Periplasmic Structure in *Saccharomyces rouxii* (Boutroux), an Osmophil

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Electron micrographs of ultrathin sections of *S. rouxii* displayed electron-dense, membrane-circumscribed structures between the protoplasmic membrane and the cell wall. These periplasmic bodies were numerous in cells from a 3-day culture and absent or rare in older cells. Periplasmic bodies were fewer and smaller (flattened) in specimens grown in a medium fortified with 10% sucrose; they were not detected in cells grown in 20% sucrose. A brief treatment with ethyl acetate caused the periplasmic bodies of young cells to become electron light. Periplasmic bodies were most prevalent in the regions of the bud scars and were often accommodated within large invaginations in the protoplasmic membrane. In general, conditions which favor the prevalence and electron density of periplasmic bodies are those which also mask the activity of β-fructofuranosidase in this species.

*Saccharomyces rouxii* (Boutroux) is employed in the fermentation process which attends the production of Miso paste and soy sauce (oriental foodstuffs). This yeast is outstanding for its ability to tolerate high concentrations of sugar or salt in the growth medium and is also commonly implicated in the spoilage of dried fruits, concentrated fruit juice, and honey. The physiology of the osmophilic yeasts has been reviewed by Onishi (10) and by Ingram (7).

A useful diagnostic parameter for *S. rouxii* is the pronounced delay which is observed in the fermentation of sucrose or raffinose as opposed to glucose or fructose (12). This is due to the eventual appearance of enzymatic hydrolysis of the oligosaccharides in aged cultures (11). Recently, Arnold (in press) has shown that the responsible enzyme is a β-fructofuranosidase (EC 3.2.1.26) which is cryptic in young cells but becomes expressed upon aging of the cell. Evidence will be presented elsewhere to show that β-fructofuranosidase in young cells of *S. rouxii* must be enclosed by the protoplasmic membrane or a special vesicular structure. The inference follows that this structure becomes modified upon aging, or (artificially) by treatment with certain organic solvents, because a brief exposure to ethyl acetate expressed the full complement of enzymatic activity. This situation may be contrasted with that in baker's yeast, *S. cerevisiae*, in which β-fructofuranosidase is always assayable in the intact cell because the enzyme is restrained only by the cell wall, which presents no barrier to substrate or buffer constituents (see, for example, refs. 1 and 3).

The extraordinary physiology of *S. rouxii* together with our recent observations on the behavior of β-fructofuranosidase in this species suggested that an ultrastructure study, with emphasis on the nature of the protoplasmic membrane, and the periplasmic region, might reveal some unique features. Correlation of ultrastructure with enzyymology is essential to an understanding of the mechanism of translocation of the periplasmic enzymes of *Saccharomyces* species generally. Also, *S. rouxii* is of inherent interest as a representative of the sugar-tolerant yeasts for which ultrastructure studies may help to explain their ability to colonize extremely concentrated media. This communication summarizes an initial exploration using ultrathin sections of *S. rouxii* cells fixed under a variety of conditions.

**MATERIALS AND METHODS**

**Yeast.** A culture of *S. rouxii* was kindly provided by H. Phaff. The strain bears the Davis collection no. 48-29 and is the subject of previous biochemical studies by the first author (in press). The yeast was grown on a gyratory shaker at 30 C in a medium containing yeast extract (0.3%), malt extract (0.3%), peptone (0.5%), and glucose (1%) (YM broth, Difco Laboratories, Detroit, Mich.). The strain was maintained on YM agar slants. Occasionally, the yeast was grown on YM broth fortified with 10 or 20% (wt/wt)
sucrose. In these, three serial transfers were made on the same medium before inoculating the test flask.

