JOURNAL OF BACTERIOLOGY, Sept. 1987, p. 4384–4387
0021-9193/87/094384-04$02.00/0

Vol. 169, No. 9

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Tunicamycin Inhibition of Epispore Formation in *Saccharomyces cerevisiae*

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Received 17 April 1987/Accepted 3 June 1987

The ascopore wall of *Saccharomyces cerevisiae* was found to contain more protein, polymeric glucosamine, and β-glucan than the vegetative cell wall, which was enriched in mannoprotein relative to ascospore walls. Tunicamycin inhibited sporulation, as judged by the absence of refractile ascospores visible by phase-contrast microscopy, but cells completed meiosis, as demonstrated by the presence of multinucleate asci. Such spores lacked the dense outer layer characteristic of normal spores. Thus, the tunicamycin effect was similar to that of glucosamine auxotrophy (W. L. Whelan and C. E. Ballou, J. Bacteriol. 124:1545–1557, 1975).

The ascospore wall of *Saccharomyces cerevisiae* consists of an inner electron-transparent layer, the endospore, and an outer electron-dense layer, the epispore (3). Before ascospore wall formation, a double membrane envelopes each of the four meiotic nuclei in the ascus, and it is between these membranes that the ascospore wall is formed (7). Both membranes are in contact with the endospore, which is formed first, but only the outer membrane is contiguous with the epispore at the time of its later appearance (17). This fact suggests a difference in the synthetic activities of the ascospore-delimiting membranes. Several sporulation-specific RNAs that appear coincidentally with the formation of the ascospore wall may encode wall proteins (14, 15).

The inner layer of the ascospore wall is composed partly of glucan and mannan (13). A glucosamine auxotroph sporulates but fails to produce the epispore layer (23), which implicates this hexosamine as an important component. Lipids may also be an important spore wall component, because myo-inositol auxotrophs sporulate poorly and produce ascospores that also lack the outer wall layer (21). The epispore layer is also believed to contain protein (5, 19), and protein cross-linking is suggested from the isolation of dityrosine from a spore coat hydrolysate (6). In this study, we investigated the yeast ascospore wall composition and assessed the effects of tunicamycin on sporulation and ascospore wall formation.

Tunicamycin was from Calbiochem-Behring and Zymolyase 5000 was from the Kirin Brewery. Other reagents were from Sigma Chemical Co. and Polysciences, Inc. *S. cerevisiae* Y55 (4) cells were grown in 2% glucose–2% peptone–1% yeast extract to stationary phase at 30°C, harvested at 5°C, and washed in 0.1 M potassium acetate (pH 7.0). The pellet was suspended in 10 liters of 0.1 M potassium acetate (pH 7.0) with 100 ml of sterile-filtered tetracycline hydrochloride (0.2 mg/ml) and maintained at 30°C with 5.0 liters of aeration per min, and the asci that formed were harvested after 4 days. Asci (1 g) were suspended in 50 mM sodium phosphate (pH 7.5) containing 2.5 mg of Zymolyase 5000 per ml and incubated at 30°C for 60 min, and the crude ascospores were collected by centrifugation, washed twice in 0.1 M Tris (pH 8.5), and treated in a Braun homogenizer. The spore walls were collected and washed by sonication 10 times in the buffer described above containing 2.0 M KCl. This treatment was followed by 10 washes in 0.1 M Tris (pH 8.5) and then 5 washes in distilled water. Vegetative walls were prepared from stationary-phase cells as described elsewhere (11).

Protein was determined (20) with bovine serum albumin as a standard on samples that were boiled for 20 min in 1.0 M NaOH. Neutral hexose was determined by the phenol-sulfuric acid method (8). Glucosamine (12) was determined on samples hydrolyzed in 6 M HCl for 8 h at 105°C. To determine mannose-glucose ratios, samples were hydrolyzed in 2.0 M trifluoroacetic acid for 2 h at 110°C, and the sugars were analyzed as alditol acetates (1).

