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Abstract:

Background: Supra- and subgingival biofilm formation is considered to be mainly responsible for early implant failure caused by inflammations of periimplant tissues. Nevertheless, little is known about the complex microbial diversity and interindividual similarities around dental implants.

Objectives: An atraumatic assessment of the diversity of microbial communities around titanium implants by single strand conformation polymorphism (SSCP) analysis of the 16S rRNA gene amplicons and subsequent sequence analysis.

Methods: Samples of adherent supra- and subgingival peri-implant biofilms were collected from ten patients. Additionally, samples of sulcusfluid were taken at titanium implant abutments and remaining teeth. The bacteria in the samples were characterized by SSCP and sequence analysis.

Results: A high diversity of bacteria varying between patients and within one patient with location was found. Bacteria characteristic for sulcusfluid and supra- and subgingival biofilm communities were identified. Sulcusfluid of the abutments showed higher abundance of *Streptococcus* species than from residual teeth. *Prevotella* and *Rothia* species frequently reported from the oral cavity were not detected at the abutments suggesting a role as late colonizers.

Conclusion: Different niches in the human mouth are characterized by specific groups of bacteria. Implant abutments are a very valuable approach to study dental biofilm development *in vivo*.

Keywords:

Oral implants, single strand conformation polymorphism, microbial diversity, implant abutments, biofilm, microbial communities

Introduction:

The clinical success of osseointegrated oral implants led to extended application. Today many conventionally prosthetic treatments have been replaced by implant supported prosthetic supraconstructions. From a clinical viewpoint this fact is supported by multitudinous studies which analyzed structural and functional improvements of the implant surface. While the problem of primary osseointegration is solved in a high quality manner, inflammation of peri-implant tissues by the presence of bacterial biofilms is considered currently as one of the principle problems in oral implantation and, therefore, responsible for early implant failure. This early implant failures are clinically characterized by inflammation of the peri-implantary mucosa (Figure 1a) and a subsequent destruction of the peri-implantary bone (Figure 1b).

The early processes of biofilm formation, like generation of an acquired pellicle by salivary biopolymers or enzymes and adherence of initial microorganisms, is described as is the relationship between biofilm formation and periodontitis or periimplantitis. For example, *Streptococcus mitis*, *S. sanguinis* and *S. oralis* are known to create the pre-conditions for the accumulation of gram-negative anaerobic bacteria [1-5]. Several studies were able to indicate, that during periimplant biofilm formation the microbial composition shifted towards a higher proportion of such gram-negative anaerobic microbiota [6-10]. However, these previously studies of microorganisms associated with diseases relied heavily upon cultivation or DNA checkerboard hybridization methods. The applied techniques are associated with the main disadvantages of a specific bacterial identification or time-consuming and fault-prone cultivation [11]. This led to the identification of several species as putative periodontopathogens based on frequency of their isolation in lesion sites. Among these are *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Bacteroides forsythus*, *Treponema denticola*, *Fusobacterium nucleatum*, *Parvimonas micra*, *Campylobacter rectus*, *Eikenella corrodens* and *Selenomonas sputigena* [12-15]. However, concentrating only on those bacteria which can currently be cultivated and ignoring particularly those which cultivation fails to capture [16] will not give a complete picture of infections leading to periodontitis or periimplantitis [17]. Because of these limitations, it is also difficult to acquire a complete understanding of the dynamic changes of oral microbes as dental biofilms turn from harmless ones into detrimental microbial communities. Answers to this challenge are culture-independent methods to monitor microbial communities over space and time.

Therefore, the objective of the present study was to assess the diversity of microbial communities, by culture-independent methods, of initial periimplant biofilms in supra- and subgingival areas, to compare them with those of the sulcus fluid and to identify microbial communities posing potential problems for infections. In

addition, a sample of sulcusfluid was taken at one remaining tooth in the same jaw to evaluate cross-infections between implants and teeth in the initial phase of biofilm formation.

Material and methods:

Subjects:

The present study was approved by the ethics committee of Hannover Medical School (No. 3791). The examination was performed with the understanding and written consent of each patient.

Qualitative analysis was based on 10 healing abutments which were inserted for 14 days into 10 patients, 6 women and 4 men, aged between 60 and 83 years (mean 70 years ± 6.7 years). Patients were selected to fulfil the following inclusion criteria: no systemic illness, no smoking, no pregnancy, no active periodontitis (probing pocket depth ≤ 4 mm), no pharmacological treatment or antibiotic therapy during or up to four months before the study. At the day of abutment insertion all patients received dental hygiene instructions but no professional tooth cleaning was performed during the observation period.

