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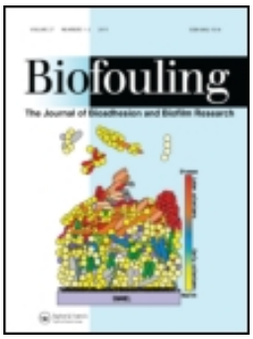

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Biofilms on tracheoesophageal voice prostheses: a confocal laser scanning microscopy demonstration of mixed bacterial and yeast biofilms

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The aim of this study was to demonstrate the presence of yeast and bacterial biofilms on the surface of tracheoesophageal voice prostheses (TVPs) by a double-staining technique with confocal laser scanning microscopy (CLSM). Biofilms of 12 removed TVPs were visualized by scanning electron microscopy, then stained with ConA-FITC and propidium iodide for CLSM. Microbial identification was by partial 16S rRNA gene analysis and ITS-2 sequence analysis. Microbial biofilms on the TVPs consisted of bacteria and filamentous cells. Bacterial cells were attached to the filamentous and unicellular yeast cells, thus forming a network. Sequence analyses of six voice prostheses identified the presence of a variety of bacterial and yeast species. *In vivo* studies showed that *Klebsiella oxytoca* and *Micrococcus luteus* efficiently attached to *Candida albicans*. CLSM with double fluorescence staining can be used to demonstrate biofilm formations composed of a mixture of yeast and bacterial cells on the surface of TVPs.

Keywords: biofilms; bacteria; yeast; biomaterials; tracheoesophageal voice prostheses

Introduction

Tracheoesophageal voice prostheses (TVPs) are indwelling silicone rubber biomaterials commonly used for speech rehabilitation after total laryngectomy and are the method of choice (Staffieri et al. 2006). Advocated for patients with laryngopharyngeal malignancies, total laryngectomy separates the digestive and respiratory tracts with definitive tracheostoma to allow breathing, but the normal voice is lost. TVPs consist in a one-way valve shunt placed between the esophagus and trachea. The mechanism allows air to pass from the trachea to the cervical esophagus and pharynx to create sounds that are further modulated to create speech. On the esophageal side of the voice prosthesis, fluids and food, as well as saliva, constitute a favorable environment for the establishment and proliferation of communities of yeasts and bacteria (Schwandt et al. 2005).

Biofilms are communities of microorganisms associated with a surface or interface where they proliferate and are encased in an extracellular matrix which consists mainly of polysaccharides (glycocalyx) and proteins, but contains also other biomolecules such as DNA (Branda et al.2005). TVPs have a propensity to harbor biofilms on their surface (Leunisse et al. 2001; Rodrigues et al. 2007). Biofilm formation is the primary cause for the failure of TVPs. Biofilms hamper the one-way mechanism and lead to leakage of fluids in the trachea (Oosterhof et al. 2005). On the tracheal side, biofilms can obstruct the trachea and increase airflow resistance during speech. Therefore, malfunctioning prostheses need frequent replacement.

Yeast and bacterial strains are recognized on the surface of TVPs. Colonizing yeast strains were identified as *Candida albicans* and *Candida glabrata* (Bauters et al. 2002). Among the culturable bacterial species, the most frequently identified are *Staphylococcus* spp. and *Streptococcus* spp., either as commensals from skin or of oral origin.

The proper demonstration of biofilms is challenging because staining both the mixture of yeasts and bacteria and the glycocalyx matrix is difficult. So far, mixed communities of yeasts and bacteria organized in a structured biofilm on the surface of TVPs have usually been visualized by scanning electron microscopy (SEM) (Neu et al. 1993). A major drawback of SEM is that the specimen must be dehydrated, which reduces the total volume of the glycocalyx matrix and alters its architecture (Akiyama et al. 2002).

