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Full Paper

Morphology of mitochondrial nucleoids, mitochondria, and nuclei during meiosis and sporulation of the yeast *Saccharomycodes ludwigii*

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The morphology of mitochondrial nucleoids (mt-nucleoids), mitochondria, and nuclei was investigated during meiosis and sporulation of the diploid cells of the ascosporogenic yeast *Saccharomycodes ludwigii*. The mt-nucleoids appeared as discrete dots uniformly distributed in stationary-phase cells as revealed by 4′,6-diamidino-2-phenylindole (DAPI) staining. Throughout first and second meiotic divisions, the mt-nucleoids moved to be located close to the dividing nuclei with the appearance of dots. On the other hand, mitochondria, which had tubular or fragmented forms in stationary-phase cells, increasingly fused with each other to form elongated mitochondria during meiotic prophase as revealed by 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] staining. Mitochondria assembled to be located close to dividing nuclei during first and second meiotic divisions, and were finally incorporated into spores. During the first meiotic division, nuclear division occurred in any direction parallel, diagonally, or perpendicular to the longitudinal axis of the cell. In contrast, the second meiotic division was exclusively parallel to the longitudinal axis of the cell. The behavior of dividing nuclei explains the formation of a pair of spores with opposite mating types at both ends of cells. In the course of this study, it was also found that ledges between two spores were specifically stained with $DiOC_6(3)$.

Key Words——DAPI; meiosis; mitochondria; mitochondrial nucleoids; *Saccharomycodes ludwigii*; sporulation

Introduction

In the life cycle of the yeast *Saccharomyces cerevisiae*, mitochondria show dynamic morphological changes of fusion and division. In particular, mitochondria show high levels of fusion to form a network of tubular structures during meiosis and sporulation, and are distributed into spores, closely associating with the dividing nuclei (Miyakawa et al., 1984, 1994; Sando et al., 1981). This implies that the association of mitochondria with dividing nuclei serves to enable efficient segregation of mitochondria into a limited volume of spores. During sporulation, a highly dynamic network of non-polarized actin cables is present underneath the plasma membrane of the mother cell (Taxis et al., 2006). Our observation also suggested that the location of actin patches close to tubular mitochondria, specifically at the branching or bending points of tubular mitochondria, is involved in the formation of a network of tubular mitochondria at meiotic prophase (Miyakawa et al., 2006). Specific proteins that give rise to fusion and fission of mitochondria are necessary for proper distribution of mitochondria into spores (Gor-

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sich and Shaw, 2004). However, it remains unclear whether the unique behavior of mitochondria, as seen in the sporulation process of *S. cerevisiae*, is a common feature to ensure the inheritance of mitochondria into spores in the sporulation of yeasts.

Saccharomycodes ludwigii is a lemon-shaped yeast that grows by bipolar budding (Kreger-van Rij, 1969). *S. ludwigii* produces four spores in an ascus with a high sporulation ratio, and the cell size is larger than that of *S. cerevisiae.* Furthermore, *S. ludwigii* has a unique feature in that two spores with opposite mating types form a pair at the poles of an apiculate cell. A pair of spores are tethered together by a narrow ledge and mate just after germination. Therefore, *S. ludwigii* serves as an interesting model not only to elucidate whether there is a common mechanism in yeasts for proper inheritance of mitochondria into spores during the sexual life cycle, but also to consider the mechanisms of intratetrad mating (Knop, 2006). In this study, we first observed the morphology of mitochondrial nucleoids and mitochondria during meiosis and sporulation of *S. ludwigii* in order to elucidate the segregation pattern of mitochondria. Next, we investigated the behavior of meiotic nuclei and demonstrated that the direction of meiotic division leads to a unique arrangement of spores.

Materials and Methods

Strains and culture. A diploid strain of *Saccharomycodes ludwigii* Hansen NBRC1721 was cultured in 100 ml of modified Burkholder's medium supplemented with 3.6% (v/v) tomato extract (Miyakawa et al., 1984) at 30°C at an initial cell concentration of OD_{660}=0.01 after preculture on YPD slant (1% yeast extract, 2% peptone, 2% glucose). Cells that reached stationary phase after 30 h were harvested by centrifugation, washed with sterilized distilled water, and inoculated into the sporulation medium (0.98% potassium acetate, 1% glucose, 0.25% yeast extract) at a cell concentration of 3×10^6 cells/ml and cultured with reciprocal shaking at 25°C (McClary et al., 1959; Yamazaki et al., 1976). More than 300 cells were counted to determine the stages of meiotic division and the sporulation ratio.

