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FULL PAPER

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Saccharomyces cerevisiae forms actin ring structures in sporulation, similarly to *Zygosaccharomyces rouxii*

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Abstract Yeast filamentous actin (F-actin) exists mainly as patches and cables. Previously, we investigated the behavior of F-actin during sporulation of *Zygosaccharomyces rouxii* and found a novel actin ring localized around the spore periphery in zygotic asci at a late stage of sporulation. To clarify whether the actin rings are also formed in sporulation in the model yeast *Saccharomyces cerevisiae*, we observed the distribution of F-actin in sporulating *S. cerevisiae* by rhodamine-phalloidin staining and confocal laser scanning microscopy. Ringlike actin structures were detected at the peripheral regions of *S. cerevisiae* spores in globose asci. When asci of *S. cerevisiae* were induced to become zygotic, actin rings were more obvious than those in globose asci. These results indicate that *S. cerevisiae* forms characteristic actin ring structures at a late stage of sporulation, similarly to *Z. rouxii*.

Key words Actin ring · Filamentous actin · *Saccharomyces cerevisiae* · Sporulation · *Zygosaccharomyces rouxii*

Introduction

The actin cytoskeleton undergoes dynamic changes in form and distribution according to various morphological events. The actin cytoskeleton of yeast cells consists of two distinct filamentous actin (F-actin) structures: patches and cables. During vegetative growth, actin patches are located near the plasma membrane, where they are thought to be involved in endocytosis and construction of the cell wall. Actin cables are long filamentous structures that run through cells and are putatively involved in the transport of vesicles and organelles. In addition to these F-actin structures, contractile actin rings arise at the division site of cytokinesis (reviewed by Pruyne and Bretscher 2000; Schott et al. 2002).

During sporulation, the actin behavior of *Saccharomyces cerevisiae* Meyen ex E.C. Hansen has been investigated intensively. Smith et al. (1995) reported that actin patches and cables develop in the cortical region during the early stages of meiosis. Doyle and Botstein (1996) reported that cortical patches of actin fused with green fluorescent protein (GFP) persist in spores throughout spore formation. Morishita and Engebrecht (2005) recently identified network-like actin filaments throughout meiosis and observed patches at the periphery of spores during the late stage of sporulation.

Previously, we studied the behavior of F-actin during sporulation in *Zygosaccharomyces rouxii* (Boutroux) Yarrow, which is phylogenetically related to *S. cerevisiae* (Wilmotte et al. 1993; de Montigny et al. 2000; Souciet et al. 2000), using confocal microscopy. These observations revealed a novel actin ring structure (Suda et al. 2005). *Zygosaccharomyces rouxii* a- and α-haploid cells undergo mutual conjugation to form diploid zygotes. The conjugated diploid cells that remain in their original zygote shape undergo meiosis and produce four haploid nuclei. After meiosis, spore walls are synthesized to form mature spores in zygotic asci. During this process, *Z. rouxii* cells form actin ring structures near the peripheral regions of spores with some actin patches.

Even with the intensive studies of actin behavior in *S. cerevisiae* sporulation, the actin ring structure observed in *Z. rouxii* has not been reported to date. To elucidate the role of actin in yeast sporulation, it is worth investigating whether this actin ring is present in the well-defined and closely related yeast, *S. cerevisiae*. Observation of a similar actin ring structure in both species would clarify the role of actin in sporulation. In the present study, we examined the behavior of F-actin in sporulation in *S. cerevisiae* using confocal microscopy. Furthermore, we investigated the relationship between the ascus shape and the actin ring because the ascus shape is different between *S. cerevisiae* and *Z. rouxii*: the former forms globose asci, whereas the latter forms zygotic asci.

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Materials and methods

Sporulation of *Saccharomyces cerevisiae*

A diploid strain of *Saccharomyces cerevisiae* (IFO 0234) was cultured to logarithmic stage in YPD (1% yeast extract, 2% peptone, and 2% glucose) medium at 25°C and sporulated in sporulation medium containing 1% potassium acetate. Haploid mating type a- (IFO 10449) and α- (IFO 10450) strains of *S. cerevisiae* were cultured separately in YPAD (1% yeast extract, 2% peptone, 0.004% adenine sulfate, and 2% glucose) medium. After reaching the logarithmic stage, haploid cells of both types were mixed and incubated for 4 h to induce zygote formation. The cultures were then transferred to sporulation medium and incubated for 16 h to produce zygotic (dumbbell-shaped) asci.

