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Fine Structure of *Cryptococcus neoformans* Grown In Vitro as Observed by Freeze-Etching

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Cryptococcus neoformans grown on culture media was observed by the freeze-etching technique. In the capsule, short fibrils were seen when freeze-etched. This organism was unique in the appearance of the cell wall, which showed two strata. The outer one was dense with particles of about 20 nm in diameter, whereas the inner one was sparse in particles. The appearance of the cell membrane of this organism differed distinctly depending on the culture media. When grown on glycerol medium, the cell membrane possessed, as do other yeasts, clear but somewhat longer and curved invaginations. The membrane of cells grown on nonglycerol medium exhibited, however, only a few invaginations of irregular shape. Instead, characteristically of this organism, the cell membrane showed round depressions of 40 to 200 nm in diameter which were the surface view of the paramural bodies. In cross-fractured cells, both types of paramural bodies were found. Some of them contained a single vesicle of about 50 nm in diameter. These seem to play a role in secreting the cytoplasmic vesicles. Data suggesting the existence of multivesicular bodies in the cytoplasm and of multivesicular lomasomes were also obtained. Some of the baglike paramural bodies showed multilayered membrane. These are thought to be plasmalemmasomes. This organism was similar to other yeasts reported in other respects.

Cryptococcus neoformans is one of a few pathogenic yeasts causing systemic disease, especially in the lung and brain. It is unique among pathogenic yeasts in capsule formation. On transfer from parasitic state to culture media, this organism changes and reduces cell and capsule size.

One of the interesting problems with this organism is the mode of production of the capsule, which plays an important role in its pathogenicity (1, 2). As for the other structures of the extracellular membrane, the production mechanism of the cell wall was partly clarified recently in studies of other organisms (5). It seems that not only the enzymes required for wall synthesis but also the precursors of the wall material are produced in the cytoplasmic vesicles and secreted in vesicular form out of the cell membrane. But only a little seems to be known about the capsule production.

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The freeze-etching technique is suitable for studying the fine structures, especially membranous ones, of yeasts (9). To our knowledge, however, observation of *C. neoformans* by using this technique has not been reported.

This paper describes the fine structure of *C. neoformans* grown in vitro as observed by freeze-etching. A subsequent paper deals with cells grown in vivo (11). The structural features found in both growth and mode of capsule production will be discussed in the latter paper.

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MATERIALS AND METHODS

Organisms. Two strains of *C. neoformans* were used. Okuno strain was isolated by us from a patient with *Cryptococcus* meningitis. IFO 608 strain (abbreviated as 608) was kindly supplied by the Institute for Fermentation, Osaka. The two strains showed considerable differences under light micros-

copy. Okuno strain possessed a conspicuous capsule when isolated from the patient; however, it scarcely showed any capsule in India ink preparations when grown in vitro. Mature cells were homogeneous in size. The 608 strain possessed a capsule when grown on nonglycerol medium. The cell size varied considerably on the same slant. For this reason, the size of intracellular structures was described in the case of Okuno strain.

Growth conditions. The organisms were usually grown at 37 C for 18 h to 3 days on a nonglycerol medium (15 g of sucrose, 15 g of glucose, and 20 g of agar per liter of potato-yeast extract made in the following way: sliced potato (20%) and raw yeast (3%) in water were boiled for 0.5 h and filtered through gauze). Sometimes the organisms were grown on a glycerol medium the same as described above except for supplement of glycerol, 10%.

Preparation of sample. The growths were fixed with 2 to 5% glutaraldehyde containing 0.067 M phosphate buffer, pH 7.4 or 6.5, for 2 to 5 h at room temperature or overnight at 4 C. Then they were immersed in 30% glycerol containing the phosphate buffer as described above for 2 to 5 days at 4 C.

Freeze-etching. A new instrument devised by Nishiura was used (10). Specimens were prepared for freeze-etching by immersing a specimen holder bearing droplets of the thick cell pellets into liquid Freon 12. The specimen holder was placed on the specimen block and cooled in liquid nitrogen completely (−196 C) and then was placed on the specimen table of the instrument. After vacuum pressure below 2×10^{-5} mm Hg was obtained, specimens were cut with a knife and, when desired, etched by elevating the temperature to −100 C for 2 min. Usually the use of liquid Freon 12 was omitted for freeze-fracture. Electron micrographs were printed in reverse to facilitate interpretation.