DAPI staining and SYBR Green staining. Cells were fixed with 5% glutaraldehyde for 90 min at room temperature by direct addition of 1/4 volume of 25% glutaraldehyde solution to the culture medium. After 3 washes with NS buffer, cells were suspended with a small volume of NS buffer (Miyakawa et al., 1984). For 4′,6-diamidino-2-phenylindole (DAPI) staining, 3 μl of cells was mixed with 3 μl of DAPI solution (2 μg/ml NS buffer) on a glass slide. Samples were squashed and observed under UV excitation. For SYBR Green staining, 10 μl of the fixed cells was mixed with 1 μl of 1 : 1,000 diluted solution of SYBR Green I nucleic acid gel stain (FMC BioProducts), stood for 1–2 h at room temperature, and then observed without squashing under G excitation (Aoyama et al., 2006).

$DiOC_6(3)$ and DAPI-$DiOC_6(3)$ double staining. In order to perform vital staining of mitochondria with 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$], 1 ml of the sporulation culture was quickly washed twice with NS buffer and resuspended in 1 ml of NS buffer. Stock solution of $DiOC_6(3)$ (10 μg/ml) was added at a final concentration of 1 μg/ml and incubated for 5 min at 30°C. Mitochondria were observed under B excitation. Double staining with DAPI and $DiOC_6(3)$ was carried out as follows: To sporulating cells suspended in NS buffer were added $DiOC_6(3)$ and DAPI, each at a final concentration of 1 μg/ml, which were left for 5 min at 30°C. Mitochondrial nucleoids and nuclei were observed under UV excitation and mitochondria were observed under B excitation (Miyakawa and Yanagamizu, 1998; Miyakawa et al., 1994).

DAPI and $DiOC_6(3)$ staining of germinating spores. Mature asci that were cultured in sporulation medium for 108 h were collected from 3 ml of sporulation medium and suspended in 1 ml of solution A (0.5 M potassium phosphate buffer pH 6.5, 2.0 M sorbitol, 0.5 mM $MgCl_2$) supplemented with Zymolyase 20 T (final concentration of 1 mg/ml) and β-mercaptoethanol (final concentration of 0.5%) and incubated for 30 min at 30°C to digest cell walls. After washing cells with solution B (0.1 M potassium phosphate buffer pH 6.5, 1.2 M sorbitol, 0.5 mM $MgCl_2$), cells were suspended in phosphate-buffered saline. Spores were dispersed with a sonicator (Kubota UP-50H, Japan) at amplitude 80%, cycle 0.5, 30 cycles. Spores were cultured in 10 ml of YPD medium at 30°C and germinating spores were observed at a time interval of 1 h after fixation with 5% glutaraldehyde. In order to stain mitochondria in mature spores and germinating spores, $DiOC_6(3)$ (final concentration of 0.2 μg/ml) was added to the sporulation medium at 48 h. For germination, mature spores that incorporated $DiOC_6(3)$ were cultured in YPD medium that contained the same concentration

of $DiOC_6(3)$.

Immunofluorescence microscopy. Cells at each stage were fixed with 3.7% paraformaldehyde for 30−60 s and next fixed with 2.5% glutaraldehyde for 1 h at 25˚C. Cells were washed with PEM buffer (Hagan and Hyams, 1988: 100 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9) 3 times, and then resuspended in PEM buffer. Cells were treated with Zymolyase 20 T (final concentration 1 mg/ml) and 0.5% β-mercaptoethanol at 30˚C for 30 min, permeabilized with 1 ml of PEM buffer and 1% Triton X-100 for 2 min, and then washed 3 times with PEM buffer. Cells were treated with 200 μl of PEM buffer and 1 mg/ml sodium borohydrate for 5 min at room temperature. Then, cells were washed 3 times with PEM buffer and suspended in 100 μl of PEMBAL buffer (PEM buffer plus 1% BSA, 0.1% NaN_3, 1% lysine) for 30 min. Cells were treated in 50 μl of PEMBAL buffer with anti-yeast α-tubulin monoclonal antibody YOL 1/34 (Serotec, Indianapolis, Ind., USA) at 1 : 100 dilution for 24 h at 4˚C. Subsequently, cells were washed with 100 μl of PEMBAL buffer 3 times, and then treated with secondary antibody, rhodamine (TRITC)-labeled goat anti-rat IgG, at 1 : 10 dilution for 24 h at 4˚C in 50 μl of PEMBAL buffer. Cells were washed with PEMBAL buffer 3 times and suspended with phosphate-buffered saline (PBS). Three microliters of cell suspension was mixed with 3 μl of DAPI (2 μg/ml NS buffer) and observed without squashing.

All observations were made with an epifluorescence microscope (BHS-RFK, Olympus Optical. Co., Ltd.) equipped with a digital camera (Nikon DIGITAL SIGHT DS-Qi1Mc).

