Oral Microbiology Immunology 2008: 23: 419–424
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

Biofilm microbial communities of denture stomatitis

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Campos MS, Marchini L, Bernardes LAS, Paulino LC, Nobrega FG. Biofilm microbial communities of denture stomatitis.
Oral Microbiol Immunol 2008: 23: 419–424. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

Introduction: Denture stomatitis is a common lesion that affects denture wearers. Its multifactorial etiology seems to depend on a complex and poorly characterized biofilm. The purpose of this study was to assess the composition of the microbial biofilm obtained from complete denture wearers with and without denture stomatitis using culture-independent methods.
Methods: Samples were collected from healthy denture wearers and from patients with denture stomatitis. Libraries comprising about 600 cloned 16S ribosomal DNA (rDNA) bacterial sequences and 192 cloned eukaryotic internal transcribed spacer (ITS) region sequences, obtained by polymerase chain reactions, were analyzed.
Results: The partial 16S rDNA sequences revealed a total of 82 bacterial species identified in healthy subjects and patients with denture stomatitis. Twenty-seven bacterial species were detected in both biofilms, 29 species were exclusively present in patients with denture stomatitis, and 26 were found only in healthy subjects. Analysis of the ITS region revealed the presence of *Candida* sp. in both biofilms.
Conclusion: The results revealed the extent of the microbial flora, suggesting the existence of distinct biofilms in healthy subjects and in patients with denture stomatitis.

Key words: denture stomatitis; internal transcribed spacer region; oral biofilms; ribosomal genes

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Accepted for publication January 28, 2008

Denture stomatitis is a common disease affecting denture wearers, characterized by an erythema confined to the area covered by the complete denture (28, 35). Its etiology seems to be multifactorial and associated with local and systemic factors (35).

The presence of a biofilm generated by a sequential process of microbial adherence, aggregation, and growth, usually associated with inadequate denture hygiene, has been suggested as an etiological factor (5, 26). Although a possible role of *Candida albicans* in denture stomatitis pathogenesis has been suggested (35), the predominance of bacterial species over yeasts in this biofilm has been reported using culture-based methods (14, 16).

The denture stomatitis biofilm is still poorly characterized, and seems to offer a protective environment for interactions among eukaryotic and prokaryotic cells (5, 7, 10, 12, 17, 27). Moreover it is considered to be an important reservoir of potential respiratory pathogens (24, 32, 33).

The study of the oral bacterial community by culture-dependent methods has been limited by difficulties in culturing many microorganisms. Also, some microorganisms do not have a sufficiently distinctive morphology to be identified by microscopy (23) and physiological studies can be time-consuming and expensive. In this context, the use of culture-independent molecular methods has been demonstrated to be of great value in oral biology (12, 13, 15, 20, 21, 24, 31), increasing our knowledge of biofilm composition and microbial interactions (12, 13).

The purpose of the present study was to characterize the bacterial and fungal microbiota in the biofilm of wearers of complete dentures with and without denture stomatitis, using culture-independent molecular methods and pooled samples.

Materials and methods

Subject population and sample collection

Ten healthy denture wearers (seven women and three men; mean age 52.7 ± 7.94 years) and 10 denture wearers with generalized (Newton stage II) denture stomatitis (six women and four men; mean age 57.1 ± 10.8 years) were studied. All subjects that participated in the study provided written consent certified by the National Research Ethics Committee, who approved the project. Subjects did not use antibiotics during the 3 months before sample collection and had no systemic diseases. No measures were taken related to the denture or oral hygiene before

sample collection to avoid interfering with the existing microbial flora. The samples were collected with a sterile swab (Catch-All; Epicentre, Madison, WI). One side was used to remove biofilm from the palatal tissue then the swab was rotated through 180 degrees and used to collect from the fitting surface of the denture. Upon immersion in 500 µl extraction solution (Quick-Extract DNA™) the samples were processed according to the manufacturer's instructions (BuccalAmp DNA Extraction Kit; Epicentre).

Study groups

These samples were pooled into two groups (healthy subjects and patients with denture stomatitis) by mixing aliquots of each individual lysate. The DNA from each pool was concentrated by ethanol precipitation in the presence of glycogen and 250 mM NaCl, washed with 80% ethanol, and dissolved in 20 µl QuickExtract DNA extraction solution (Epicentre) (4).