RESULTS

Cytoplasmic organelles. As shown in Fig. 1, the cytoplasmic structures of *C. neoformans* were similar to other yeasts reported (9). The diameter of the nucleus and its pores were revealed to be about 1.6 μm and 80 nm, respectively. The cell diameter was about 3 to 4 μm. Generally, vacuoles were less than 1 μm in diameter, and the inside view was full of particles, whereas the outer surface was sparse in them, which was contrary to the cell membrane. The stationary-phase cells contained relatively large globular organelles with multilayered shell, which are reported to be storage organelles (9).

Noteworthy is the existence of vesicles about 50 nm in diameter, which were not associated with bud formation.

Capsule. Freeze-fracture did not reveal the capsule at all. However, freeze-etching revealed the capsule, as is shown in Fig. 2. The round part surrounding the wall differed in the etching pattern from the other part. Many short fibrils, about 100-nm long, radiated in the capsule.

Wall particle. Two strata could be distinguished in the cell wall (Fig. 2 and 4). The outer stratum was dense in particles about 20 nm in diameter (wall particle), whereas the inner one was sparse in them. The outer stratum was 40 to 80-nm thick and constituted the minor part of the wall which was about 200-nm thick. The wall particles were different in size from the cell membrane particles, measuring about 10 nm in diameter (Fig. 4).

Cell membrane. The appearance of the cell membrane changed with the growth media. When grown on glycerol medium, cells possessed clear invaginations as shown in Fig. 3. Two types of invaginations could be found and were not intermingled in one cell. One was long (0.3 to 2 μm), wide (50 nm), deep, and curved, and the other was short (0.2 to 0.4 μm), narrow (15 nm), shallow, and also curved.

The features of the cell membrane of *C. neoformans* grown on nonglycerol medium were markedly different from those of other yeasts reported (9) and studied in some detail.

Firstly, some cells exhibited round depressions of 40 to 200 nm in diameter as shown in Fig. 6. The proportion of cells showing round depressions varied considerably in each culture. Figure 7 shows circular invaginations which were not restricted to the small region of cell surface as in the case of bud formation (8).

Secondly, the cell never possessed many of the "ordinary" invaginations as shown in Fig. 4 and 5. Furthermore, in Okuno strain the shape of the invaginations were irregular, crooked, and varied. Okuno strain also showed many wrinkles, which were caused by shallow concavity and convexity of the cell membrane (Fig. 5). The wrinkle could be distinguished from invaginations as the former bore particles of the cell membrane while the latter did not. Small number of wrinkles were also found in cells grown on glycerol medium. The 608 strain differed from Okuno strain in that it had rather clear invaginations, if any, but did not show many wrinkles.

Paramural body. In the cross-fractured cell grown on nonglycerol medium, many paramural bodies were found which were the cross-sectional view of the round depressions of the cell membrane. Judging from the shape and size, *C. neoformans* seems to have two types of paramural bodies. The first is the spherical invaginations about 100 to 150 nm in diameter containing one vesicle about 50 nm in diameter (Fig. 1 and 9). The second was a baglike

