Do different implant surfaces exposed in the oral cavity of humans show different biofilm compositions and activities?

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Osseointegrated dental implants play an important role in restorative dentistry. However, plaque accumulation may cause inflammatory reactions around the implants, sometimes leading to implant failure. In this *in vivo* study the influence of two physical hard coatings on bacterial adhesion was examined in comparison with a pure titanium surface. Thin glass sheets coated with titanium nitride (TiN), zirconium nitride (ZrN) or pure titanium were mounted on removable intraoral splints in two adults. After 60 h of intraoral exposure, the biofilms were analyzed to determine the number of bacteria, the types of bacteria [by applying single-strand conformation polymorphism (SSCP analysis) of 16S rRNA genes], and whether or not the bacteria were active (by SSCP analysis of 16S rRNA). The results showed that bacterial cell counts were higher on the pure titanium-coated glass sheets than on the glass sheets coated with TiN or ZrN. The lowest number of bacterial cells was present on the ZrN-coated glass. However, the metabolic activity (RNA fingerprints) of bacteria on TiN- and ZrN-coated glass sheets seemed to be lower than the activity of bacteria on the titanium-coated surfaces, whereas SSCP fingerprints based on 16S rDNA revealed that the major 16S bands are common to all of the fingerprints, independently of the surface coating.

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The excellent biocompatibility of titanium surfaces as an implant material results mainly from its surface properties. While problems in the osseous healing of implants appear to be largely solved, the adsorption of biomolecular pellicles, and the subsequent accumulation and metabolism of bacteria on these surfaces, is still the main stimulus for the induction of inflammatory processes. Several studies have demonstrated that both the quality and the quantity of plaque adhesion on the implant surface are important for the long-term success of dental implants (1, 2).

The initial adhesion and the colonization of bacteria to an implant surface are considered to play a key role in the pathogenesis of infections related to biomaterials (3, 4). It has been reported that different implant materials promote selective adherence during early plaque formation (5, 6). An *in vivo* study, exposing different implant materials on gingiva to the oral microbial community, showed that streptococci were the predominant colonizing micro-organisms and that the number of viable plaque-forming bacteria was dependent on the surface properties of the implant material (7). On intraoral hard tissues, *Actinomyces* species and streptococci are considered to be early colonizers, preparing the environment for late colonizers that require more demanding growth conditions. Many of these bacteria, such as *Fusobacterium*, *Capnocytophaga* and *Prevotella* species, which bind to streptococci, are also known to be involved in periodontal infections. It is therefore very important to develop implant surfaces (around the transmucosal portion) that reduce the number of initially adhering bacteria, thereby minimizing plaque formation and subsequent inflammation of the soft tissues.

In order to reduce or avoid the development of bacterial plaques on the implant material, different materials with different surface characteristics and various surface treatments have been previously tested in the oral cavity (8–10). Hard coatings [mainly titanium nitride (TiN)] were used to reduce plaque formation on the metal parts of partial dentures (11, 12). In our previous *in vitro* study, bacterial colonization was examined on titanium implant surfaces modified with a TiN or ZrN coating, thermal oxidation or laser radiation (8). When compared with polished titanium, significantly fewer adherent bacterial colonies were found on titanium

surfaces coated with inherently stable titanium hard materials such as TiN and zirconium nitride (ZrN). SCARANO and co-workers (9) confirmed our findings in an *in vivo* evaluation of bacterial adhesion to TiN-coated and uncoated titanium implants.

A variety of molecular methods have been used to determine the species composition of bacterial communities (10). An array of nucleic acid-based methods – such as probe hybridization of extracted bulk DNA (13) or individual cells (10), 5S rRNA community fingerprinting (14), the cloning of 16S rRNA genes (15) and sequence-specific separation of 16S rRNA gene fragments by denaturing gradient gel electrophoresis (DGGE) (16), temperature gradient gel electrophoresis (TGGE) (17) and single-strand conformation polymorphism (SSCP) analysis (18) – are now available to investigate mixed colonies of bacteria. Frequently, ribosomal RNAs and their genes are the target molecules of these techniques. Bacterial RNA concentration may be related to activity or growth owing to the fact that ribosomal RNA comprises typically > 80% of the cellular RNA content (19), i.e. it is directly correlated to the number of ribosomes per cell which, in turn, is a measure of its protein-synthesizing capacity (20). Thus, RNA-based fingerprints might represent the community structure of metabolically active members (21), whereas DNA-based fingerprints reflect the numerically abundant members of the respective community. To access both nucleic acids, we adapted, in this investigation, a protocol described recently by WENDEROTH and co-workers (22) that allowed the simultaneous extraction of RNA and DNA from bacteria of biofilm communities growing in biliary stents.