Results

Morphology of mitochondrial nucleoids (mt-nucleoids) and mitochondria during sporulation culture

Cells that were cultured in the sporulation medium were observed by DAPI staining and phase-contrast microscopy. The time course of a representative sporulation culture is shown in Fig. 1. The final sporulation ratio reached about 50% after 70 h of culture. The first meiotic division began after 36 h of culture. Half of the sporulating cells passed through the first meiotic division at 43.5 h and the second meiotic division at 44.5 h after the onset of cultivation. Mature asci appeared after 45 h of culture and finally about 50% of cells transformed into asci at 70 h of culture (Fig. 1). The time course suggested that the second meiotic division followed 1 h after completion of the first meiotic division and mature asci appeared 7−8 h after the second meiotic division.

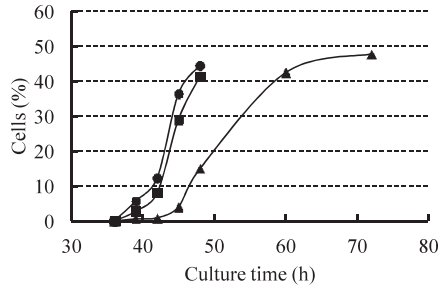

Fig. 1. Development of meiotic process of *S. ludwigii*.
Percentages of cells that passed first meiotic division (●), second meiotic division (■), and exhibited mature asci (▲) are shown.

Mt-nucleoids appeared as discrete dots that were dispersed in cytoplasm of stationary-phase cells (cells at 0-h sporulation) (Fig. 2A, arrow). As the first meiotic division commenced, mt-nucleoids began to assemble in a region around dividing nuclei (Fig. 2B). After completion of the first meiotic division, the mt-nucleoids assembled near each of two nuclei located at each pole of the cell (Fig. 2C). During the second meiotic division, mt-nucleoids were also located close to dividing nuclei (Fig. 2D). After the second meiotic division, a pair of spores were located at each pole of the cells, around which mt-nucleoids were observed as fluorescent dots (Fig. 2E). In squashed spores, mt-nucleoids aligned along the inner surface of the spore wall (Fig. 2F). A pair of spores that were produced at both poles of lemon-shaped cells were held together with a structure called a ledge (Fig. 2G, arrow). In contrast to the strings-of-beads appearance of mt-nucleoids in sporulating cells of *S. cerevisiae* (Miyakawa et al., 1984, 1994), mt-nucleoids in *S. ludwigii* constantly appeared as discrete dots throughout the sporulation culture.

Mitochondria were clearly stained with $DiOC_6(3)$ during the early stage of sporulation culture (Fig. 3A). Mitochondria appeared as either small particles or short tubular structures at the cell periphery in stationary-phase cells (Fig. 3A). As sporulation culture proceeded, mitochondria fused to each other and became more tubular and thick (Fig. 3BC). During first and second meiotic divisions, the distinct tubular structure of mitochondria became obscure. During these stages, mitochondria assembled in the region near dividing nuclei (Fig. 3DE). After completion of the second

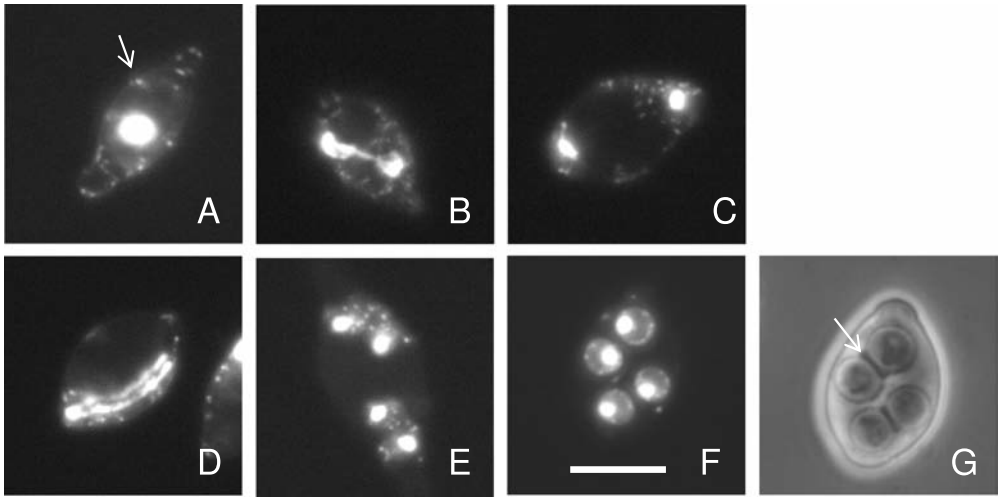

Fig. 2. Fluorescence and phase-contrast photomicrographs of cells during meiosis and sporulation after DAPI staining.

(A) A stationary-phase cell that was inoculated to the sporulation medium; (B) a cell that underwent first meiotic division at 42 h; (C) a cell that completed first meiotic division at 45 h; (D) a cell that underwent second meiotic division at 45 h; (E) a cell that completed second meiotic division; (F) a mature ascus; (G) a phase-contrast image of (F). An arrow indicates an mt-nucleoid in (A) and a ledge between two spores (G), respectively. Bar 10 μm.