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More recently, Buijssen et al. (2007) showed the successful application of confocal laser scanning microscopy (CLSM) for the visualization of microbial communities of yeast and lactobacilli on voice prostheses using probe (DNA) specific fluorescence *in situ* hybridization (FISH) staining. Applying CLSM technology provides the possibility to preserve the preserve the architecture of the biofilm because of the lack of dehydration. Recently, Karnia et al. (2007, 2008) developed a double-staining technique combined with CLSM to allow simultaneous imaging of the structural elements of a mucosal biofilm. This double-staining technique can demonstrate biofilms in a preserved 3-D architecture by showing bacterial cells and the glycocalyx with fluorescent-labeled lectins that bind to carbohydrates of the matrix. In contrast to Buijssen et al. (2007), the authors' staining technique is not organism specific, but a more general staining method for overall or initial analyses. This technique of bacterial biofilm visualization may help in visualizing biofilms on biomaterials such as TVPs, especially biofilms containing a mixture of yeasts and bacteria. Indeed, local conditions determine the formation, structure and behavior of biofilms (Wolcott and Ehrlich 2008). Both yeasts and bacteria, the host inflammatory response, the host constituents, and the microenvironment can modify the composition of the glycocalyx of the extracellular matrix.

This study aimed to demonstrate that bacterial and yeast biofilm formations on indwelling silicone rubber TVPs can be investigated by a double-staining technique with CLSM for visualizing both the glycocalyx matrix and the communities of microorganisms.

Methods

In vivo *indwelling biomaterial collection*

Twelve TVPs (Provox® 2, Atos Medical, Sweden) were obtained from patients with laryngectomy during routine outpatient replacement of the prostheses because of failure due to leakage through the shunt. Because anonymized tissue was used that was left over from surgical procedures, stored, and analyzed in the Institute of Biology, the Committee of Medical Ethics of Leiden University was not needed. Six and five specimens were assigned to SEM or CLSM analyses, respectively. One specimen was used to show the feasibility of identifying bacterial, yeast, or fungal species on the surface of the biomaterial.

Scanning electron microscopy

The specimens for SEM were washed in phosphate-buffered saline (PBS) and fixed with 1.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4, for 24 h at 4°C on a rotary shaker. The samples were then dehydrated through a graded series of acetone solutions (70, 80, 90, 96, and 100% acetone) for 20 min at room temperature. The critical-point-dried specimens were then oriented, mounted on metal stubs and sputter coated with gold by the use of a Polaron 5000 Sputtering System (Watford, England) before imaging. The specimens were examined under a JSM6400 scanning electron microscope (JEOL, Tokyo, Japan) with digital imaging capabilities. The images were collected at an acceleration voltage of $\sim$5.0 kV, a filament current of $\sim 10^{-10}$ A, and a working distance of $\sim$15 mm. All images were digitized as high-resolution TIFF files (resolution 635 dpi), then converted to high-quality JPEG files by use of Adobe Photoshop 7.0 (Adobe Systems Inc, San Jose, CA).

Confocal laser scanning microscopy

For CLSM, to preserve the biofilm architecture, the biomaterial specimens were immediately snap-frozen in cold isopenthane on dry ice, stored at –80°C, and processed for double staining. They were washed three times with PBS, then stained with propidium iodide (PI), 15 μM, for 5 min at room temperature to stain bacterial or yeast cells red. After a wash with PBS, the biomaterials were incubated with 50 μg ml^{-1} Concanavaline A fluorescein isocyanate-conjugated (ConA-FITC, C7642, Sigma, St. Louis, MO) for 5 min at room temperature to stain the glycocalyx green. The biomaterials were then successively washed in PBS and demineralized water, then cut with a custom-made device to obtain 1-mm sections of TVPs along the axis. After sections were embedded in Gelvatol/DABCO, they were examined under an Axioplan upright microscope (Zeiss, Germany) equipped with a Biorad MRC1024ES scan head (Hercules, CA) with a krypton/argon laser for visualization of ConA-FITC (excitation 488 nm and emission 522 DF 32 nm) and PI (excitation 568 nm and emission 605 DF 32 nm). Digital images of the CLSM optical sections were collected by use of Lasersharp 2000 software (Biorad, Hercules, CA). 2D-images as shown in the figures were constructed by overlay of a Z-series scanning, with varying distances of 0.5–3.0 μm depending on the enlargement. The overlay of merged red and green images in TIFF format were converted to high-quality JPEG files by use of Adobe Photoshop 7.0.

