would include a racemization of one of the chiral centers.

Lds proteins are required for the binding of bisformyl dityrosine to dit1Δ spores

The binding assays so far described were performed with *dit1*Δ spores washed with high salt. Subsequently, the assays were performed with *dit1*Δ spores washed with detergent (0.5%

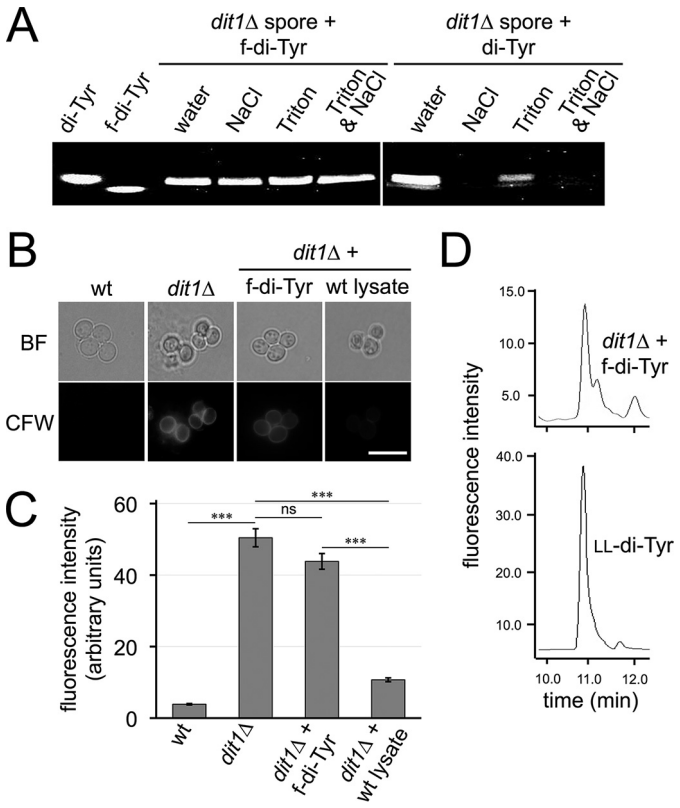

Figure 7. Binding assays of bisformyl dityrosine and dityrosine to *dit1*Δ spores. *A*, *dit1*Δ spores were incubated with bisformyl dityrosine (*f-di-Tyr*) or dityrosine (*di-Tyr*) and washed with water, 0.6 M NaCl, 0.5% Triton X-100, or 0.6 M NaCl and 0.5% Triton X-100. The spores were hydrolyzed with 6 N HCl, and the lysates were subjected to SDS-PAGE. *B*, wild-type spores, *dit1*Δ spores, or *dit1*Δ spores incubated with bisformyl dityrosine (*f-di-Tyr*) were washed with 0.6 M NaCl and stained with CFW. Images were obtained by fluorescence (*CFW*) or bright-field (*BF*) microscopy. *Scale bar*, 5 μm. *C*, quantification of the fluorescence intensities of CFW of the spores described in *B*. Data presented are the mean of three independent samples. *Error bars* represent S.E. ***, $p < 0.001$; *ns*, not significant. *D*, hydrolytic lysate of *dit1*Δ spores treated with LL-bisformyl dityrosine (*dit1*Δ + *f-di-Tyr*) was subjected to HPLC. LL-Dityrosine (LL-*di-Tyr*) is shown as a control.