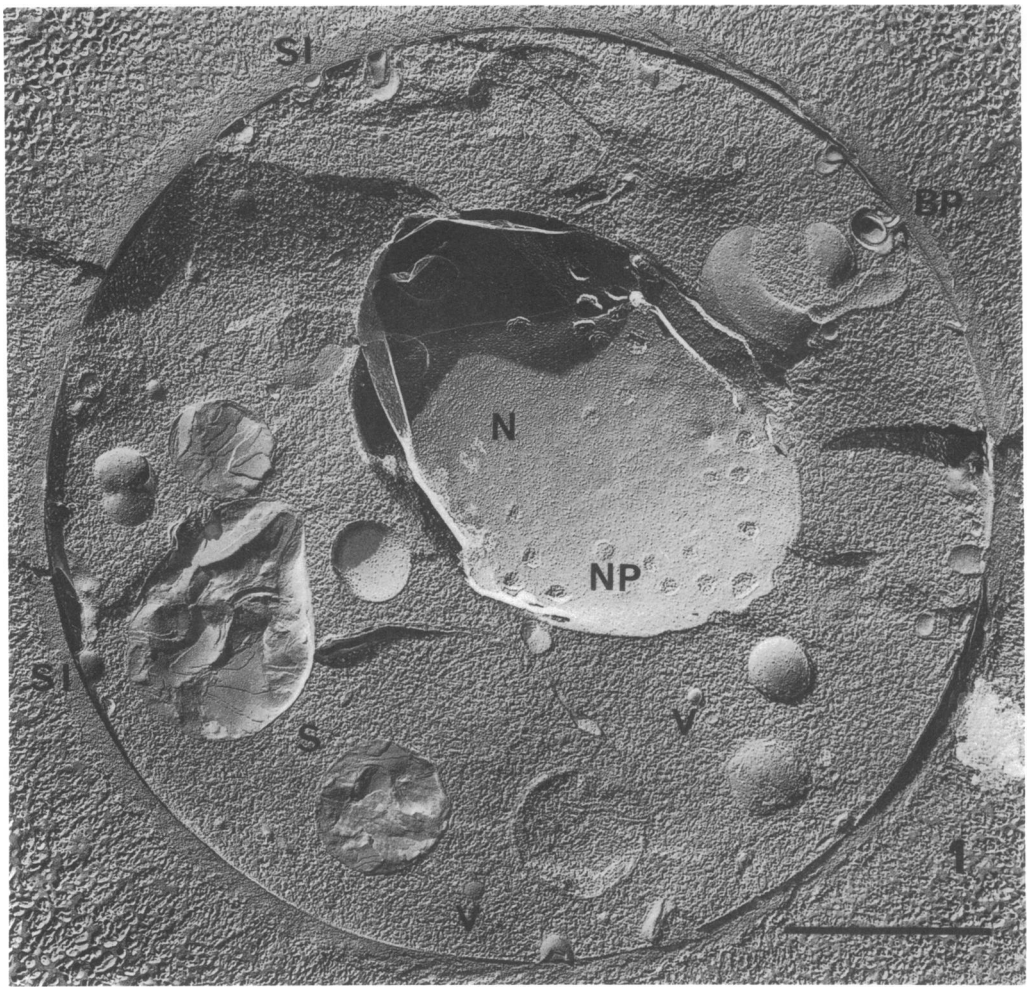

FIG. 1. *Freeze-etched cell showing nucleus (N), nuclear pores (NP), storage organelles (S), vesicles (V), spherical invaginations (SI), and baglike paramural bodies (BP). This figure and Fig. 2 and 8–10 indicate the 608 strain. Other figures show the Okuno strain. The bar in this and in all subsequent figures indicates 1 μm.*

paramural body which was about 200 to 600 nm in diameter. Usually inner structures of the paramural bodies were not revealed. However, spiral-shaped or multilayered membrane continuous with the cell membrane was observed as shown in Fig. 8 and 9. In the cytoplasm not only vesicles of 50 nm in diameter, but also multivesicular bodies were observed as shown in Fig. 10.

DISCUSSION

The freeze-etching technique revealed several new features of this organism concerning cellular envelope.

The microfibrils of the capsule reported in a thin-sectioning study (3) are recognized by freeze-etching but not by freeze fracture. This fact seems to show that the capsule is highly hydrated.

The outer part of the cell wall accumulates the wall particle; and was not observed in other genera of yeasts such as *Saccharomyces*, *Pichia*, *Candida*, and *Torulopsis* in our study (unpublished data) nor in other papers reported (4, 9). The wall particle is not attributable to artifact shrinkage of the capsular materials to the outermost part of the cell wall, since they are found deep enough from the wall surface. Furthermore, particles of the same size are also found in the inner part of the capsule (11).