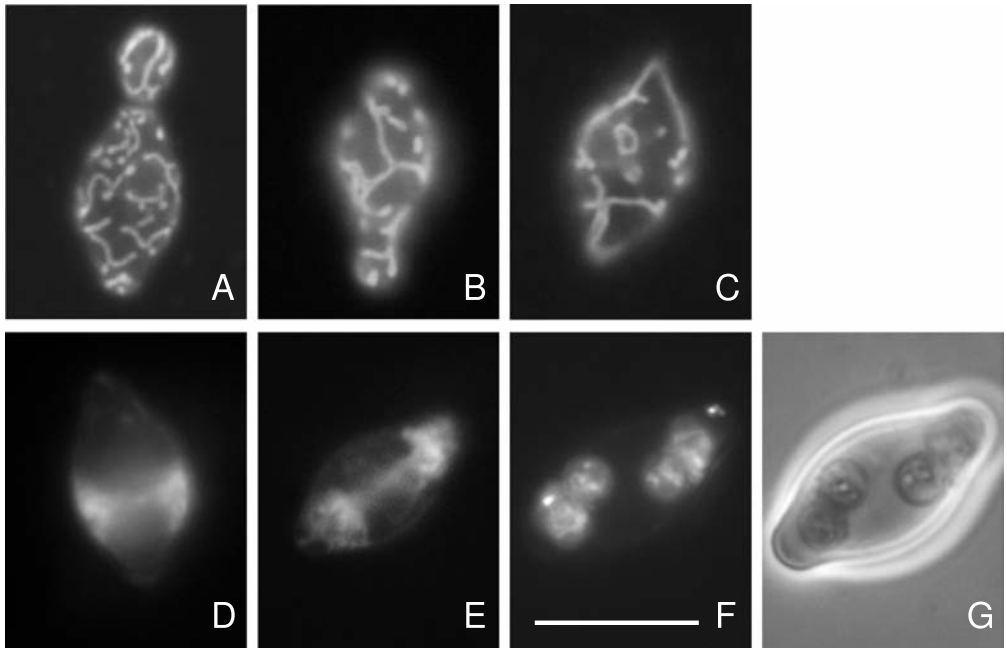

Fig. 3. Fluorescence and phase-contrast photomicrographs of cells during meiosis and sporulation after $DiOC_6(3)$ staining.

(A) A stationary-phase cell that was inoculated to the sporulation medium; (B) a cell at meiotic prophase that was cultured for 12 h; (C) a cell at meiotic prophase that was cultured for 24 h; (D) a cell that underwent first meiotic division at 42 h; (E) a cell that probably completed second meiotic division at 45 h; (F) a mature ascus; (G) a phase-contrast image of (F). Bar 10 μm.

meiotic division, almost all mitochondria were incorporated inside the spore wall. The presence of circular mitochondria that encompassed the nucleus, which were seen just before spore formation of *S. cerevisiae*, was not distinct in *S. ludwigii* (Miyakawa et al., 1984, 1994, Fig. 3F).

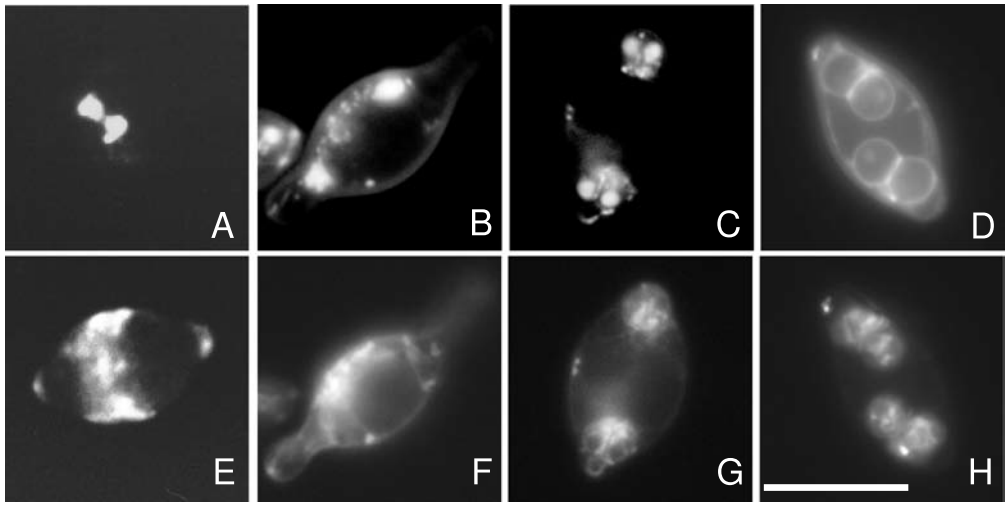

Fig. 4. Double staining of sporulating cells with DAPI and $DiOC_6(3)$.

