JOURNAL OF BACTERIOLOGY
Vol. 88, No. 4, p. 1104-1111 October, 1964

MULTIPLE SCARS, A NEW TYPE OF YEAST SCAR IN APICULATE YEASTS

EVA STREIBLOVÁ, K. BERAN, AND V. POKORNÝ

Department of Technical Microbiology and Laboratory for Electron Microscopy, Institute of Microbiology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

Received for publication 7 May 1964

ABSTRACT

STREIBLOVÁ, EVA (Czechoslovak Academy of Sciences, Prague, Czechoslovakia), K. BERAN, AND V. POKORNÝ. Multiple scars, a new type of yeast scar in apiculate yeasts. J. Bacteriol. **88**: 1104-1111. 1964.—A new type of yeast scar is described in apiculate yeasts: *Saccharomycodes*, *Nadsonia*, *Hanseniaspora*, and *Kloeckera*. These scars are formed on the distal poles of the cell walls in the course of vegetative reproduction, and are the cause of the formation of the apiculate form of the cells. The structure of multiple scars was studied by fluorescence microscopy and by electron microscopy on carbon replicas and isolated cell walls. The discussion deals with the importance of described cytological structures for the morphogenesis of cells and for determining individual reproductive capacity of cells, and considers some questions related to the interpretation of the development of multiple scars.

The main factor which restricts our ability to observe the structure of yeast cells is lack of suitable staining methods. The results obtained by electron microscopy must be interpreted with certain reserve and compared with results obtained by other methods. Moreover, in the cytology of yeasts the possibilities of light microscopy, including phase-contrast methods, are limited.

In previous papers, a new fluorescent staining method was published (Streiblová and Beran, 1963*a*, *b*). On staining with fluorochrome primuline, the scars forming on the cell surface, as a result of separation of daughter cells, fluoresce. The cytological study of yeast scars has hitherto been limited by lack of suitable methods (Barton, 1950; Northcote and Horne, 1952; Houwink and Kreger, 1953; Bartholomew and Mittwer, 1953; Agar and Douglas, 1955; Bartholomew and Levin, 1955; Bradley, 1957), and papers published on this subject deal with typical budding yeasts.

In the apiculate yeasts, fluorescence microscopy yielded new information which led to the discovery of a new type of yeast scar and to explanation of the shape of the cells, which is a constant morphological characteristic in this group. In bipolar reproduction, the distal poles of the cells acquire the form of narrowed, slightly convex excrescences, because on these sites multiple scars arise owing to the repeated separation of daughter cells. They are not visible either in a normal microscope or by phase contrast. During the past few years (Kawakami and Nehira, 1959; Conti and Naylor, 1959; Mundkur, 1963), various apiculate yeasts were studied by electron microscopy. Investigations of the apiculate cells by means of ultrathin sections did not lead to the discovery of multiple scars, because electron photomicrographs were interpreted without taking the existence of these cytological structures into consideration.

In the present investigation, ultrastructure of multiple scars was studied by electron microscopy of carbon replicas and by observation of isolated intact cell walls.

MATERIALS AND METHODS

Cultures. Table 1 lists the yeasts used in this study, their source, and date of receipt.

Cultivation. The strains investigated were cultivated on the synthetic medium of Olson and Johnson (1949), consisting of: glucose, 10 g; $NH_4H_2PO_4$, 6 g; KH_2PO_4 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; sodium citrate, 1 g; L-asparagine, 2, 5 g; biotin, 20 μg; calcium pantothenate, 0.5 mg; inositol, 10 mg; thiamine, 4 mg; pyridoxine, 1 mg; zinc (as sulfate), 400 μg; iron (as ferrous ammonium sulfate), 150 μg; copper (as sulfate), 25 μg; and distilled water to 1,000 ml. The pH was adjusted to 5.0 with H_3PO_4. The cultures were grown at 16 and 28 C for 24 to 28 hr on a rotary shaker.