C. neoformans shows many paramural bodies which have wide distribution among fungi,

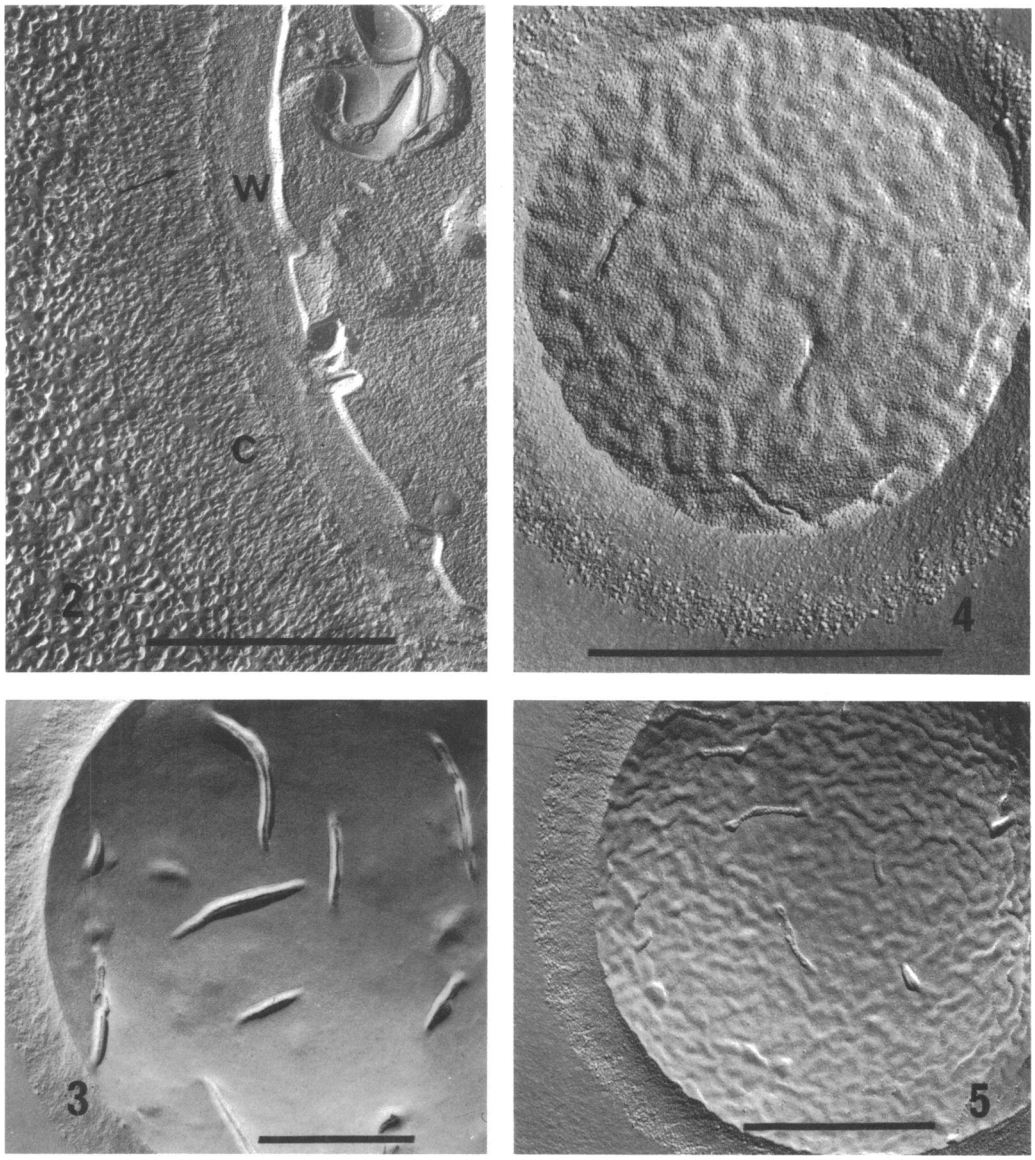

FIG. 2. *Portion of freeze-etched cell showing the capsule (C). Many fibril forms, about 100-nm long (arrow), radiate in the capsule (about 0.3-μm thick) surrounding the wall (W). The outer part of the wall accumulates particles, whereas the inner one is smooth. (See also Fig. 4.)*

FIG. 3. *Inside view of the freeze-fractured cell membrane showing long, wide, deep, and somewhat curved invaginations. Only this figure shows cells grown on glycerol medium. All the other figures indicate cells grown on nonglycerol medium.*

