Arch. Microbiol. 128, 157–161 (1980)

Archives of
Microbiology

The Structural Organization of the Tibi Grain as Revealed by Light, Scanning and Transmission Microscopy

Marielise Moinas, Marc Horisberger, and Heinz Bauer*

Nestle Research Department, CH-1814 La Tour-de-Peilz, Switzerland

Abstract. Tibi grains consist of a unique and very stable symbiotic association of *Lactobacillus brevis*, *Streptococcus lactis* and *Saccharomyces cerevisiae* embedded in a dextran matrix. The structural organization of the grain was examined by light, scanning and transmission electron microscopy. The grain was constituted of an outer compact layer and an inner spongy structure. The outer layer was more densely populated by the microorganisms than the inner layer but dextran, stained on frozen thin sections with fluorescein-conjugated concanavalin A, was more abundant in the inner layer.

Key words: *Lactobacillus brevis* – *Streptococcus lactis* – *Saccharomyces cerevisiae* – Concanavalin A – Symbiosis – Tibi.

Tibi is an acidic, mildly alcoholic drink prepared by fermentation of a sucrose solution by Tibi grains of "Tibi Complex". Figs, dates, raisins, lemons or ginger roots probably provide growth factors and contribute to the pleasant taste of the beverage. Microbiologically, the Tibi grains are a symbiotic association of *Lactobacillus brevis*, *Streptococcus lactis* and *Saccharomyces cerevisiae* (Horisberger, 1969). After 1 or 2 days of fermentation at room temperature, the liquid (drink) can be decanted, the grains washed under running tap water and reused for further fermentation of fresh medium. Fermentation and transfer of the grains can be carried out under non sterile conditions practically without any risk of contamination. Thus it appears that this very stable symbiotic association does not permit an extensive growth of intruding foreign microorganisms.

The Tibi grains are translucent and contain a matrix of dextran consisting mainly of a backbone of α-D (1→6)-linked glucopyranosyl residues with (1→3)-linked side chains (Horisberger, 1969). The grains originate from Mexico where they occur in the form of hard granules on the disk-shaped leaves of the *Opuntia* plant (Lutz, 1899; Daker and Stacey, 1938). They have been described under several different names (Kebler, 1921) and have to be distinguished from the better known Kefir grains used for the conversion of milk into the Russian beverage Kefir (Schultz, 1946; La Rivière et al., 1967).

In spite of a very early and comprehensive description of the Tibi grains (Ward, 1892), to our knowledge no electron optical study has been carried out on this unique biological system. The aim of this paper is to present some aspects of the structural organization of the Tibi grains as revealed by light, scanning and transmission electron microscopy.

Materials and Methods

Materials

Concanavalin A (con A) (lyophilized, 3 × cryst) was purchased from Miles Laboratories Ltd., Bucks, England and conjugated with fluorescein isothiocyanate (Calbiochem., Los Angeles, Calif.) according to the procedure of Tkacz et al. (1971). For staining thin sections, fluorescein-conjugated con A was used at a concentration of 160 µg/ml in 0.15 M NaCl-0.05 M Tris, pH 7.2 made 1 mM in $CaCl_2$ and $MgCl_2$ (buffer A).

Parofor N was from Farbenfabriken Bayer AG, Leverkusen, Federal Republic of Germany.

Cultivation of Tibi grains

Tibi grains were grown under non sterile conditions in a medium containing 60 g sucrose, two dry figs and a quartered lemon per 2 l of tap water (Horisberger, 1969). After 48 h at room temperature in an open jar, the grains were harvested and prepared for microscopical

Abbreviation: Con A, concanavalin A

* Present address: Vitoreco, Food Development, CH-8310 Kemptal, Switzerland

Offprint requests to: M. Horisberger, Société d'Assistance Technique pour Produits Nestlé S.A. CH-1814 La Tour-de-Peilz, Switzerland