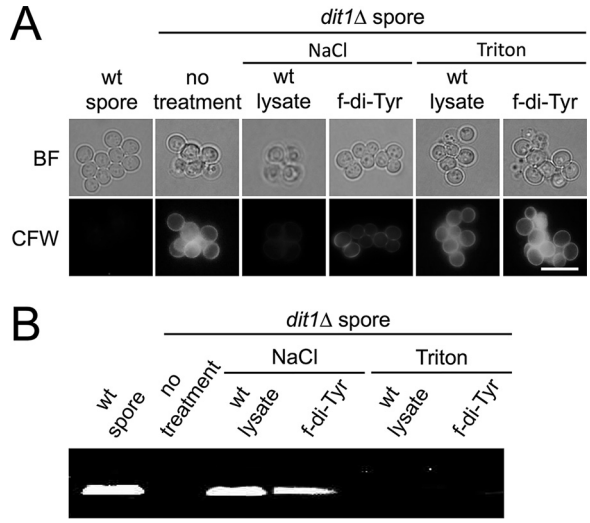

Figure 8. Effect of detergent wash on the binding of dityrosine-containing molecules and bisformyl dityrosine to *dit1*Δ spores. *A*, *dit1*Δ spores washed with 0.6 M NaCl or 0.5% Triton X-100 were incubated with wild-type ascal lysate or bisformyl dityrosine (*f-di-Tyr*) and washed with 0.6 M NaCl. The spores were stained with CFW, and images were obtained by fluorescence (*CFW*) or bright-field (*BF*) microscopy. Wild-type spores and *dit1*Δ spores (no treatment) washed with 0.6 M NaCl were used as controls. *Scale bar*, 5 μm. *B*, the spores described in *A* were hydrolyzed with 6 N HCl, and the lysates were subjected to SDS-PAGE.

Triton X-100). The spores were first incubated with wild-type ascal lysate and then stained with CFW. As shown in Fig. 8*A*, detergent-washed spores were susceptible to CFW staining even after incubation with wild-type ascal lysate. The SDS-PAGE assay revealed that neither dityrosine-containing molecules nor bisformyl dityrosine bound to detergent-washed *dit1*Δ spores (Fig. 8*B*).

These results indicate that some molecule associated with the spore wall via hydrophobic interaction is involved in the binding of bisformyl dityrosine. Lds1, Lds2, and Rrt8 (for simplicity, they will be collectively referred to as the Lds proteins) are paralogous proteins that reside in lipid droplets (20, 24). Lipid droplets containing Lds proteins are associated with the outside of the spore wall, and importantly, the triple deletion (*lds*Δ mutation) causes a loss of the dityrosine layer (20). The effect of loss of Lds proteins on the binding of bisformyl dityrosine and dityrosine-containing molecules to the spore wall was examined. *dit1*Δ*lds*Δ spores washed with high salt were stained by CFW similarly to untreated *dit1*Δ spores, showing that the mutant spores contain the chitosan layer (Fig. 9*A*). As with *dit1*Δ spores washed with detergent, *dit1*Δ*lds*Δ spores incubated with the wild-type ascal lysate were stained by CFW (Fig. 9, *A* and *B*). Dityrosine was not detected in hydrolytic lysate of *dit1*Δ*lds*Δ spores incubated with bisformyl dityrosine or dityrosine-containing molecules by the SDS-PAGE and HPLC assays (Fig. 9, *C* and *D*). These results demonstrate that Lds proteins are required for the binding of bisformyl dityrosine to the chitosan layer.

Discussion

In the present study, we demonstrate that bisformyl dityrosine can bind to *dit1*Δ spores and be stably incorporated into the spore wall *in vitro*. In several aspects, this incorporation resembles *in vivo* incorporation. As discussed below, our results provide insight into the mechanism of dityrosine layer formation.

Modification of bisformyl dityrosine and its isomerization

In the wild-type ascal lysate, dityrosine molecules exist as conjugates to some other molecule rather than a free form of bisformyl dityrosine. Presumably the dityrosine-containing molecules are an intermediate in the assembly of the dityrosine layer. The modifying residues appear to include a hydrophobic non-protein molecule because the dityrosine-containing molecule is liberated by a detergent wash, and it is resistant to proteinase K treatment. These characteristics are reminiscent of the previously reported spore wall component χ (20). Although component χ is an unidentified molecule, solid-state NMR analysis suggested that it is a non-protein molecule with reduced carbons, consistent with a hydrophobic nature. The dityrosine-containing molecules bound to *dit1*Δ spores can be extracted by detergent but not by high-salt solution. This property should be useful to concentrate it for further characterization. Structural analysis of the dityrosine-containing molecule may reveal the critical component of the spore wall.