In studies with tunicamycin, cells were grown in 0.1 M potassium acetate–2% peptone–1% yeast extract (pH 5.5) to a density of 4×10^7 cells per ml and suspended in sporulation medium plus tunicamycin at the same density. Sporulation was carried out at 30°C in 10-ml flasks containing 2 ml of culture and shaken at 250 rpm. The percent sporulation was determined as described elsewhere (10).

Photographs were taken on a Zeiss Photomicroscope III. Mithramycin staining and acridine orange counterstaining were done as reported elsewhere (22). For transmission electron micoscopy, specimens were fixed with glutaraldehyde and osmium tetroxide, dehydrated, and then embedded in resin. Samples were sectioned and stained with uranyl acetate and lead citrate before they were examined.

Differences in vegetative and ascospore wall composition. The most striking differences between ascospore and vege-

TABLE 1. Composition of vegetative and ascospore cell walls

Component	% in:	
	Vegetative walls	Ascospore walls
Protein	14	20
Glucose	29	55
Mannose	59	14
Glucosamine	1.4	15

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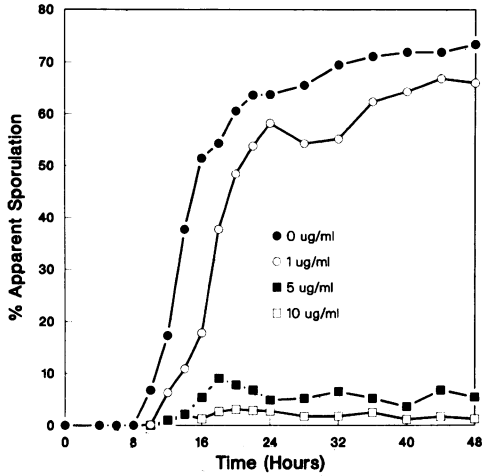

FIG. 1. Effect of tunicamycin on sporulation. Cells were harvested from growth medium, washed, and suspended in 0.1 M potassium acetate at the tunicamycin concentrations shown in the figure. Percent apparent sporulation was determined by the appearance of refractile ascospores under phase-contrast microscopy.

tative cell walls were in the content of glucosamine, which was 10 times higher in ascospore walls than in vegetative walls, and the ratio of glucose to mannose, which was high in ascospore walls and low in vegetative walls (Table 1). Most of the mannose was solubilized by treatment with Zymolyase, which is characteristic of mannoproteins (3). We conclude that the spore wall is enriched in glucan and a glucosamine-containing polymer, whereas the vegetative wall is enriched in mannoprotein (3).

Inhibition of epispore coat formation but not meiosis by tunicamycin. Tunicamycin inhibits the formation of asparagine-linked carbohydrate chains of yeast mannoproteins (16) and arrests cell division in vegetative cultures (2). Tunicamycin at 1 μg/ml had little effect on sporulation, whereas 5 μg/ml almost completely inhibited the process (Fig. 1).

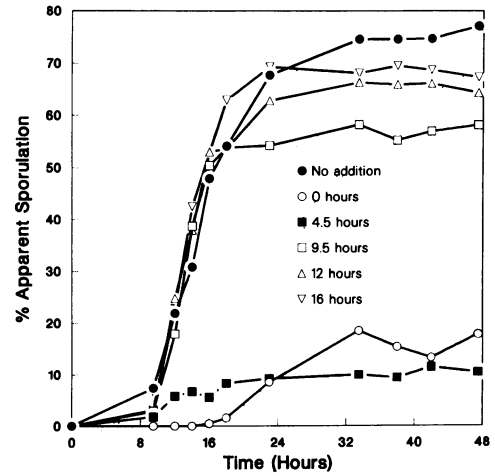

FIG. 2. Effect of the time of addition of tunicamycin on sporulation. Logarithmic-phase cells were suspended in 0.1 M potassium acetate. At the times indicated in the figure, portions of the culture were transferred to 0.1 M potassium acetate containing 10 μg of tunicamycin per ml. Percent apparent sporulation was determined as described in the legend to Fig. 1.

