FREEZE-FRACTURING IN NORMAL VACUUM REVEALS RINGLIKE YEAST PLASMALEMMA STRUCTURES

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ABSTRACT

The fine structure of the regular arrays of subunits seen on both plasmalemma fracture faces in resting and starved *Saccharomyces cerevisiae* (baker's yeast) has been compared using different freeze-fracture replication methods. Freeze-cleaving was carried out at 173°, 133°, and 108°K under a vacuum of 2×10^{-7} torr (2.6×10^{-7} mbar) or under liquid nitrogen at atmospheric pressure.

Independent of the preparation conditions (fracturing temperature, and whether cleaved under vacuum or liquid nitrogen), resting and starved yeast show a significant difference in the morphology of the subunits forming the regular arrays.

The regularly arranged particles of the P face of the plasmalemma of starved yeast have a clear craterlike structure which has previously been reported to be demonstrated only by freeze-etching at very low temperatures in ultrahigh vacuum. A complementary structure is seen on the plasmalemma E face.

Prolonged exposures of fracture faces under the protection of liquid nitrogen-cooled shrouds have shown that, because of the consequent drastic reduction of condensable gases in the specimen area, no detectable condensation contamination of exposed fracture faces occurs within 15 min at a specimen temperature of 108°K. This shows that a complicated ultrahigh vacuum technology is not required for high resolution freeze-etching.

KEY WORDS freeze-fracturing · baker's yeast · plasmalemma structure · paracrystalline arrays · physiological state of yeast · fracturing temperatures

Recently Gross, Bas, and Moor (3) reported that the topographic resolution and complementarity of morphological details of yeast plasmalemma fracture faces can be improved significantly by freeze-cleaving at 77°K and maintaining a vacuum of 2×10^{-9} torr (UHV) during fracturing and Pt/C shadowing. The authors used three different sets of experimental conditions for vacuum and cleavage temperature: (*a*) 10^{-6} torr, 173°K; (*b*) 10^{-9} torr, 173°K; and (*c*) 10^{-9} torr, 77°K. Micrographs and optical diffraction patterns were presented to show the apparent effects of higher vacuum and lower temperature on the topography of the paracrystalline arrays on E and P faces of yeast plasmalemma. It was stated that only when the cleavage process takes place at 77°K and 10^{-9} torr do the particles of the paracrystalline regions exhibit a craterlike structure which corresponds with a ringlike depression on the E face. Furthermore, neither structure was seen when membranes were fractured at 173°K in a vacuum of either 10^{-9} or 10^{-6} torr. The published optical diffraction pattern of the regular arrays shows third- and fourth-order spots from fracture faces obtained by cleaving at 77°K and shadowing at 10^{-9} torr. However, when cleaving was done at 173°K, only diffuse spots were observed which did not extend beyond the second order. From this, Gross et al. (3) conclude both that: (*a*) "The insignificant difference between applying high vacuum or UHV at 173°K on the one hand, and the

great improvement achieved by additional lowering the specimen temperature on the other hand, indicates that the structural record is primarily temperature-dependent"; and, (*b*) "If the specimen is cleaved at 173 K, the temperature might rise so much that membrane lipids are softened, enabling lateral dislocation of protein complexes (particles) and lipid molecules. This may lead to disorder in the arrangement of particles and to closing of pits in the lipid matrix of the opposite half of the fractured membrane. UHV conditions are necessary only to keep the fracture face clean."

However, from our own experiments, we consider that both the structural differences in the individual subunits themselves, and the long-range order in the hexagonally arranged membrane particles, are primarily due to a specific physiological state of the yeast rather than to whether the fracture takes place at very low temperature or whether UHV is used.

MATERIALS AND METHODS

In accordance with Gross (2) and Gross et al. (3), freshly pressed baker's yeast (*S. cerevisiae*) was chosen as the test specimen. The yeast was obtained from a local yeast factory and was kept at 4°–6°C for no longer than 24 h before use. The pressed yeast was either resuspended in distilled water at 4°–10°C to give a thick paste (resting culture) and immediately frozen, or resuspended in distilled water (1 g pressed yeast/100 ml water) and kept in suspension at 20°–23°C for 24 h in a 500 ml sintered glass filter flask with a sterile airflow of 300–400 ml/min (starved culture). Cells were harvested by gentle centrifugation and immediately frozen. Freezing was performed by dipping the loaded specimen holders designed for the complementary replica technique (7) into nitrogen slush (9).

To allow valid comparisons, in all experiments resting and starved yeast cells were processed (cleaved) simultaneously and each experiment was repeated 4–6 times. Freeze-fracturing and replication of the sample were carried out in the fully automatic Leybold Heraeus Bioetch 2005 freeze-etching unit (Leybold-Heraeus Vacuum Products Inc., Monroeville, Pa.) (6, 10). The unit was equipped with both a complementary replica device and a device for a contamination-free transport of specimens freeze-cleaved under liquid nitrogen or helium (10) into the vacuum. Specimens cleaved under high vacuum (2×10^{-7} torr) at temperatures of 173°, 133°, and 108°K were replicated immediately after fracturing. To test contamination rates within the plant, some specimens were exposed to vacuum up to 15 min at constant temperatures within the liquid nitrogen-cooled shrouds.

