

FULL PAPER

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## Novel actin ring structure in sporulation of *Zygosaccharomyces rouxii*

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**Abstract** The filamentous actin (F-actin) during sporulation of *Zygosaccharomyces rouxii* was visualized with rhodamine-phalloidin, and then the behavior was observed using confocal laser scanning microscopy. During spore formation, we found a novel actin ring structure that has not been reported in other yeasts and molds in sporulation. The ring surrounded each meiotic nucleus at the peripheral regions of spores. Three-dimensional observation suggested that the ring was not an artificial structure produced by spherical structure sectioning. The period and location of the ring's appearance suggest that the ring may have some relation to the spore membrane or wall development. In addition, this ring structure was more stable than other F-actin structures against latrunculin A, an F-actin disrupting agent.

**Key words** Actin ring · Filamentous actin · Latrunculin A · Sporulation · *Zygosaccharomyces rouxii*

### Introduction

Filamentous actin (F-actin) is an essential cytoskeletal protein that plays an important role in the cell division of yeasts. Vigorous studies have been made to clarify the structural changes and functions of F-actin that correspond to the various stages of cell division (reviewed by Pruyne and Bretscher 2000; Schott et al. 2002). Regarding meiosis and subsequent spore formation, several F-actin structures have been reported. Smith et al. (1995) observed actin patches and cables that developed in the cortical region of *Saccharomyces cerevisiae* Meyen ex E.C. Hansen during the early stages of meiosis. Using mutants with the actin gene, they

demonstrated that these F-actin patches and cables were closely related to the rearrangement of mitochondria. Doyle and Botstein (1996) showed that cortical actin patches remained in spores throughout spore formation. Petersen et al. (1998) and Toya et al. (2001) indicated that, in *Schizosaccharomyces pombe* Linder, F-actin appeared as concentrated dots at the projection tip during cell conjugation and karyogamy, around the nucleus during meiosis, and within the spores during spore maturation. In addition to these structures of F-actin, Thompson–Coffe and Zickler (1993, 1994) found a cage structure of F-actin in *Sordaria macrospora* Auersw and *Neurospora crassa* Shear & B.O. Dodge during their ascospore formation. They inferred that this cage structure was closely related to spore formation of these fungi because the cage enclosed the spore at the late stage of sporulation.

We investigated the behavior of F-actin during sporulation in an ascomycetous yeast *Zygosaccharomyces rouxii* (Boutroux) Yarrow to compare with that in other yeasts and molds which have already been reported. During that study, we discovered a novel ring structure that surrounded four nuclei resulting from the meiosis, but its function remains unclear. The present study describes the process of formation of this ring structure. Furthermore, the nature of this ring structure is discussed on the basis of the effect of latrunculin A, an F-actin-disrupting agent, on the ring.

### Materials and methods

Two strains of *Z. rouxii*  $\alpha$  (IFO 1876) and  $\alpha$  (IFO 1877), which differ from one another in the mating types, were used for the present study. NaCl was supplied to all media because vegetative growth is enhanced by the presence of NaCl (Watanabe and Takakuwa 1984) and because sporulation only occurs in environments containing NaCl (Wickerham and Burton 1960; Mori and Onishi 1967). First, *Z. rouxii*  $\alpha$  and  $\alpha$  were cultured separately in growth medium (YPD<sub>N</sub>; 1% yeast extract, 2% peptone, 2% glucose, and 5% NaCl) at 25°C. When both cultures reached

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the early stationary phase, we mixed the cells of  $\alpha$  and  $\alpha$  in sporulation medium (MEN; 5% malt extract and 5% NaCl). The mixture was placed onto an MEN agar plate because *Z. rouxii* sporulation was not induced in the liquid medium. After about 48 h, we were able to observe spores in a zygotic ascus.

Filamentous actin was stained by the modified method described by Balasubramanian et al. (1997). The cells under sporulation were fixed in 3.5% paraformaldehyde for 2 h at 25°C. After fixation and washing with phosphate-buffered saline, the cells were permeabilized in 1% Triton X-100 solution for 1 min and were washed immediately. Permeabilized cells were then incubated in 0.075  $\mu$ M rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) for 2 h at 25°C. The cells were washed and stained using 10  $\mu$ M TO-PRO 3 (Molecular Probes), a fluorescent dye to stain nuclear DNA, in Fluoroguard antifade reagent (Bio-Rad Laboratories, Hercules, CA, USA). Fluorescence images were then taken with a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany) equipped with a 100 $\times$  oil immersion objective (Zeiss Plan-Neofluar; NA = 1.3). Rhodamine fluorescence was excited and detected using a 543-nm He/Ne laser and a 560- to 615-nm bandpass emission filter, respectively. TO-PRO3 fluorescence was excited and detected using a 633-nm He/Ne laser and a 650-nm long-pass emission filter, respectively. Images were taken as optical sections (0.2 to 0.3- $\mu$ m intervals) through the entire cell and then projected onto a single image. Although TO-PRO3 exhibited deep red fluorescence ordinarily, the color was converted to light blue to distinguish it from rhodamine fluorescence. Differential interference contrast (DIC) images were taken simultaneously. To show the three-dimensional structure of F-actin in detail, the acquired optical section images were reconstructed to three-dimensional rotation images. Then, the reconstructed images were observed from various angles (30° intervals) by rotation. All images were converted into TIFF files and processed with Adobe Photoshop 7.0.

