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## INITIAL BIODETERIORATION OF SILICONE RUBBER BY *C. ALBICANS* AND *C. TROPICALIS* STRAINS ISOLATED FROM VOICE PROSTHESES

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### Abstract

Silicone rubber voice prostheses in patients after total laryngectomy become rapidly colonized by a biofilm, consisting of yeasts and bacteria. An especially troublesome feature of these biofilms is the ingrowth into the silicone rubber by colonizing yeasts. Microcolonies of ingrowing yeasts have been frequently observed on explanted voice prostheses after 3-4 months use. Incubation of silicone rubber samples in a modified Robbins device with a culture of *Candida tropicalis*, isolated from a Groningen button voice prosthesis, while passing the yeasts through a cycle of feasts (7 days) and famine (also 7 days) demonstrated, by electron microscopy, the onset of the ingrowth features as seen *in vivo*. In this paper, the *in vitro* capacities of four *C. albicans* and four *C. tropicalis* strains, all isolated from explanted voice prostheses, are compared. All eight strains showed onset of the ingrowth features observed *in vivo*: sometimes in the form of a small group of yeasts growing into a hole-like defect or, at other times in the form of clearly visible imprints in the silicone rubber, left after detachment of adhering yeasts during preparation of the samples for electron microscopy.

**Key words:** Biodeterioration; silicone rubber; *C. albicans*, *C. tropicalis*, voice prosthesis.

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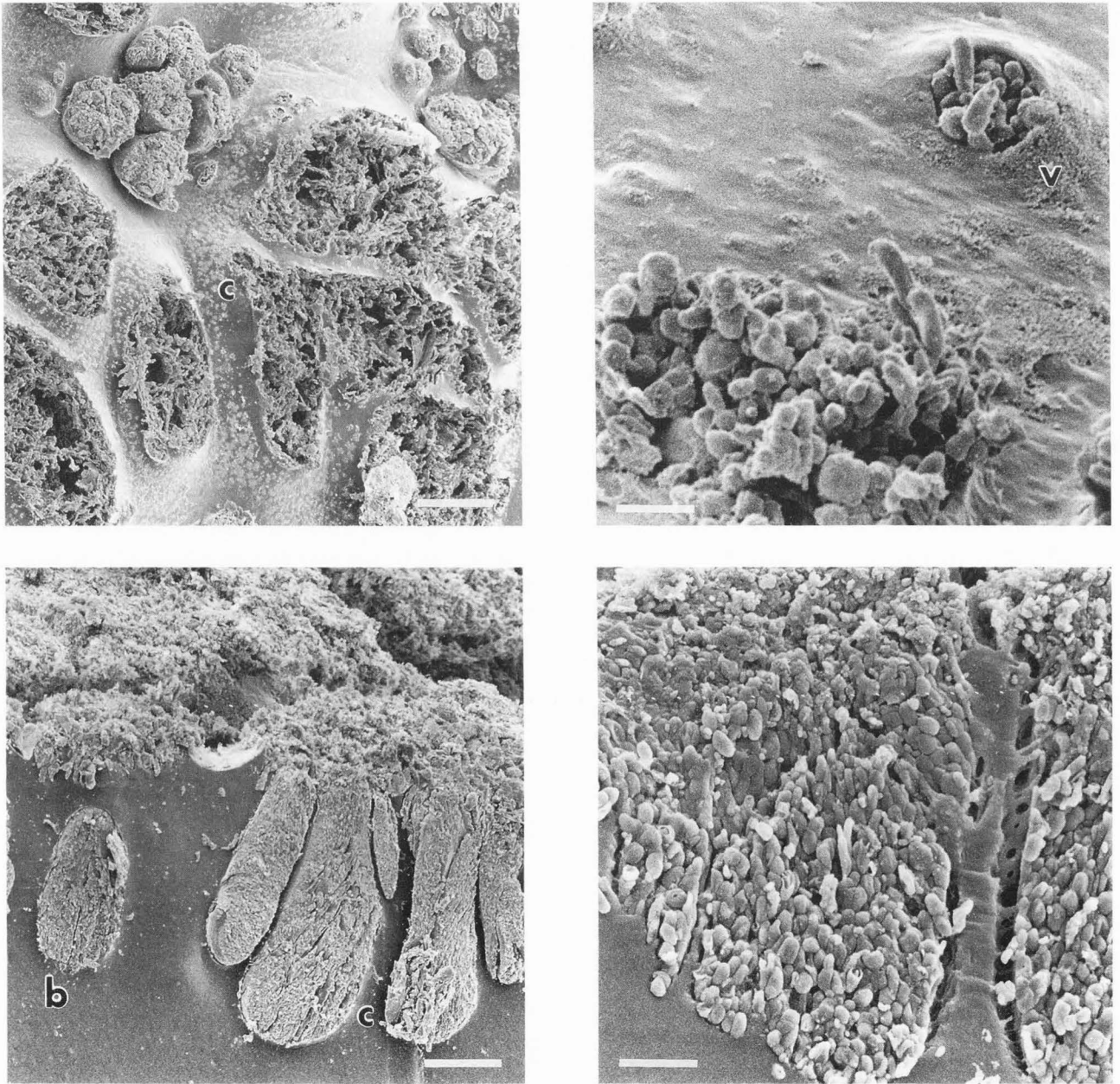
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### Introduction