Image analysis

Three investigators (REK, GL, and GB) evaluated the images independently in a blind retrospective manner according to previously published criteria to determine whether a specimen contained a bacterial biofilm

(Kania et al. 2007, 2008). These criteria were adapted to search for biofilms containing a mixture of bacteria, unicellular yeast-like cells or filamentous fungal cells as follows: (i) the presence on the biomaterial surface of bacterial, yeast, or filamentous fungal cells recognized by size, morphology, and for CLSM, by red fluorescent PI staining; (ii) the presence of glycocalyx shown on CLSM images by bright-green fluorescence with ConA-FITC staining; (iii) the presence or absence of artifacts of dehydration and cutting for SEM and CLSM, respectively; and (iv) the absence of microorganisms without extracellular matrix or microorganisms located outside the specimen that could account for potential contamination during section preparation.

Isolation of bacterial, yeast and fungal strains

To identify bacterial, yeast, and fungal species on the biomaterial surface, a sample scraped from both sides of six TVPs stored at −80°C was streaked on agar plates with brain heart infusion (BHI), Luria Bertani (LB), potato dextrose (PD), Sabouraud dextrose or mycological (Difco) medium. After incubation for 1–3 days at 37°C, plates were examined for colony morphology by eye and by use of a stereomicroscope (Leica MZ 12 equipped with a Leica DC 500 Camera, Bensheim, Germany) for the presence of bacterial, yeast, or fungal colonies. For DNA extraction bacteria and yeast strains were cultured in liquid LC-medium. Strains were preserved at −80°C in 15% glycerol-LC medium.

Isolation of bacterial chromosomal DNA

Bacterial chromosomal DNA was isolated as described by de Souza et al. (2003) with some adaptations. Briefly, cells of 0.5-ml overnight LB-culture grown at 37°C were harvested and resuspended in 550 μl TE buffer (Tris 10 mM, EDTA 5 mM, pH 8.0) amended with lysozyme (2 mg ml^{-1}). The mixture was incubated at 37°C for 30 min, then 20 μl proteinase K solution (2 mg ml^{-1} in TE) and 5 μl RNAase were added and gently mixed. Subsequently, 60 μl of a 10% sodium dodecyl sulfate (SDS) solution were added, and the mixture was incubated for 15 min at 65°C. Then, 100 μl of 5 M NaCl and 80 μl of CTAB/NaCl (0.3 M CTAB, 0.7 M NaCl) were added, and the mixture was incubated for 10 min at 65°C. The mixture was extracted with 600 μl chloroform/isoamyl alcohol (24:1, v/v). DNA was precipitated from the water phase by the addition of 0.6 volume (400 μl) isopropanol. The precipitate was washed twice with 70% ethanol and dried. The DNA-containing pellet was dissolved in 30 μl Milli-Q water by incubating for 15 min at 65°C.

Isolation of yeast chromosomal DNA

Yeast cells from a 5-ml overnight liquid LB-culture grown at 37°C were collected by centrifugation. The cell pellet was transferred to a mortar and frozen by the addition of liquid nitrogen. To break the cell walls, cells were ground to obtain a fine white/grey powder. Extraction buffer was prepared by mixing 0.8 g tirisonaphtalene sulphonic acid in 40 ml H_2O (TNS), 4.9 g p-aminosalycilic acid in 40 ml H_2O (PAS) and 1 M Tris-HCl, pH 8.5, 1.25 M NaCl, 0.25 M EDTA (5 × RNB) in a ratio of 6:6:3, v:v:v. Ground cells were resuspended in 400 μl extraction buffer and 400 μl phenol/chloroform. Phases were separated by centrifugation for 15 min at 4 C, then 500 μl of the water phase were removed and added to 500 μl phenol–chloroform to remove protein traces. After centrifugation, 350 μl of the water phase were collected, to which 35 μl 3 M Na-acetate pH 4.8 were added. After mixing, 875 μl 96% ethanol were added. The extract was vortexed and incubated on ice for 10 min. Precipitated DNA was collected by centrifugation for 10 min at maximal speed at 4°C; the pellet was washed with 70% ethanol, dried, and finally resuspended in 100 μl H_2O.