The aim of the present study was to determine whether the antimicrobial surfaces TiN and ZrN (8) were as good as or better than a pure titanium surface (in general used as an implant surface) for reducing bacterial adhesion in the oral cavity. For this purpose, the species composition, activity, and cell number of the bacterial biofilms formed on the different implant surfaces were evaluated using molecular methods.

Material and methods

Preparation of surfaces

Square glass sheets (1 mm in thickness and 0.7–0.9-cm^2 in surface area) were coated with either TiN or ZrN by physical vapor deposition (PVD); a pure titanium coating (by PVD) served as control. PVD coating, performed in the Federal Institute for Materials Research and Testing (BAM, Berlin, Germany) was carried out in an HTC 625 Multilab ABS coating system (HAUZER Techno Coating; Venlo, The Netherlands) with unbalanced magnetron sputtering, as described previously (8). The medium thickness of the coatings was 2.4 ± 0.3 μm (titanium), 1.8 ± 0.2 μm (TiN) and 2.2 ± 0.2 μm (ZrN). The coating did not change the roughness of the original glass surface; R_a values of the coated surface were close to zero.

Two volunteers (both female, 23 and 26 yr of age, volunteers A and B, respectively), in excellent systemic health, participated in this study. The participants were selected on the basis of good periodontal health, without menstruation during the experiment, and had a high standard of oral hygiene, with plaque index and sulcus bleeding index scores close to zero. In each of the two participants a removable acrylic splint was adapted to the premolar-molar region of each quadrant of the upper jaw. The splints were self-retaining and did not require etching or bonding to the teeth surfaces. Glass sheets were attached with dental resin to the buccal surfaces of the splints, their position is represented in Table 1. The splints were worn for a time-period of 60 h, and were removed only for meals and toothbrushing (during which they were stored in an environment of 100% humidity). Volunteers were requested to maintain their normal eating habits during the entire experiment. Neither cleaning procedures nor agents for chemical plaque control were applied to the glass sheets for the complete duration of the test period. After 60 h, all specimens were removed from each splint and processed immediately. The entire set of glass sheets provided are shown in Table 1.

For bacterial cell counting, the glass sheets were overlaid in 4% formaldehyde and stored at 4°C until the beginning of the experiments. Bacteria on glass sheets which were used for molecular biological procedures (nucleic acid extraction) were frozen immediately at −70°C.

Bacterial cell counts

The formol-treated glass sheets were transferred in a fresh 1.5-ml Eppendorf tube (Eppendorf, Hamburg, Germany) and overlaid with 1 ml of phosphate-buffered saline (PBS) buffer (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.5). Subsequently, the glass sheets were vortexed and sonicated each for 1 min (4-mm needle diameter; Labsonic U 2000 set at 50 W and 0.5-s pulses; B. Braun, Melsungen, Germany). Two 100-ml volumes of supernatant were transferred to two fresh tubes following which one was diluted 1 : 10 and the other 1 : 100 with 0.01 M sodium pyrophosphate. The dilutions were filtered through an Anodisc aluminum oxide filter (Whatman, Kent, UK) by vacuum filtration (< 10 kPa). The filter was placed sample side up in a Petri dish on a drop of 1 : 1000-diluted SYBR Green I solution (Molecular Probes, Leiden, the Netherlands). After a staining period of 5 min in the dark, the filter was placed back on the filter holder and the dye solution was sucked off. The filter was mounted on a glass slide with a drop of antifade solution (50% glycerol, 50% PBS, 0.5% ascorbic acid) and a 25-mm^2 coverslip. Cells were enumerated under blue excitation by using a Zeiss epifluorescence microscope (Axioplane; Zeiss, Göttingen, Germany). At least 200 cells from at least 10 eye fields were used to generate tif files. Cell numbers were estimated by using the IMAGE PRO PLUS software package (Media Cybernetics, Silver Spring, CA, USA). To compare the bacterial cell numbers of the different discs, the numbers were related to a disc size of 100 mm^2.