In liquid culture, the microorganism reaches stationary phase in approximately 3 days. Cells from a 3-day (or younger) culture are here referred to as young cells. The expression of β-fructofuranosidase, which is minimal in young cells, becomes apparent in cultures which are typically at least 9 days old (Arnold, in press). Cultures were harvested by centrifugation, and the cells were washed twice in 0.25 M sodium acetate buffer (pH 6). Cells were resuspended in the same buffer to approximately 20% (wet weight per vol) and fixed on the day of harvest, unless otherwise indicated. The β-fructofuranosidase assay and the methylene blue test for viability have been described previously (2).

For the ethyl acetate treatment, 0.1 volume of the organic solvent was added to a 20% (wet weight per vol) suspension of S. rouxii cells in 0.25 M sodium acetate buffer (pH 6). After 15 min at 30 C, the suspension was diluted with another volume of the same buffer and centrifuged. The pellet was washed in 10 volumes of the same buffer and finally suspended to 20% (wet weight per vol). This treatment is sufficient to express all of the β-fructofuranosidase activity of younger cells.

Preparation for electron microscopy. Primarily, cells were fixed at 4 C with 3% glutaraldehyde in 0.1 M s-collidine buffer (pH 6.8) for 24 h. After exhaustive washing in 0.9% NaCl, they were postfixed for 20 h at 4 C with 1% osmium tetroxide in 0.1 M s-collidine buffer (pH 7.4). Other portions of the cells were fixed for 15 min at 4 C in a mixture containing 1% acrolein, 1% glutaraldehyde, and 1% tris-(1-azidinyl) phosphine oxide (TAPO) (Polysciences Inc., Warrington, Pa.) according to Djabenko and Cassone (4). Thirdly, cells were fixed for 10 min at 4 C in 0.6% potassium permanganate.

After fixation, all specimens were embedded in 2% Noble agar to facilitate handling. Small agar cubes containing the cells were dehydrated in a graded series of ethanol and embedded in Epon 812 (6). Ultrathin sections were cut on an LKB Ultrotome III with a diamond knife and placed on uncoated copper grids of 300 mesh. Staining was 5 min with lead citrate followed by 10 min with 3% uranyl acetate in methanol. Electron micrographs were made on an Hitachi instrument (model HU-11B-1).

For cells harvested from 20 or 10% sucrose-fortified media, the glutaraldehyde fixative was also fortified with the appropriate sucrose concentration. In these cases, the subsequent saline washes were a graded series of sucrose-fortified solutions.

RESULTS

Figure 1 illustrates the ultrastructural appearance of S. rouxii after fixation in glutaraldehyde-osmium. Although two bud scars are evident in the polar regions, the histochemical techniques employed in this study resulted in poor visualization of the cell wall which has an average thickness of about 300 nm. Generally, cells were observed to contain a single nucleus, scattered mitochondria, occasional lipid storage bodies, and one or more vacuoles.

A striking feature of the S. rouxii cross section is the occurrence of membrane-circumscribed structures of somewhat increased electron density, which are located between the protoplasm and the cell wall. We refer to these structures as periplasmic bodies. These structures were consistently observed in young cells as exemplified by Fig. 1–4. Periplasmic bodies were observed in cells fixed in glutaraldehyde-osmium (Fig. 1 and 2), TAPO (Fig. 3), and also permanganate (Fig. 4). The periplasmic bodies were irregular in shape and variable in cross section. An estimate of cross-sectional areas was obtained by weighing traced outlines of these bodies and comparing the values with the weight of a defined area of the same tracing paper. The average area of the 14 periplasmic bodies discernible in Fig. 2 is 0.022 μm², and, in that particular plane of section, the periplasmic bodies occupy an area equivalent to 11% of the area of the protoplasm. In several instances these structures were bounded by a clearly delineated limiting membrane (see in particular Fig. 5). Adjacent periplasmic bodies in the same electron micrograph might be interpreted as representing cross sections of papillae (i.e., projections from the protoplasm). The possibility remains that the circumscribed structures represent sections through a tortuous papilla.

The periplasmic bodies generally were concentrated in the regions of the bud scars. However, see Fig. 1 for additional peripheral distribution, and, in the absence of serial sections, it is not possible to state conclusively that they are restricted to the vicinity of bud scars.