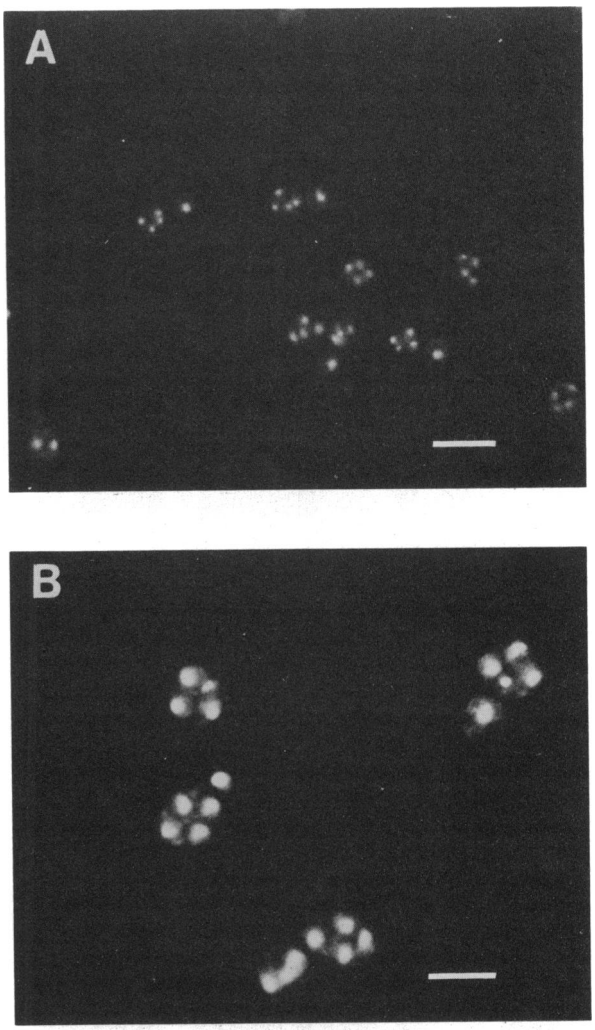

FIG. 3. Nuclear staining of cells sporulated in the presence of tunicamycin. Cells were sporulated for 24 h with 10 μg of tunicamycin per ml and then stained in mithramycin, counterstained in acridine orange, and examined by fluorescence microscopy at low and high magnifications. (A) Bar, 10 μm. (B) Bar, 6 μm.

Addition of tunicamycin at 9.5 h after the initiation of sporulation had little effect (Fig. 2), but addition as late as 4.5 h after the cells were placed on sporulation medium was as effective as addition at zero time, and the effect was not reversed by washing the cells in sporulation medium alone. Thus, tunicamycin acts before asci appear, 8 to 10 h before in this strain (4), and at later times the sporulating cells no longer appear to be susceptible even though the number of asci continues to increase for 24 h.

Tunicamycin-treated cells lacked refractile ascospores under phase-contrast microscopy, but staining with mithramycin revealed multinucleate asci, so meiosis had occurred (Fig. 3). Examination of the culture by thin-section electron microscopy revealed that the asci contained spores that lacked the outer wall layer and were easily deformed (Fig. 4A). In contrast, asci from untreated cultures contained rigid spherical spores with a densely stained outer wall layer (Fig. 4B).