Sporulation of *Zygosaccharomyces rouxii*

Globose asci were induced in *Zygosaccharomyces rouxii*, as described by Mori (1973) with some modifications. Haploid mating type a- (IFO 1876) and α- (IFO 1877) strains of *Z. rouxii* were cultured separately in growth medium (YPDN; 1% yeast extract, 2% peptone, 2% glucose, and 5% NaCl) at 25°C. When both cultures reached the early stationary phase, the two cell types were mixed in sporulation medium (MEN; 5% malt extract and 5% NaCl) and plated onto MEN agar plates to induce zygote formation. The cultures including zygotes were harvested and transferred to fresh growth medium 12 h later. The zygotes were cultured as diploid round cells in the growth medium for 24 h. The diploid cell cultures were transferred onto MEN agar plates and incubated for 36 h to produce globose (round) asci.

Fluorescent staining

Cells undergoing sporulation were stained as described previously (Suda et al. 2005), with slight modifications, to visualize the actin cytoskeleton. The sporulating cells were fixed in 3.5% paraformaldehyde for 2 h. The globose asci were treated with 1% Triton X-100 solution to improve the permeability of the cells to fluorescent dyes. For improvement of staining efficiency and ease of detection of spore morphogenesis on differential interference contrast (DIC) images, *S. cerevisiae* cells forming zygotic asci were digested with 0.1 mg/ml Zymolyase 20T (Seikagaku, Tokyo, Japan) for 30 min at 37°C. The F-actin was then stained with 0.075 μM rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) for 2 h at 25°C. Finally, the nuclear DNA was visualized using 5 μM SYTO16 or 10 μM TO-PRO3 (Molecular Probes) along with Fluoroguard Antifade reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Fluorescent images were then obtained using a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany) equipped with a 100× oil immersion objective (Zeiss Plan-Neofluor; NA = 1.3). Rhodamine fluorescence was excited with a 543-nm He/Ne laser and detected through a 560- to 615-nm bandpass emission filter. The SYTO16 fluorescence was excited with a 488-nm argon laser and detected through a 505- to 530-nm bandpass emission filter. The TO-PRO3 fluorescence was excited with a 633-nm He/Ne laser and detected through a 650-nm-long pass emission filter. Images were acquired as optical sections (0.2- to 0.3-μm intervals) through an entire cell and then projected onto a single image. Although TO-PRO3 ordinarily exhibited deep red fluorescence, the color was converted to light blue to distinguish it from rhodamine fluorescence. DIC images were acquired simultaneously. To show the three-dimensional structure of F-actin in detail, the acquired section images were reconstructed to three-dimensional rotation images and observed from various angles (30° intervals) of rotation. All images were converted into TIFF files and processed using Adobe Photoshop 7.0.

Results and discussion

Under nitrogen starvation, but in the presence of a nonfermentable carbon source, such as acetate, the *S. cerevisiae* diploid cell undergoes meiotic division and forms four haploid spores in globose ascus. Figure 1 shows the F-actin distribution during the late stage of *S. cerevisiae* sporulation. We obtained fluorescent images as optical sections and stacked them into single images to analyze the distribution of F-actin throughout the cells. Fibrous actin structures that resembled rings were faintly stained with actin patches around the periphery of each spore. We observed the sectioned images before stacking onto a single image to analyze the actin structures in detail (Fig. 2). These images indicated ringlike structures around the periphery of each spore with some actin patches. At a depth of 1.0 μm, the ring part became detectable (Fig. 2). By lowering the focal plane, we found that a fibrous structure extended continuously to a depth of 2.8 μm. In the process of lowering the focal plane, fibrous actin ring structures also began to appear at other spore peripheries. The emerging position and timing of this structure appeared to be consistent with that of the actin ring of *Z. rouxii* (Suda et al. 2005). This result indicates that the actin ring is formed in the sporulation of *S. cerevisiae* globose asci as well as *Z. rouxii* zygotic asci.