Table 1

Position of coated glass sheets mounted on acrylic splints in the oral cavity (numbers reflect the position of teeth in each quadrant)

	Titanium (Ti)	Titanium nitride (TiN)	Zirconium nitride (ZrN)
Volunteer A	16 and 27	17	26
Volunteer B	17 and 27	16	26

Nucleic acid extraction

Total DNA and RNA were obtained using the Fast DNA Spin kit for soil, according to the manufacturer's instructions (BIO 101; Q-biogene Alexis, Grünberg, Germany). After adding the binding matrix and shaking for 1 min at room temperature, the suspension was transferred into the catch tube and centrifuged at 14,000 g for 30 s. The centrifuged solution contained the RNA. RNA was precipitated with 1 volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH 4.0), dried in a speed vac and resuspended in 50 μl of diethylpyrocarbonate-treated water. The elution volume of DNA was also 50 μl. DNA and RNA concentrations were determined in a GenQuant analyzer (Amersham Biosciences Europe, Freiburg, Germany).

Primer selection and 16S polymerase chain reaction (PCR) and reverse transcription (RT)–PCR

The primers used for the amplification of bacterial 16S rDNA (18) and to determine their positions in the *Escherichia coli* 16S rRNA gene were as follows: forward primer Com1 (5′-CAGCAGCCGCGGTAATAC-3′, positions 519–536); and reverse primer Com2-Ph containing a 5′-terminal phosphate group (5′-CCGTCAATTCC TTTGAGTTT-3′, positions 907–926). Each PCR reaction was performed in a total volume of 50 μl in 0.1-ml microreaction tubes (Eppendorf), containing $1 \times$ PCR buffer with 1.5 mM $MgCl_2$, deoxynucleoside triphosphate solution (200 μM each dATP, dCTP, dGTP and dTTP), primers Com1 and Com2-Ph (0.5 μM each), 5 U of DNA polymerase (HotStar Taq; Qiagen, Hilden, Germany) and 10 ng of genomic DNA template. Thermocycling in an Eppendorf Mastercycler (Eppendorf) started with an initial denaturation for 15 min at 95°C, followed by 30 cycles at 94°C for 90 s, 40 s at 50°C, and 40 s at 72°C, and a final elongation for 10 min at 72°C.

RT–PCR was performed with the One-Tube RT-PCR Kit from Qiagen, according to the protocol of the manufacturer. After the RT step (30 min at 50°C), the PCR conditions used were those described above. The purity and size of the PCR products were analyzed by agarose gel electrophoresis (1.5% agarose, $1 \times$ Tris-Borate-EDTA (TBE), pH 8.0) running buffer, and ethidium bromide staining (23) of 4 μl of the reaction mixture.

Single-strand conformation polymorphism (SSCP) fingerprinting analysis

The preparation of single-strand DNA, gel electrophoresis, and silver staining of the polyacrylamide gels was carried out according to SCHWIEGER & TEBBE (18). For image analysis, gels were digitized to create tif files. Cluster analysis of the 16S rDNA fingerprints was performed using the software package GELCOMPARE II (Applied Maths, Kortrijk, Belgium). The background was first subtracted using rolling circle correction (circle diameter 30 pts), and lanes were normalized. Only bands with an intensity that was ≥2% of the total lane intensity were considered as a band.

Isolation and sequencing of single products from SSCP community profiles

Selected amplicons, revealed by silver staining of the polyacrylamide gels, were excised using sterile scalpels, transferred to an Eppendorf tube, and mixed with 50 μl of elution buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl, 0.1% Triton-X-100, pH 9.0). After incubation for 20 min at 95°C, 5–20 μl of the band solution, depending on the band intensity, were subjected to PCR amplification using Com primers and the conditions described above. The reamplified PCR products were purified using the Qiaquick MinElute-Gel Purification Kit (Qiagen, Hilden, Germany) and sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and one of the Com primers (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were analyzed on an Applied Biosystems 377 genetic analyzer.