Production and analysis of 16s-DNA polymerase chain reaction fragments

Standard molecular biology techniques were performed as described by Sambrook and Russel (2001). To amplify 16s-DNA from bacterial cells, two universal primers for 27fm (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1522R (5′-AAG GAG GTG ATC CAG CCG CA-3′) were used (Weisburg et al. 1991). The reaction mixture contained 2 μl of isolated chromosomal DNA (see above) as a template and 100 pmol of each primer. A general polymerase chain reaction (PCR) mixture was prepared with Taq polymerase. A hot start at 95°C for 3 min was followed by 35 cycles at 95°C for 40 s, 55°C for 20 s, and 72°C for 45 s. Finally, the mixture was incubated at 72°C for 10 min and stored at 4.0°C. The presence of 16-sDNA PCR products (∼500 bp) was analyzed on 1% agarose gels. The presumed 16-sDNA PCR product was purified from agarose gel by use of a Qiagen® gel purification kit. One-twentieth of the purified product was used as a template in a second PCR reaction under the conditions described above to obtain sufficient amounts of PCR product for sequencing and to reduce background noise of non-specific products created in the first PCR reaction using the whole chromosome as a template. The PCR product of the second reaction was purified by use of a Qiagen PCR purification kit. Products of bacterial isolates were compared by

amplified rDNA restriction analysis (ARDRA) analysis (Vaneechoutte et al. 1998) with *Hpa*2, *Taq*1 and *Sau*3A1, respectively, according standard incubation conditions recommended by the manufacturer (New England BioLabs). PCR products were sequenced with the universal 16s primers mentioned above at ServiceXS (Leiden, The Netherlands). Sequences were searched by BLAST X on the NCBI website (http://www.ncbi.nlm.nih.gov/).

Production and analysis of 18S interspacer region 2 (ITS2; 550-600 bp)

The ITS2 region was amplified by PCR with the primers for ITS3 (5′-GCATCGATGAAGAACG CAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′) (Ciardo et al. 2006). The reaction mixture contained 2 μl of isolated yeast chromosomal DNA (see above) as a template and 100 pmol of each primer. A general PCR mixture was prepared with *Taq* polymerase (Sambrook and Russel 2001). A hot start at 95°C for 6 min was followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Finally, the mixture was incubated at 72°C for 10 min and stored at 4.0°C. The presence of ITS-2 PCR products ($\sim$550-600 bp) was analyzed on 1% agarose gels. The presumed ITS-2 PCR product was purified from agarose gel by use of a Qiagen gel purification kit. To enrich the ITS2 DNA, one-twentieth of the purified product was used as a template in a second PCR reaction under the conditions of the first PCR reaction. Products of different isolates were compared by restriction patterns after incubation with *Hpa*2, *Taq*1, and *Sau*3A1 according to standard incubation conditions recommended by the manufacturer (New England BioLabs). PCR products were sequenced with the universal ITS-3 primer at ServiceXS (Leiden, The Netherlands). Sequences were searched by BLAST X on the NCBI website (http://www.ncbi.nlm.nih.gov/).

Results

Yeast and bacterial biofilm visualization

Of the 11 TVPs investigated for imaging, all showed the presence of biofilms according to the above-mentioned criteria.

Scanning electron microscopy

SEM was used first to visualize biofilm formations on the surface of TVPs (Figure 1). At low magnification, packed irregular formations were observed on the flat surface or in small cavities of the biomaterial (Figure 1A). At high magnification, these packed formations showed bacterial shaped cells and hyphal structures (Figure 1B). In addition to bacterial microcolonies, larger round-shaped structures resembling unicellular yeast-like cells were scattered over the surface of the TVPs (Figure 1C). Some surface areas showed a scaffolding network of bacterial cells, fungal hyphae and unicellular yeast-like cells (Figure 1D, E, and F). Bacterial cells on the outer surface were clearly visualized at the single cell level (Figure 1D). Densely packed formations of microorganisms appeared in some areas with bacterial cells, hyphal structures, and unicellular yeast-like cells (Figure 1E). Large numbers of cells were located at the inner part of the TVP (Figure 1E and F). Proliferation of fungal hyphae was largely on the pharyngeal side (Figure 1F).