0302-8933/80/0128/0157/$01.00

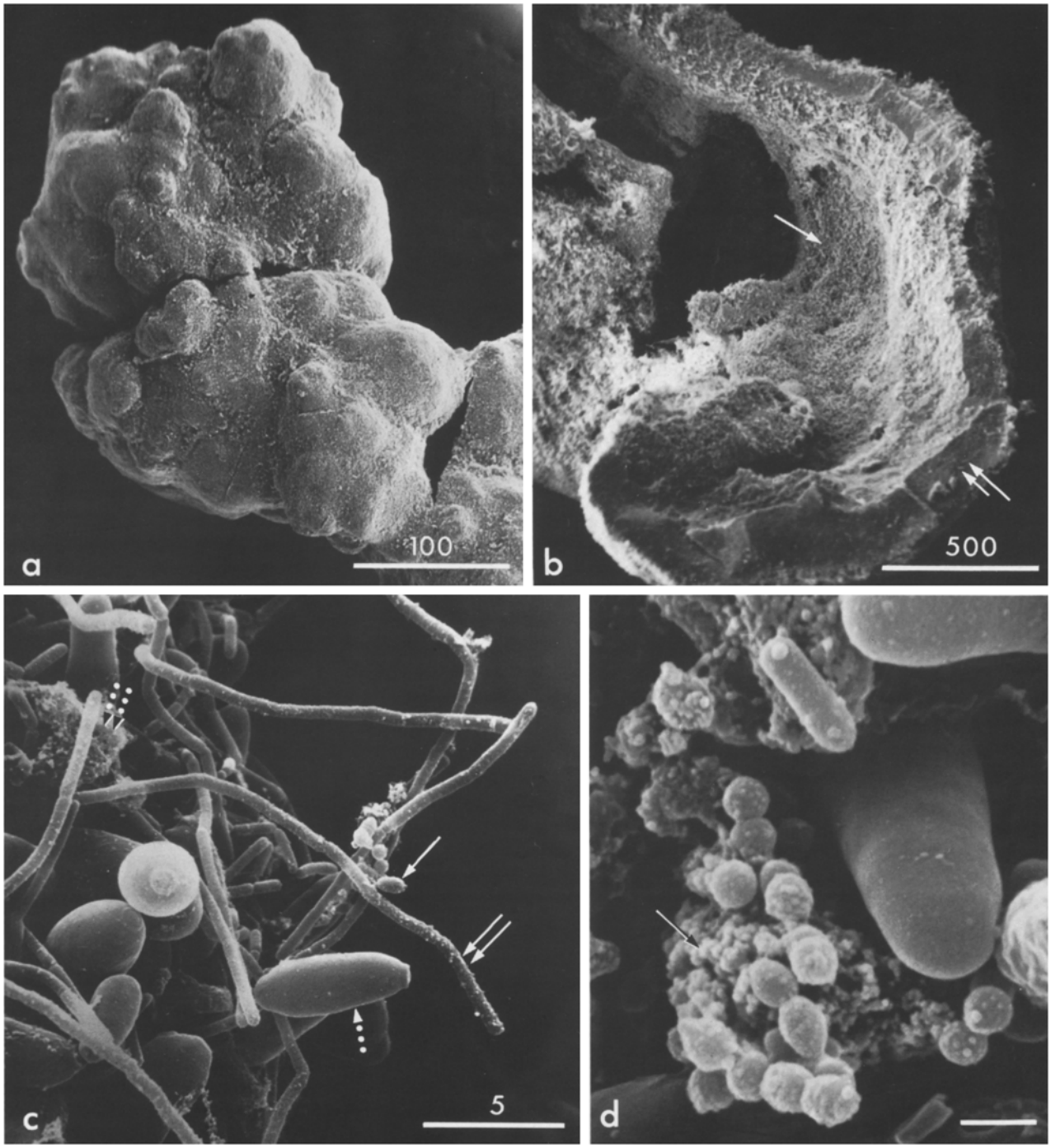

Fig. 1 a–d. Scanning electron micrographs of a Tibi grain. **a** A Tibi grain in the process of fission. **b** A fractured Tibi grain with its inner spongy structure (*arrow*) and an outer compact layer (*double arrow*). **c** The outer layer contains streptococci (*arrow*), lactobacilli (*double arrow*), yeasts (*dotted arrow*) and dextran (*double dotted arrow*). **d** The streptococci are embedded in dextran (*arrow*). The bar represents 1 μm or a multiple of it