Cells were vitally stained with DAPI (A–D) or $DiOC_6(3)$ (E–H). Cells underwent first meiotic division at a direction perpendicular to their longitudinal axis (AE) or parallel to their longitudinal axis (BF). (CG) A cell that completed second meiotic division; (DH) a mature ascus. Bar 10 μm.

In order to elucidate the spatial relationship between dividing nuclei and mitochondria, cells were vitally double-stained with DAPI and $DiOC_6(3)$ (Fig. 4). When the first nuclear division occurred in the direction perpendicular to the longitudinal axis of cells, mitochondria assembled around dividing nuclei at the central region of cells (Fig. 4AE). On the other hand, when the first meiotic division occurred parallel to the longitudinal axis of cells, mitochondria also assembled between dividing nuclei (Fig. 4BF). After the second meiotic division, mitochondria assembled at both poles of cells, in which two nuclei were closely located (Fig. 4CG). As seen in Fig. 3F, mitochondria exclusively appeared in spores of mature ascus (Fig. 4DH).

Direction of meiotic division

The first meiotic division unexpectedly took place in various directions irrespective of cell shape (Fig. 2, Fig. 4). We classified the direction of the first meiotic division into the following categories: (1) parallel to the longitudinal axis of cells, (2) diagonal to the longitudinal axis of cells, and (3) perpendicular to the longitudinal axis of cells. To obtain clear DAPI staining requires the squashing of yeast cells on a glass slide. On the other hand, SYBR Green staining does not require the squashing of cells. Therefore, we subsequently stained cells with SYBR Green I to determine the direction of meiotic division without artifacts due to squashing (data not shown). Observation of cells showed that 47% of them underwent meiotic division in the longitudinal direction, 23% of cells in the diagonal direction, and 30% of cells in the perpendicular direction. In contrast, all second meiotic divisions took place in the longitudinal direction. To confirm the result of SYBR Green I staining, immunofluorescence microscopy using anti-tubulin antibody was performed to determine the direction of nuclear division and the length of spindles (Fig. 5). At meiotic prophase, microtubules were associated with a single SPB located on the nuclear envelope (data not shown). At the first meiotic division, elongation of spindles occurred parallel to the longitudinal axis of cells (Fig. 5AEI), diagonal to the longitudinal axis of cells (Fig. 5BFJ), or perpendicular to the longitudinal axis of cells (Fig. 5CGK). The proportions of cells exhibiting each direction in the first meiotic division coincided well with those determined by SYBR Green staining. On the other hand, at the second meiotic division, two spindles elongated from pole to pole of the cells, parallel to their longitudinal axis (Fig. 5DHL). On the basis of the above results, a diagram of meiotic division is depicted in Fig. 6 to explain the formation of a pair of spores with opposite mating types.

Germination and zygote formation

Pairs of spores tethered by a ledge were isolated from the ascus and cultured in YPD medium (Fig. 7AD). Germination of spores began 2 h after culture and small protuberances appeared at the same side of the two spores (Fig. 7BE). These protuberances fused with each other to form a conjugation tube at 4 h, im-