However, the actin ring structure of *S. cerevisiae* differed somewhat from that of *Z. rouxii*. The ring structure of

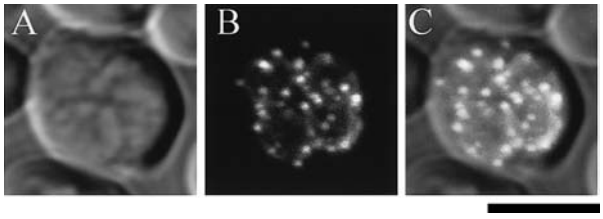

Fig. 1. F-actin distribution in *Saccharomyces cerevisiae* at the late stage of sporulation. **A** Differential interference contrast (DIC) image; **B** F-actin; **C** merged image of **A** and **B**. Sporulating cells were fixed and stained with rhodamine-phalloidin. *Bar* 5 μm

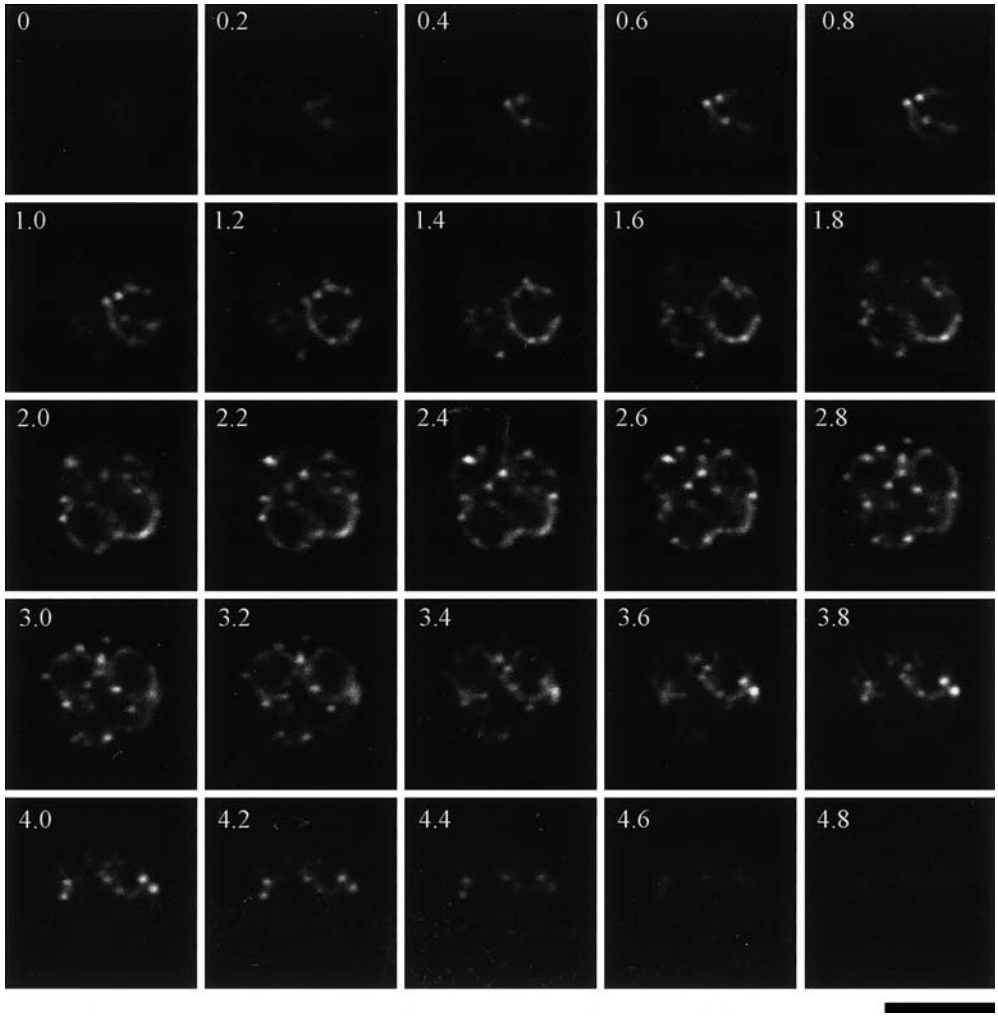

Fig. 2. Sectioned images before stacking onto a single image (Fig. 1). Images are optical sections (0.2-μm intervals) through one entire cell. *Bar* 5 μm