Polymerase chain reaction amplification of the 16S ribosomal RNA gene

Polymerase chain reaction (PCR) amplification was carried out for each pool using a universal primer set for bacteria (D88 and E94) and clones supposed to represent novel phylotypes were sequenced entirely by using additional primers (B34 and F20) as described previously (23). The PCR amplification was set up as suggested previously (23), using 1 U Platinum *Pfx* DNA polymerase™ (Invitrogen, Carlsbad, CA). The PCR products of about 1500 base pairs (bp) were examined by agarose gel (1%) electrophoresis, after staining with ethidium bromide.

PCR amplification of the internal transcribed spacer region of fungal ribosomal DNA

Universal primers for fungi [internal transcribed spacer (ITS) regions ITS3 and ITS4] were used for PCR amplification according to the previously described conditions (8).

Construction of libraries

To optimize cloning efficiency, the PCR products were incubated with *Taq* DNA polymerase and dATPs to add 3′ A-overhangs to the blunt-ended PCR products generated by the *Pfx* DNA polymerase. Next, the amplicons were cloned using the TOPO-TA Cloning Kit™ (Invitrogen, San Diego, CA), according to the manufacturer's instructions. Clone isolation followed standard procedures (29) and the selected colonies were stored at −80°C after overnight growth in 96-well microplates containing Luria–Bertani ampicillin broth with 8% glycerol. Purified plasmid DNA was prepared from cultures grown in 96-well, deep-well plates, by using a microwave-based boiling procedure (22). The plasmid DNAs were analyzed for integrity by electrophoresis on 1% agarose gels.

DNA sequencing and phylogenetic analysis

Sequencing reactions followed the protocol provided by the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1™ (Applied Biosystems, Foster City, CA). The reactions were analyzed using the ABI Prism 3100 Genetic Analyzer™ (Applied Biosystems). Sequences presenting at least 500-bp (bacteria) and 200-bp (fungi) nucleotides of good quality were used to determine the identity or approximate phylogenetic position. Full sequences were obtained for clones that were less than 98% similar to the closest known bacterial and fungal sequences. The sequences were compared to those in the NCBI GenBank database using the BLASTN algorithm version 2.2.16 (2), and were aligned using CLUSTALX version 1.8 (34). Phylogenetic analyses based on the 16S ribosomal DNA (rDNA) sequences were performed using MEGA software, version 3.1 (19), using the neighbor-joining method (30). The same method was used for the phylogenetic analyses based on the ITS region sequences, using the MEGA2 software (18). In both cases, distance matrices were calculated using Jukes–Cantor algorithm (11). Chimeric sequences, (about 10% of the clones), were identified using Bellerophon (http://foo.maths.uq.edu.au/~huber/bellerophon.pl) and not included in the alignments. Species richness (supplemental data) was evaluated with a non-parametric richness estimator, Chao 1 (6), using ESTIMATES, version 7 (htpp://purl.oclc.org/estimates).

Nucleotide sequence accession numbers

Full bacterial DNA sequences were deposited at GenBank under accession numbers AY672070–76, AY672079, AY672084, and AY672086–89.

Results

The sequences obtained for 16S rDNA with at least 500 bp (600 clones) and at least 200 bp for ITS fungal region (192 clones) were compared to the GenBank database. Based on nearest relative (BLASTN) and by phylogenetic affiliation, sequences with ≤97% similarity with known GenBank sequences were designated phylotypes (25). Sequences included in the same phylotype had ≥97% identity to each other. To determine the extent to which the data reflect the diversity of the sample, the species richness of patients with and without denture stomatitis was evaluated using the Chao 1 estimator (6). Based on these analyses, almost 100% of the estimated bacterial species number was detected in the healthy and diseased sites available. The distribution of known bacterial species and novel phylotypes identified in this study are presented in two phylogenetic trees (Figs 1 and 2). The clones amplified revealed 82 different bacterial phylotypes. As shown in Fig. 1, 26 bacterial phylotypes were found only in healthy subjects, with a strong representation of the genus *Streptococcus* (27%). Thirty-two bacterial phylotypes were exclusively found in the biofilm from individuals with denture stomatitis (Fig. 2), and were represented mainly by species within the genera *Streptococcus* (23%), *Atopobium* (16%), and *Prevotella* (11%). Thirty-one bacterial phylotypes were common to samples from both healthy and diseased subjects (highlighted in bold type in Figs 1 and 2), and were represented mainly by species within the genera *Streptococcus* and *Veillonella*, comprising 21–43% of the clones. About 50% of the bacterial flora was represented by not-yet-cultivated phylotypes.