Voice restoration in patients after total laryngectomy can be done by placing a shunt-valve between the trachea and the oesophagus. Because the oesophagus is not a sterile environment, the valve is rapidly colonized by microorganisms. The life-time of silicone rubber voice prostheses is greatly limited by the development of a biofilm on the valve-side of the prostheses causing increased air-flow resistance or leakage of the valve, ultimately leading to replacement of the prosthesis within 3-4 months on average (Nijdam *et al.*, 1982; Hilgers and Schouwenberg, 1990; Van den Hoogen *et al.*, 1996). Microbiological analysis of the organisms colonizing "Groningen-button" voice prostheses (Neu *et al.*, 1994b) demonstrated that these biofilms generally consist of yeasts and bacteria, most notably streptococci from the oral cavity and staphylococci from the skin. In a case study (Neu *et al.*, 1994a) on the succession of colonization of an ESKA-Herrmann prosthesis it was found that *Candida albicans* was exclusively present in the initial biofilm, whereas the predominant yeast strain in more mature (older than eight days) biofilms was *Candida tropicalis*.

Electron microscopy on sectioned, explanted voice prostheses showed that the yeast strains had the capacity to grow into the silicone material (see also Fig. 1) and to cause various types of defects in the silicone rubber, recently classified by Neu *et al.* (1993). Neither the mechanism of ingrowth of yeasts into the silicone rubber, nor the reason why the yeasts grow into the material are known. Experiments with a *C. tropicalis* strain, growing on silicone rubber disks in a modified Robbins device (Busscher *et al.*, 1994) demonstrated that by creating a cycle of feast and famine in the device, i.e., flowing medium or phosphate buffered saline through the device, cells could be stimulated to adapt a filamentous growth form and penetrate the silicone rubber, as the onset of the ingrowth features seen *in vivo*. To our knowledge, no other *in vitro* experiments have been done, demonstrating ingrowth of yeasts into silicone rubber.



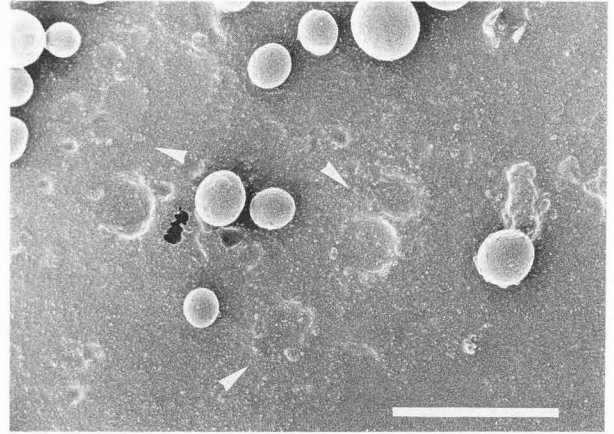
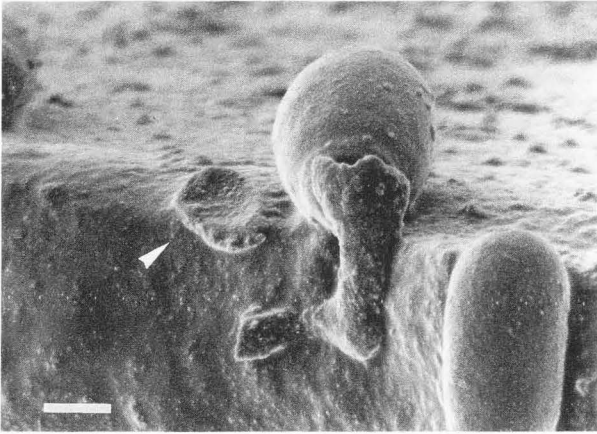
**Figure 1.** Scanning electron micrographs of sectioned, explanted "Groningen button" shunt valves explanted 4-7 months after use, showing biodeterioration of the silicone rubber by colonizing yeasts. TOP micrographs: Crater (c) and volcano (v) types of defects according to the classification by Neu *et al.* (1993). BOTTOM micrographs : Bag (b) and comb (c) defects (Neu *et al.*, 1993). The bars equal 100  $\mu\text{m}$  (left) and 10  $\mu\text{m}$  (right).