All patients were partially edentulous and had at least one oral two-piece implant made of titanium (Astra Tech, Mölndal, Sweden), which had been inserted three months before investigation. Two weeks after abutment surgery, the previously existing abutments were removed and new sterile healing abutments (Astra Tech, Mölndal, Sweden) for the analysis were inserted (Figure 2). Thereby no trimming of surrounding gingiva was performed.

Sampling:

Two weeks after insertion of the new sterile healing abutments, samples of adherent biofilm and crevicular fluid were taken. The periimplant saliva was removed by use of an air spray and cotton rolls. First, samples of crevicular fluid were taken with sterile paper points at abutments and remaining teeth. Afterwards, samples of adherent supragingival biofilm on the abutments were taken with sterile sponges. The samples of adherent subgingival biofilm could be collected after abutment removal. All samples were stored in sample tubes (Eppendorf AG) at -80 °C.

DNA analysis:

105 DNA was extracted using the Fast-DNA-Spin-Kit for soil (Bio 101, CA, USA) following the
106 instructions of the manufacturer. The primers chosen for the amplification of bacterial 16S rRNA genes were
107 forward primer Com1 (5'CAGCAGCCGCGGTAATAC3') and reverse primer Com2-Ph
108 (5'CCGTCAATTCCTTTGAGTTT3' with 5'-terminal phosphate group) as published by Schwieger and Tebbe
109 [18]. To obtain single-stranded DNA the phosphorylated strand of the PCR products was digested by lambda
110 exonuclease (New England Biolabs, Schwalbach, Germany) following the protocol of Schwieger and Tebbe, and
111 further purified using the Mini-elute-Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer, and
112 the remaining single stranded DNA was dried under vacuum. The DNA was then resuspended in denaturating
113 SSCP loading buffer (47.5 % formamide, 5 mM sodium hydroxide, 0.12 % bromophenol blue and 0.12 % xylene
114 cyanol), incubated at 95°C for 2 min and immediately cooled on ice. After 5 min, samples were loaded onto the
115 gels and subjected to electrophoresis. The samples were electrophoresed in a MDE gel (9 ml 2x MDE (FMC
116 Bioproducts, Rockland, Maine), 3 ml 10x TBE buffer, 18 ml bidest. water, 10% APS 120 µl and 12 µl TEMED).
117 Gels (21 cm) were cast (spacers of 0.5 mm) and run with 1x TBE buffer. Gels were run at 400 V for 16 h at
118 20°C in a Macrophor electrophoresis unit (LKB Bromma, Sweden) and subsequently silver stained [19].
119
120 *Sequence analysis:*
121 Single bands were excised from the gels and eluted in extraction-buffer (10 mM Tris-buffer, 5 mM KCl,
122 1.5 mM MgCl2 x 6 H2O, 0.1 % Triton X 100, pH 9.0) at 95°C for 15 min. Extracts were centrifuged (12000 x g,
123 1 min) and the DNA in the supernatant fluid was used for PCR with the primers described above. The PCR-
124 product was cleaned (Mini-elute-Kit, Qiagen, Hilden, Germany) and sequenced with a sequencing kit
125 (DYEnamic ET Terminator cycle sequencing kit by Amersham Biosciences, Freiburg, Germany) and both
126 primers. The product was cleaned with the Dye Ex Spin Kit (Qiagen, Hilden, Germany) and the sequence
127 analyzed on an ABI PRISMTM 337 DNA-Sequencer and 3100 Genetic Analyser. Comparisons of the sequences
128 were performed using the BLAST program and the databases of EMBL [20] and GenBank [21]. The
129 phylogenetic analysis was done using the MUSCLE version 3.6 software for sequence alignments [22]. Tree
130 topologies were reconstructed with neighbour-joining algorithm (complete deletion of gaps/missing data,
131 maximum composite likelihood model, bootstrap: 1000 replicates) (Figure 3) with the software MEGA 4 [23].
132 All reference sequences used in the tree are from validated bacterial species: each has been cultured,
133 characterized, deposited with at least two recognized culture collections, and named under the procedure
134 described in the International Bacteriological Code of Nomenclature (ICNB), 1990 revision [24].The sequences

of the oral rDNA bands obtained from patients 1 to 10 (31 sequences, indicated in Figure 3) have been deposited in the GenBank database under accession numbers FN870024-FN870053 and FR666873.

Statistics:

Statistical analyses were done using the STATISTICA 6 software. For correlation analysis and discriminate analysis the presence/absence of a species was used. Discriminant analysis was performed applying the forward stepwise method setting the tolerance to minimum (10^{-10}).