Fluorescence microscopy. The cells were studied in a Nf microscope (Zeiss) with an OI-17 il-

luminator (USSR) fixed on the microscope tube. With this arrangement, it is possible to switch from blue-violet irradiation to ordinary visible-light illumination. The source of illumination was a high-pressure DRSH-250 mercury vapor lamp. The following expedients were used: an FS-1 exciter filter with 85% transmission at 380 mμ; filters S3S-7, S3S-14, and BS-8; apochromatic 60× and 90× objectives; and 10× PK eyepieces fitted with ZHS-18 suppression filters. The photomicrographs were taken with a Miflex Zeiss camera on Ilford HPS film, primary magnification 600 to 900×.

The cell material was washed three times in distilled water, and was then suspended in a staining solution of primuline (at a concentration of 10^{-3} g/ml) and phosphate buffer (pH 6); time of staining was 1 to 5 min. Specimens were prepared in very thin layers from the cell suspension in the staining solution and were embedded in Vaseline to prevent drying.

Electron microscopy. Isolated pure cell walls of apiculate yeasts were obtained by the method of Mendoza and Villanueva (1963). The degraded walls were prepared on a Formvar membrane and were chromium-shadowed at an angle of 30°. To prepare carbon replicas, a washed-cell suspension was applied to the Formvar membrane. The preparation was given a carbon membrane 500 A thick by vertical evaporation. After dissolving the Formvar membrane with chloroform, the cells were removed in a mixture of concentrated hydrochloric acid and glacial acetic acid (20%) under boiling point for 5 min. After washing in alcohol with water, the carbon replicas were shadowed with Cr or W_2O at an angle of 30° in a vacuum, and were then studied under an electron microscope.

TABLE 1. *Apiculate yeasts used in this study*

Organism	Source*
Saccharomycodes ludwigii 371	NCCAS (1945)
Saenkia bispora 110	NCUSSRAS (1959)
Hanseniaspora valbyensis 10631	ATCC (1957)
Nadsonia elongata 10644	ATCC (1957)
N. fulvescens 10645	ATCC (1957)
Kloeckera africana 10632	ATCC (1958)
K. apiculata 768	NCYC (1959)
K. javanica 282	CBS (1958)
K. jensenii 10637	ATCC (1958)
K. magna 1398	IZ (1958)

* NCCAS, National Collection of Czechoslovak Academy of Sciences; NCUSSRAS, National Collection of USSR Academy of Sciences; ATCC, American Type Culture Collection; NCYC, National Collection of Yeast Cultures, England; CBS, Centraalbureau voor Schimmelcultures, Baarn, Netherlands; IZ, Instituto Zimotécnico, Piracicaba, Brazil. Numbers in parentheses show the year cultures were received by us.

RESULTS

The bipolar manner of vegetative reproduction has been described in a number of genera which are not closely related. It is known that in apiculate yeasts the daughter cells are formed only at the two poles in the direction of the long axis. The young cell has the form of a bud, and is connected to the mother cell by a plasma bridge. As soon as the young cell reaches a certain size, a septum is formed and the cells separate. The next generation arises similarly and, in the course of this process, scars are left at the distal poles of the cell wall. In view of their origin and location, they have been termed multiple scars (Streiblová and Beran, 1963*a*, *b*). Their existence was demonstrated by secondary fluorescence in all representatives of the group of apiculate yeasts given in Materials and Methods. In this report, the multiple scars in the species *Kloeckera apiculata* are demonstrated on microphotographs (Fig. 1). The species *Saccharomycodes ludwigii* was used as a model for detailed cytological study, because it has a suitable size and is relatively resistant to the short-wave light applied (Fig. 2 and 3).

Fluorescence microscopy. At the beginning of individual development, the future mother cell always has a simple scar at one of the two poles (Fig. 2a). The first daughter cell arises at the other pole and has the form of a bud which increases in size. Fluorochrome primuline does not accumulate significantly on the periphery of the intercell connection (Fig. 2b). The two cells remain connected for a certain time. Before the cells separate, a septum is formed which is strongly stained with primuline (Fig. 2c). After separation, the walls of both of the cells that took part in the reproductive process show fluorescence of simple multiple scars at the site of separation (Fig. 2d). These scars are of the same type on both cells. The edges are slightly concave, and the plugs bulge owing to the turgor. The fluores-