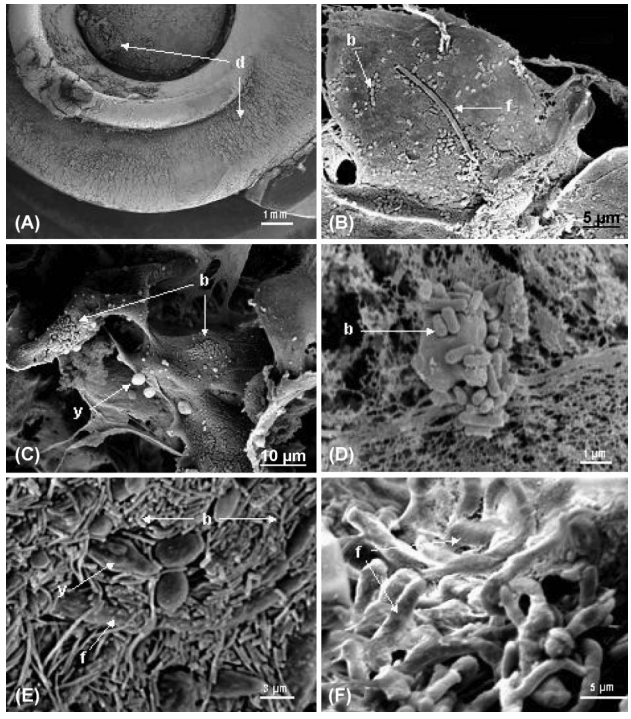

Figure 1. SEM images showing dense formations of aggregates on the surface of normal-appearing biomaterial attached to the surface of a TVP ('d'-labeled arrow) (A). Bacterial cells ('b'-labeled arrow) and filamentous cells ('f'-labeled arrow) appeared in some extracellular networks (B). Colonies of unicellular bacterial and yeast ('y'-labeled arrow) cells were seen on the surface of extracellular formations (C). High magnification of bacteria building up a colony on the surface of inorganic material (D). Pharyngeal side of voice prosthesis showing large amounts of interconnected bacteria (E). Proliferation of filamentous cells embedded in a network of extracellular matrix (F).

Confocal laser scanning microscopy

To study biofilm formations on the TVP in more detail, samples of the TVPs were double-stained with red fluorescent PI (binding to DNA) to identify

microorganisms and green fluorescent ConA-FITC (binding to α–D-mannose) to identify glycocalyx components. CLSM at low magnification showed large amounts of red and green fluorescence in the outer surface of the TVP samples, clearly showing the thickness of the biofilm (Figure 2A). At higher magnification, microorganisms were visualized at the single-cell level (Figure 2B–F). Well-delineated mixed biofilms were observed on the outer surface of the biomaterial (Figure 2B and C). Mixed biofilm contained bacteria, fungal hyphae (filamentous cells) and unicellular yeast-like cells interconnected in 3-D scaffolding systems (Figure 2B–F). The combination of green fluorescent staining for the glycocalyx matrix and red fluorescent staining for the bacteria, yeast-like cells, and hyphae demonstrated the coexistence of mixed communities of microorganisms in a scaffolding network composed of extracellular matrix (Figure 2B,C, and E).