examination following washing with water. Stock cultures have been kept in good conditions for over 10 years in our laboratory.

Microscopy

Tibi grains or small blocks of grains were fixed in 0.2 M cacodylate buffer, pH 7 containing 2% glutaraldehyde and then in 0.2 M phosphate buffer containing 2% OsO_4. The blocks were dehydrated in 30, 50, 70, 90 and 100% ethanol and examined in a Cambridge Stereoscan S4–10 at an accelerating voltage of 30 Kv following metallisation with gold.

The fixed blocks were also stained with 0.5% uranyl acetate in water and dehydrated as described above. The blocks were impregnated overnight in a 1:9 (v/v) mixture of methyl methacrylate and n-

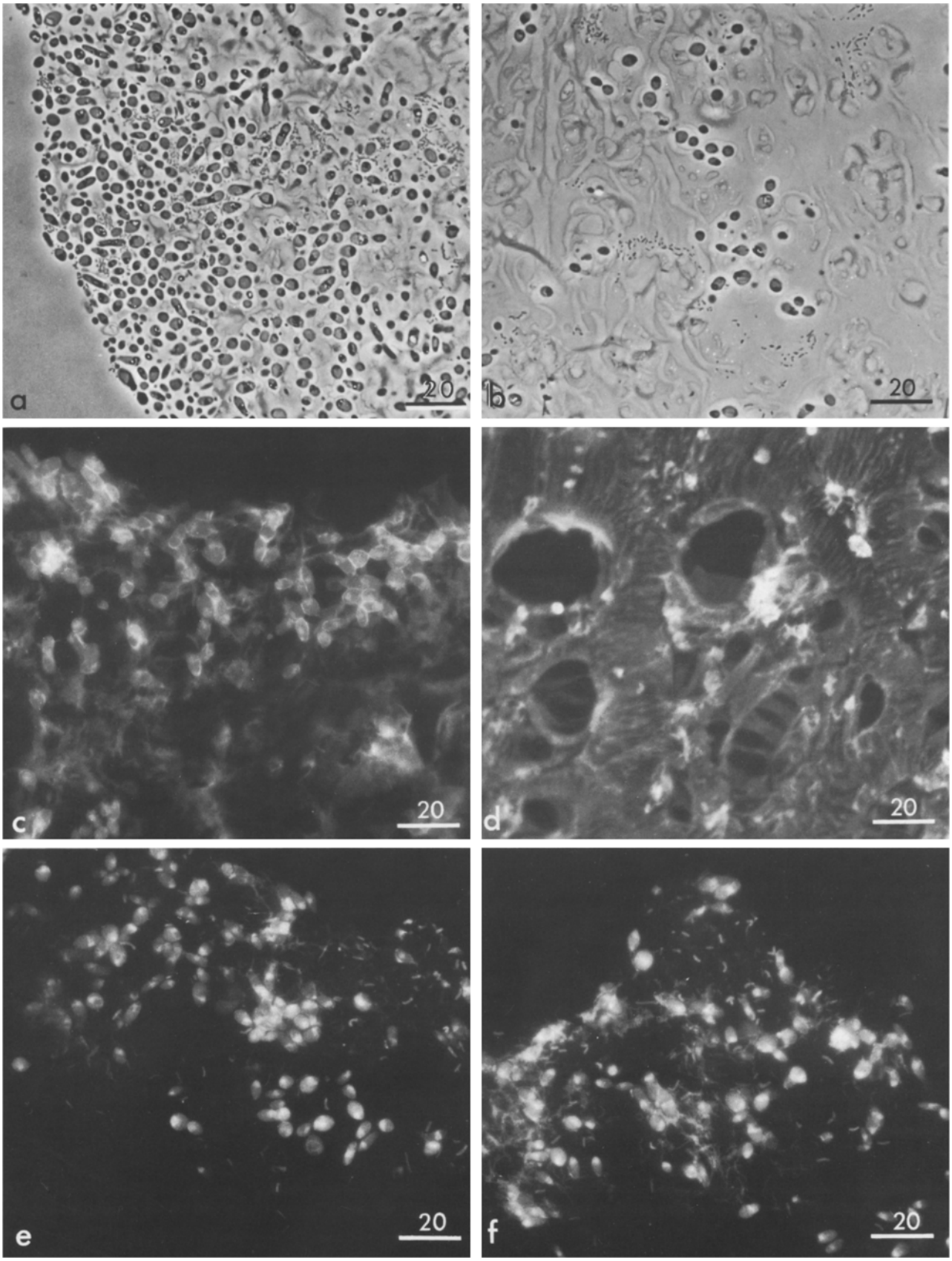

Fig. 2. Phase contrast microscopy of Tibi grain outer (**a**) and inner (**b**) layer. Fluorescence microscopy of Tibi grain stained with fluorescein-conjugated conA [outer layer (**c**) and inner layer (**d**)]. Primary fluorescence of Tibi grain (**e**) and a control in the presence of methyl α-D-mannopyranoside (**f**)