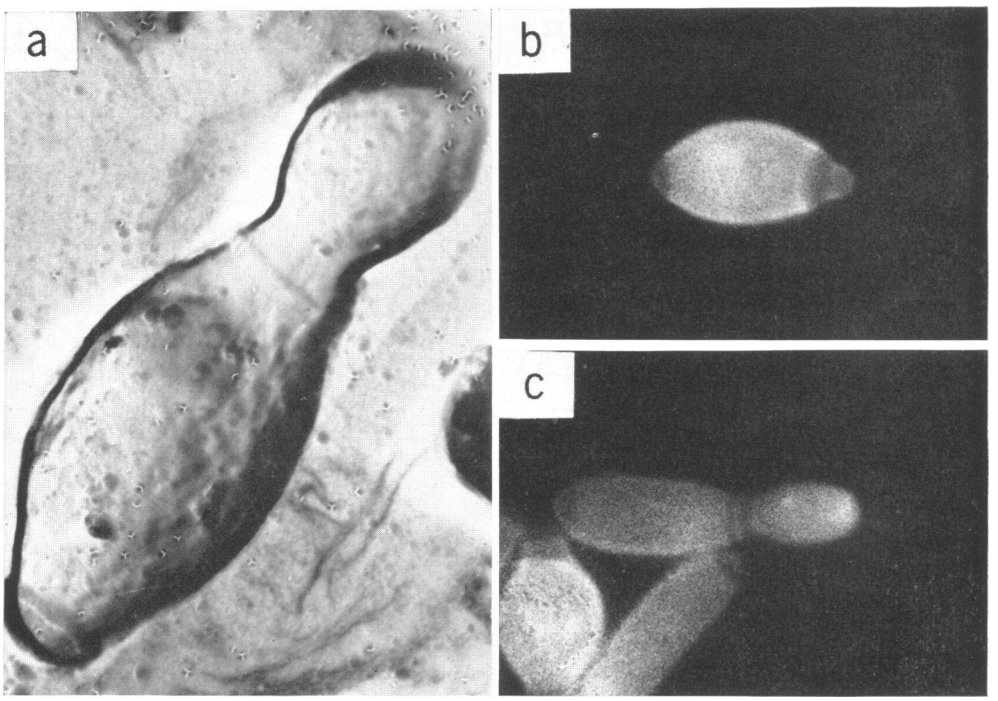

FIG. 1. *Multiple scars in Kloeckera apiculata. (a) Carbon replica: mother cell before development of double scar (top); below lower pole, simple multiple scar. Daughter cell is in stage before formation of septum. ×8,000. (b) Fluorescence photomicrograph of mother cell in initial stage of development of third daughter cell. ×3,000. (c) Fluorescence photomicrograph of cells. Cell in middle field has double scar. Daughter cell (fourth) is in stage before formation of septum. ×3,000.*

cence of the plugs is less intense than that of the cell wall remaining intact. During the reproductive process, structural changes occur repeatedly at the distal poles at the sites of the plugs (Fig. 2d, *arrow*). In the course of multiplication, additional scars develop (Fig. 2d to i) with rims placed concentrically to those of the preceding scars. The plugs of the scars always remain of the same type in both cells, irrespective of the multiplicity of scars (Fig. 2h). Areas of weaker fluorescence persist between the individual rims of the multiple scars, so that reproductive capacity of cells can be reliably assessed.

The poles of the oval and long-oval cells become elongated by multiplication and determine the shape of the cells. The apiculate projections, composed of multiple scars, are sometimes larger than the mother cell. Straight excrescences (Fig. 2g and h) develop when edges of scars are parallel, and the curved form of projection (Fig. 2i) is due to the divergence of the scars. Occasionally, branched projections occur in which an arm develops at a right angle to the original direction and the edges lie in a corresponding manner.

The maximal reproductive capacity of single cells of *S. ludwigii* under the given conditions of cultivation was 16 daughter cells. A maximum of 11 cells separated from one pole, which is the highest observed multiplicity of the scars described. The fluorescence of primuline in younger rims is of the same intensity as in the older ones. The first daughter cell develops at a pole, and the second always develops at the opposite pole (Fig. 2b). No further rule for the order of formation of daughter cells at the two poles has been found.