The aim of the present paper is to compare the capacities of four *C. albicans* and four *C. tropicalis* strains, all isolated from explanted voice prostheses, to grow into silicone rubber after a cycle of feast and famine.

## Materials and Methods

### Yeast strains

The yeast strains used in this study were isolated from explanted "Groningen button" (GB) voice prostheses (Neu *et al.*, 1994a). Four *C. albicans* GB 1/2, GB 1/9, GB 3/4 and GB 9/4 and four



**Figure 2.** Imprints of yeast strains on silicone rubber samples from the Robbins device after a 14 days cycle of feast and famine. LEFT micrograph: Section through a silicone rubber sample with *C. tropicalis* GB 9/9. RIGHT micrograph: Imprints (arrows) left by *C. albicans* GB 1/2 yeasts on silicone rubber. The bar equals 10  $\mu\text{m}$ .

*C. tropicalis* GB 9/2, GB 19/4, GB 9/9 and GB IV B4 were used. A preculture of the yeast strains was grown for 24 h, at 37°C in ambient air in a defined growth medium (glucose 7.5 g/l;  $(\text{NH}_4)_2 \text{SO}_4$  3.5 g/l, L-asparagine 1.5 g/l, L-histidine 10 mg/l, DL-methionine 20 mg/l, DL-tryptophane 20 mg/l,  $\text{KH}_2\text{PO}_4$  1 g/l,  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$  500 mg/l, NaCl 500 mg/l,  $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$  500 mg/l, yeast extract 100 mg/l,  $\text{H}_3\text{BO}_3$  500  $\mu\text{g/l}$ ,  $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$  400  $\mu\text{g/l}$ , Fe (III)  $\text{Cl}_3$  120  $\mu\text{g/l}$ ,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  200  $\mu\text{g/l}$ , KI 100  $\mu\text{g/l}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  40  $\mu\text{g/l}$ ). After overnight growth, this preculture was used to inoculate the Robbins device.

#### Silicone rubber

Silastic Medical Grade Silicone Rubber (Q7-4750, Dow Corning) kit was purchased and samples of 6.3 mm diameter and 1.0 mm thickness were produced following the procedures suggested by the manufacturer. Briefly, equal proportions of part A and part B were thoroughly blended together and injected into a mold at room temperature through a 3 mm diameter opening with a force of 3 tons. Subsequently, the silicone rubber was immediately cured at 200 °C for 50 minutes. Finally, samples were cleaned in a 2% RBS 35 (Fluka Chemie AG, Buchs, Switzerland) detergent solution under simultaneous sonication (5 min, 150 W) and thoroughly rinsed in Millipore grade water and absolute ethanol (> 96%).

#### The modified Robbins device and experimental set-up

A modified Robbins device (Costerton *et al.*, 1986, Busscher *et al.*, 1994), in which 10 silicone rubber samples could be simultaneously inserted was used. First, the device was inoculated with an

overnight culture of the *Candida* strain studied which was left in the device for 3 h, after which the flow was started. The device was perfused for 7 days with defined growth medium and subsequently, also for 7 days, with phosphate buffered saline (10 mM potassium phosphate and 140 mM NaCl, pH 7.0) in order to mimic the dynamic nutrient conditions (varying availability of nutrients) occurring *in vivo*. The temperature of the device was maintained between 34 and 37°C during all experiments.