From 192 clones amplified with fungal universal primers ITS3 and ITS4, 87 from healthy subjects and 105 from disease biofilm, three different fungal species were identified (Fig. 3). *C.albicans* was found in samples from both healthy and diseased subjects. Only *C.albicans* was detected in samples from patients with denture stomatitis whereas a higher diversity was found in healthy subjects: *C.albicans* (22%), *Candida glabrata* (54%), and *Candida tropicalis* (24%).

Discussion

To our knowledge, this is the first study using culture-independent molecular methods to characterize the microbial composition of the biofilm present in

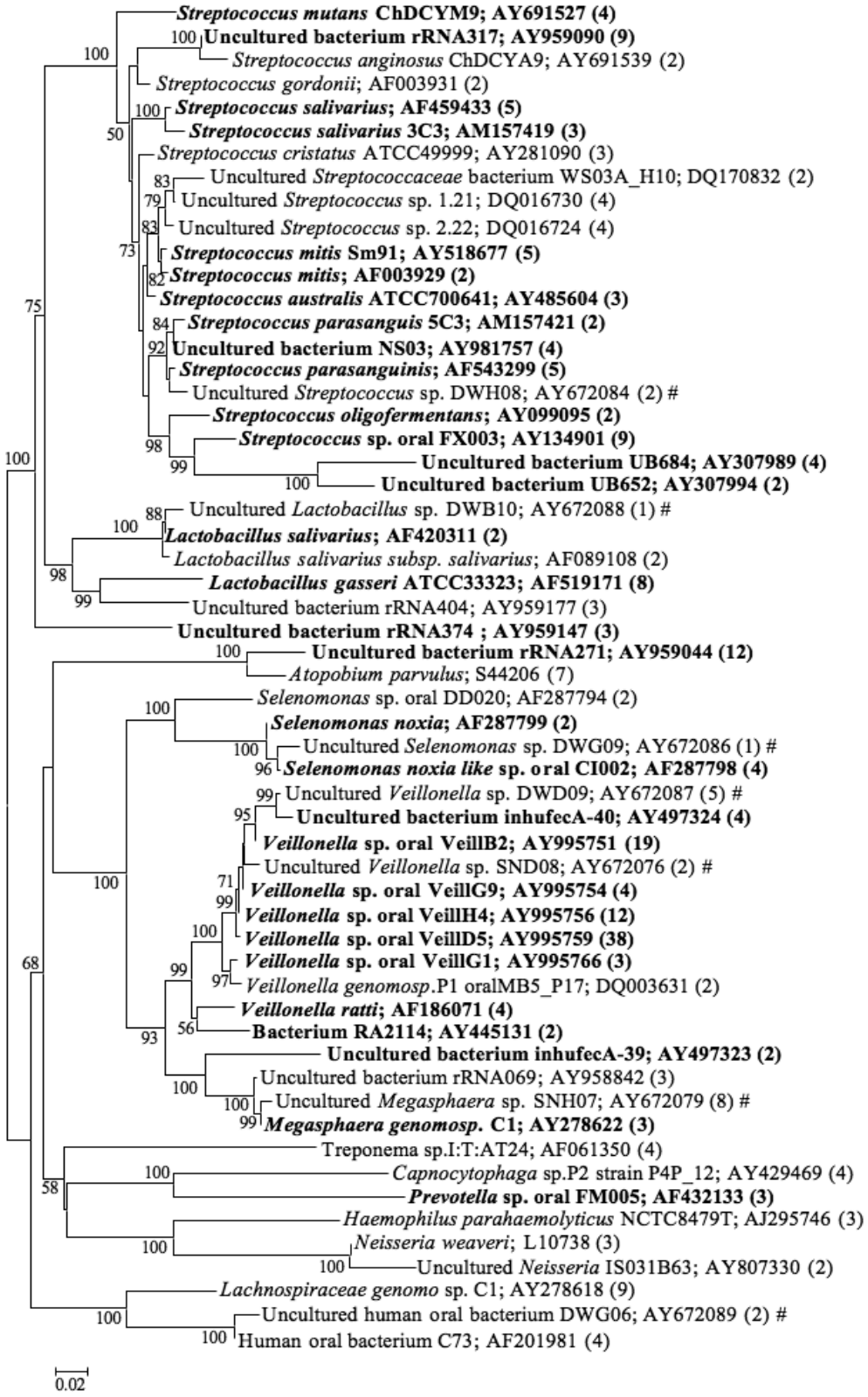

Fig. 1. Neighbor-joining tree based on 16S ribosomal DNA sequences, showing the phylogenetic relationship between phylotypes found in subjects without denture stomatitis (healthy). Matrix of distance was calculated using Jukes–Cantor algorithm. Bootstrap values are based on 1000 replicates, (values higher than 50% are shown). Symbol # represents full sequences obtained in this study. Bold types represent organisms found in both groups (healthy and disease). The number of clones representing each phylotype is shown between parentheses.