An unidentified organelle of potential interest is shown in Fig. 3. We have used the term "vacuole-like structure" to describe this membrane-circumscribed region which contains a myelin-like figure and other membranous structures. Of particular interest is the apparent continuity of this organelle with the periplasmic space via a thin channel. Although a rare observation, similar structures were observed in a few other sections. Demonstration of this communication into the cytoplasm might be a function of the plane of section. A possible relationship between this structure and the ontogeny of the periplasmic bodies is of course speculative at this time.

The integrity of the protoplasmic membrane was best preserved in selected sections which had been fixed by the TAPO method (Fig. 3).
However, deep invaginations in the protoplasmic membrane were common with all fixatives. The extent of these invaginations and their possible relationship to the periplasmic bodies is not established, but in many instances (Fig. 1) the infoldings of the protoplasmic membrane accommodate adjacent periplasmic bodies.

It would be of interest to know the relationship between the prevalence of the periplasmic bodies and the expression of β-fructofuranosidase upon aging of the cells. Such relationships have been difficult to establish since we have not synchronized our cultures, and aged samples would of course contain a spectrum of cell types. Our experience has been that periplasmic bodies were observed in those cells retaining a high degree of integrity of internal structure (e.g., intact nucleus, mitochondria, vacuoles, etc.). On the other hand, cells which were internally degenerate were usually devoid of periplasmic bodies. Bud cells in older cultures of S. rouxii were frequently more elongated and were sometimes arranged in chains of unseparated cells. Figure 6 illustrates a longitudinal section of a cell from a 9-day-old culture in which 79% of the cells were judged dead by the methylene blue test and 100% of the β-fructofuranosidase activity was expressed. A bud cell remains attached to the mother cell with clear delineation of the septal area. The cytoplasm of both mother and daughter cell is degenerate as evidenced by the poor differentiation of the organelles. No periplasmic bodies are observed in the plane of section.

Figure 7 shows cross sections of portions of two cells from a 3-day culture of S. rouxii which was briefly treated with ethyl acetate (as described in Methods) prior to fixation. The features are the maintenance of invaginations in the protoplasmic membrane and the washed-out appearance of residual periplasmic bodies. The electron density of the latter is less than the ground substance (in contrast to untreated cells, see Fig. 1-5) although the limiting membrane remains distinct.

Several sections of cells from a culture of S. rouxii grown 3 days in 20% sucrose medium were examined for periplasmic bodies. They were not detected in any of the samples, although retention of internal structure and protoplasmic
Fig. 2. Section of a cell from a 2-day culture of S. rouxii. Note the prevalence of periplasmic bodies (PB) in the vicinity of the three bud scars (BS). CW, Cell wall. Glutaraldehyde-osmium fixation. ×37,000. Bar represents 0.25 μm.

Fig. 3. Section of a budding cell from a 2-day culture of S. rouxii. Note the periplasmic bodies (PB) near a bud scar (BS) in the mother cell. The daughter cell shows deep invaginations (IV) in the protoplasmic membrane. Note the channel (C) which appears to connect a vacuole-like structure (VS) with the periplasmic space. TAPO fixation. ×80,600. Bar represents 0.20 μm.
Fig. 4. Section of an S. rouxii cell from the same culture as the cell shown in Fig. 1. IV, Invagination; PB, periplasmic body; BS, bud scar. Permanganate fixation. ×52,400. Bar represents 0.25 μm.

Fig. 5. A group of five periplasmic bodies (PB). Note the limiting membrane (LM). Glutaraldehyde-osmium fixation. ×104,300. Bar represents 0.10 μm.
**Fig. 6.** Section of a budding cell from a 9-day culture of *S. rouxii*, which had been stored at 4°C for several weeks. The methylene blue test indicated only 21% of the cells were viable, and 100% of the β-fructofuranosidase activity was expressed (cf. Fig. 1). Note the staining of the septum (S) and the presence of lipid storage bodies (LB) in an otherwise degenerate cytoplasm (C). The elongated bud cell (BC) is also degenerate. Glutaraldehyde-osmium fixation. ×63,500. Bar represents 0.25 μm.