FIG. 4 and 5. *Outside and inside view of the freeze-fractured cell membranes. See the irregularly shaped invaginations and many wrinkles of the cell membrane. The wall particles, about 20 nm in size, are dense in the outer stratum of the wall. The cell membrane particles are about 10 nm in size (Fig. 4).*

algae, and higher plants (6). Only two papers, however, seem to have been published concerning paramural bodies of the yeasts. Edwards et al reported mesosomes of cell membrane origin (plasmalemmasome) in this organism (3). Moor described spherical invaginations with single vesicles containing enzymes appearing during bud formation (8). Features of spherical invaginations of this organism coincide well with the vesicular secretion of the protease

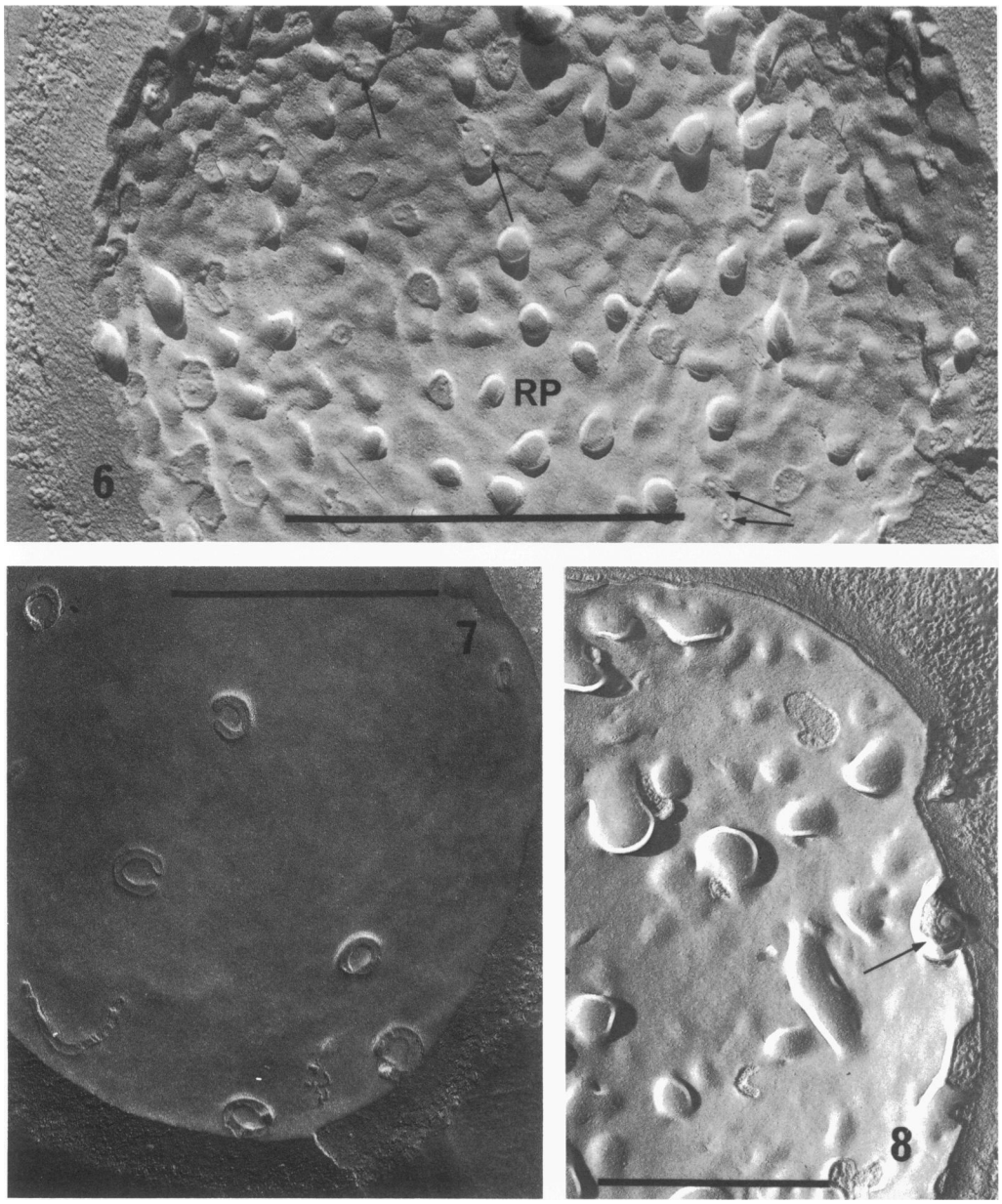

FIG. 6. *Inside view of the freeze-fractured cell membrane showing round protrusions (RP) which actually are depressions of the membrane when viewed from outside. Cells with such numbers of round protrusions as this are not encountered often. About one-third of the protrusions are cut off, and particles resembling cell wall particles are seen (arrows). Leftside arrow indicates a hole the size of a single wall particle.*