Results

Bacterial cell counts

Four glass sheets with different surface coatings (two each of titanium, TiN and ZrN), in two different volunteers (A and B), were used to assess variation in bacterial cell numbers. Because they had the same surface and the same position in the oral cavity, identical surfaces were dealt with in parallel. The total bacterial cell counts of the eight different samples incubated in the oral cavity differed, ranging from 1.2×10^7 (ZrN-A26) to 9.8×10^{10} (Ti-B17) cells per glass sheet (Fig. 1). Bacterial cell counts were higher on all titanium-coated glass sheets than on TiN- or ZrN-coated glass. The fewest bacterial cells were present on the ZrN-coated glass sheet in both volunteers.

SSCP fingerprints based on 16S rDNA and 16S rRNA

Total DNA and RNA extraction was performed in order to compare bacterial communities of biofilms on glass sheets coated with different surfaces. 16S rDNA genes were amplified by the PCR, and 16S rRNA was amplified by RT–PCR. The amplicons generated were subjected to SSCP analysis to obtain fingerprints of the bacterial community of the biofilms (Fig. 2A, PCR products of the 16S rRNA genes; Fig. 2B, RT products of the 16S rRNA). Each band in the fingerprints represents the amplicon of a different rRNA or rDNA, which should represent a different type of bacterium. Fig. 2A shows the corresponding numbers and positions of bands from volunteer B (lanes 1 and 2) with slight
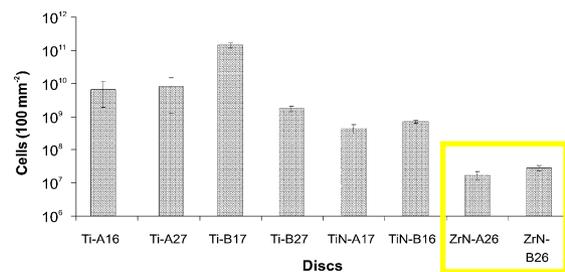

Fig. 1. Bacterial cell counts, determined by SYBR Green I staining and epifluorescence microscopy, of the biofilms grown on discs with different coatings. Ti, titanium; TiN, titanium nitride; ZrN, zirconium nitride.

differences compared to the fingerprints of volunteer A (lanes 3 and 4).

Figure 3 summarizes the number of bands estimated for the rDNA fingerprints and rRNA fingerprints. With regard to rDNA, each biofilm of the eight surfaces examined contains ≥9 bands (up to 20), with 3–5 major bands representing the most abundant community members. The major 16S bands are common to all of the fingerprints.

Obviously, the SSCP fingerprints obtained from 16S rRNA (Fig. 2B) showed a strictly different pattern, in terms of intensity and number of bands, than the DNA-based SSCP fingerprints (Fig. 2A). The band numbers estimated in the rRNA fingerprints range from 7 to 23. In order to identify the bacteria, the main bands from the SSCP fingerprints were excised, labeled (see the rhombus shapes of Fig. 2A), and the polynucleotide sequences of the fragments determined. For each sequence type, the nearest phylogenetic relative in the GENBANK database was traced (Table 2). Bands from the same positions in different fingerprints gave identical sequences, showing that similar organisms – streptococci seem to be the predominant colonizing micro-organisms – represent the most abundant members in biofilms on glass sheets coated with different surfaces. However, *Gemella* sp. was only identified on the coated glass sheets of volunteer B.
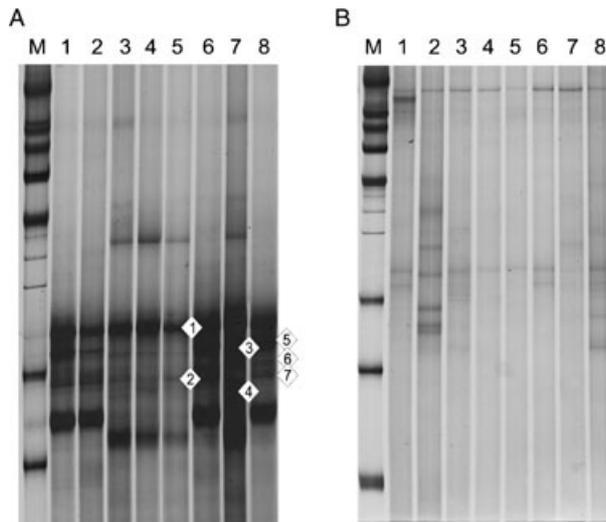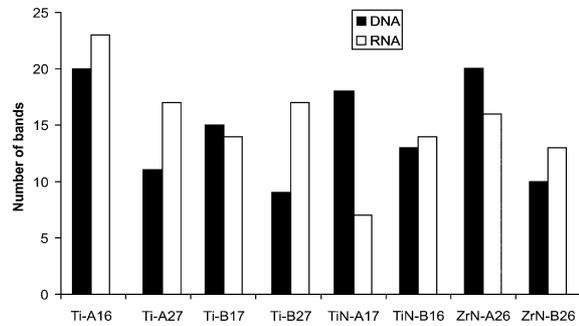