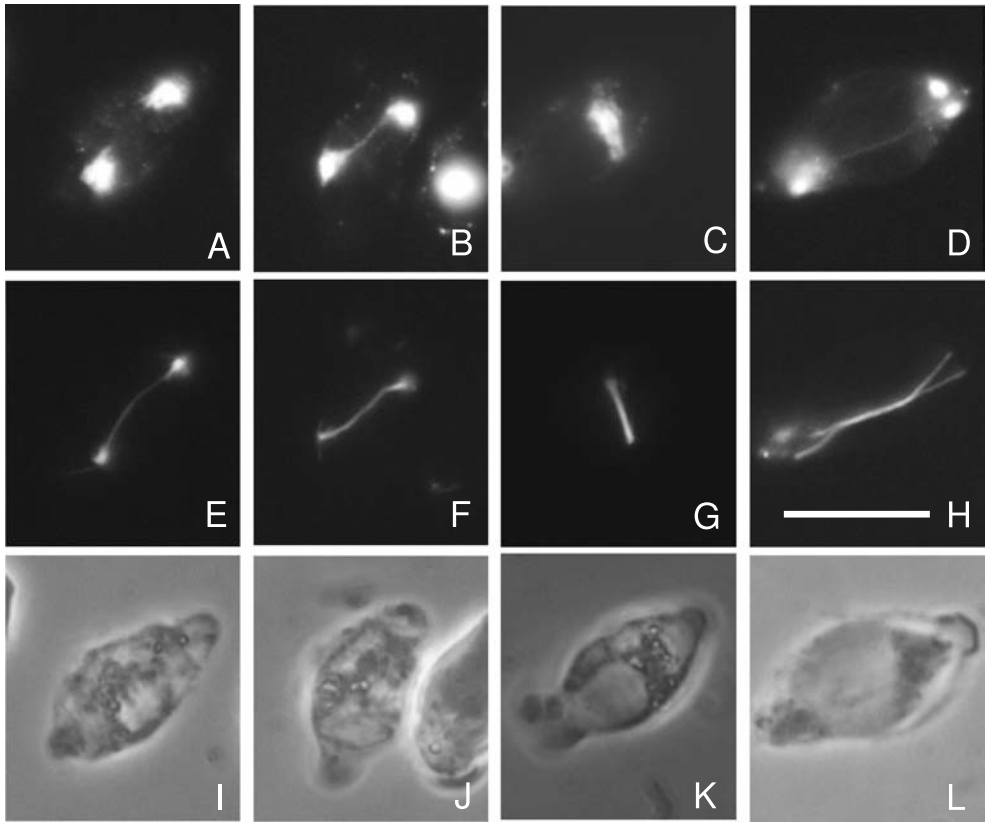

Fig. 5. Anti-tubulin immunofluorescence microscopy of *S. ludwigii*.

Upper row (A–D), DAPI staining; middle row (E–H), anti-tubulin staining; lower row (I–L), phase-contrast microscopy. Cells underwent first meiotic division parallel to their longitudinal axis (AEI), diagonal to their longitudinal axis (BFJ), or perpendicular to their longitudinal axis (CGK). (DHL) A cell that underwent second meiotic division. Bar 10 μm.

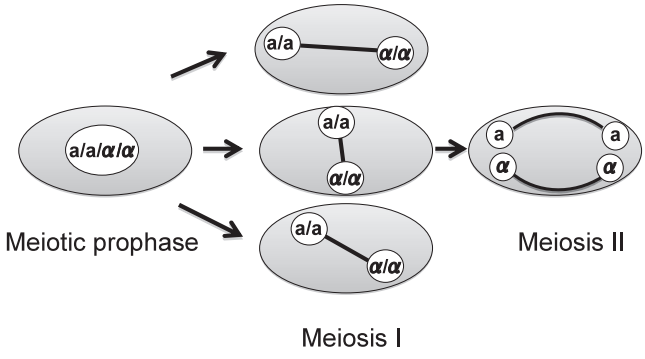

Fig. 6. A model for meiotic division of *S. ludwigii*.

mediately following nuclear fusion at the central region of the conjugation tube (Fig. 7CF). Several mt-nucleoids were also discerned in the conjugation tube. Conjugation tubes became thicker, in which complete fusion of haploid nuclei and doubling of the DNA content took place at 8 h (Fig. 7GJ). Zygotes at these stages appeared as a V-shape or a Y-shape owing to collapse of tethering of spores by the ledge (Fig. 7HK). At 9 h after culture, an ellipsoidal diploid cell separated from the zygote (Fig. 7IL). As far as we observed for fifty pairs of spores in YPD medium, all pairs of spores mated with each other, indicating that a pair of spores have opposite mating types. During the course of observation, we found that ledges located between two spores were specifically stained with $DiOC_6(3)$ and appeared as a fluorescent annulus (Fig. 8).

As mature spores did not incorporate $DiOC_6(3)$ (Fig. 8), we added $DiOC_6(3)$ (final concentration of 0.2 μg/ml) directly to the sporulation medium at 48 h just before spore maturation, and continued sporulation culture. This treatment enabled us to stain mitochondria in mature spores (Fig. 9AD). In mature spores, mitochondria were visible as tubular structures, and the tubular mitochondria elongated to fuse to each other in zygotes at the time of zygote formation (Fig. 9BE). The tubular mitochondria further elongated toward the new bud at the time of bud emergence during the germination culture (Fig. 9CF).