The finding that the yeast ascospore wall is enriched in glucosamine relative to vegetative cells agrees with earlier

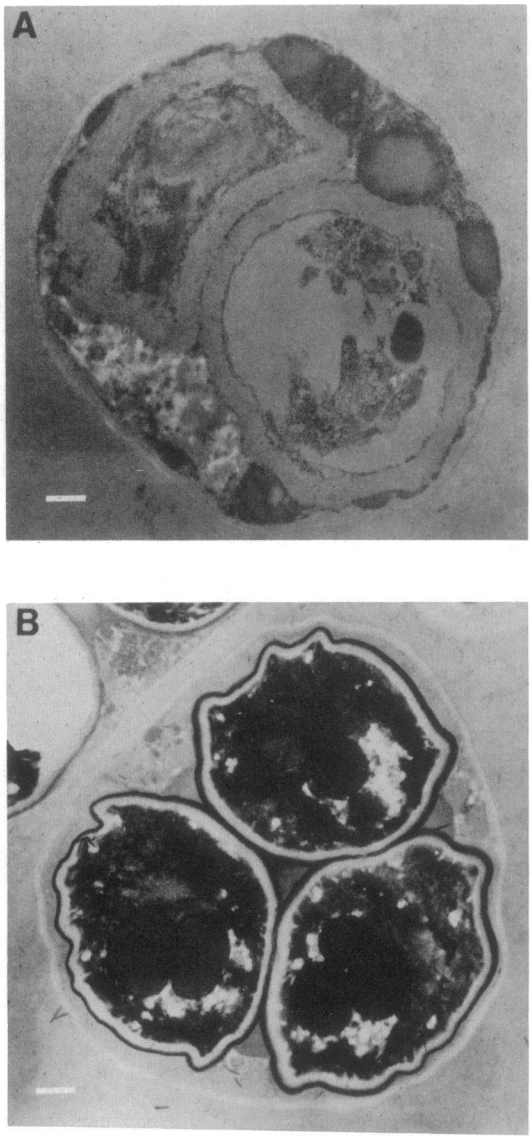

FIG. 4. Electron micrographs of tunicamycin-treated cells. Cells sporulated in the presence of tunicamycin (10 μg/ml) (A) and in its absence (B) were examined by electron microscopy. Bar, 0.25 μm.

studies that revealed an enhanced synthesis of glucosamine coincident with spore wall formation (4). A recent report (P. Briza, G. Winkler, A. Ellinger, and M. Breitenbach, Abstr. 12th Int. Conf. Yeast Genet. and Mol. Biol., 1984, p. 273) of a chitinous layer in the ascospore wall is also consistent with a high glucosamine content. Conversely, enrichment in glucan content, relative to that in vegetative walls, suggests that mannoprotein synthesis and deposition are relatively more active in the vegetative cell.

The tunicamycin effect, which is exerted before the appearance of mature spores, cannot be demonstrated when the drug is added to the sporulation medium after 9.5 h, by which time the first mature spores have appeared. Formation of mature asci continues for at least 24 h, so the synthesis of spore walls must also continue for some time. Therefore, the inhibition in synthesis of the epispore may result from an earlier block that affects the synthesis of a protein or a precursor pool of wall components. The ineffectiveness of tunicamycin addition after 9.5 h could also reflect a reduced uptake of the drug, since by that time the ascospore membranes that have formed might be impermeable. The pH change that occurs in a sporulating culture (10), however, should not interfere with the uptake of the inhibitor because similar effects have been obtained with a strongly buffered medium (18). Some tunicamycin isomers inhibit protein synthesis (9), but the cells should not sporulate at all if general protein synthesis is altered. Thus, a specific inhibition of synthesis of the inducible glucosamine synthetase or interference with another step of epispore polymer formation may be the site of tunicamycin action.

The effect of tunicamycin on ascospore wall synthesis is reminiscent of the effect of glucosamine auxotrophy, in which nonrefractile ascospores showing normal nucleation by Giemsa staining but lacking the epispore layer are produced (23). Spores from the glucosamine auxotroph are readily digested by β-glucanase, in contrast to wild-type spores, which demonstrates that the epispore protects the underlying glucan layer. Thus, tunicamycin may interfere with a glucosamine-dependent step required for synthesis of the epispore.

These studies were supported by Office of Naval Research grant 85-K-0363, National Science Foundation grant DMB-8400251, and Public Health Service grant AI-12522 from the National Institutes of Health.

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