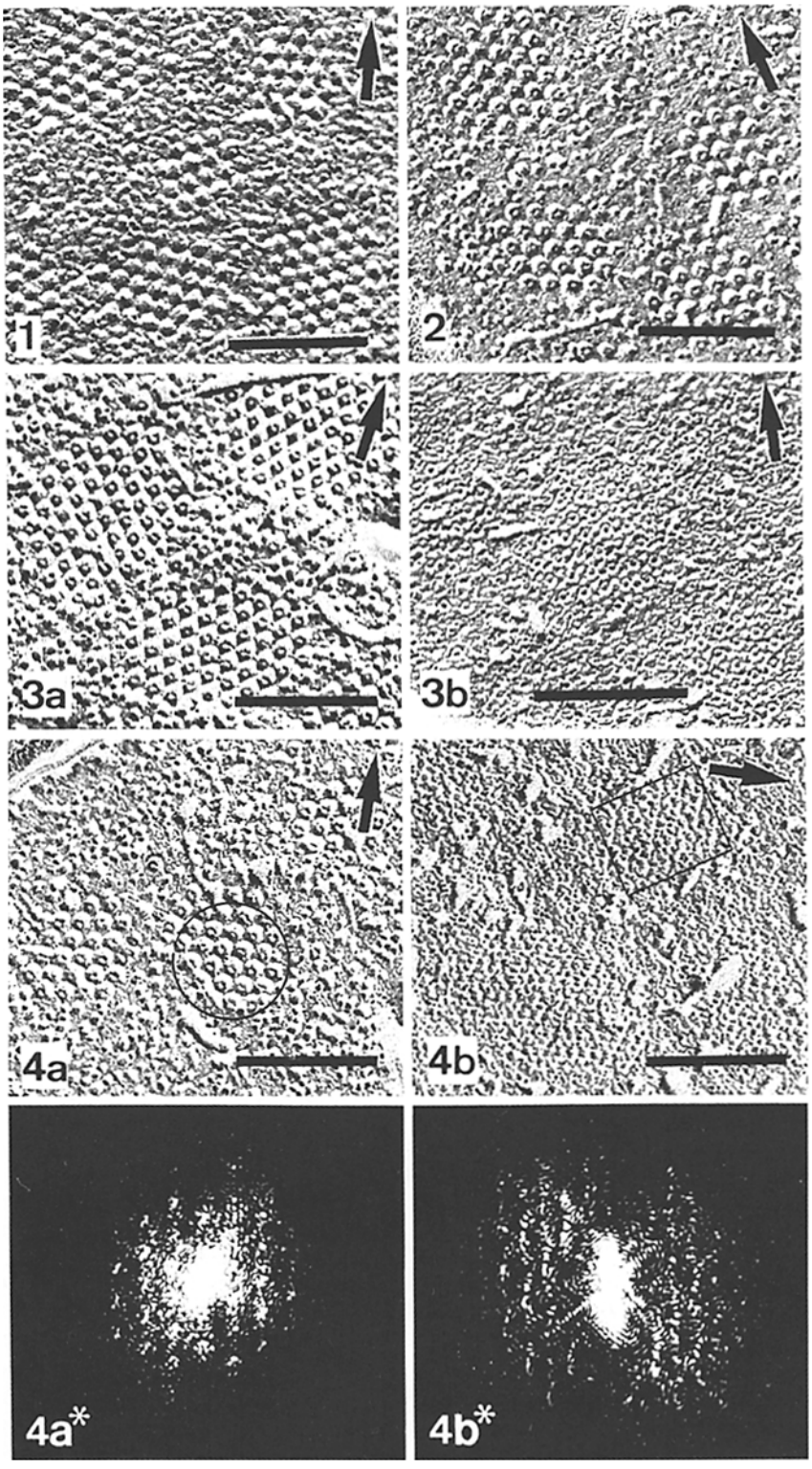
To differentiate between structural changes of the fracture faces induced by either the evaporation or the cleavage process, specimens freeze-cleaved under liquid nitrogen were warmed up to 108° and 173°K, respectively, before coating. This was done under the protection of a lid mechanism (10) attached to the specimen cup of the Bioetch 2005 while completely enclosed by the liquid nitrogen-cooled shrouds. A 2-nm layer of platinum-carbon was evaporated using the method described by Glitsch (1) at a deposition rate of 0.4 nm s^{-1} and an angle of 45° after reaching a vacuum of $>2 \times 10^{-7}$ torr (measured outside the cooled shrouds). 20 nm of carbon was then deposited using thermal evaporators following the standard procedure for the Bioetch 2005. Both the Pt/C and the C evaporators were preheated to allow degassing at a stage in the automatic cycle when the specimen was still enclosed within the liquid nitrogen-cooled shrouds (10).