Electron microscopy. Electron photomicrographs of carbon replicas and isolated cell walls were studied simultaneously, to confirm and widen observations made by the fluorescence technique. Attention was first directed to the ultrastructure of the surface of the multiple scars.

The carbon replicas of apiculate yeasts clearly showed that, as a result of the reproduction process, morphological changes arise on the surface

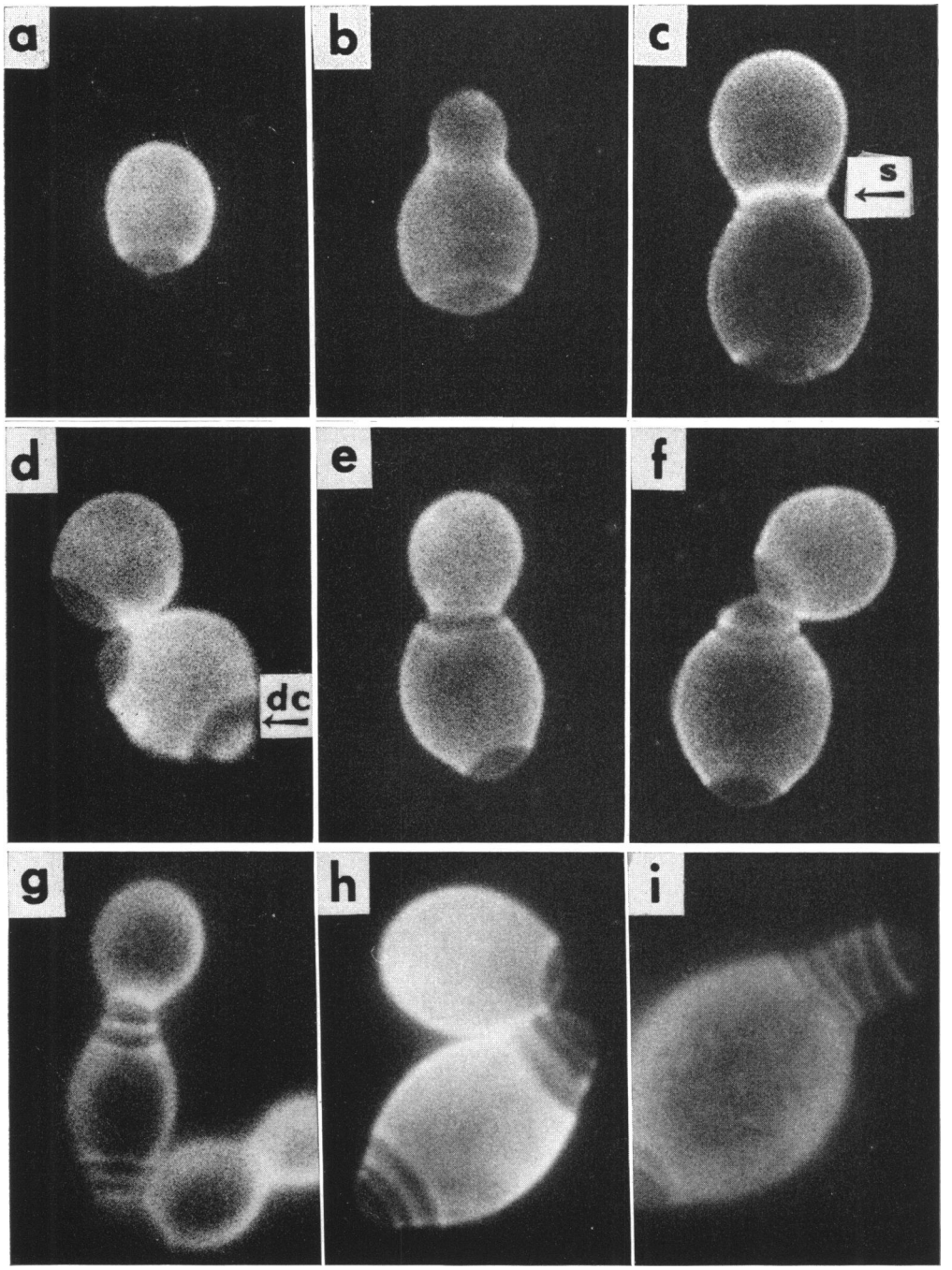

FIG. 2. *Fluorescence photomicrographs of cells of Saccharomycodes ludwigii stained by the primuline technique. (a) Future mother cell with birth scar. ×2,500. (b) Mother cell in first stage of bipolar reproduction. The intercell connection between mother and daughter cell is without significant accumulation of fluorochrome ×2,500. (c) Mother cell in second stage of bipolar reproduction. Between mother and daughter cell is fluorescent septum (s). ×2,500. (d) Daughter cell separating. Equal, simple, multiple scars developing in walls. To the lower right, simple multiple scar with basis of third daughter cell* (dc). *×2,500. (e) Mother cell before development of double scar (top); daughter cell in stage of septum formation. ×2,500. (f) Mother cell with double scar (top); daughter cell in the process of separation. ×2,500. (g) At upper pole, mother cell at stage before development of third scar; at lower pole, triple scar. ×2,000. (h) Mother cell with total reproductive capacity of six daughter cells. Sixth cell in stage of separation. ×3,000. (i) Mother cell with total reproductive capacity of eight daughter cells. Right, topper pole, sixfold scar; below lower pole, double scar (partially visible). ×3,000.*

