Electron Micrography of Bud Formation in 
Metschnikowia krissii

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The fine structure of bud formation of Metschnikowia krissii was studied by means of ultramicrotomy and transmission electron microscopy. Bud protrusion and development were observed by scanning electron microscopy. Bud formation in this yeast takes place by an extension of a small localized area of the existing parent wall. The parent cell and its bud are initially separated by the plasmalemma, creating an intercellular site within which the generation of new cell wall (bud and birth scar areas) occurs centripetally. When the dividing wall is complete and new cell wall material is formed, a narrow cleavage plane becomes increasingly defined. This cleavage plane apparently proceeds laterally toward the direction of the existing outer walls which rupture, resulting in the separation of the bud from the parent cell. The bud scar is prominently convex in shape; the birth scar is less conspicuous and initially concave in shape. Comparison of bud formation in M. krissii is made with that observed in Saccharomyces cerevisiae and Rhodotorula glutinis.

Recent studies by Marchant and Smith (11, 12) and Kreger-van Rij and Veenhuis (8) indicate differences in the mechanism of bud formation between Saccharomyces cerevisiae and Rhodotorula glutinis. In the case of S. cerevisiae, buds are initiated by a bulging extension of the mother cell wall followed by deposition of new wall material leading to centripetal septum formation. In contrast, R. glutinis new cell wall material is formed internally which causes a slight distention and eventual rupture of the outer wall layers with emergence of the new bud from the inner layer(s) and newly-synthesized wall. Fine structure of budding in Candida albicans and C. scottii also has been studied by Shannon and Rothman (19) and McCulley and Bracker (9), respectively.

Although Metschnikowia is well established as an ascomycetous yeast, a study of the fine structure involved in budding might elucidate its relatedness to other ascomycetes and if not, it is valuable in the elucidation of the basic physiology and life style of the yeasts.

MATERIALS AND METHODS

Cultures used for this study include: M. krissii, (UCD, FS&T 61-31) the type strain isolated from seawater off La Jolla, Calif.; S. cerevisiae (UCD, FS&T 70-50) cloned by several single cell and single ascospore isolations from a Montrocchet champagne yeast strain; and R. glutinis (UCD, FS&T 68-255) the type strain isolated from the atmosphere by Pringsheim. Specimen preparation was as described below.

Scanning electron microscopy. Vegetative cells were obtained by growing the culture for 20 to 24 h at 24°C in liquid yeast autolysate glucose broth (0.5% yeast autolysate, Pfizer, and 2% glucose) on a Rollo-drum (model TC 5, New Brunswick Scientific Co., New Brunswick, N.J.) rotating at 27 rpm for aeration. Cells were centrifuged, washed two times with 20 mM phosphate buffer, pH 7.0, which was used in all subsequent washes. Washed cells were suspended in 1 ml of buffer and 1 ml of 2% osmic acid was added, then they were refrigerated overnight in a screw-top vial. Cells were then washed three times with phosphate buffer and dehydrated in a graded ethanol series (20, 40, 80, 100, 100%). From absolute ethanol the cells were infiltrated with amylacetate three times, enclosed in filter paper, and dried by the critical point method (2). Dried cells were mounted on aluminum specimen mounts which were prepared by spraying first with T.V. Tube-Koat conductive coating (G. C. Electronics, Hydro-Metals, Rockford, Ill.) followed by spreading a drop of Scotch tape adhesive (Minnesota Mining and Manufacturing Co., St. Paul, Minn.), dissolved in benzene. The specimens were coated with 20 nm of silver followed by 20 nm of gold. All specimens were examined with the Cambridge Stereoscan Mark II A scanning electron microscope (SEM) operating at 10 kV.

Transmission electron microscopy. Yeast cells were washed twice in phosphate buffer and sedi-
mented by centrifugation for 10 min at 1,600 rpm. The cells were fixed in 1.5% K2MnO4 for 10 min at room temperature (24°C). Postfixation was done in 1% osmic acid in Veronal-acetate buffer (17) for 2 h at 4°C. After washing three times with the phosphate buffer at 20-min intervals to remove excess osmic acid, the fixed cells were dehydrated in a graded series (30, 50, 70, 90, 100, 100%) of acetone solutions. The dehydration process was interrupted at 70% acetone (containing 2% uranyl acetate), where it was allowed to stand overnight at 4°C for prestaining purposes. Subsequent to dehydration, the cells were embedded in Maraglass (6). Polymerization was allowed to proceed for 3 to 4 days at 60°C in a vacuum oven.