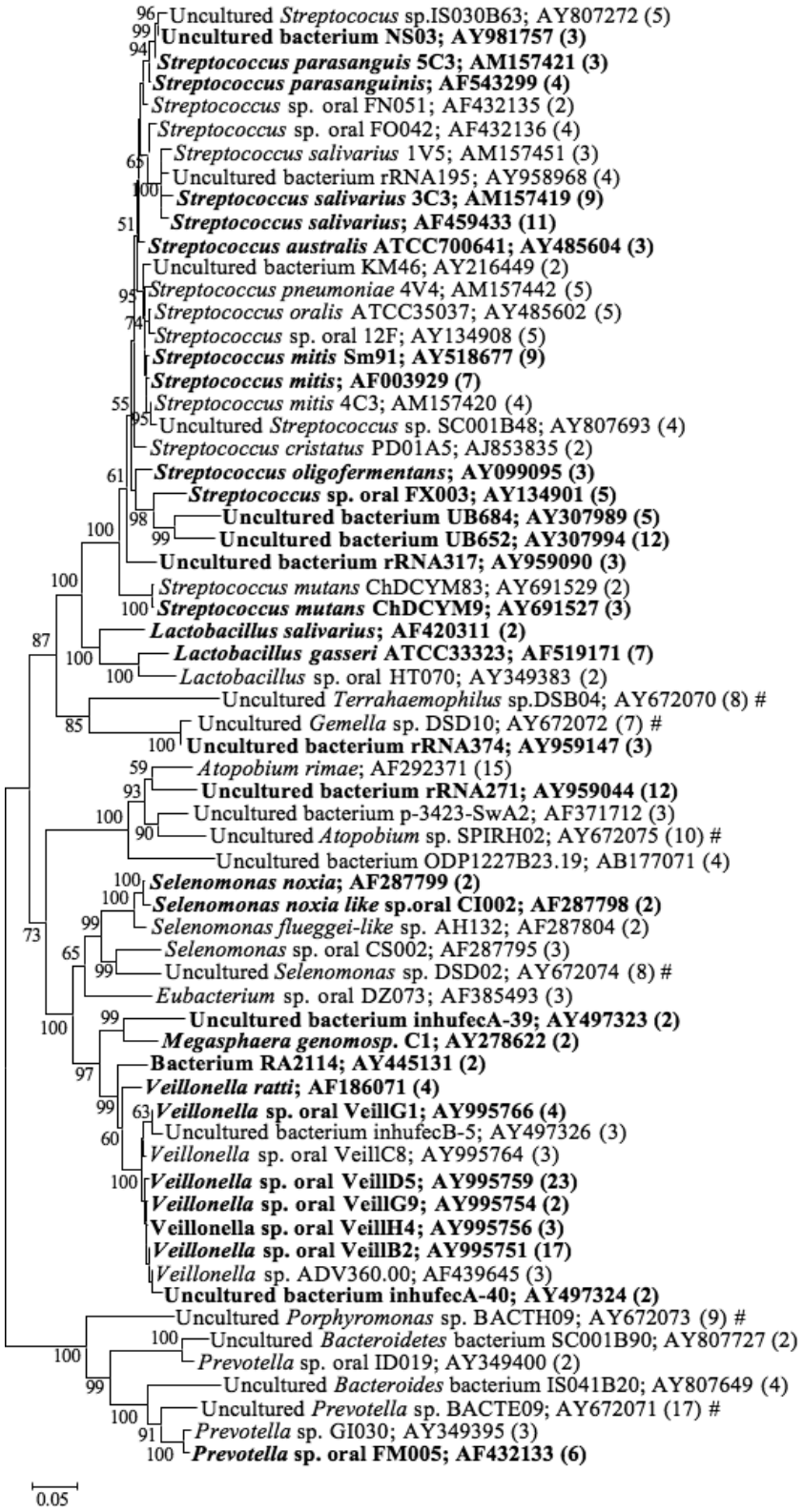

Fig. 2. Neighbor-joining tree based on 16S ribosomal DNA sequences, showing the phylogenetic relationship between phylotypes found in subjects with denture stomatitis (disease). Additional information is described in Fig. 1.

denture wearers with and without denture stomatitis. Biofilms are defined as structured microbial communities that are attached to a surface and encased in a matrix of exopolymeric material (12, 27). It is now estimated that a significant proportion of all human microbial infections involve biofilm formation (7, 27). A complex and mixed bacterial–fungal biofilm imparts increased resistance to antifungal or antibacterial therapies, serving as a reservoir for future infections (5, 27). A large amount of culture-independent data is available from biofilms on enamel surfaces (13); however, little is known about denture-related microbial communities.

This study is a large-scale analysis, including more than 700 clones (among bacterial and fungal species) examined from a restricted oral niche. For this analysis, we used universal primers to amplify bacterial (16S rDNA) and fungal (ITS region) DNAs. The ITS primer is used, as universal fungal primer, to amplify the fungal 5.8S rDNA and the adjacent ITS region (8) while 16S rDNA primers are used as universal bacterial primers (15, 23, 24). These primers generate amplicons of 400 bp (ITS) and 1500 bp (16S rDNA). These regions are very informative for species identification and the 200 bp for fungi and 500 bp for bacteria selected for analysis are enough for discrimination.

The individual samples were pooled into two groups (from subjects with and without denture stomatitis) to provide a representative sample of the microbial diversity of the denture biofilm, and establish a basis for further individual and quantitative analysis. Unfortunately, pooling all the healthy and all the disease samples together does not provide information on sample-to-sample variability and, more importantly, on how this variability could contribute to the bacterial and