FIG. 7. *Inside view of freeze-fractured cell membrane with circular invaginations about 150 nm in diameter. These seem to indicate early stage of formation of spherical invaginations which secrete the vesicles outside the cell membrane.*

FIG. 8. *Inside view of freeze-etched cell membrane associated with structures different from those seen in Fig. 6 to 7 because of their larger size and irregular shape. Note the spiral membrane revealed (arrow).*

found in hyphae of *Neurospora crassa* (7). The circular invagination seen in Fig. 7 probably shows the early stages of spherical invagination. The shallow invaginations are thought to be the ghosts remaining after the secretion of vesicle. Judging from the large number of

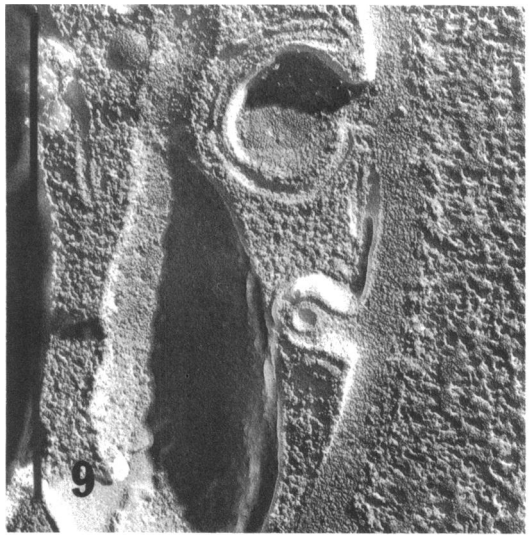

FIG. 9. *Cross-fractured paramural bodies. Both membranous and vesicular bodies are shown (freeze-etched).*

FIG. 10. *Portion of freeze-fractured cells showing a multivesicular body (arrow) in the cytoplasm. See also the cell membrane (CM) and vesicles (V) in the ctyoplasm which are about the same size as those seen in the multivesicular body.*

spherical invaginations found, *C. neoformans* seems to have a higher activity of secretion than do other yeasts.

C. neoformans also possesses many baglike paramural bodies which are larger than spherical invaginations in size. Some of them are revealed to have multilayered or spiral membranes continuous with cell membrane and could be called plasmalemmasomes. Interestingly, this organism also shows multivesicular bodies in the cytoplasm. This fact, in connection with the multivesicular paramural bodies found in an in vivo study (11), suggests the presence of multivesicular lomasomes, said to be distinctly absent in yeasts (6).

Strangely, *C. neoformans* grown on nonglycerol medium does not possess many "ordinary" invaginations. This feature of cell membrane is more prominent in the cells grown in vivo (11). Except for round depressions, they have an almost smooth cell membrane. In other yeasts, the cell membrane of small buds is unsculptured, as in *C. neoformans*. However, during the bud maturation more and more invaginations appear and become a prominent feature of the stationary-phase cell (9). It may be that the invagination, once made, remains, in general, unaltered, and thus the cell accumulates invaginations as it matures. The invaginations of *C. neoformans* once made, however, might well be abolished in the course of the formation of the round depressions which are found so often in the cells grown in the parasitic state and on nonglycerol medium. The cells grown on glycerol medium do not possess many round depressions. As a result, the invaginations, once made, could remain as in other yeasts.

This organism shows another interesting cell membrane feature. Okuno strain grown on nonglycerol medium possesses irregularly shaped invaginations and many wrinkles, but when grown in vivo or on glycerol medium this strain has rather clearly shaped invaginations and only a small number of wrinkles, as do other yeasts. The irregularly shaped invaginations may, therefore, be caused by the influence of wrinkle formation upon clear, preexisting invaginations. These seem to provide a dynamic aspect of the cell membrane of this organism.

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