S. cerevisiae was less prominent than the *Z. rouxii* ring. This difference may be caused by the differences in type of ascus between the two yeasts. To investigate this suggestion, we induced zygotic asci in *S. cerevisiae* and then followed the behavior of F-actin throughout sporulation (Fig. 3). The opposite mating types conjugated to form diploid zygotes. Before mitotic nuclear division, the diploid cells were induced to sporulate by transferring to potassium acetate medium. At the karyogamous stage with a single nucleus (Fig. 3B), actin patches and fine cables were dispersed all over the zygote except for the nuclear region (Fig. 3C,D). The diploid cells then underwent meiosis to produce four haploid nuclei (Fig. 3F). Dense actin patches accumulated near each haploid nucleus (Fig. 3G,H), but spores were undetectable in DIC images (Fig. 3E). An actin ring structure surrounding the nucleus subsequently appeared at the spore periphery (Fig. 3K,L,O,P). Actin patches were also localized along with the rings. At this stage, spore edges were faint in DIC images (Fig. 3I,M). The ring structures eventually disappeared and only patches remained in mature spores (Fig. 3S,T). The behavior of F-actin in zygotic asci of *S. cerevisiae* resembled that of *Z. rouxii* throughout sporulation, and the actin ring was detected in a similar position and at a similar timing to *Z. rouxii*.

We reconstituted the actin distribution in three-dimensional images to demonstrate more clearly the ring structure distribution of F-actin. Figure 4 shows the rotation images of actin rings obtained at various angles (30° intervals). At an angle of 30°, the ring was somewhat smaller than that at 0°. With rotation, the ring structures changed to barlike structures (60°–120°), and then appeared again at 150° and 180°. In zygotic asci of *S. cerevisiae*, the actin rings were more obvious than those in globose asci. In *S. cerevisiae* globose asci, the spores partially overlap each other. In contrast, the spores in zygotic asci are somewhat more distant from each other than those in globose asci and are resident in almost the same plane. This feature may allow observation of the fine actin distribution and enable detection of an obvious actin ring in zygotic asci.

Although the actin ring structure seemed to differ somewhat between globose and zygotic asci, the phenomenon that forms the actin ring was common to the two ascus shapes in *S. cerevisiae*. Therefore, we next analyzed the behavior of F-actin in *Z. rouxii* after inducing its globose asci. Figure 5 shows clearly that actin rings were formed in the peripheral regions of each spore. This result confirmed the formation of the actin ring in both globose and zygotic asci in the two yeasts.