Sections were cut with a Sorval ultramicrotome MT2 and with a DuPont diamond knife mounted at an included angle of 45°. The angle of the knife holder was set to +3 to achieve the proper cutting angle without causing contortions or compressions of the sections. Sections were stained in 2% uranyl acetate for 10 min. Examination of the sections was done with an AEI electron microscope (type 3B) with an accelerating voltage of 60 kV and a 25-μm objective aperture for maximal contrast.

RESULTS

Figures 1 to 14 are electron micrographs of a thin section of cells. Corresponding SEM micrographs are of intact cells illustrating the different stages in bud formation. It is believed that these figures, which were selected from many micrographs, closely approximate the actual steps in the budding process of Metschnikowia krissii.

The early stages in the formation of a bud are shown in Fig. 1, 2, and 3. The bud is formed initially as a small protuberance of the cell wall. The electron opacity of the bud wall in thin sections (Fig. 1, 2) differs considerably from the rest of the wall, but the bud wall remains continuous with the parent wall throughout all stages of bud formation. The bud surface, viewed by SEM, becomes readily distinguishable from that of the mother cell (Fig. 5). This agrees with the description of McLary and Bowers (10) and Marchant and Smith (12) on the budding process of Saccharomyces cerevisiae. A localized thickening (electron-light area) is formed between the inner wall and the cell membrane and forms a constriction in the vicinity of the juncture between the bud and the mother cell (Fig. 1–4). Very little structural detail can be seen in the cytoplasmic matrix of the premature bud (Fig. 1, 2 and 3) although ribosomal particles are conspicuous during all stages of bud formation.

Figure 4 illustrates a budding cell acquiring a full complement of cellular organelles. The nucleus has nearly divided into the two areas of the cell, but the plasmalemma has not yet delimited the new bud cell, as is depicted in Fig. 6. The separation of the two cell compartments by the electron-light material creates a primary wall upon which the new cell wall material appears to be deposited (Fig. 7).

Figures 7 to 10 and 12 are electron micrographs showing the appearance of the dividing wall at different stages. The bud scar wall does not appear to be a continuation of the existing mother cell wall, differing in staining character. Contrast Fig. 6 with Fig. 8 to 10 and 12. The new wall material is deposited on both sides of the primary wall, becoming in fact two appressed walls separated by a layer of electron-light material. It is our opinion that the wall formation is in much the same fashion as in S. cerevisiae (12) rather than as in R. glutinis (11). The cleavage plane appears as a thin, fairly uniform electron-light line developing laterally. The cleavage plane occupies a median position, remaining fairly equidistant from each cell as it develops. Occasionally, invaginations are found associated with the cross wall (Fig. 8). They contain particles similar in appearance to those seen in the cross wall itself. The significance of these invaginations and particles to the formation of the new cell walls is not known, although crenulated areas have been noted in other dividing cells and young buds (9).

A mother cell with a mature bud is shown in Fig. 10. The fracture in the existing wall is clear and oriented close to the bud. Mature buds may become as large in size as the parent cell (Fig. 12, 13). The invaginations associated with the newly forming cell wall are no longer evident. The convex plug which forms the central portion of the bud scar does not appear to be a continuation of the parent cell wall. This is illustrated in Fig. 10 to 12 which show the prominent bud scar. The unmistakable convexity of the bud scar area at this stage of maturity is at variance with the situation in S. cerevisiae (10) and C. albicans (19) but agrees with the observations of Barton (3) and Agar and Douglas (1). The area of the bud which ultimately gives rise to the birth scar is less conspicuous (Fig. 10, 12). The SEM micrographs, however, reveal the area of the new birth scar to be a pronounced indentation (Fig. 14) complementing the convex shape of the bud scar. With growth the birth scar area expands and becomes distended indicating a high degree of plasticity in young walls. The bud scar is very prominent (Fig. 5, 11), remains the same size, and may be a factor in the aging of the cell.
FIG. 1. Thin section of M. krissii showing a pronounced protrusion initiating a bud on a vegetative cell. Note the localized dark staining character of the protrusion. Arrows indicate electron-light material present throughout bud formation.