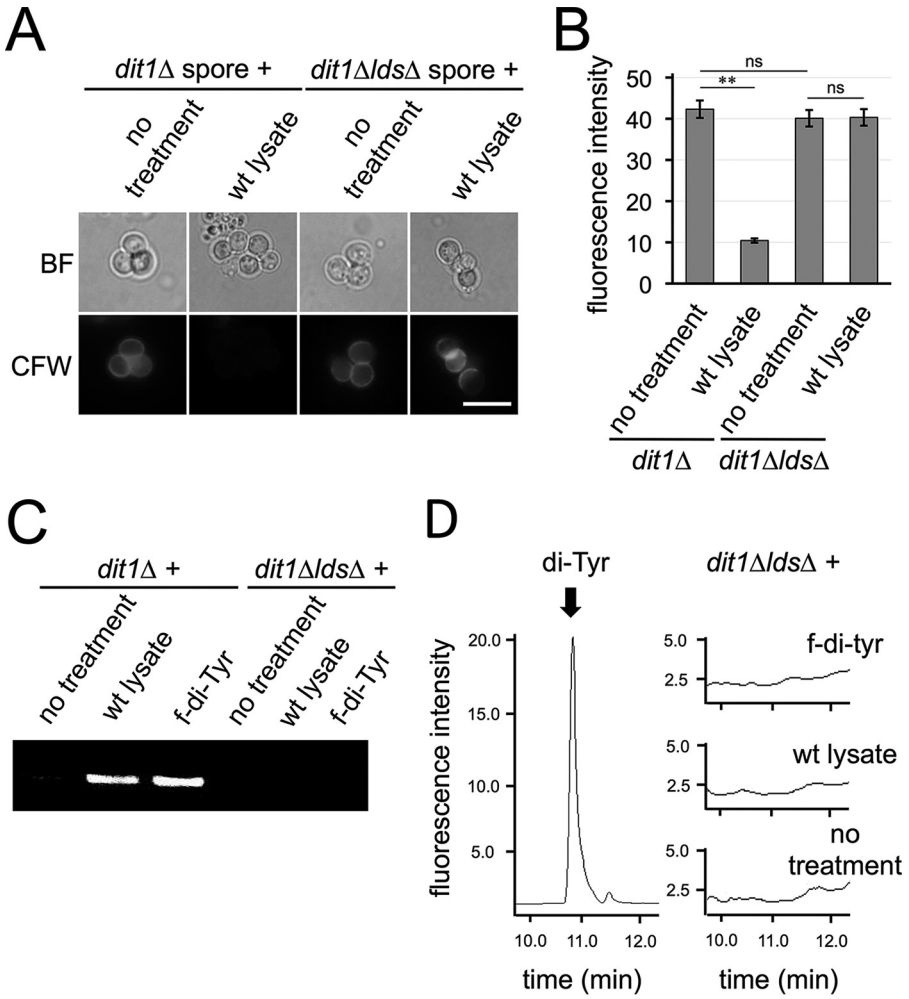

Figure 9. Effect of *lds*Δ mutations on the binding of dityrosine-containing molecules and bisformyl dityrosine to *dit1*Δ spores. *A*, *dit1*Δ or *dit1*Δ*lds*Δ spores incubated with wild-type ascal lysate were washed with 0.6 M NaCl and stained with CFW. Images were obtained by fluorescence (*CFW*) or bright-field (*BF*) microscopy. *dit1*Δ or *dit1*Δ*lds*Δ spores washed with 0.6 M NaCl (no treatment) were used as controls. *Scale bar*, 5 μm. *B*, quantification of the fluorescence intensities of CFW of the spores described in *A*. Data presented are the mean of three independent samples. *Error bars* represent S.E. **, $p < 0.005$; *ns*, not significant. *C*, *dit1*Δ or *dit1*Δ*lds*Δ spores incubated with wild-type ascal lysate (*wt lysate*) or bisformyl dityrosine (*f-di-Tyr*) were washed with 0.6 M NaCl. The spores were hydrolyzed with 6 N HCl, and the lysates were subjected to SDS-PAGE. Hydrolytic lysates of *dit1*Δ or *dit1*Δ*lds*Δ spores washed with 0.6 M NaCl (no treatment) were also subjected to SDS-PAGE as controls. *D*, *dit1*Δ*lds*Δ spores incubated with wild-type ascal lysate (*wt lysate*) or bisformyl dityrosine (*f-di-Tyr*) were washed with 0.6 M NaCl, and hydrolysates were subjected to HPLC. Dityrosine (*di-Tyr*) was also subjected to HPLC as a control.