Fig. 3. Specific number of bands obtained from polymerase chain reaction (PCR) and reverse transcription (RT)–PCR single-strand conformation polymorphism (SSCP) fingerprints from bacterial biofilm communities (all bands with more than 2% relative abundance).

Differences in minor bands reflect microdiversity in biofilm communities on titanium hard coatings

In order to assess the relatedness of the biofilm communities on the differently coated glass sheets, the SSCP fingerprint patterns were digitized, normalized, and subjected to cluster analysis by the GELCOMPARE II software. The dendrogram shown in Fig. 4A reveals two main groups of communities on the different coatings. The fingerprints obtained from volunteer A grouped together, and the other group was built by the fingerprints of volunteer B. Only the fingerprint obtained from disc ZrN-A26 clustered to the group of volunteer B.

A different dendrogram, which compared the 16S rRNA-based fingerprints, is shown in Fig. 4B. It was obvious that the similarities between the RNA-based fingerprints were much higher than between the DNA-based fingerprints. The fingerprints that were exposed at the same teeth or beside were clustered together independently of the volunteer and the surface coating. Only samples ZrN-A26 and ZrN-B26 did not group together (Fig. 4A).

Discussion

Osseointegrated dental implants play an important role in restorative dentistry. However, plaque accumulation may cause inflammatory reactions around the implants, sometimes leading to implant failure. While there is

Fig. 2. Original (not normalized) single-strand conformation polymorphism (SSCP) fingerprints from bacterial biofilm communities were generated using (A) polymerase chain reaction (PCR) products of the 16S rRNA genes and (B) reverse transcription (RT) products of the 16S rRNA. The bands indicated with a number and a rhombus shape were sequenced. The sequence-derived phylogenetic assignments are given in Table 2. Lane M, DNA ladder; lane 1, Marker III (Boehringer); lane 2, Ti-B27; lane 3, Ti-B17; lane 4, Ti-A16; lane 5, Ti-A27; lane, 6; TiN-A17; lane 7, ZrN-A26; and lane 8, ZrN-B26. Ti, titanium; TiN, titanium nitride; ZrN, zirconium nitride.

Table 2

Phylogenetic assignments of sequences from disc biofilms

Band no.	Accession no.	Closest relative in the GenBank database	% Similarity
1	AF003932	*Streptococcus oralis*	96%
2	AF439645	*Veillonella* sp.	97%
3	AY099095	*Streptococcus oligofermentans*	98%
4	Y13366	*Gemella* sp.	97%
5	AY005042	*Streptococcus* sp.	98%
6	AJ295853	*Streptococcus mitis*	98%
7	AF371939	Uncultured bacterium	99%

A

Similarity (%)

80   85   90   95   100

TiN-B16
ZrN-B26
Ti-B27
Ti-B17
ZrN-A26
Ti-A16
Ti-A27
TiN-A17

B

Similarity (%)

20   40   60   80   100

Ti-A27
ZrN-B26
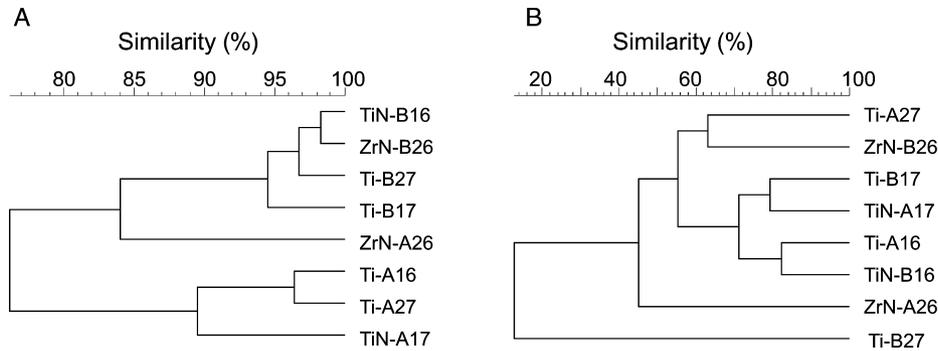Ti-B17
TiN-A17
Ti-A16
TiN-B16
ZrN-A26
Ti-B27