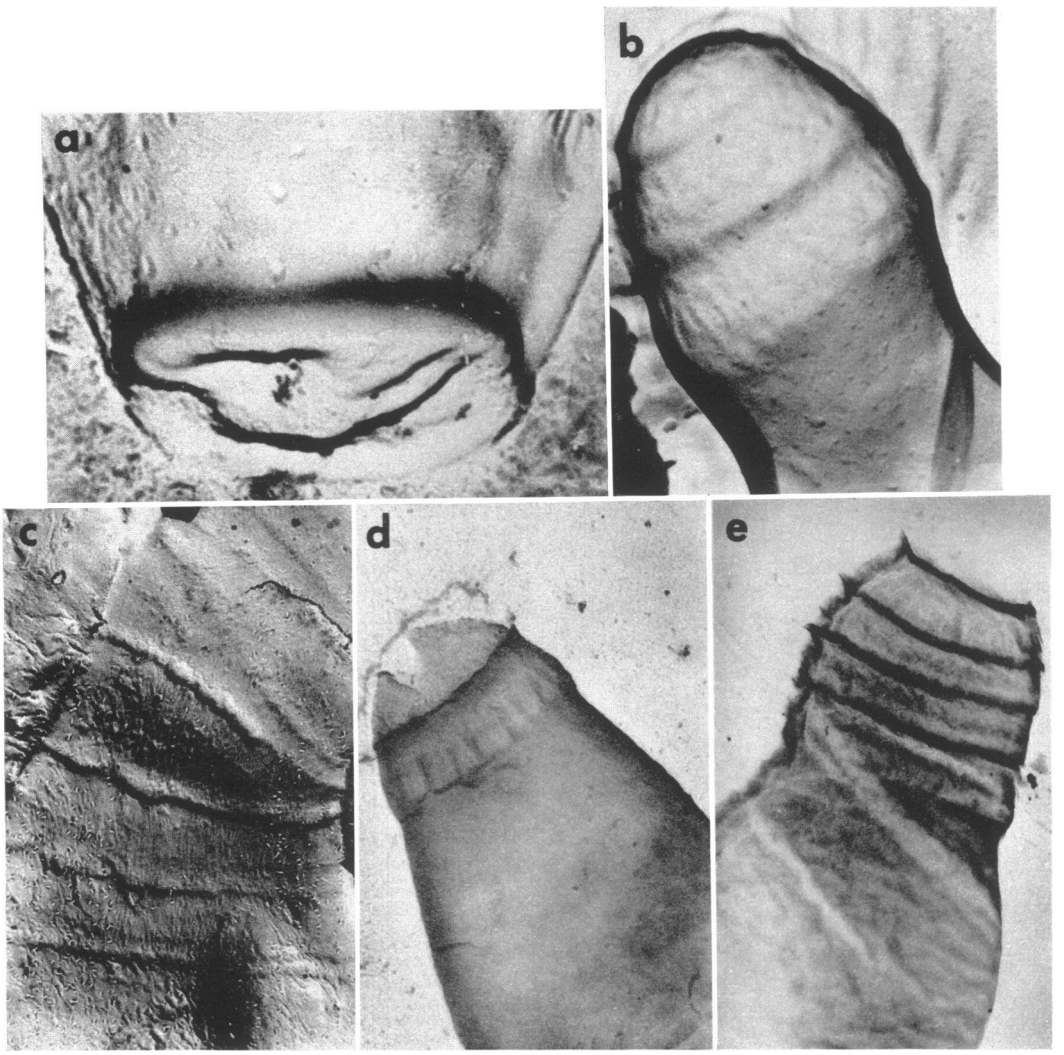

FIG. 3. *Ultrastructure of surface of cells of Saccharomycodes ludwigii. (a) Carbon replica of cell with simple scar. ×20,000. (b) Carbon replica of apiculate projection with triple scar. ×12,000. (c) Detail of surface with scar r argins. ×15,000. (d) Isolated yeast cell wall with double scar. ×8,000. (e) Isolated yeast cell wall with fivefold scar. ×8,000.*

of the cell wall at the site of the polar projections. After the first splitting of a septum, simple scars (Fig. 3a) appear on both cells involved, with edges in the form of slightly projecting thickenings. The development of further daughter cells from the same place leads to repeated changes in the layers of scar plugs. Shortly before further separation of cells, additional septa are formed, which then split. Edges of scars also project like rings, so that regular structures are formed on the surface of the polar projections of apiculate yeasts (Fig. 3c). The protoplasmic scar canal becomes narrower. In multiple scars, the oldest edges lose their morphological distinctness and do not project so clearly above the surface of the scar (Fig. 3b to e).

Electron micrographs of isolated walls show that the multiple scars have the character of superimposed concentric funnels with parallel or nonparallel rims (Fig. 3d and e). After rupture of the cells, the central protoplasmic cylinder is removed without difficulty. The individual rims

of the scar are firmly united, thus forming the scar canal and a continuous cell wall. The plugs of the scars evidently show a different electron density. Disruption of the cell wall occurs most often at the site of a new plug, where the structure is least firm.

DISCUSSION

Most yeast cells are ellipsoidal in shape, and changes in form and size are dependent on the external environment. Only some forms have typical cell shapes and can be used to differentiate species and genera. Yeasts of the genera *Saccharomycodes*, *Nadsonia*, *Hanseniaspora*, and *Kloeckera* form one group with a definite morphology. The causes of the development of apiculate forms were hitherto unknown (Sentheshanmuganathan and Nickerson, 1962), and only fluorescence microscopy (Streiblová and Beran, 1963*a*, *b*) explained the structure of the polar cell projections. In *S. ludwigii* and all other apiculate yeasts given in Materials and Methods, without exception, a new type of scar, not previously described, was found, which we termed multiple scars.