*dit1*Δ spores incubated with the ascal lysate became resistant to CFW staining. By contrast, this phenomenon was not observed with the spores incubated with bisformyl dityrosine. These results support the idea that modification of dityrosine is required to construct the proper spore wall. The dityrosine-containing molecules bind to the spore wall in two ways: hydrophobic and covalent interactions. The dityrosine-containing molecules bound hydrophobically can make *dit1*Δ spores resistant to CFW staining. Their hydrophobic interactions may obscure the binding site of CFW. The dityrosine-containing molecules covalently incorporated into the spore wall did not inhibit CFW staining. One explanation for this phenomenon is that, without modification, dityrosine molecules incorporated into the spore wall via the covalent interaction do not inhibit CFW staining.

Production of DL-dityrosine is a characteristic event that happens during dityrosine layer assembly (18, 19). Notably, a portion of LL-bisformyl dityrosine molecules bound to *dit1*Δ spores appeared to be isomerized to the DL-form. This result further supports the notion that the *in vitro* assay faithfully mimics the physiological assembly process. D-Amino acids have been found as components of the bacterial cell wall; they are produced by an enzymatic reaction (25). If the isomerization of dityrosine is mediated by such an enzyme, it should be localized to the spore wall, although no such enzyme has yet been identified. As an alternative possibility, the isomerization may occur via a non-enzymatic reaction. Such an example has been reported for aspartic acids in human α-crystallin (26). In this model, the isomerization may be dependent on the ambient condition in the spore wall because the DL-form of dityrosine is not found in the spore cytosol (18). DL-Dityrosine was detected from the dityrosine-containing molecules in wild-type as well as *chs3*Δ ascal lysate. Thus, it is a covalent association that involves change in the chirality.

Requirements for the incorporation of dityrosine into the spore wall

In our experimental condition, bisformyl dityrosine, but not dityrosine, was incorporated into *dit1*Δ spores. Thus, the

formyl groups are required for the interaction. Apart from the formyl groups, no other modification to dityrosine is required for the interaction. Bisformyl dityrosine did not bind to *dit1*Δ*chs3*Δ or *dit1*Δ*lds*Δ spores. These results are in good agreement with previous genetic and cytological data, suggesting that the *in vitro* system faithfully reproduces at least some aspect of normal wall assembly.

The chitosan layer is required for the incorporation of dityrosine to the spore wall in the *in vitro* system. However, dityrosine-containing molecules did not bind tightly to pure chitosan particles. Thus, chitosan is not sufficient for the reaction. Another component that is required for the incorporation is proteins associated with the spore wall, including Lds proteins. *lds*Δ mutations abolish the binding of bisformyl dityrosine to the spore wall despite the presence of the chitosan layer. During the course of spore formation, Lds proteins are colocalized with a subset of lipid droplets that attach to the outside of the prospore membrane or spore wall (20). Although the function of Lds proteins remains to be determined, our results clarify that they are required for attachment of bisformyl dityrosine to the chitosan layer. Proteinase K treatment as well as detergent wash of *dit1*Δ spores also blocked incorporation. One explanation for this phenomenon is that Lds proteins were liberated from the spore wall by these treatments. However, we cannot rule out the possibility that the Lds proteins are more indirectly involved in dityrosine layer assembly. It is possible that other components of the lipid droplet or spore wall might be required for the process. Previous studies have identified several other proteins that are localized to the spore wall and involved in dityrosine layer formation (20, 21). Further investigations using this binding assay could reveal whether such proteins are involved in this process.

Possible mechanism of the dityrosine layer assembly in the spore wall

Because the cell wall is generally constructed in the extracellular space, small energy-carrying molecules, such as ATP, are not used for assembly of cell wall materials (27, 28). Thus, it is possible that the dityrosine layer is created without using small energy-carrying molecules. In accordance with this concept, bisformyl dityrosine was incorporated into *dit1*Δ spores without any soluble cytosolic materials. How then are bisformyl dityrosine molecules covalently attached to the chitosan layer and cross-linked to form the dityrosine layer? In several organisms, phenolic compounds are used as cross-linkers to produce macromolecules in the cell wall or extracellular matrices. Examples of such phenolic compounds include monolignols in plant cells (29), dihydroxy-L-phenylalanine in the squid beak (30), and tyrosine of the fertilization envelope in sea urchin eggs (31). As a common mechanism, these phenolic compounds are cross-linked to each other or to other molecules via oxidation. In these reactions, the oxidation is mediated by extracellular oxidases. Thus, a similar oxidation mechanism may be utilized to assemble the dityrosine layer. If so, the spore wall should include an enzyme that can oxidize bisformyl dityrosine. In this regard, it is intriguing that a previous screening identified a multicopper oxidase family gene as a possible candidate involved in dityrosine layer formation (20), although its role in the process has not yet been studied.