Fig. 4. Cluster analysis of the *(A)* DNA-based and *(B)* RNA-based single-strand conformation polymorphism (SSCP) fingerprints shown in Fig. 2.

considerable information about bacterial adhesion to enamel, little is known about the mechanisms of bacterial interactions with implant materials in the oral cavity. In the oral cavity, implants frequently become colonized by the opportunistic bacteria that form biofilms on implant surfaces. Biofilm-initiated diseases are by no means unique to the oral cavity. POTERA (24) has argued that 65% of infections which affect humans are caused by organisms growing in biofilms. Infected implants may exhibit reduced efficacy and serve as reservoirs for systemic infections. Many *in vivo* and *in vitro* studies (25) suggest that the composition of the supragingival bacterial community around healthy teeth differs from that around implants.

In this study we attempted to relate microbial community structure and activity of biofilms on different surfaces that were exposed to the oral cavity. Microbial community structure and activity were determined by culture-independent PCR-based SSCP fingerprints (18). One of the great advantages of this method is the direct comparison of the DNA and RNA SSCP fingerprints, because both nucleic acids came from an identical sample. Nevertheless, PRATTEN *et al.* reported a discrepancy between culture-based and molecular biological techniques (26) in that they were able to detect bacteria by using a PCR-based method but not by culture, and vice versa. VON WINZINGERODE *et al.* (27) reported that the extraction of RNA and DNA separately showed different extraction efficiencies in the yield, which could be a pitfall in the PCR and/or RT–PCR, and the PCR itself can amplify bias owing to the number of cycles of replication (28). However, because all samples were treated in a similar manner in this study, between-sample comparisons were possible (29, 30). In this study, parallel DNA- and RNA-based fingerprints were used to compare the abundant bacteria with the metabolically active ones. HURT and co-workers (31) postulated that the simultaneous recovery of RNA and DNA offers the potential for comparing the differences of microbial activities among different samples. This assumption has been confirmed by MUTTRAY & MOHN (32), who tested the DNA/RNA ratio for describing bacterial communities. These two complementary techniques of culture-independent community analysis helped to distinguish bacterial populations that were merely present (rDNA

profiles) from those that were metabolically active (rRNA profiles) (21, 33). Therefore, we used SSCP fingerprints based on rRNA and rDNA for differentiating between metabolically active bacteria (34) and abundant bacteria (35) organized in biofilms.

Within this study, the influence of three different surface coatings (titanium, TiN and ZrN on glass sheets) on the colonization of plaque-building bacteria was examined in the oral cavity. Bacterial cell number was estimated by DNA staining; activity and plaque composition were investigated using PCR amplification of 16S rRNA/rDNA, SSCP fingerprinting of the amplicons, and sequencing of the major SSCP species. Bacterial cell counts were higher on all titanium-coated glass sheets than on TiN- or ZrN-coated glass. The lowest number of bacterial cells was counted on the ZrN coating in both volunteers. These results are in accordance with the findings of GROESSNER-SCHREIBER *et al.* (8), who showed in an *in vitro* experiment that the number of adherent bacterial colonies *(Streptococcus sanguis, S. mutans)* was significantly reduced on titanium surfaces coated with inherently stable titanium hard materials, such as ZrN, compared to a polished titanium surface. Similar results were achieved by TiN coating and by a thermic oxidation. These results were confirmed by an *in vivo* study of SCARANO *et al.* (9), who showed that bacterial adhesion on implant surfaces coated with TiN was significantly lower compared to an uncoated implant surface.