fungal phylogenetic diversity in health and disease. For this reason, the present study should be complemented by future studies but, at this time, we provide a global overview about the denture stomatitis biofilm, extending existing data in this field. It is important to emphasize that, until now, using culture-dependent methods, the bacterial diversity related to denture wearers with and without denture stomatitis recorded in previous studies ranged from 11 to 30 species (14, 16) and we were able to identify species within all the genera identified by these authors. In these studies, the predominant organisms were *Streptococcus* species, *Veillonella parvula*, *Lactobacillus* species, and *Bacteroides* species. In the present work, the number of bacterial species identified expands considerably our knowledge about microbial diversity in these oral biofilms. From the 82 bacterial species, two were novel phylotypes (unclassified),

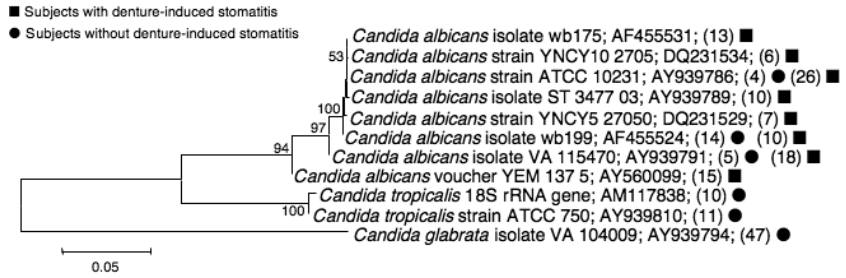

Fig. 3. Neighbor-joining tree based on the internal transcribed spacer (ITS) region of the fungal ribosomal DNA sequences showing the fungal species identified in subjects with and without denture stomatitis. The number of clones representing each phylotype is shown in parentheses. Codes correspond to GenBank accession numbers. Bootstrap values are based on 1000 replicates (values higher than 50% are shown).

representing potential new species. Besides, the present study, tried to provide a global 'qualitative assay' showing which organisms were involved in healthy denture wearers and in subjects with denture stomatitis. For this reason, we analyzed the biofilm from palatal tissue and from the corresponding fitting surface of the denture together to sample the complete composition of these two closely related sites. No relevant differences related to composition of the microorganisms placed on palatal tissue and the fitting surface of the denture had been found in a previous study using conventional microbiological approaches (14), confirming our choice to analyze them together.