As mentioned earlier, the formyl group is required to link dityrosine to *dit1*Δ spores in our assay. Further investigations are needed to determine whether this linkage is mediated by an oxidative reaction. A previous study suggested that the formyl group was involved in linking dityrosine molecules either directly or indirectly (8). However, the same study also suggested that dityrosine molecules could be linked to the spore wall without using the formyl groups. Thus, the dityrosine layer may be assembled via several different linkages. Our *in vitro* binding assay may only reproduce the formyl group-dependent linkage.

Fig. 10 shows a possible model of dityrosine layer formation to summarize the present study. The dityrosine-containing molecules are produced as a precursor for construction of the dityrosine layer probably in the ascal cytosol. DL-Bisformyl dityrosine could be formed during this process (Fig. 10*A*). The dityrosine-containing molecules bind to the chitosan layer via hydrophobic interaction so that spores become resistant to the CFW staining. Some spore wall-resident protein is involved in their binding (Fig. 10*B*). The dityrosine-containing molecules can be covalently incorporated onto the chitosan layer, which is mediated by some protein (enzyme) attached to the spore wall. A portion of the dityrosine-containing molecules remain in the ascal cytosol without linking to the spore wall (Fig. 10*C*).

Our *in vitro* assay can be used to investigate further components involved in dityrosine layer formation. Apart from the biological interest, assembly of the dityrosine layer may be intriguing from the point of view of material science. Given that bisformyl dityrosine is linked to the chitosan layer, it may be applied to modify or cross-link chitosan-derived materials.

Experimental procedures

Yeast strains

Yeast strains and oligonucleotide primers used in this study are listed in Tables 3 and 4, respectively. AN120 (32) was used as the wild-type strain. To construct the *dit1*Δ*chs3*Δ double mutant (HW193), a DNA fragment to disrupt *CHS3* was amplified by PCR using pFA6a-KanMX6 (33) as a template and HXO 505 and HXO 506 as primers. This fragment was transformed into the *dit1*Δ haploids (34), *chs3*Δ deletions were confirmed by PCR, and the resulting strains were mated to generate the diploid *dit1*Δ*chs3*Δ cells. The *lds1*Δ*lds2*Δ*rrt8*Δ*dit1*Δ mutant (HW325) was constructed in a similar way. *LDS1* was disrupted using pFA6a-HIS3MX6 (33) as a PCR template, and HXO 619 and HXO 620 were used as primers. *LDS2* and *RRT8* are neighboring genes, and they were disrupted simultaneously by using pFA6a-KanMX6 as a template and HXO 623 and HXO 624 as primers. ANA262 (35) was used as a *chs3*Δ mutant.

Yeast culture and sporulation

Yeast culture and sporulation were performed as described previously (36). Briefly, yeast cells derived from a single colony were grown overnight in 5 ml of YPAD liquid medium (1% yeast extract, 2% peptone, 2% dextrose, 0.003% adenine), and then 1 ml of the culture was added into 200 ml of YPA medium (1% yeast extract, 2% peptone, 2% potassium acetate) and grown for