Many other *in vitro* and *in vivo* studies have demonstrated that the surface texture has a significant impact on *de novo* plaque formation (36–38). The specific roughness of a provided surface is believed to be the main factor allowing bacteria to adhere to the surface, and a reduction of surface roughness is accompanied with reduced plaque formation (39, 40). In our previous study (8), determination of surface roughness for Ti-TiN, Ti-ZrN, TiO_2 and polished titanium discs revealed similarly low R_a values of 0.14–0.2 μm. To eliminate the influence of surface roughness in this study, coated glass sheets were used as substrates (with an R_a value close to zero).

The results of 16S rDNA/rRNA determination of the bacterial biofilms grown on the different coatings revealed similar main bands but showed small differences between glass sheets obtained from volunteer A and

volunteer B. Each volunteer had a more or less unique SSCP fingerprint on the different coatings (Fig. 2). Comparison of the fingerprints revealed two main clusters representing the glass sheets obtained from volunteer A and B, probably reflecting nutritional, genetic, and lifestyle differences of the two volunteers (Fig. 4). As metabolically active bacteria may contain a greater number of ribosomes than quiescent bacteria (20), rRNA fingerprints are considered to reflect the composition of the metabolically active members of the community (21, 33), whereas the rDNA fingerprints reflect the population composition of the community (35). It seems that microniches occur within the oral cavity. This might be the reason for grouping together the RNA fingerprints obtained from the same exposure position, independently of the specific surface and volunteer. These results are in accordance with reports from other authors who have not established any major differences in the microbial spectrum from amalgam, titanium, porcelain, or composit resin in *in vivo* studies over a period of 24 h (7, 41, 42).

Sequencing of single products from SSCP community profiles revealed 7 abundant bacterial species: 4 *Streptococcus* species (*S. oralis*, *S. oligofermentans*, *S. mitis* and one without species affiliation), *Veillonella* sp., *Gemella* sp., and an uncultured bacterium. The 16S rRNA gene sequence of this abundant plaque bacterium was highly similar (98%) to existing sequences of an uncultured bacterium in databases. These data have been confirmed by HEAD *et al*. (43). It seems that, in our study, *Streptococci* were the predominant organisms and represent the main early colonizers of all surface coatings examined. No *Actinomyces* species were found on any of the three different coatings. Our findings are in accordance with several other studies. An *in vivo* study exposing different implant materials on gingiva to the oral flora showed that streptococci were the predominant colonizing micro-organisms and that the number of viable plaque-forming bacteria was dependent on the surface properties of the material (7). It appears that bacterial adherence to ceramic material or to coatings with a ceramic-like character (as hard coatings) is lower than adherence to titanium alloys (44).

However, on intraoral hard tissues, *Streptococci* and *Actinomyces* species are considered to be early colonizers, preparing the environment for late colonizers that require more demanding growth conditions (45). On the other hand, a study conducted by WOLINSKY *et al*. (46) showed that plaque-forming bacteria, such as *A. viscosus*, adhered to enamel in numbers five times higher than they adhered to titanium surfaces. In contrast, *S. sanguis* adhered similarly to enamel and titanium. It might be that in our study all of the different titanium coatings prevented early colonization (within the first 60 h) of *Actinomyces* species. It has been reported that different implant materials promote selective adherence during early plaque formation (5, 6). Other studies have examined bacterial adhesion and colonization on a variety of biomaterials and have found, in general, that factors such as type of bacterium, concentration, growth phase,

and surface properties of the materials, all affect colonization to varying degrees (46, 47).

In conclusion, molecular analysis of the bacterial biofilms revealed the same main early colonizers on all surfaces examined, independently of the surface coatings. Minor differences (e.g. *Gemella* sp., which only appeared in volunteer B) seem to be dependent on the volunteer and not on the surface coating. Analysis of the rDNA and rRNA fingerprints showed a different number of bands, with the rRNA fingerprints showing slightly greater diversity. Differences in rRNA bands appear to be dependent on the volunteer and the position of the glass sheets in the oral cavity. However, both of the titanium hard coatings investigated in the present study seem to reduce initial bacterial adherence, with the ZrN coating showing the lowest number of adhering bacteria compared to pure titanium. These results are in accordance with other studies (8, 9). The physico-chemical reasons for the different bacterial cell numbers on the titanium hard coatings are still not fully known, but strongly confirm their suitability as implant coatings to decrease peri-implant soft tissue inflammation. Further studies will elucidate when the detected bacteria adhere to the specific surface and where they are within the plaque biofilm.

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