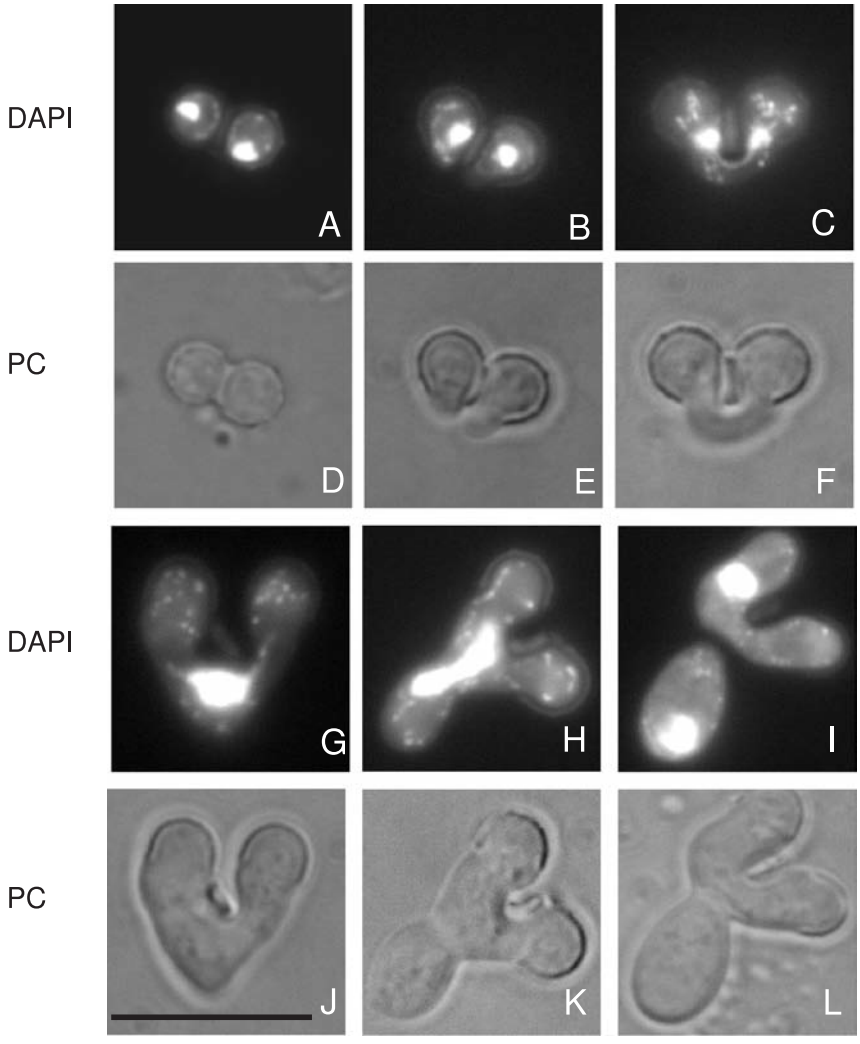

Fig. 7. Fluorescence and phase-contrast photomicrographs of germinating spores after DAPI staining.

A–C and G–I show DAPI staining images, and D–F and J–L show phase-contrast images. (AD) A pair of spores isolated from ascus; (BE) germinating spores with short protuberances at 2 h; (CF) a pair of spores that underwent mating to form a zygote at 4 h; (GJ) a zygote that just completed nuclear fusion at 6 h; (HK) a zygote that commenced nuclear migration into a new bud at 8 h; (IL) a zygote that completed first budding to produce a diploid cell at 9 h. Bar 10 μm.

Discussion

Mitochondria in sporulating cells of *S. cerevisiae* fuse with each other to form a network of tubular mitochondria at meiotic prophase, in which mt-nucleoids align to exhibit a string-of-beads appearance. Mitochondria move to be located close to the dividing nuclei, and finally each of four circular mitochondria is distributed into four spores (Gorsich and Shaw, 2004; Miyakawa et al., 1984, 1994). In *S. ludwigii*, fusion of mitochondria is also facilitated during meiotic prophase to form long tubular mitochondria that surround the periphery of cells, as well as mitochondria of *S. cerevisiae*. The feature that mitochondria in *S. ludwigii* assemble near the dividing nuclei during first and second meiotic divisions also resembles the behavior of mitochondria in *S. cerevisiae*. On the other hand, a few differences were also observed. The contour of tubular mitochondria that assembled around nuclei became obscure in *S. ludwigii* at the stage of first and second meiotic divisions (Fig. 3DE), whereas tubular mitochondria were clearly visible throughout meiotic division of *S. cerevisiae* (Miyakawa et al., 1984, 1994). As tubular mitochondria were clearly visible in cells that

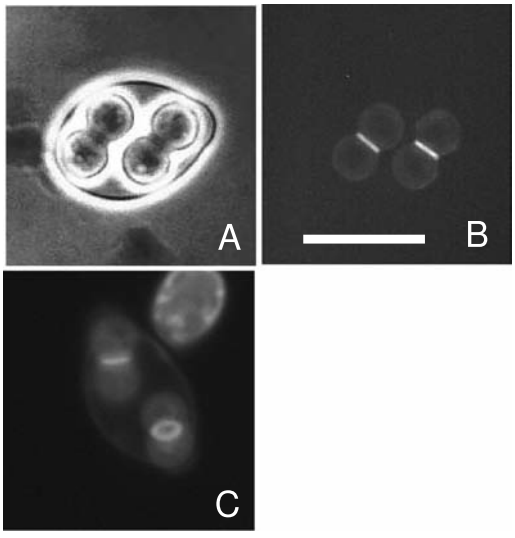

Fig. 8. Ascospores of *S. ludwigii* stained with $DiOC_6(3)$.

(A) A phase-contrast image of a mature ascus; (B) $DiOC_6(3)$ staining image of (A). (C) $DiOC_6(3)$ staining image of another ascus. Bar 10 μm.