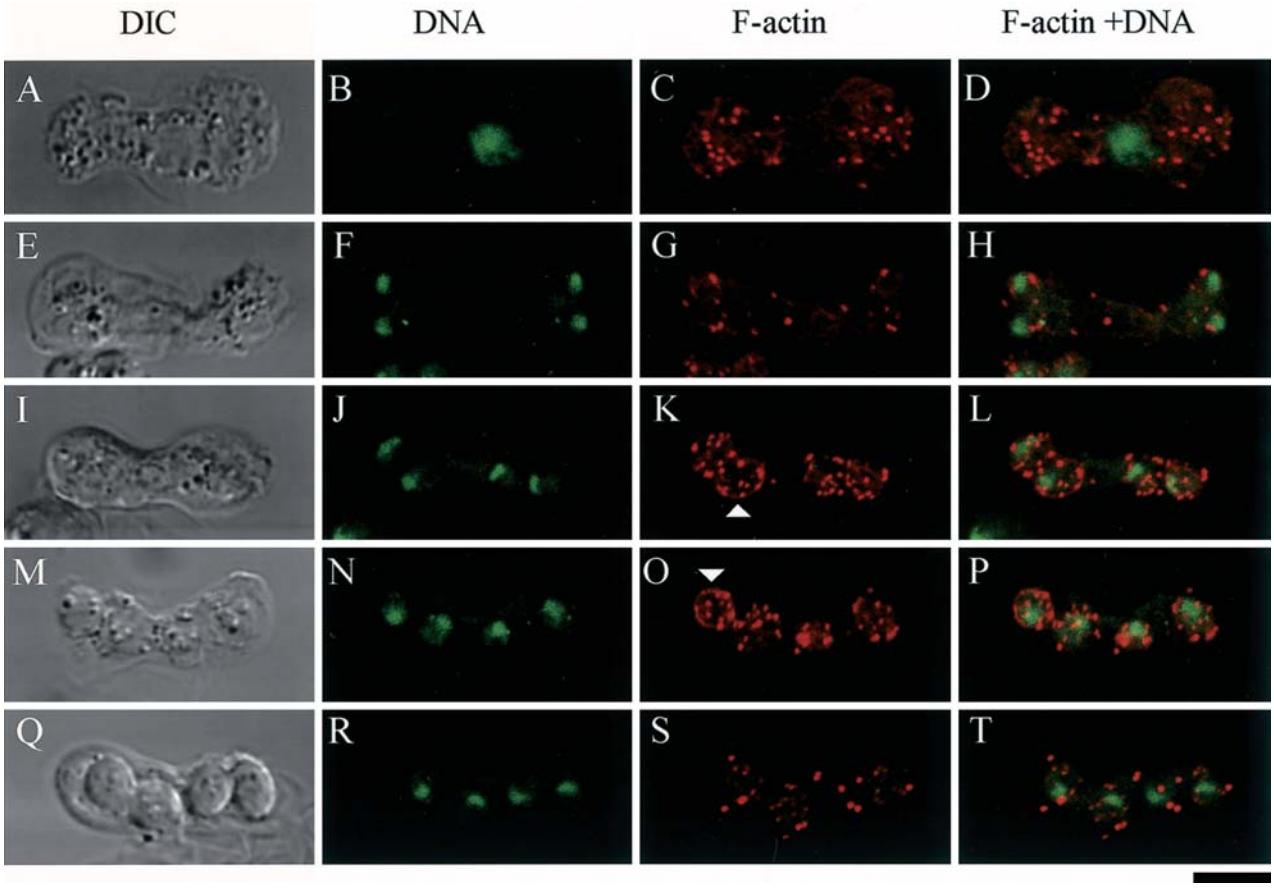

Fig. 3. F-actin distribution in sporulation of *Saccharomyces cerevisiae* forming zygotic asci. *First column*, DIC images; *second column*, nuclear DNA stained with SYTO 16; *third column*, F-actin stained with rhodamine-phalloidin; *fourth column*, merged images of second and third columns. *Saccharomyces cerevisiae* zygotes formed by a- and α-cell conjugation were incubated in sporulation medium and then fixed and stained as described in Materials and methods. **A–D** A zygote with a single nucleus in karyogamy; **E–H** a cell with four divided nuclei; **I–L**, **M–P** cells during spore formation; **Q–T** a cell during spore maturation. *Arrowheads* in **K** and **O**, actin ring structures. *Bar* 5 μm

There have been a number of intensive studies of the sporulation process in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* Linder (reviewed by Shimoda 2004; Neiman 2005), and some proteins were shown to form ring structures. During meiosis II, double-layered membranes, termed prospore membranes in *S. cerevisiae* or forespore membranes in *S. pombe*, extend along each meiotic nucleus and encapsulate to produce four haploid nuclei. The leading-edge proteins, Ady1p, Don1p, and Ssp1p in *S. cerevisiae* (Knop and Strasser 2000; Moreno-Borchart et al. 2001; Nickas and Neiman 2002) and Meu14 in *S. pombe* (Okuzaki et al. 2003), form ring structures at the lip of the growing prospore/forespore membrane. Furthermore, at the early stage of meiosis II, septin proteins also form ring structures at the leading edge of the prospore membrane (Fares et al. 1996; De Virgilio et al. 1996). The roles of these proteins are not entirely clear, but they are thought to be involved in the synthesis of spore membranes and walls (Moreno-Borchart et al. 2001; Tachikawa et al. 2001; Nickas and Neiman 2002; Okuzaki et al. 2003). The ring formation of the leading-edge proteins implies a relationship with the actin ring. However, the timing of the appearance is different between the actin ring described in this study and the leading-edge proteins; the actin ring was observed after meiotic division, whereas the leading-edge proteins were observed throughout meiosis II. The actin ring is unlikely to colocalize and interact directly with the leading-edge proteins, although we do not yet have direct evidence to support this. Further studies are needed to clarify the relationship between the actin ring and leading-edge proteins.