FIG. 2. Thin section of M. krissii showing a developing bud. Note the thickened area (electron light) at the neck region between the mother and daughter cells. This region is believed to be the initiation site for the synthesis of new wall materials and forms the collar of the bud scar.

FIG. 3. Thin section of M. krissii showing an intermediate stage in the formation of a daughter cell. Typical of budding cells at this stage is the presence of numerous ribosomal particles. Arrows indicate characteristic electron-light material.
SEM micrographs of *S. cerevisiae* and *R. glutinis* (Fig. 15 to 18) are included for comparison with micrographs of *M. krissii* and the transmission electron microscope (TEM) micrographs published by Marchant and Smith (11, 12). In *S. cerevisiae* the bud wall is continuous with the mother cell wall throughout the budding process (Fig. 15). The bud cell is large and well formed before the cleavage fissure is apparent. After separation from the mother cell (Fig. 16) the young cell does not appear to have an indentation in the area of the birth scar as does *M. krissii*, or it expands rapidly. The birth scar area is plastic and the bud scars are rigid which is identical to the situation in *M. krissii*. This is well illustrated in Fig. 15 showing a bud scar formed upon a portion of the birth scar which had been distended by growth.

The surface of *R. glutinis* is covered with mucilage as stated by Marchant and Smith (11) and as seen in SEM micrograph (Fig. 17). The surface of the cell is homogeneously granular in contrast to the smooth wall surface of *S. cerevisiae*. It was not possible, however, to detect any indication of the ruptured outer mother cell wall layers in any stage of budding, either young or old, as is shown in TEM figures of Marchant and Smith (11). Young and old
Fig. 6. Thin section of M. krissii showing the complete delineation of the two cells by the plasmallemma. Note the electron-light nature of the intracellular site.

Fig. 7. Thin section of M. krissii showing differential staining properties of the new wall(s) separating the cells.

Fig. 8. Thin section of M. krissii showing the presence of crenulated invaginations on each side of the partition. A cleavage plane (electron-light line) between the two cells can be distinguished.
Fig. 9. Thin section of M. krissii showing a distinct electron transparent line situated medially between the cells. Note that the outer cell wall is continuous.

Fig. 10. Thin section of M. krissii showing the typical configuration of the daughter and mother cell wall, just prior to separation. Arrows refer to the cleavage of the existing wall which is oriented closer to the bud wall than the mother cell wall.

Fig. 11. Thin section of M. krissii showing a prominent bud scar. Note that the wall material which plugs the central portion of the scar is not continuous with the existing wall of the mother cell.
Fig. 12. Thin section of M. krissii at the time of separation showing the daughter cell as large as the mother cell. Arrows indicate the site where cleavage has developed.

Fig. 13. Scanning electron micrograph of M. krissii showing surface features of a comparable cell (Fig. 12). Arrows indicate bud cleavage site.
cells, as inferred by size, display little difference in surface appearance. As the bud enlarges, a neck region can be distinguished but no cleavage fissure is seen.

In all cases, birth scars appear to diffuse by cell growth and enlargement to some extent. Perhaps the birth scar in *R. glutinis* is less distended because vegetative cells of *R. glutinis* are more elongated than those of *S. cerevisiae* and *M. krissii*. The area of a bud scar(s) site in *R. glutinis* can be seen as a raised plate-like scar (Fig. 18), but not by the predominant ring and protuberance characteristic of *M. krissii* and *S. cerevisiae*. Again, bud scar areas appear less plastic in nature, retaining the same dimensions during and after bud formation.