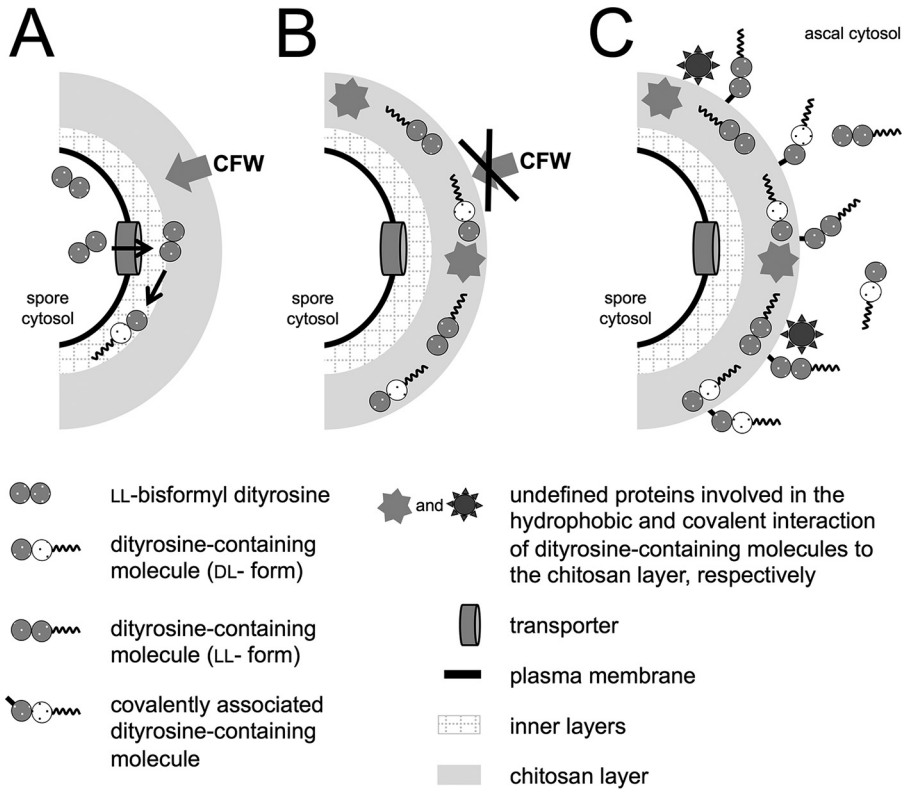

Figure 10. Possible model of dityrosine layer formation. Refer to the text for details. *A*, transport and modification of bisformyl dityrosine. *B*, hydrophobic binding of the dityrosine-containing molecules to the chitosan layer. *C*, covalent incorporation of the dityrosine-containing molecules to the chitosan layer.

Table 3
***S. cerevisiae* strains used in this study**

Strain	Genotype	Source/Ref.
AN120	*MATα/MAT***a** *ARG4/arg4-NspI his3ΔSK/his3ΔSK ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1::hisG/trp1::hisG ura3/ura3*	32
AN117-4B	*MATα ura3 leu2 trp1 his3ΔSK arg4-NspI lys2 ho::LYS2 rme1::LEU2*	32
AN117-16D	*MAT***a** *ura3 leu2 trp1 his3ΔSK lys2 ho::LYS2*	32
AN262 (*chs3*Δ)	*MATα/MAT***a** *ARG4/arg4-NspI his3ΔSK/his3ΔSK ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1::hisG/trp1::hisG ura3/ura3 chs3Δ::his5*$^+$*/chs3Δ::his5*$^+$	35
HW3 (*dit1*Δ)	*MATα/MAT***a** *ARG4/arg4-NspI his3ΔSK/his3ΔSK ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2RME1/rme1::LEU2 trp1::hisG/trp1::hisG ura3/ura3 dit1Δ::his5*$^+$*/dit1Δ::his5*$^+$	34
HW193 (*dit1*Δ*chs3*Δ)	*MATα/MAT***a** *ARG4/arg4-NspI his3ΔSK/his3ΔSK ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1::hisG/trp1::hisG ura3/ura3 chs3Δ::his5*$^+$*/chs3Δ::his5*$^+$ *dit1Δ::his5*$^+$*/dit1Δ::his5*$^+$	This study
HW325 (*lds1*Δ *lds2*Δ *rrt8*Δ *dit1*Δ)	*MATα/MAT***a** *ARG4/arg4-NspI his3ΔSK/his3ΔSK ho::LYS2/ho::LYS2 leu2/leu2 lys2/lys2 RME1/rme1::LEU2 trp1::hisG/trp1::hisG ura3/ura3 lds1Δ::his5*$^+$*/lds1Δ::his5*$^+$ *lds2Δrrt8Δ::kanMX6/lds2Δrrt8Δ::kanMX6*	This study

Table 4
Oligonucleotide primers used in this study

Name	Sequence
HXO 505	CCATTTTCTTCAAAGGTCCTGTTTAGACTATCCGCAGGAAAGAAATTAGACGGATCCCCGGGTTAATTAA
HXO 506	CAACCATATATCAACTTGTAAGTATCACAGTAAAAATATTTTCATACTGT GAATTCGAGCTCGTTTAAAC
HXO 619	ATAGTGTAGGGAACAAAGGTACATTATACAAACAATAACAACAACAGGAACGGATCCCCGGGTTAATTAA
HXO 620	ATAGCTAGGTAATTTTAATCTGGGGAGAGAAATGGTGAACTTTTTTCAATGAATTCGAGCTCGTTTAAAC
HXO 623	TCTTAGTTATAACATTAGAACTATAAGAGCTCCTAAGACACCAAGCAAACCGGATCCCCGGGTTAATTAA
HXO 624	TAGTTAAGGAATATATAATCACACTTCTACATAAATTTGCAGTTTTAGGCGAATTCGAGCTCGTTTAAAC

24 h. The cells were harvested by centrifugation, washed with sterilized water, resuspended in 100 ml of 2% potassium acetate medium, and cultured for 24 h.