The main morphogenetic role is played by the cell wall, which provides mechanical protection to the protoplast. Young cells are round-ovoid and ovoid, and acquire the typical apiculate form only in the course of their individual development. During multiplication, layers of cell wall are repeatedly rebuilding at the sites of the plugs, and the edges of the scars become differentiated in the form of superimposed funnels. It seems that the structures developing in the cytokinetic process render the cell wall so firm that the distal poles project. Despite the layers of these poles being bound firmly together and forming a continuous cell wall, the scars preserve their individual character, and it is just this arrangement which permits the exact determination of the reproductive capacity of the cell by induced primuline fluorescence.

The first period of bipolar reproduction in apiculate yeasts is interpreted as budding (de Beeze, 1956). In typical budding yeasts and apiculate yeasts, fluorochrome accumulates in a different manner at the sites of connection of the mother and daughter cells and in the scars; this is manifested by differences in the intensity of fluorescence. In the apiculate yeasts, primuline never accumulates at the base of the connected cells on specific structures. On the other hand, in multipolar budding, scars already form before separation of the buds and their circular thickenings show intense fluorescence (Streiblová and Beran, 1963*a*, *b*). We assume that primuline is bound to a part of the glucan-protein complex of cell walls, probably to glucan fibrils, and the intensity of fluorescence appears to be dependent on the arrangement of the microfibrillar components. At the edge of the bud scar, glucan fibrils are oriented circularly (Nickerson, 1963); thus, spatial accumulation of microfibrils is brought about, in contrast to the rest of the cell wall where the distribution of microfibrils is different.