**DISCUSSION**

The initial step in budding must somehow involve a loosening of the firm arrangement of the glucan and mannan components of the existing wall to make allowance for the limited expansion of the cytoplasm at the onset of bud formation. Nickerson (16) and Moor (14) suggested that the weakening of the wall could be accomplished by an enzyme, reductase, which breaks the protein-disulfide linkages. Necas and Svoboda (15) and Marchant and Smith (11) found that the staining properties of the bud wall differed significantly from those of the mother cell wall. Employing fluorescent-antibody staining technique, Chung et al. (5) determined the nature of cell wall duplication in *S. cerevisiae*. They indicated that the wall at the small region of bud formation is actually “opened” or perforated, upon rearrangement and deposition of new cell wall components that are being pushed into it. Consequently, the bud wall differs from the existing wall. The expansion of the wall and the synthesis of new wall material must take place at the same time to maintain the protoplast inside the thick cell wall.

The increased electron density of the young bud wall can be taken as an indication of changes in the cell wall chemistry. Enzymatic expansion of the existing cell wall could be
reflected in this difference in staining features. It is also surmised that the local thickening (electron-light area) of the bud wall at the constricted region of the budding cell is somehow associated in maintaining the particular shape of budding cells and is involved in the typical bud scar formed in *S. cerevisiae* and *M. krissii*. The electron-light material is not observable in the early stages of budding in *R. glutinis*.

The precise triggering mechanism for the formation of a bud is not known with certainty. Moor (14) suggested that the vesicles he observed in the young bud could carry the enzymes which initiate bud formation. Marchant and Smith (11) and McCulley and Bracker (9) observed similar structures in the growing bud of *R. glutinis* and *C. scottii*, respectively. Marchant and Smith felt the proposed vesicles could be implicated in cell wall synthesis. Similar structures (crenulated invaginations) were observed in the young bud of *M. krissii*, in addition to the prominent ribosomal particles. Such repeated observations are valuable, but the exact significance to the inception of bud formation is uncertain.
Electron microscope studies of ultrathin sections (12) and freeze-etched sections (13) revealed that in all stages of bud formation in S. cerevisiae the bud wall remained continuous with the existing mother cell wall. In contrast, R. glutinis (11) and other heterobasidiomycetous yeasts (8) bud by rupturing the outer cell wall layers and developing the inner layer(s) into the daughter cell wall.

The present study has shown that the bud formation in M. krissii is similar to S. cerevisiae in that the bud wall appears to be continuous with the parent wall. This is particularly apparent in the early stages of budding. The bud wall remains substantially thick indicating that the synthesis of cell wall materials must be actively taking place to keep up with the rapidly expanding bud. Kreger-van Rij and Veenhuis (8) found differences and believed that the bud wall protruded from inside the mother cell and was not continuous with the mother cell wall. In M. krissii we consider the bud wall as continuous and in this matter agree with the interpretations of Marchant and Smith (12) and Matile et al. (13). Differences from S. cerevisiae were observed in M. krissii in that the electron-light material was not located closer to the mother cell but was equidistant between the developing walls at all stages.

The development of the dividing wall formation is somewhat more difficult to demonstrate. However, on the basis of the morphological evidence presented in this study, it is conceivable that the synthesis of new cell wall material must be initiated in the intercellular site between the parent and daughter cells at the region of constriction. When the bud is formed, the electron-light material, by growing centripetally, separates the two cells, and new cell wall material is then deposited on each side to form the birth and bud scar walls. The cleavage plane appears to be initiated internally at the midsection of the new cell materials on the “bud side” of the electron-light material. The cleavage proceeds centrifugally and separates the original cell wall close to the bud, leaving a relatively inconspicuous birth scar (Fig. 10, 12) with no thick “retaining ring.” The plasticity of the bud wall and subsequent young cell is demonstrated by stretching of the birth scar to the sizes seen in mature cells.
The bud scar consists of the remnants of the original cell wall, electron-light material, and the central plug of new cell wall material. Evidence is presented that the convex plug which seals the constricted region of the mother cell is definitely not continuous with the parent wall. Houwink and Kreger (7) found chitin on krissii which showed the chitinase treatment did not destroy the integrity of the cell envelope but did largely eliminate the bud scar rims. They concluded that the chitin is located in a ring around the bud scar and sandwiched between two layers of glucan. These findings agree with the present study on bud formation in Metschnikowia krissii which is believed similar to that described for Saccharomyces cerevisiae.

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LITERATURE CITED