Preparation of ascal lysates

Yeast spores cultured for 24 h in potassium acetate medium were used to prepare ascal lysate. 1.5 g (wet weight) of asci were suspended in 10 ml of phosphate-buffered saline (PBS) and sonicated on ice with a probe-type sonicator (Sonics, Newtown, CT) for 1.5–2 h. Individual spores and debris were removed by centrifugation (21,800 × *g*, 30 min). The ascal lysates were kept at 4 °C for further tests.

CFW staining and quantification

52 mg (wet weight) of spores were suspended in 200 μl of distilled water, and 20 μl of CFW (1 mg/ml) were added (Sigma-Aldrich). The mixture was incubated at 30 °C for 30 min, washed twice with sterile water, and resuspended in 1 ml of

distilled water. The fluorescence quantification was performed with a Synergy H4 Hybrid multimode microplate reader (BioTek Instruments, Winooski, VT) with 380-nm excitation and 475-nm emission. For quantification, spore suspensions were diluted 20-fold in distilled water, and 200 μl of the diluted suspension were used for measurement.

Synthesis of LL-dityrosine and N,N′-bisformyl-LL-dityrosine

LL-Dityrosine and LL-*N*,*N*′-bisformyl dityrosine were synthesized by the oxidative reaction of L-tyrosine or *N*-formyl-L-tyrosine (Sigma-Aldrich) as described before (37). For this, 2 ml of Tris-HCl (0.3 M, pH 8.5), 1.5 ml of L-tyrosine or *N*-formyl-L-tyrosine (2 mg/ml), 0.1 ml of hydrogen peroxide (0.003%), and 0.5 ml of horseradish peroxidase (1 mg/ml; Sangon, Shanghai, China) were mixed and incubated at 20 °C for 1 h. LC-MS was performed to verify that LL-dityrosine and LL-*N*,*N*′-bisformyl dityrosine were synthesized.

Preparation of chitosan particles

Chitosan particles were prepared as described (38) with some modifications. Commercial chitosan (Sigma-Aldrich) was dissolved (3 mg/ml) in 5% acetic aqueous solution. Sodium tripolyphosphate was dissolved in distilled water at a concentration of 1 mg/ml. 5 ml of the chitosan solution were added drop-by-drop to 5 ml of sodium tripolyphosphate solution under magnetic stirring (1,000 rpm, 60 min) at room temperature. Finally, the particles were separated by centrifugation at 20,000 × *g* for 15 min and washed twice with distilled water. The chitosan particles were resuspended with 5 ml of distilled water and kept at 4 °C for further analysis.

Dityrosine-binding assay

To release spores from asci, the ascal wall was first digested by β-glucanase (lyticase; Sigma-Aldrich). For ascal wall digestion, ascospores were suspended in 10 ml of spheroplast buffer (50 mM potassium phosphate buffer, pH 7.5, 1.4 M sorbitol, 40 mM β-mercaptoethanol) and mixed with 100 μl of β-glucanase stock solution (1 mg of β-glucanase dissolved in 1 ml of 50% glycerol). After 1.5-h incubation at 37 °C, asci were sonicated briefly and washed twice with water containing 0.6 M NaCl or 0.5% Triton X-100.

For the binding assay, 52 mg of spores released from asci or chitosan particles were suspended in 1 ml of ascal lysate or dityrosine or LL-*N*,*N*′-bisformyl dityrosine solutions. The suspensions were incubated at 30 °C with shaking (220 rpm) for 24 h. After the incubation, spores were collected by centrifugation at 4,770 × *g* for 1 min and washed with water containing 0.6 M NaCl and/or 0.5% Triton X-100.