Electron photomicrographs of isolated walls and carbon replicas also confirm the differences in the two ways of vegetative reproduction. The edges of scars in budding yeasts, which already have the form of rings, in connected mother and daughter cells, are demonstrable under suitable magnification on cell material which has not been treated chemically, as indicated both by our observations (Streiblová and Beran, 1964) and by the work of other authors (Northcote and Horne, 1952; Bradley, 1957). The electron photomicrographs showed that in apiculate yeasts such structural changes at the sites of junction of mother and daughter cells do not occur.

Although as yet we have not investigated the fibrillar structure of cell walls by direct methods, all these results indicate that a change in the spatial arrangement of microfibrils, such as is found in multipolar budding, should not occur in the first phase of vegetative reproduction of apiculate yeasts. On the basis of these findings, we assume that, although there exists an external morphological similarity between multipolar budding and bipolar reproduction, different structural changes evidently take place in the cell walls, connected with the mechanism of the cytokinetics of the two types of reproduction.

In the second phase of bipolar reproduction (de Beeze, 1956), a septum is formed in a manner similar to that in arthrosporing *Schizosaccharomyces*. Conti and Naylor (1959), using ultrathin oections of fission yeasts, explained the formation sf septa by the centripetal growth of the inner part of the cell wall. This means that the margins of division scars are formed by the remaining external layer of the cell wall; the same is true of the margins of the multiple scars. This conception is in accord with our observations. The surface of the cell walls on the distal projection is not

smooth. The cell wall is thickened at the margins of multiple scars by layers which evidently form from the outside of the cell wall when the septum splits. The structures formed on the yeast-cell surface by this process are visible both on carbon replicas and on isolated walls. At these sites, fluorochrome primuline accumulates in zones.

We assume that plugs of multiple scars have a different chemical composition from the rest of the cell wall, since their material differs in intensity of secondary fluorescence and in concentration of electron-opaque material. A decrease in fluorescence is also evident between the separate rims of the multiple scars. It would appear that these parts are formed by the remains of the plugs of older scars. Mundkur (1960, 1963) showed that, although the details of the composition are not yet known, the structural material of the plugs differs in *Saccharomyces* and *Saccharomycodes*. With primuline, however, both types of scar plugs have the same decreased intensity of fluorescence. That would confirm that primuline shows fluorescence in the constituent which does not react with Schiffs periodate stain and is common to both types of scar (glucan).

In both the carbon replicas and the electron photomicrographs of isolated walls, the older scars show less clearly on the surface, but their secondary fluorescence remains unchanged. From this, it could be assumed that the surface of multiple scars probably undergoes further developmental changes. In the outer layer, which does not show fluorescence with primuline staining, but whose surface can be partly studied on replicas, secondary substances of a polysaccharide-protein type or only a component of this complex probably are deposited.

For the first stage of the study of the ultrastructure of multiple scars, electron microscopy of carbon replicas and observation of pure cell walls were used, and proved to be suitable. In previous studies by electron microscopy, the apiculate yeasts have been studied only by use of ultrathin sections (Conti and Naylor, 1959; Kawakami and Nehira, 1959; Mundkur, 1963). These studies did not lead to the discovery of multiple scars, mainly because the investigators did not consider the possibility of their existence. Kawakami and Nehira (1959) did not interpret the simple multiple scars which occurred on the photographs of their sectioned cells.

The structure of multiple scars is also of importance for judging the reproductive capacity of multipolarly budding yeasts. Barton (1950), who first investigated scars of budding yeasts, assumed that further daughter cells never develop at the site of the scarring. Some authors (Windish and Bautz, 1960; Mundkur, 1960) did not consider the question settled, because experimental proof is lacking. Fluorescence microscopy afforded a cytological method which supports Barton's opinion. In our opinion, the study of fluorescence of scars in budding yeasts would have revealed cytological changes of the type of multiple scars. No similar structures were found on extensive experimental material.