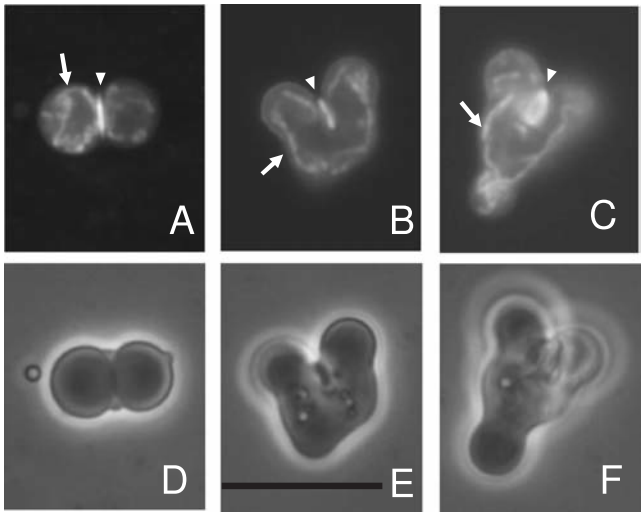

Fig. 9. Fluorescence and phase-contrast photomicrographs of germinating spores after $DiOC_6(3)$ staining.

A–C show $DiOC_6(3)$ staining images, and D–F show phase-contrast images. (AD) A pair of spores isolated from ascus; (BE) a zygote after 6-h germinating culture; (CF) a zygote after 8-h germinating culture. Arrows indicate tubular mitochondria and arrowheads indicate ledges between two spores. Bar 10 μm.

had not yet entered the first meiotic division in the same culture, this phenomenon is likely to be specific for mitochondria in cells that have entered meiotic division. Although distinct rings of mitochondria were found to be incorporated into spores in the case of *S. cerevisiae* (Miyakawa et al., 1984, 1994), this is not the case in *S. ludwigii* (Fig. 3F).

Differences in the morphology of mt-nucleoids were also discerned between *S. cerevisiae* and *S. ludwigii*. Mt-nucleoids in *S. ludwigii* appeared as discrete dots throughout the sporulation process, in contrast to the string-of-beads appearance of mt-nucleoids of *S. cerevisiae*. The mt-nucleoids in *S. cerevisiae* are so numerous that they align as a string of beads inside the tubular mitochondria, in which recombination of the mitochondrial genome may frequently take place. In contrast, it is possible that mt-nucleoids of *S. ludwigii* are not so abundant in tubular mitochondria that individual mt-nucleoids are visible as separate dots during sporulation.

S. ludwigii has a unique feature that a pair of spores with opposite mating types are formed at both poles of lemon-shaped cells. Interestingly, crossing over and genetic recombination do not occur during meiosis of *S. ludwigii* (Yamazaki et al., 1976). Yamazaki et al. (1976) postulated a scheme of meiotic division that explains the formation of a pair of spores. However, observation of nuclear division has not been reported yet. They postulated that first meiotic division occurs parallel or diagonal to the longitudinal axis of cells. The resultant two nuclei divide parallel to the longitudinal axis of the cell. As a result, a pair of nuclei with non-sister chromatids move to be located at each pole of cells. The present observations demonstrate that this scheme is principally correct (Fig. 6). The first meiotic division can take place in any direction parallel, diagonal, or perpendicular to the longitudinal axis of cells. However, the second meiotic division always takes place parallel to the longitudinal axis of cells with long spindles extended from one pole to the other. This leads to the formation of a pair of spores with opposite mating types.

Early electron microscopic observation demonstrated that a narrow and distinct ledge is present between the spores of each pair (Kreger-van Rij, 1969). The electron-dense material is visible between the spores of each pair, and the ledge tethers two spores of *S. ludwigii*. Freeze-fracture replicas showed that the ledge is a raised annulus (Simmons and Ahearn, 1985). However, the components of the ledge have not been investigated yet. The maturation of spore walls occurs through deposition of the different layers of spore wall materials between the two membrane bilayers of prospore membrane (Taxis et al., 2006). Our preliminary observation suggested that the components of the ledge are different from those of the spore

wall because the ledge is specifically stained with $DiOC_6(3)$. Nothing is known about whether the mitochondrial membrane is involved in ledge formation. Furthermore, it remains to be determined whether ledge formation is induced only between a pair of spores with different mating types or simply between spores that are located side by side at a cell pole independent of mating type. Determination of the mechanism of ledge formation will raise interesting issues in terms of how intratetrad mating is regulated. *S. ludwigii* has seven chromosomes, but genetic recombination does not occur as a result of meiosis (Yamazaki and Oshima, 1996; Yamazaki et al., 1976). We could observe 16 bivalent chromosomes of *S. cerevisiae* that specifically appeared at the meiotic prophase by DAPI staining (Kuroiwa et al., 1984, 1986). If bivalent chromosomes are formed during the meiotic prophase of *S. ludwigii*, seven chromosomes should be visualized by careful observation of cell nuclei at the meiotic prophase.

Acknowledgments

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