**Fig. 7.** The result of a brief pretreatment with ethyl acetate as described. Cells were from a 3-day culture. Note the washed-out appearance of periplasmic bodies (PB) and the retention of a deep invagination (IV) in the protoplasm. CW, Cell wall; BS, bud scar. Glutaraldehyde-osmium fixation. ×51,300. Bar represents 0.25 μm.
membrane invaginations indicated acceptable fixation and staining technique. A cross section of a budding cell from a culture of *S. rouxii* grown in 10% sucrose is shown in Fig. 8. Periplasmic bodies are evident at the septal area which is consistent with cells grown in normal medium. The periplasmic bodies of cells grown in 10% sucrose were as electron dense as the ground substance, but they were generally smaller than counterparts from unfortified media and exhibited a flattened appearance in cross section. The limiting membrane of the periplasmic bodies is quite distinct (Fig. 8). It is worth noting that the growth rates, and yields after 3 days, were not significantly different for unfortified and 10% sucrose-fortified media. On the other hand, the growth rate in 20% sucrose-fortified medium was reduced, and the yield (dry weight basis) after 3 days was about half of the unfortified control.

**DISCUSSION**

The ultrastructure of *S. rouxii* is, for the most part, comparable with that of other *Saccharomyces* species (8). The novel feature is the occurrence of relatively large and numerous periplasmic bodies. Their demonstration after three different methods of cell fixation argues against an artifactual origin. The description of size, shape, and distribution of periplasmic bodies will be strengthened by a study using freeze-fracturing techniques.

An organelle of comparative interest is the lomasome which has been described in filamentous fungi (9). Apart from a periplasmic location, these organelles bear little resemblance to the periplasmic bodies we describe in *S. rouxii*. The vacuole-like structure, which was encountered rarely (Fig. 3), has some similarity to a structure described (5) in *Debaryomyces hansenii* (Zopf), which is a salt-tolerant yeast.

Our preliminary studies indicate a reverse relationship between the concentration of sucrose in the growth medium and the prevalence of periplasmic bodies. Cells grown in 20% sucrose-fortified medium were apparently devoid of periplasmic bodies; those cells from 10% sucrose medium exhibited fewer and smaller (flattened) periplasmic bodies. Because growth was decreased at the highest sucrose concentration, and it was demonstrated that periplasmic bodies were most prevalent in the budding

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**Fig. 8.** Section of a cell from a 3-day culture of *S. rouxii* in YM broth fortified with 10% sucrose. Note the flattened appearance of the periplasmic bodies (PB). S, Septum; CW, cell wall; IV, invagination. Glutaraldehyde-osmium fixation. ×80,100. Bar represents 0.1 μm.
regions of cells in normal medium, we cannot exclude the possibility of a functional relationship between periplasmic bodies and the budding mechanism.

In connection with the sugar tolerance of this species, the demonstrated invaginations in the protoplasmic membrane provide a sizeable increase in the space which is available to media constituents of moderate molecular weight. This may be important in the evaluation of solute distribution studies on this species.

The location of the enzyme β-fructofuranosidase within S. rouxii cells is still far from defined. However, observations reported herein provide circumstantial evidence which is in sympathy with biochemical results. For example, the enzyme is cryptic in 3-day cells (in which periplasmic bodies are most prevalent) and is partly expressed in aged cells (in which periplasmic bodies are rare or absent). A brief treatment with ethyl acetate "washes out" the periplasmic bodies and is accompanied by complete expression of the enzyme. Further studies are required in which mild disruption of cells, followed by fractionation methods on the centrifuge, might give some insight into the behavior, function, and enzyme content of periplasmic bodies.

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