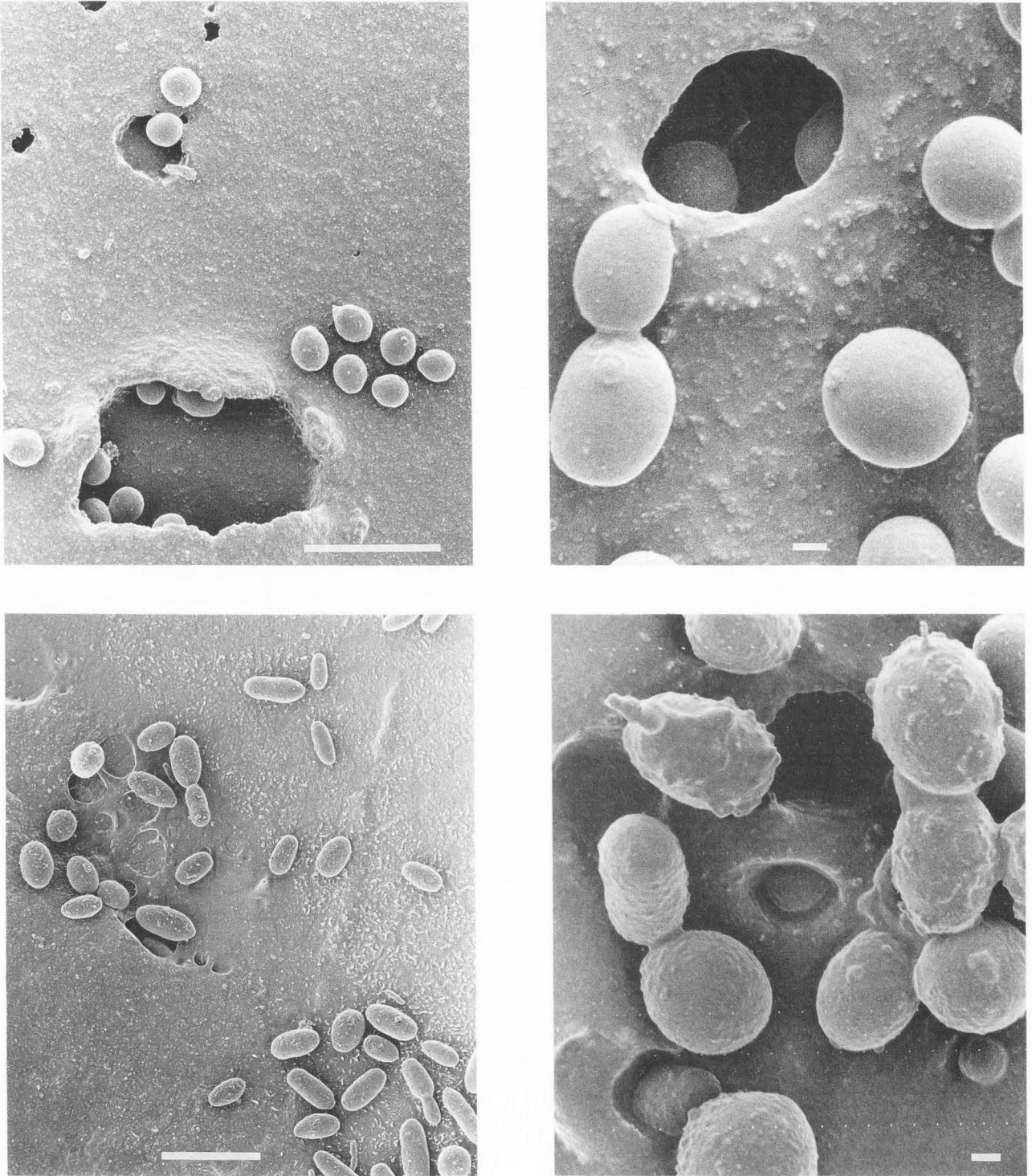
The samples were removed from the device after 14 days and prepared for electron microscopy.

#### Scanning electron microscopy

The samples from the Robbins device were flushed with 6.8% sucrose and 0.1 M cacodylate buffer (pH 7.4), fixed and stained in 2% glutaraldehyde and 0.2% ruthenium red in 0.1 M cacodylate buffer at 4°C and flushed again. Post-fixation and staining was carried out in 1%  $\text{OsO}_4$  and 0.2% ruthenium red in cacodylate buffer by gently shaking for 3 h at room temperature. Buffer washes and dehydration involved the following rinsing procedures: 20 min in 6.8% sucrose in 0.1 M cacodylate buffer; 3 x 10 min bidistilled water; 20 min in respectively 30, 50 and 70% ethanol and 4 x 30 min in 100% ethanol. After critical-point drying with  $\text{CO}_2$  for 4 h, the specimens were mounted on SEM stubs and sputter-coated with gold/palladium (15 nm). SEM observations were taken, made using the JEOL 6301, with different magnifications at 15-25 kV.