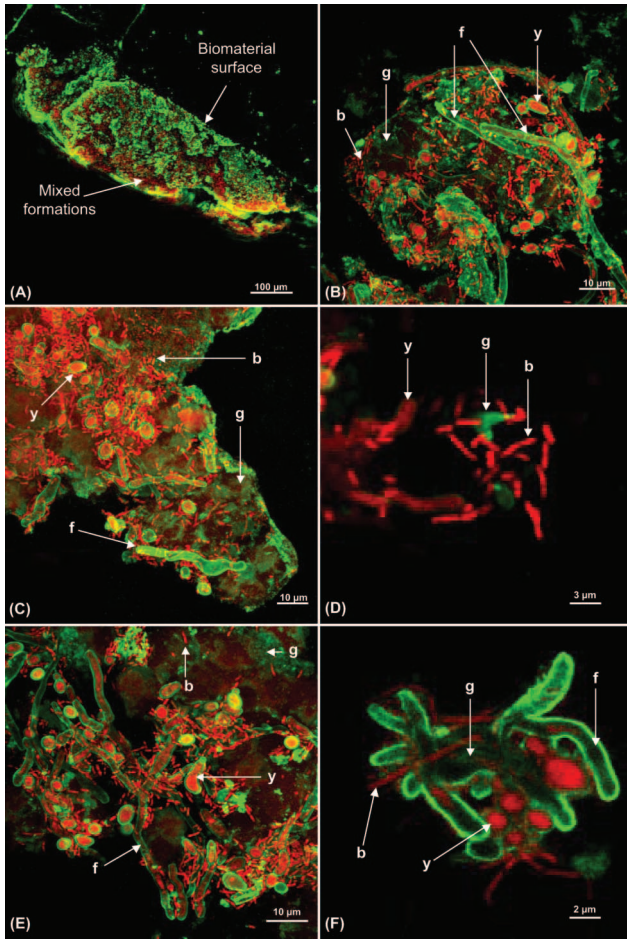

Figure 2. Mixed biofilm formations on indwelling biomaterials (TVPs) demonstrated with CLSM after double-staining. Biofilm formation was ascertained by the combination of red and bright green fluorescent staining for bacteria, unicellular yeast and filamentous cells ('b', 'y', and 'f'-labeled arrows, respectively) and the glycocalyx ('g'-labeled arrow). Biofilm formations were located on the air–biomaterial interface (A). Microorganisms building up the scaffolding network of mixed biofilms (B). Mixed biofilms densely packed and encased in the glycocalyx matrix (C). Combination of bacteria (in the process of division) and glycocalyx prolonging yeast formations (D). Representative image of mixed biofilm formation demonstrated by interconnected bacteria, yeast and fungi, which were encased in the scaffolding network of glycocalyx (E). Optical section (high magnification) of densely packed interconnected microorganisms showing typical 3-D architecture of mixed biofilm (F).

Bacterial and yeast identification

To identify (at least part of) the microorganisms present in the observed biofilms at the species level by DNA sequence analysis, microorganisms were cultured from six TVPs frozen specimen differentiating between the esophageal side and the tracheal side. After incubation for 1–3 days at 37°C, colonies appeared on agar plates cultured with media for bacterial and/or fungal growth. From the BHI and LB plates, morphologically different colonies were chosen for identification on the basis of 16S rDNA sequence homology (Table 1). From biofilms of three prostheses *Lactobacillus* spp., eg *Lactobacillus rhamnosus/pentosus, Lactobacillus casei/paracasei, Lactobacillus lactis*, and *Lactobacillus plantarum* were identified at the esophageal inner side of the prosthesis. Other bacterial species identified included *Streptococcus salivarius, Streptococcus agalactiae, Klebsiella oxytoca, Staphylococcus aureus, Staphylococcus warneri/pasteuri, Staphylococcus lugdunensis, Staphylococcus* sp. (identifiable to genus level), *Microbacterium oxydans*, and *Micrococcus luteus*.

Morphologically similar white colonies appeared on PD, Sabouraud and Mycological media after incubation for approximately 2 days. From each plate, a single colony was chosen and grown in liquid medium. After overnight growth, cells were examined by CLSM after double-staining with PI and ConA-FITC. These analyses showed the presence of large, round cells, with some germinating and showing the outgrowth of a filamentous structure with a green-stained cell wall, which indicated the presence of a yeast strain (Figure 3A and B). Chromosomal DNA was isolated from all cultures, and for each, a PCR fragment was obtained with universal fungal primers for the amplification of the ITS2. Sequences of these PCR fragments were identical and showed in the case of four prostheses (TVP1, 4, 5, and 6) highest homology (99.7%) to *C. albicans* and in the case of TVP2 and TVP3, *C. glabrata* (99.7) and *Candida tropicalis* (100%) respectively (Table 1). DNA sequences of the strains (16S rDNA of the bacterial strains and the ITS2 region of *Candida* strains) are

Table 1. Identification of microbial flora present in voice prostheses.

TVP	Esophageal side		Tracheal side	
	Bacteria[a]	Fungi[b]	Bacteria[a]	Fungi[b]
1	*S. salivarius, K. oxytoca, S. aureus, M. luteus*	*C. albicans*	nd	nd
2	*S. pasteuri/warneri*[c], *L. rhamnosus/pentosus, M. oxydans*	*C. glabrata*	*S. pasteuri/warneri*[c]	–
3	*S. aureus, S. salivarius*	*C. tropicalis*	*S. aureus*	*C. tropicalis*
4	*S. salivarius*	*C. albicans*	*S. salivarius*	*C. albicans*
5	*L. casei/paracasei*[c]	*C. albicans*		*C. albicans*
6	*K. oxytoca, S. aureus, L. casei/paracasei*[c], *L. lactis, L. plantarum*	*C. albicans*	*S. aureus, S. lugdunensis S. agalactiae, Staphylococcus* sp.[d], *K. oxytoca*	*C. albicans*

[a]Bacterial identification was enabled by sequence homology analysis of the 16S rRNA gene; [b]Fungal identification was enabled by sequence homology analysis of the ITS2 region; [c]16S rRNA gene analysis did not allow the differentiation between two closely related species; [d]16S rRNA gene homology analysis was able to assign to the genus level but not to the species level.