In the treatment of latrunculin A (Lat A), the cells under the sporulation on an MEN agar plate were suspended in MEN broth. Then, Lat A (Wako Pure Chemical, Tokyo, Japan) dissolved in dimethyl sulfoxide (DMSO) was added to the suspension so that the final concentration of Lat A became 200  $\mu$ M. These cells were incubated for 5 and 15 min after Lat A addition at 25°C and stained by rhodamine-phalloidin and TO-PRO3. Images of the stained cells were then acquired by confocal laser scanning microscopy as already described. The parameters used for the creation of an image were kept uniform to compare rhodamine brightness among images of different incubation times.

## Results and discussion

Figure 1 shows the process of sporulation of *Z. rouxii*. Because the cell cycle was not synchronized, cells of various stages were observed at the same time using confocal microscopy observation. The stages of cells were determined

from nucleus behavior and DIC images. Actin dynamics throughout sporulation were followed by visualization with rhodamine-phalloidin. Shmoo, a peculiar structure for the conjugation, proliferated from a cell of each mating type to that of another mating type (Fig. 1.1). We easily recognized that dense actin patches are localized at the growing tip of the shmoo (Fig. 1.3). After conjugation, the two nuclei came close to each other through the isthmus of the zygote (Fig. 1.6); many actin patches occurred at the isthmus region (Fig. 1.7). Thereupon, these nuclei became a single nucleus through karyogamy, creating a diploid nucleus (Fig. 1.10). In this step, relatively small actin patches seemed to be scattered all over the zygote and the actin cables expanded along the longitudinal cell axis (Fig. 1.11). As the nucleus divided into four (Fig. 1.14), actin patches disappeared and faint F-actin distributed all over the zygote (Fig. 1.15). When the nucleus division finished (Fig. 1.18), bright actin dots appeared near each nucleus (Fig. 1.20). The bright dots of F-actin around each nucleus became more numerous (Fig. 1.24). When the spore shape became visible in the DIC image (Fig. 1.25), ring structures of F-actin appeared around each nucleus (Fig. 1.28). Observations of DIC and F-actin images suggest that the rings usually appear at the peripheral region of spores without changing their diameter dynamically. In addition to the ring structure, a few actin patches were located near the rings. With spore maturation (Fig. 1.29), the ring structures disappeared and actin patches increased (Fig. 1.31).

The ring structure is not a section of spherical structure of F-actin. It was shown to be a prominent ring structure because images were taken as optical sections (0.2 to 0.3- $\mu$ m intervals) through the entire cell and then projected onto a single image using confocal microscopy. Furthermore, three-dimensional images also indicate this fact. Figure 2 shows three-dimensional images of the actin ring viewed from various angles. These images suggest that the ring structure is not an artifact caused by observation of the single section of the images (Fig. 2).

The role of the actin ring is obscure. The period and location of the appearance of the ring suggest that the ring may be related to spore membrane or wall development. Alternatively, the ring may have the function of arranging mitochondria around spore nuclei. Miyakawa et al. (1984) found that mitochondria distribute as a ring structure in the sporulation of *S. cerevisiae*. The position of the mitochondrial ring appears to be consistent with the observed actin ring in *Z. rouxii*.

The actin ring that occurred during sporulation seems to be characteristic of *Z. rouxii*. So far, an actin ring structure in yeasts has been shown in mitosis, but not in sporulation. In vegetatively growing cells, the actin ring appears at the division site in *S. cerevisiae* (Kilmartin and Adams 1984; Epp and Chant 1997; Bi et al. 1998; Lippincott and Li 1998) and *S. pombe* (Marks and Hyams 1985; Kitayama et al. 1997; Arai and Mabuchi 2002). These actin rings, which include myosin II protein, serve to separate these cells by constricting the ring (cytokinesis). The ring structure observed in the present study is distinct from them in both period of occurrence and function. In filamentous ascomyc-

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