The actin ring may participate in mitochondrial distribution during sporulation. After meiosis, mitochondria are distributed as a ring structure around each spore nucleus in *S. cerevisiae* (Miyakawa et al. 1984). As mitochondrial movement in *S. cerevisiae* is dependent on the actin cytoskeleton in meiosis (Smith et al. 1995), the actin ring structure may contribute to mitochondrial ring formation.

There have been few studies specifically examining fine actin structures at the peripheries of spores, and therefore many researchers may not be aware of the actin ring structure. Smith et al. (1995) mainly addressed actin behavior in meiosis. Doyle and Botstein (1996) observed only patches of GFP-fused actin. Although Morishita and Engebrecht (2005) observed actin behavior throughout sporulation and demonstrated actin structures at the peripheries of spores,

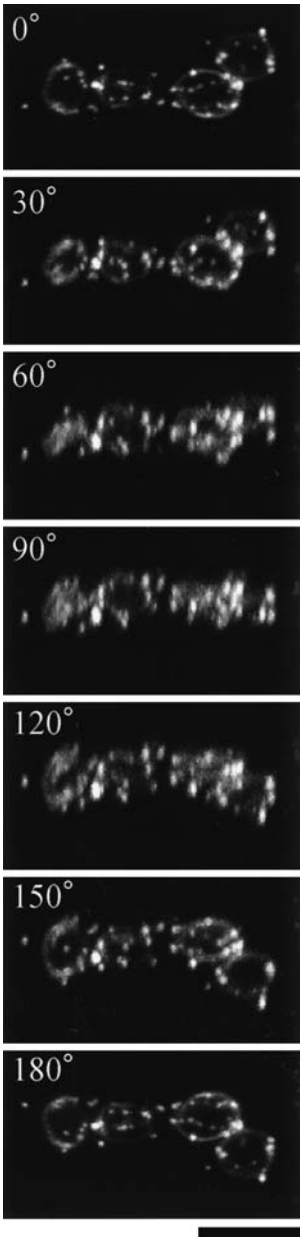

Fig. 4. Three-dimensional images of the actin ring structure in a zygotic ascus of *Saccharomyces cerevisiae*. Optical sectioned images were projected onto three-dimensional images and rotated at 30° intervals. *Bar* 5 μm

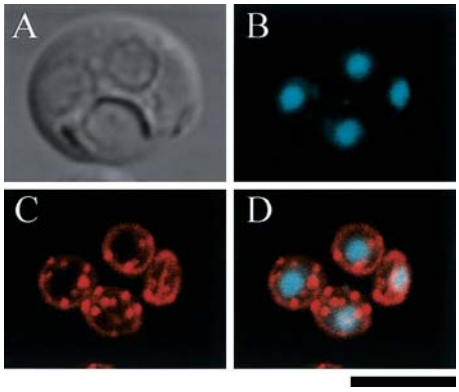

Fig. 5. F-actin distribution in globose asci of *Zygosaccharomyces rouxii*. **A** DIC image; **B** F-actin; **C** nucleus DNA; **D** merged images of **B** and **C**. Sporulating cells were fixed and stained with rhodamine-phalloidin and TO-PRO3 to visualize F-actin and nuclear DNA, respectively. *Bar* 5 μm

they did not pay attention to the presence of ringlike actin structures. They did not use three-dimensional observation techniques, such as confocal microscopy, to observe actin structures, and therefore they may have been unable to detect the actin structure as a ring.

The present study demonstrated that the actin ring structure is formed not only in *Z. rouxii* but also in *S. cerevisiae* during sporulation. This is the first report of the existence of the actin ring structure in the sporulation of *S. cerevisiae*. This finding is important to clarify the mechanism of spore morphogenesis in the model yeast *S. cerevisiae*.

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