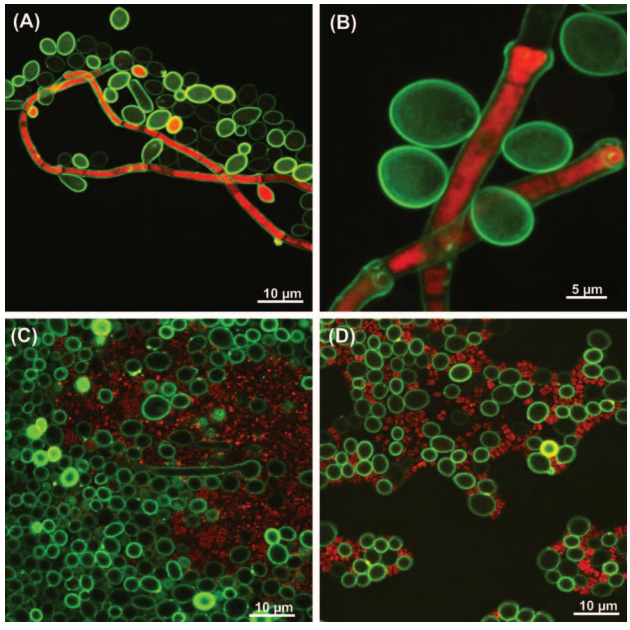

Figure 3. CLSM images of *C. albicans* grown in liquid medium after double-staining with PI and ConA-FITC. (A and B) *C. albicans* and hyphal cells from pure cultures. (C and D) co-incubation of *C. albicans* (green) and *K. oxytoca* (red; C) and *M. luteus* (red; D) in liquid medium resulting in attachment and clumping of cells.

available in Genbank (accession numbers FJ424513, FJ424514, FJ424515, FJ424516 and FJ424517; the sequences of microorganisms isolated from TVP numbers 2–6 (Table 1) are presently under submission). To assess the ability of the isolated bacterial strains to attach to *C. albicans*, overnight cultures of each bacterium were mixed with a *C. albicans* liquid culture, incubated for 1 h and analyzed by CLSM after double-staining with PI and ConA-FITC. Most frequently, the bacterial cells *K. oxytoca* and *M. luteus* attached to *C. albicans* (Figure 3C and D).

Discussion

Use of CLSM with double staining revealed biofilm formations containing a mixture of yeast and bacterial cells on the surface of indwelling TVPs. CLSM with double staining allowed for visualization of microorganisms (fungi and bacteria) at the single-cell level, as well as the glycocalyx matrix in a 3-D architecture as part of the biofilm. The presence of living bacteria and yeast was verified by cultures obtained from snap-frozen TVPs. 16S-rDNA sequence analysis for bacteria and ITS2 for fungal cells revealed a variety of Gram-negative and Gram-positive bacterial species as well as three different *Candida* species (Table 1).

So far, electron microscopy (SEM and/or TEM) has mainly been used to demonstrate biofilm formations on the surface of biomaterials such as indwelling silicone rubber TVPs (Neu et al. 1993), endotracheal tubes removed from intubated neonates (Zur et al. 2004), and frontal recess stents in patients with chronic rhinosinusitis (Perloff and Palmer 2004). In these studies, the structure of the biofilm matrix could not be studied mainly because of the dehydration process, which is required for preparation of the samples. Because the glycocalyx is highly hydrated (typically 95% to 99%), the dehydration process does not allow for visualizing an intact glycocalyx matrix.

In this study, the use of SEM and CLSM was compared for the visualization of microbial biofilms formed on the inner part of TVPs (Figures 1 and 2).

In addition, a double-staining fluorescent technique was used to differentiate cells and the sugar-containing glycocalyx, a technique previously developed for visualizing bacterial biofilms on adenoids and tonsils (Kania et al. 2007, 2008). CLSM allowed for visualizing the glycocalyx (green), with the microorganisms in the biofilm embedded in a preserved 3-D architecture (Figure 2). Both SEM and CLSM showed the presence of filamentous structures and large, round cells as part of the biofilm (Figures 1 and 2), which suggested the presence of fungal cells, specifically yeast-like cells.