Hydrolytic liberation of dityrosine

Dityrosine was released from the spore wall or dityrosine-containing molecules by the method described (18). Briefly, after incubation in a dityrosine source, 52 mg (wet weight) of spores were suspended in 200 μl of 6 N HCl. The tubes were incubated at 95 °C for 5 h with an open lid. The dried hydrolysates were resuspended with 200 μl of distilled water, spun down by centrifugation at 21,800 × *g* for 5 min, and passed through a 0.45-μm microfilter. For hydrolysis of liquid samples, ascal lysates were frozen in a −80 °C freezer for 30 min and then freeze-dried in an Eyela FD-1000 freeze dryer (Tokyo Rikakikai, Tokyo, Japan) at −50 °C for 24 h under a pressure of 25 pascals. Acidic hydrolysis was then performed as described above.

Detection of dityrosine by SDS-PAGE

5 μl of hydrolysates were loaded on a 10% acrylamide gel. The hydrolysate of wild-type spores was diluted 20 times with water. 10 μl of synthesized LL-*N*,*N*′-bisformyl dityrosine or dityrosine were used as controls. After electrophoresis, the gel was directly observed under UV light. Images were captured by a GelDoc imager (Bio-Rad). ImageJ software (ImageJ 1.48V, Wayne Rasband, National Institutes of Health) was used to measure the fluorescence intensity of the dityrosine signals.

HPLC analysis of dityrosine

The samples were analyzed with a Discovery C_{18} column (150 mm × 4.6-mm inner diameter, 5-μm particles) (Sigma-Aldrich) using a Waters separation module e2695 HPLC system. 10 μl of samples were loaded. The column was developed with a gradient of CH_3CN in 0.01 M trifluoroacetic acid (0–50% CH_3CN in 55 min). The flow rate was 1 ml/min. 285-nm excitation and 425-nm emission were used for detection.

To quantify LL-dityrosine, chemically synthesized LL-dityrosine was purified by HPLC as described above, and standard solutions (1, 10, 30, 50, 80, and 100 μg/ml) were prepared. These standards were applied to HPLC, and a calibration curve was generated by plotting their peak area. The concentration of LL-dityrosine in the samples was determined based on this calibration curve.

Microscopy images

Microscopy images were obtained using a Nikon Eclipse Ti-E inverted microscope equipped with a DS-Ri camera and NIS-Element AR software (Nikon, Tokyo, Japan).

Microscopic quantification of dityrosine on the spore wall

Quantitation of dityrosine fluorescence was performed as described before (21). Spores were suspended in 5% NH_4OH to raise the pH. A Zeiss Axio-Observer Z1 microscope (Oberkochen, Germany) equipped with a Hamamatsu ER-G camera and a customized dityrosine filter (excitation, 320 nm; emission, 410 nm) (Omega Optical, Brattleboro, VT) were used for this measurement. Images were collected, and fluorescence intensity at the spore periphery was measured using Zeiss Axiovision software (version 4.7). To calculate fluorescence values, fluorescence intensity was measured at two points along the edge of a spore, and fluorescence intensity at a point just outside the spore was measured as a background control. The background value was subtracted from the average of the values obtained from the spore periphery to generate a fluorescence level for each spore.

Proteinase K treatment

52 mg of wet spores were suspended in 1 ml of Tris-HCl (30 mM, pH 8.0), and 200 μl of proteinase K (1 mg/ml) were added. The mixture was incubated at 37 °C for 2 h (for Fig. 6, *A* and *B*) or the indicated times (for Fig. 6, *C*, *D*, and *E*). The spores were collected by centrifugation at 4,770 × *g* for 1 min and washed twice with sterile water. For the ascal lysate, 1 ml of the lysate

described above was freeze-dried. The lyophilized powder was treated with the same conditions as the wet spores. After the incubation, the suspension was heated at 95 °C for 1 h to stop the reaction, and then the suspension was freeze-dried again and resuspended in 1 ml of PBS.

Statistics

Data presented are the mean ± S.E. of the indicated numbers of independent samples. Statistical significance was determined with Student's *t* test (two-tailed, heteroscedastic) using Microsoft Excel software. Differences between the analyzed samples were considered significant at $p < 0.05$.

Author contributions—H. N. and X.-D. G. designed the experiment. H. N., A. M. N., and L. D. B. wrote the manuscript. L. D. B. and O. M. performed experiments. Z. L. provided technical assistance.

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