Our data show that the healthy and diseased biofilms are distinct. This specificity is characterized in our pooled study by the presence of *Prevotella* sp. (about 9% of the clones) only in the pool from patients with stomatitis. They differ also in the relative abundance of *Veillonella* sp. clones in health (∼33%) or disease (∼19%) or *Atopobium* sp. in health (∼3%) and disease (∼8%), Additionally, the bacterial phylotypes found in the healthy denture wearers, represented by the phyla *Firmicutes* (such as species of *Streptococcus*, *Gemella*, *Selenomonas*, and *Veillonella*), *Proteobacteria* (e.g. *Neisseria* spp.) and the *Bacteroides* (e.g. species of *Prevotella* and *Capnocytophaga*) were also related to the maxillary anterior vestibule of healthy subjects identified previously (1, 21, 24).

Considering the fungal presence, *C. albicans* was found in both groups, as expected from previous work (14). Interestingly, *C. albicans* was the only yeast detected in the pool from denture wearers with disease in our samples. In contrast to a previous culture-dependent report (3), the present work found a greater fungal diversity in subjects without stomatitis. These data reinforce the hypothesis that *C.albicans* is the fungal species most commonly associated with biofilm formation (7, 17, 27). Moreover, although, the Chao 1 estimator analysis suggests a complete description of species diversity for the bacteria, the number of clones analyzed for the eukaryotic study (192 clones) may be insufficient to prevent a sampling bias. Still, because *C.albicans* is a normal inhabitant of the oral cavity, a direct cause and effect relationship between this fungus and denture stomatitis cannot be established. Additionally, *C. albicans* may play a role in the disease, because the yeast population increases with the severity of the stomatitis, as shown in a previous culture-dependent study (16). This finding has to be further analyzed by future quantitative and individual studies.

Data presented in this work confirm the polymicrobial nature of the studied biofilms, probably with important interactions between the bacterial and eukaryotic members to characterize the biofilm community and eventually to trigger disease. The differences found between biofilms from healthy and diseased subjects suggest the existence of a specific biofilm involved in denture stomatitis. At this moment, local and general predisposing factors related to denture stomatitis are described in the current literature, but there is not enough comprehensive information about host factors (e.g. immune system) and microbial status and how they tip the balance from health to disease. Moreover, biofilm formation involves the activation of genes that are essential for survival and confer biofilm-related properties, such as increased resistance to immune cell detection, environmental trauma, and antimicrobial compounds (5, 7, 9, 10, 12, 17).

Further studies at the individual level, analyzing diversity, quantitative molecular identification of particular species, and interactions among members of the resident microbiota and the host factors involved in the transition of the fundamental biofilm from commensal to pathogenic, are necessary for progress in the understanding of this common oral disease.

Acknowledgments

We thank Leila M. Toffoli and Júlio Cesar Moreira for expert technical help, and Dr Vicente P.P. da Cunha for his collaboration with sample collection. This study was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP grants 01/12111-4 and 02/13994-0), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

1. Aas JA, Paster BJ, Stokes LN, Olsen I, Floyd ED. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol 2005: **43**: 5721–5732.
2. Altschul SF, Madden TL, Schaffer AA et al. Gapped BLAST and PSI_BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997: **25**: 3389–3402.
3. Barbeau J, Seguin J, Goulet JP et al. Reassessing the presence of *Candida albicans* in denture-related stomatitis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003: **95**: 51–59.
4. Birnboim HC. A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol 1983: **100**: 243–255.
5. Chandra J, Mukherjee PK, Leidich SD et al. Antifungal resistance of *Candida* biofilms formed on denture acrylic *in vitro*. J Dent Res 2001: **80**: 903–908.
6. Chao A. Estimating the population size for capture–recapture data with unequal catchability. Biometrics 1987: **43**: 783–791.
7. Douglas LJ. *Candida* biofilms and their role in infection. Trends Microbiol 2003: **11**: 30–36.
8. Fujita SI, Lasker BA, Lott TJ, Reiss E, Morrison CJ. Microtitration plate enzyme immunoassay to detect PCR-amplified DNA from *Candida* species in blood. J Clin Microbiol 1995: **33**: 962–967.
9. García-Sánchez S, Aubert S, Iraqui I, Janbon G, Ghigo JM, d'Enfert C. *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. Eukaryot Cell 2004: **3**: 536–545.
10. Jenkinson HF, Douglas LJ. Interactions between *Candida* species and bacteria in mixed infections. In: Brogden KA, Guthmiller JM, ed. Polymicrobial diseases. Washington, DC: ASM Press, 2002: 357–373.
11. Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN, ed. Mammalian protein metabolism. New York: Academic Press, 1969: 21–132.