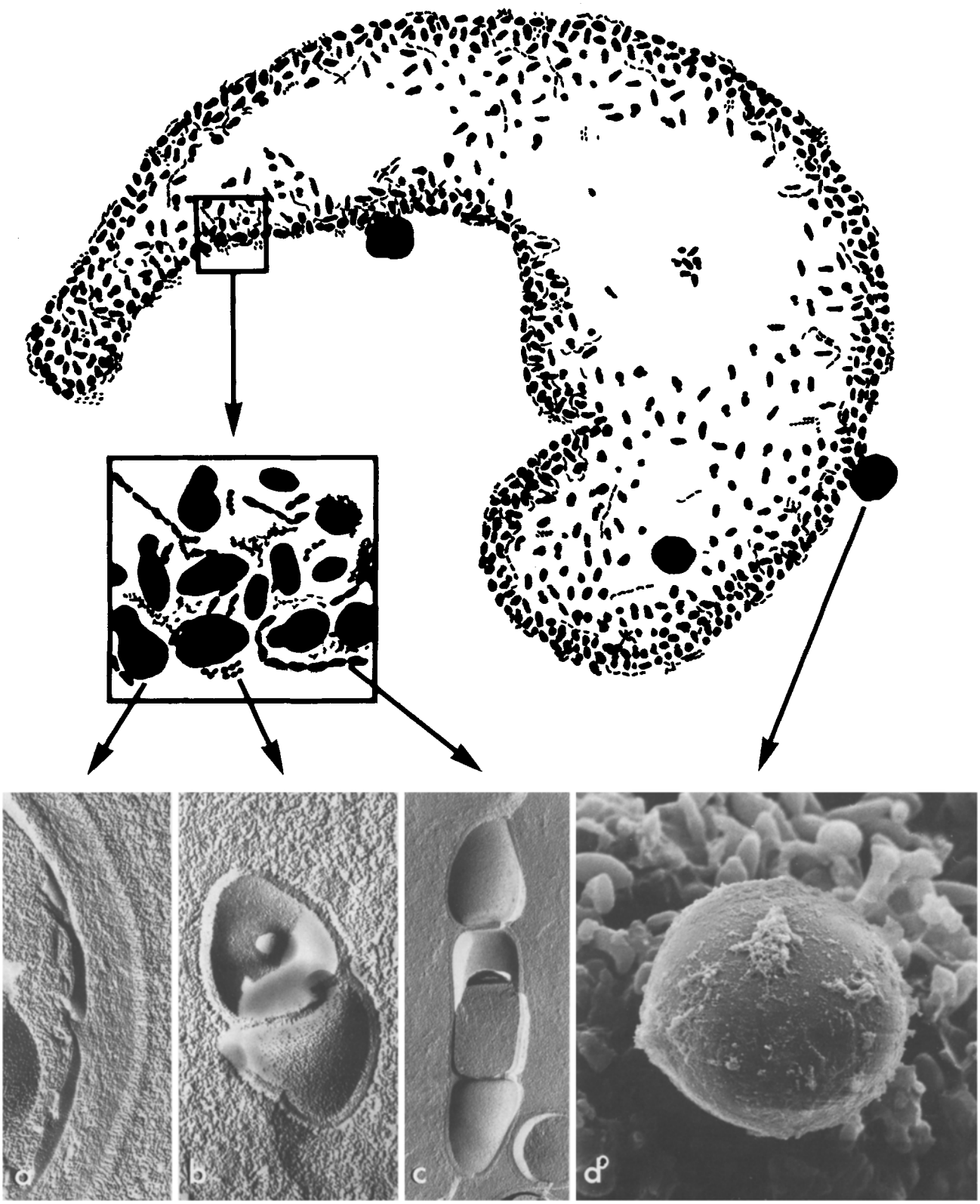

Fig. 3. General structure of the Tibi grain. Micrographs of freeze-etched *Saccharomyces cerevisiae* (**a**), *Streptococcus lactis* (**b**) and *Lactobacillus brevis* (**c**) and scanning electron micrograph of a rough element of unknown origin (**d**). Magnification: (a) × 34,000; (b) × 50,000; (c) × 20,000; (d) × 2000

butyl methacrylate and then incubated in the same mixture containing in addition 0.5% Parofor N as the catalyst. The polymerization was allowed to proceed for 4 days at 45°C. The use of Parofor N eliminated problems with prepolymerization and formation of bubbles. Sections of the blocks (0.5 μm thick) were examined under a phase contrast Leitz Orthoplan Microscope.

For fluorescence microscopy, grains were cut at −12° in a Leitz Cryomat, the sections (5 μm thick) were collected on 3% formaldehyde and dried at 50°C. The sections were stained with fluorescein-conjugated con A for 45 min, rinsed with buffer A and with water. As a control, mehtyl α-D-mannopyranoside (5 mg/ml) was added to the staining solution. The sections were examined under a Ploemopak 2 Leitz Orthoplan Microscope equipped with filter G.

Freeze-etching of small blocks of Tibi grains was performed in a Balzers BA 360 M and the replica were examined in a Philips EM 300 electron microscope.

Results and Discussion

The general structure of a Tibi grain in the process of fission was revealed by scanning electron microscopy (Fig. 1a). The grains were irregular in shape. They are reported to have a maximal diameter of 8–10 mm (Porchet, 1934; Stadelmann, 1957). In presence of the growth medium, the grain increased in size and then divided. The fission was determined by the inner pressure of CO_2. When the grain was fractured, one could distinguish an outer compact layer and an inner spongy structure (Fig. 1b). Due to CO_2 formation during fermentation the grain was hollow. At a higher magnification, the outer layer of the grain was shown to contain streptococci, polymorph lactobacilli, yeasts and the dextran generated by the lactobacilli (Fig. 1c). The streptococci were embedded in the dextran (Fig. 1d). Similar observations were made when the inner layer of the grain was examined with the exception that the lactobacilli were much shorter.