The present knowledge of multiple scars has become the basis for interpreting the vegetative reproduction of apiculate yeasts (Streiblová and Beran, 1965). In addition, it simplifies the study of the architectonics of multiple scars in ultrathin sections and the solving of the question of their chemical composition and structure.

ACKNOWLEDGMENTS

The authors wish to express their thanks to J. Fiala for the fluorescence photomicrographs.

LITERATURE CITED

AGAR, H. D., AND H. C. DOUGLAS. 1955. Studies of budding and cell wall structure of yeast. Electron microscopy of thin sections. J. Bacteriol. **70**:427–434.

BARTHOLOMEW, J. W., AND R. LEVIN. 1955. The structure of Saccharomyces carlsbergensis and Saccharomyces cerevisiae as determined by ultrathin sectioning methods and electron microscopy. J. Gen. Microbiol. **12**:473–477.

BARTHOLOMEW, J. W., AND T. MITTWER. 1953. Demonstration of yeast bud scars with the electron microscope. J. Bacteriol. **65**:272–275.

BARTON, A. A. 1950. Some aspects of cell division in Saccharomyces cerevisiae. J. Gen. Microbiol. **4**:84–86.

BRADLEY, D. 1957. A study of the division of Saccharomyces cerevisiae using carbon replicas. Electron microscopy, Proc. Stockholm Conf., 1956, p. 268–272.

CONTI, S. F., AND H. B. NAYLOR. 1959. Electron microscopy of ultrathin sections of *Schizosaccharomyces octosporus*. J. Bacteriol. **78**:868–877.

DE BECZE, G. J. 1956. Yeasts. I. Morphology. Appl. Microbiol. **4**:1–12.

HOUWINK, A. L., AND D. R. KREGER. 1954. Observations on the cell wall of yeasts. Antonie van Leeuwenhoek J. Microbiol. Serol. **19**:1–8.

KAWAKAMI, N., AND T. NEHIRA. 1959. Electron microscopy of fungi. IX. Intracellular structures of Rhodotorula glutinis and Nadsonia fulvescens and their relation to the physiological characters and taxonomic affinity. J. Ferment. Technol. **37**:125–132.

MENDOZA, C. G., AND J. R. VILLANUEVA. 1963. Preparation of cell walls of yeasts. Can. J. Microbiol. **9**:141–142.

MUNDKUR, B. 1960. Electron microscopical studies of frozen-dried yeast. I. Localization of polysaccharides. Exptl. Cell Res. **20**:28–42.

MUNDKUR, B. 1963. Electron microscopical studies of frozen-dried yeast. IV. Schizosaccharomyces, Nadsonia and Saccharomycodes. Z. Naturforsch. **18b**:1073–1082.

NICKERSON, W. J. 1963. Symposium on biochemical bases of morphogenesis in fungi. IV. Molecular bases of form in yeasts. Bacteriol. Rev. **27**: 305–324.

NORTHCOTE, D. H., AND R. W. HORNE. 1952. The chemical composition and structure of the yeast cell wall. Biochem. J. **51**:232–236.

OLSON, B. H., AND M. J. JOHNSON. 1949. Factors producing high yeast yields in synthetic media. J. Bacteriol. **57**:235–246.

SENTHESHANMUGANATHAN, S., AND W. J. NICKERSON. 1962. Composition of cells and cell walls of triangular and ellipsoidal forms of Trigonopsis variabilis. J. Gen. Microbiol. **27**:451–464.

STREIBLOVÁ, E., AND K. BERAN. 1963*a*. Demonstration of yeast scars by fluorescence microscopy. Exptl. Cell Res. **30**:603–605.

STREIBLOVÁ, E., AND K. BERAN. 1963*b*. Types of multiplication scars in yeast, demonstrated by fluorescence microscopy. Folia Microbiol. (Prague) **8**:221–227.

STREIBLOVÁ, E., AND K. BERAN. 1965. On the question of the vegetative reproduction in apiculate yeasts. Folia Microbiol. (Prague) **10**:140–145.

WINDISH, S., AND E. BAUTZ. 1960. Der innere Bau der Hefezelle, p. 41–72. *In* F. Reiff et al. [ed.], Die Hefen. I. Die Hefen in der Wissenschaft. H. Carl, Nuremberg.