#### Results and Discussion

All silicone rubber samples removed from the



**Figure 3.** Hole-like defects in silicone rubber created by various yeast strains in a modified Robbins device after a 14 days cycle of feast and famine. TOP micrographs: *C. albicans* strains GB 1/2 (left) and GB 9/4 (right). BOTTOM micrographs: *C. tropicalis* strains GB 9/9 (left) and GB 19/4 (right). The bars equal 10  $\mu\text{m}$  (left) and 1  $\mu\text{m}$  (right).

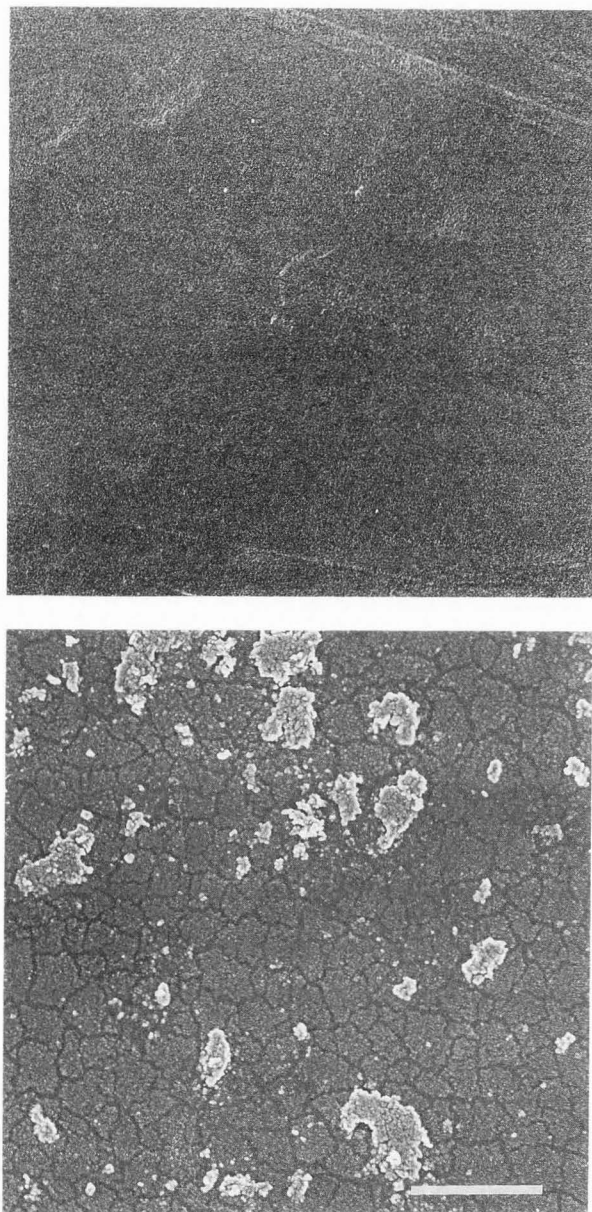
Robbins device after 14 days showed a thick biofilm observable with the naked eye, regardless of the type of yeasts strain involved. After rinsing, which was necessary for the preparation of SEM, most of the biofilm was detached leaving only those yeast cells in direct contact with the silicone rubber.

Scanning electron micrographs showed that the onset of biodeterioration of silicone rubber by yeast cells could be either in the form of imprints on the silicone rubber, made by adhering yeasts that had detached during preparation for electron microscopy (see Fig. 2) or in the form of hole-like defects, created by groups of yeasts growing in the silicone rubber (see Fig. 3). Imprints and hole-like defects were equally observed for *C. albicans* as for *C. tropicalis*. The appearance of initial biodeterioration by yeasts is not dependent on which of the two species is involved.

A control experiment was performed to test whether exposure of silicone rubber to growth medium and PBS buffer would yield dissolution of, e.g., the silica filling particles, present in medical grade silicone rubber for enforcement purposes therewith creating hole-like defects. To this end, silicone rubber samples were exposed to growth medium followed by PBS buffer in a similar modified Robbins device experiment as described before, but without addition of the microorganisms. Fig. 4 demonstrates an electron micrograph of the exposed silicone rubber, clearly indicating that the hole-like defects do not originate from exposure of the silicone rubber to medium or buffer only. This unequivocally proves that the initial biodeterioration effects seen are associated with the presence of yeasts on the silicone rubber.

The *in vitro* biodeterioration of silicone rubber shown in Figs. 2 and 3 are extremely minor when compared with the defects found on explanted prostheses (see Fig. 1). However, the *in vitro* experiments only extend over a time-scale of 14 days while the *in vivo* defects are formed during several months. Thus it can easily be envisaged that the imprints and hole-like defects are indeed the onset of the biodeterioration of silicone rubber as seen *in vivo*.