Sections of glutaraldehyde-fixed grains were examined by phase contrast microscopy. The outer layer of the grain was much more densely populated by microorganisms embedded in the polysaccharide (Fig. 2a) than the inner layer (Fig. 2b).

The lectin con A combines specifically with a variety of branched polysaccharides having α-D-glucopyranosyl, α-D-mannopyranosyl, β-D-fructofuranosyl or α-D-arabinofuranosyl residues at non reducing terminal positions. Various dextrans are known to bind con A (Goldstein and Hayes, 1978).

When frozen sections of the Tibi grain were stained with fluorecein-conjugated con A, the outer layer (Fig. 2c) contained less dextran than the inner layer which was heavily stained (Fig. 2d). The primary fluorescence of the outer layer is shown in Fig. 2e. When the outer layer of the grain was incubated with the fluorescent lectin in the presence of methyl α-D-mannopyranoside, an inhibitor of con A, the fluorescence of the polysaccharide decreased (Fig. 2f).

The section of a whole grain was examined by light microscopy and photographed. The drawing summarises the results (Fig. 3). The whole outer layer of the grain was more densely populated by microorganisms than the inner part. Large areas were empty due to the presence of gas. The three different types of microorganisms were examined by freeze-etching (Fig. 3a–c). Some granules of unknown origin were occasionally found inside and on the surface of the grain (Fig. 3d). These elements were present in a smooth and rough form. Contrary to the smooth form, the rough form was stained by fluorescein-conjugated con A. This is probably due to the deposit found on the rough form which was very similar to the dextran deposit found on streptococci.

Tibi grains are a unique symbiotic association of microorganisms embedded in dextran. The association appears to be very stable since it has been cultivated from the original grains for over 10 years without interruption.

Acknowledgements. The authors thank Prof. A. Camus (National Institute of Agronomics, Paris) for the gift of Tibi grains, Mrs. L. Cavani for subculturing the grains for 10 years, Mrs. M. Beaud for the photographic work and Mr. V. Fryder for the scanning electron micrographs and the drawing of Fig. 3.

References

Daker, W. D., Stacey, M.: CCLI. Investigation of a polysaccharide produced from sucrose by *Betabacterium vermiformé* (Ward-Meyer). Biochem. J. **52**, 1946–1948 (1938)

Goldstein, I. J., Hayes, C. E.: The lectins: carbohydrate-binding proteins of plants and animals. Adv. Carbohydr. Chem. Biochem. **35**, 127–340 (1978)

Horisberger, M.: Structure of the dextran of the Tibi grains. Carbohydr. Res. **10**, 379–385 (1969)

Kebler, L. F.: California bees. J. Am. Pharm. Assoc. **10**, 939–943 (1921)

La Rivière, J. W. M., Kooiman, P., Schmidt, K: Kefiran, a novel polysaccharide produced in the Kefir grain by *Lactobacillus brevis*. Arch. Mikrobiol. **59**, 269–278 (1967)

Lutz, L.: Recherches biologiques sur la constitution du Tibi. Bull. Soc. Mycol. France **15**, 68–72 (1899)

Porchet, B.: Etude d'une boisson fermentée, à base de figues. Mitt. Lebensmittelunters. Hyg. **25**, 235–244 (1934)

Schultz, M.: Beitrag zur Kenntnis der Kefirpilzsymbiose. Milchwissenschaft **1**, 19–27 (1946)

Stadelmann, E.: Die Symbiose Tibi. Bull. Soc. Fribourgeoise Sci. Nat. **47**, 16–19 (1957)

Tkacz, J. S., Cybulska, E. B., Lampen, J. O.: Specific staining of wall mannan in yeast cells with fluorescein-conjugated concanavalin A. J. Bacteriol **105**, 1–5 (1971)

Ward, H. M.: The ginger-beer plant, and the organisms composing it: a contribution to the study of fermentation-yeasts and bacteria. Phil. Trans. Roy. Soc. London **183**, 125–197 (1892)

Received May 14, 1980