Cultivation, CLSM studies (Figure 3) and sequence analysis of the ITS2 region revealed *C. albicans* in these observed structures, the growth of which is characterized by switching between a filamentous and a yeast form. ConA is a lectin protein that binds to mannose present in sugar-containing entities such as glycoproteins and polysaccharides and is therefore not specific for the glycocalyx. Since fungal cell walls contain mannose residues, cell walls of *C. albicans* were stained by ConA-FITC and visualized in green by CLSM (Figures 2 and 3). The cell wall forms a barrier to PI (which stains DNA and RNA), because the red staining was frequently absent after incubation with PI, indicating that many cells survived the freezing process and remained intact. The staining of fungal cell walls by ConA-FITC was an additional marker for distinguishing cells of bacterial and fungal origin. The presence of dark areas within the biofilm can be explained by (i) existing water channels, (ii) heterogenous production of the matrix and the different exopolysaccharides within the biofilm, and (iii) absence of ConA-FITC binding to the matrix (Akiyama et al. 2002; Costerton et al. 2003). Additionally or alternatively, 4′,6-diamidino-2-phenylindole (DAPI) stain (DNA binding) would be useful to distinguish living and dead cells when appropriate laser channels are available in the CLSM.

The presence of biofilms consisting of a mixture of bacteria and *Candida* spp. has been regularly observed and seems to be the main cause for the malfunctioning of voice prostheses (Millsap et al. 1998). *Candida* spp. were identified from biofilms present in the six voice prostheses, for which the microbial flora was characterized. In total three different *Candida* spp. were identified (Table 1), one species per prosthesis. CLSM images show the close interaction between the bacterial cells and the *Candida* cells (Figure 2). The images suggest that *C. albicans* filaments form a 3-D network that functions as a supportive substratum for bacteria, allowing for the formation of a thick biofilm with a high concentration of bacterial cells. The biofilms showed that both the micro-environment, as well as the architecture, are favorable for the colonization of indwelling biomaterials such as TVPs.

Sequence analyses of the partial 16S rDNA gene identified several *Lactobaillus* spp. in three of the six voice prostheses (Table 1) supporting the report of Buijssen et al. (2007), which showed that *Lactobacillus* spp. are frequently present in the biofilms of voice prostheses. The *Lactobacillus* spp. were only detected at the esophageal side, indicating the direct relationship with nutrition. The variance of bacterial species identified from the six different TVPs was large (Table 1). *K. oxytoca, S. aureus, S. lugdunensis S. pasteuri/warneri, M. oxydans, M. luteus, S. agalactiae*, and *S. salivarius*, can be part of the normal upper respiratory flora. However, some bacterial species such as *K. oxytoca, S. aureus, S. lugdunensis*, and *S. agalactiae* are potential human pathogens that can cause pneumonia and/or endocarditis, especially in immune compromised patients. Unusually high concentrations of microorganisms are formed, for a potential reservoir of (opportunistic) pathogens that could infect the lower respiratory tract (Paju and Scannapieco 2007).

It is unfortunate that the silicone rubber shaft and flange of TVPs allow for growth of yeasts, which will ultimately lead to irreversible damage to the valve seating. Colonization of the esophageal flange plays an important role in prosthesis dysfunction by interfering with appropriate valve closure and opening. Besides the mass effect of the deposits, the ingrowth of the microorganisms into the surface of the silicone rubber components of TVPs may affect their functioning also. Prevention of biofilms in TVPs is required to prevent malfunctioning and potential infection. Strategies can include coating the prostheses, the development of biomaterials that are less prone to biofilm formation, and the use of probiotics (Free et al. 2001). Mixing pure cultures of bacteria and *Candida* showed that attachment of *M. luteus* and *K. oxytoca*, at least, could be reconstituted under the present test conditions (Figure 3C and D) and can be used as a test system for studying the molecular determinants involved in initial attachment. Such studies will provide information on how the interactions between *Candida* and bacteria are initiated and how the formation of mixed microbial populations is established.

Conclusions

Biofilm formations on indwelling silicone rubber TVPs can be investigated by double-staining with CLSM for visualizing both the glycocalyx and a mixture of yeasts and bacteria. In addition, double-staining can be used as a test system for treatment by studying the molecular determinants involved in the attachment of microorganisms on the surface of indwelling biomaterials.

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