12. Kolenbrander PE. Oral microbial communities: biofilms, interactions, and genetic systems. Annu Rev Microbiol 2000: **54**: 413–437.
13. Kolembrander PE, Palmer RJ Jr, Rickard AH et al. Bacterial interactions and successions during plaque development. Periodontol 2000 2006: **42**: 47–79.
14. Koopmans AS, Kippuw N, de Graaff J. Bacterial involvement in denture-induced stomatitis. J Dent Res 1988: **67**: 1246–1250.
15. Kroes I, Lepp PW, Relman DA. Bacterial diversity within the human subgingival crevice. Proc Natl Acad Sci U S A 1999: **96**: 14547–14552.
16. Kulak Y, Arikan A, Kazazoglu E. Existence of *Candida albicans* and microorganisms in denture stomatitis patients. J Oral Rehabil 1997: **24**: 788–790.
17. Kumamoto CA. *Candida* biofilms. Curr Opin Microbiol 2002: **5**: 608–611.
18. Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. Tempe, AZ: Arizona State University, 2001.
19. Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 2004: **5**: 150–163.
20. Lillo A, Ashley FP, Palmer RM et al. Novel subgingival bacterial phylotypes detected using multiple universal polymerase chain reaction primer sets. Oral Microbiol Immunol 2006: **21**: 61–68.
21. Marchini L, Campos MS, Silva AM, Paulino LC, Nobrega FG. Bacterial diversity in aphthous ulcers. Oral Microbiol Immunol 2007: **22**: 225–231.
22. Marra MA, Kucaba TA, Hillier LW, Waterston RH. High-throughput plasmid DNA purification for 3 cents per sample. Nucleic Acids Res 1999: **27**: e37.
23. Paster BJ, Boches SK, Galvin JL et al. Bacterial diversity in human subgingival plaque. J Bacteriol 2001: **183**: 3770–3783.
24. Paster BJ, Olsen I, Aaas JA, Dewhirst FE. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. Periodontol 2000 2006: **42**: 80–87.
25. Paulino LC, Tseng CH, Strober BE, Blaser MJ. Molecular analysis of fungal microbiota in samples from healthy human skin and psoriatic lesions. J Clin Microbiol 2006: **44**: 2933–2941.
26. Radford DR, Challacombe SJ, Walter JD. Denture plaque and adherence of *Candida albicans* to denture-base materials *in vivo* and *in vitro*. Crit Rev Oral Biol Med 1999: **10**: 99–116.
27. Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL. *Candida* biofilms: an update. Eukaryotic Cell 2005: **4**: 633–638.
28. Reichart PA. Oral mucosal lesions in a representative cross-sectional study of aging Germans. Community Dent Oral Epidemiol 2000: **28**: 390–398.
29. Sambrook J, Russel DW. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2001.
30. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987: **4**: 406–425.
31. Siqueira JF Jr, Rôças IN, Cunha CD, Rosado AS. Novel bacterial phylotypes in endodontic infections. J Dent Res 2005: **84**: 565–569.
32. Sumi Y, Miura H, Sunakawa M, Michiwaki Y, Sakagami N. Colonization of denture plaque by respiratory pathogens in dependent elderly. Gerodontology 2002: **19**: 25–29.
33. Sumi Y, Kagami H, Ohtsuka Y, Kakinoki Y, Haruguchi Y, Miyamoto H. High correlation between the bacterial species in denture plaque and pharyngeal microflora. Gerodontology 2003: **20**: 84–87.
34. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997: **25**: 4876–4882.
35. Wilson J. The aetiology, diagnosis and management of denture stomatitis. Br Dent J 1998: **185**: 380–384.