RESULTS AND DISCUSSION

Our results clearly demonstrated that, independent of the cleavage temperature within the examined ranged from 173° to 77°K or the preparation conditions (freeze-cleaving under vacuum or under liquid nitrogen), the resting and the starved yeast did show significant differences in the topography of the regularly arranged membrane structures. In resting baker's yeast (Fig. 1) cleaved at temperatures from 173° to 77°K, the regularly arranged particles on the P face appear as dome-like structures, although there are usually a few particles in the arrays which have a central depression. It was also noted that the arrays of ringlike depressions on the E face of resting yeast (not shown) were less regular when compared with those found on starved yeast. At all cleavage temperatures, regularly arranged particles with a characteristic craterlike structure were revealed on the P face of starved yeast (Figs. 2, 3*a*, and 4*a*) and complementary ringlike depressions with a hexagonal arrangement of depressions surrounding each ring were seeen on the E face (Figs. 3*b*, and 4*b*).

Independent of cleavage temperature, the diffraction patterns of the regular arrays on both plasmalemma fracture faces of starved yeast did show fourth- (Fig. 4*a**) and even sixth- (Fig. 4*b**) order spots.

Our observations provided no indication of any significant temperature-dependent change in either the fine structure of the particles or the long-range order of the regular arrays in plasmalemma fracture faces of resting yeast. From scanning a large number of starved yeast cells, it was noted that not all plasmalemma P faces exhibited


1
2
3a
3b
4a
4b
4a*
4b*

regular arrays with the craterlike particles. Occasionally, even in individual cells, arrays with varying proportions of domelike and craterlike particles were observed. This heterogeneity, which was found independent of the preparation conditions, is seen as an additional feature supporting the idea that the revelation of crater-shaped particles is neither related to the cleavage temperature nor specifically to a reduction of condensative contamination, but that it arises from a specific property of the yeast cells themselves. We therefore conclude that the image of the regular arrays of particles on plasmalemma fracture faces is related, at least in part, to the physiological state of the yeast.

As has already been discussed by Sleytr and Robards (4, 5), both physiological conditions and the application of cryoprotectants can strongly affect the image of yeast plasmalemma fracture faces—and particularly the amount of plastic deformation of membrane-associated or intrinsic components. A heterogeneous deformation of particle-associated components could easily account for the less regular appearance of the paracrystalline arrays of resting yeast.

Since it has been shown on chemically and morphologically defined model systems that cleavage characteristics can be temperature dependent (4), our comments on the results of Gross et al. (3) do not necessarily question the usefulness of lowering the cleavage temperature for certain specimens. Furthermore, not an ultrahigh vacuum, but a clean vacuum, free of condensable vapor, is certainly required for a high-resolution replication of the fracture face. As can be seen from our experiments, where fracture faces were exposed for up to 15 min at 108°K within the liquid nitrogen-cooled shrouds before replication (Fig. 3), no detectable contamination occurs.

We consider these results to be striking evidence in support of the view that only the local vacuum conditions around the specimen are important and not necessarily a general improvement of the vacuum in the whole system involving complicated UHV technology (5). In other words, it is not the total pressure (3) but the partial pressure of condensable gases in the specimen area that is important. Good shielding with a cold trap (cold shroud) that is kept at a temperature below that of the specimen (8) can protect the fracture most efficiently from contamination both by gases produced by desorption from warmer surfaces and by gases in the system that condense at temperatures below that of the specimen itself.

Finally, we would like to emphasize that even specimens freeze-cleaved under liquid gases show the same structural details on fracture faces (Fig. 2) as specimens cleaved under high vacuum, provided proper shielding is present during transfer into the evaporation unit.

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Note Added in Proof: Recently Dr. S. Bullivant (personal communication) using a simple system of fracturing under liquid nitrogen and protection with a tunnelled cold buck (Bullivant, S. and A. Ames, 1966, *J. Cell*

FIGURE 1 Plasmalemma P face of resting yeast, freeze-fractured under liquid nitrogen and warmed to 108°K under vacuum before replicating. The regular arrays are composed of dome-shaped particles. Arrows in all micrographs indicate direction of shadowing. Bars, 0.1 μm. × 200,000.

FIGURE 2 Plasmalemma P face of starved yeast, freeze-fractured under liquid nitrogen and warmed to 108°K under vacuum before replicating. The craterlike particles can be seen. × 200,000.

FIGURE 3 Plasmalemma of starved yeast, freeze-fractured at 108°K and exposed for 10 min within the liquid nitrogen-cooled shrouds. (*a*) P face showing the craterlike structure of the subunits in the regular array. (*b*) E face with complementary ringlike depressions. × 200,000.

FIGURE 4 Plasmalemma of starved yeast, freeze-fractured at 173°K without etching. Both the P face (*a*) and the E face (*b*) exhibit the same structure as seen in Figs. 3*a* and *b*; Figs. 4*a** and 4*b** are the corresponding optical diffractograms, illustrating fourth- and sixth-order spots, respectively. Figs. 4*a* and *b*: × 200,000.

Biol., **24:**435) has demonstrated clearly both the P-face crater shaped particles and the E-face rings. This supports our assertion that it is the partial pressure of condensable gases in the specimen area that is important and that complicated UHV technology is not required. Dr. R. L. Steere (personal communication) has also demonstrated the same structure on E and P faces using a cold shroud device.

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