Silicone rubber has long been considered as an inert biomaterial. Recently, this notion has been questioned (Press *et al.*, 1992; Fisher, 1992) due to the problems arising with silicone-filled breast implants. Also in dental materials science, it has been frequently observed that silicone rubber denture liners are apt to colonization and biodegradation by yeasts (Gettleman *et al.*, 1983) similar to silicone rubber voice prostheses (Neu *et al.*, 1993; Izdebski *et al.*, 1987; Mahieu *et al.*, 1986). In this study we used four *C. albicans* and four *C. tropicalis* strains to show that



**Figure 4.** Scanning electron micrograph of silicone rubber prior to (TOP) and after (BOTTOM) exposure in a modified Robbins device to a cycle of flowing with medium (7 days) followed by PBS buffer (7 days) without addition of microorganisms. The bar equals 10  $\mu\text{m}$ .

both species can be stimulated to grow into the silicone rubber and therewith deteriorate the silicone rubber. It is emphasized, that in this study yeasts do not necessarily have to adapt a filamentous growth form in order to penetrate the silicone rubber, in contrast to previous observations (Busscher *et al.*, 1994). Unfortunately, as in our previous studies, we

are still in the dark with respect to offering an explanation or mechanism for the biodeterioration of silicone rubber by yeasts.

### Acknowledgement

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### Discussion with Reviewers

**D. Stickler:** Is it possible that *in vivo* the activity of white blood cells might also contribute to the degradation of the silicone voice prostheses?

**Authors:** Possibly, in other parts of the human body but we do not consider this likely in the oropharyngeal region.

**R.E. Baier:** Please comment on the magnitude of the rinsing force, which detached most of the biofilm. Could such rinsing forces be experienced *in vivo* during use of the G.B. or similar prostheses, perhaps by gargling or regurgitation?

**Authors:** The largest rinsing force is exerted when the sample is passed through the liquid air interface and can be approximated to be  $10^8$  N per organism, which is similar to the rinsing forces experienced *in vivo* during the use of a voice prosthesis. However, *in vivo* this force may not be adequate to stimulate detachment as the organisms are anchored more strongly into and on the silicone rubber, compare Fig. 1 with Figs. 2 and 3.

**R.E. Baier:** Do the organisms stimulate truly material degradation or possible material displacement? That is, the images may record results of biomechanics rather than biochemical events.

**Authors:** We do not know, but we believe that biochemical events are involved possibly in combination with biomechanical events because the organisms have to be passed through a cycle of feast and famine in order to stimulate degradation.

**R.E. Baier:** Have breakthrough or near-surface root porosities been checked in cross sections or in water/buffer only systems?

**Authors:** We have searched in cross-section for defects, with great success on *in vivo* samples (see Fig. 1) in which case defects are abundant. Looking for defects in *in vitro* samples during the onset of the degradation process is like searching for a needle in a haystack and Fig. 2 is the only good result we have obtained so far.

**M. Mittelman:** What metabolic features of the *Candida* spp. studied might be associated with the observed silicone biodeterioration?

**Authors:** We do not know.

**M. Mittelman:** Did the authors simulate the biological attack of silicone using organic acids and/or other fungal metabolites in an abiotic test system?

**Authors:** We did not.

**M. Mittelman:** Could the observed deterioration have resulted from a change in bulk phase medium pH mediated by the yeast?

**Authors:** We do not know.

**M. Mittelman:** Is there a relationship between implant age and the severity of biodegradation?

**Authors:** There is no relationship, because biodegradation as such is not a clinical reason for replacement. Clinically, prostheses are only removed when biofilm formation and concurrent biodeterioration has extended to the valve causing either blocking or leakage. Sometimes biofilm formation starts in or near the valve in which case loss of function occurs within weeks. On other instances in the same patient biofilm formation may extend slowly towards the valve and loss of function only occurs after several months.

**T. Fassel:** Do the authors care to speculate on any means of mechanisms of how the yeasts are causing the biodeterioration of the silicone rubber?

**Authors:** We do not.

**T. Fassel:** Do the yeasts via biodeterioration and creation of defects "prepare" the prosthesis for subsequent colonization by bacteria and development of yeast-bacteria biofilms?

**Authors:** We do not know, but believe that if any sequence in the colonization of the prosthesis occurs it is likely that the bacteria prepare the prosthesis for yeast adhesion rather than vice versa.