Results

All samples showed a large number of SSCP-bands and, hence, possess a high diversity of bacteria varying between patients and within one patient with location (Figure 4). Sequence analyses of 101 SSCP-bands led to the identification of 31 different bacterial taxa belonging to 5 different bacterial phyla (Firmicutes, Actinobacteria, Proteobacteria (Beta-, Gamma- and Epsilon-), Bacteroidetes and Fusobacteria). Remarkable were the diversity of *Veillonella*, *Streptococcus*, *Parvimonas*, *Fusobaterium* and *Neisseria* species detected. Species have only been identified down to the species level if they matched exactly with the sequence of the type strain (Figure 3). In the genus *Veillonella* gene sequences matching those of *Veillonella parvula* (*Veillonella parvula* 54-5) and *Veillonella atypica* (*Veillonella atypica* 133-5) could be identified. A third sequence (*Veillonella* sp. 112-3) was closer related to *Veillonella dispar*. As has been expected, many biofilm bacteria fell into the genus *Streptococcus*. Here *Streptococcus mitis* 42-1 and *Streptococcus intermedius* 63-1 could be identified together with two unknown *Streptococcus* species which were related to *Streptococcus intermedius* and *Streptococcus constellatus* (*Streptococcus* sp. 41-2 and 113-3). No sequence belonging to *Streptococcus mutans* was found. The genus *Fusobacterium* comprises strictly anaerobic bacteria and species of this genus are well known from the human mouth. The species *Fusobacterium naviforme* 72-3 and *Fusobacterium canifelinum* 113-8 were identified from the corresponding SSCP amplicons together with a third *Fusobacterium* sequence (*Fusobacterium* sp. 63-3) which was related to *Fusobacterium naviforme*. As for the former two species of the genus *Neisseria* have been reported as common members of dental biofilms in humans and *Neisseria mucosa* 133-2 was found together with the unknown *Neisseria* species 71-2 related to *Neisseria polysaccharea*. *Parvimonas micra*, widely distributed in human dental biofilms, was also detected in this study (*Parvimonas micra* 52-1). From one patient however, two distinct *Parvimonas* sequences have been identified (*Parvimonas* sp. 72-1 and 72-3) belonging to two undescribed *Parvimonas* species (Figure 3).

To detect interdependencies between bacteria species we correlated the occurrence of all species identified. Significant correlations were found between *Veillonella* species 112-3 and *Neisseria* species 71-2, *Haemophilus parainfluenzae* 112-5 and *Campylobacter* sp. 113-6, between *Veillonella parvula* 54-5 and *Oribacterium* species 54-3 and *Centipeda periodontii* 54-1 and between *Veillonella* sp. 133-5 and *Campylobacter gracilis* 54-7 and *Selenomonas noxia* 134-1. *Streptococcus mitis* 42-1 was significantly associated with *Lautropia mirabilis* 74-1, while the occurrence of *Streptococcus intermedius* 63-1 correlated with that of *Campylobacter* sp. 113-6. *Fusobacterium canifelinum* is closely related to *Fusobacterium nucleatum* which is known to have many interactions with other bacteria in dental plaques, but we found only a significant correlation between its occurrences with the two *Campylobacter* species.

We addressed the question whether the microbial communities are controlled by the individual host or the environment controls the composition of the community. To achieve this goal we used the bacteria detected in a sample to discern between the different types of samples. Discriminant analysis is used to find a combination of features (here bacteria species) which characterize or separate classes of objects (here sampling sites or patients). The resulting combination can be used to reduce the dimensionality. We applied discriminant analyses to identify such correlations and could not find microbial communities specific for the individual patients (data not shown). However, discriminant analyses identified occurrences of *Lactobacillus delbrueckii* 92-2, *Parvimonas* sp. 72-1, *Haemophilus parainfluenzae* 112-5 and *Streptococcus mitis* 42-1 as discriminative between the subgingival bacteria and the other samples. The occurrences of *Parvimonas micra* 52-1 and *Neisseria mucosa* 133-2 were characteristic for the supragingival microbial communities, while those of the sulcusfluid from the abutments were characterized by the presence of *Burkholderia* sp. 113-1 (Figure 5a). In figure 5b the contribution of every discriminative strain to the plot shown in figure 5a was plotted for the first two coefficients factors. This presentation allows the fast identification of strains to a given sample set, e. g. *Parvimonas micra* 52-1 and *Neisseria mucosa* 133-2 contributed most to supragingival biofilms while *Parvimonas* sp. 72-1 was characteristic for subgingival biofilms. For the sulcusfluid from the residual teeth no discriminative bacteria could be identified as expected from the central position of this group in figure 5a. Comparing the sulcusfluid around the abutments and around the residual teeth only small differences in the microbial diversity was seen. One remarkable difference, however, was the higher diversity and the much higher frequency of *Streptococcus* sequences found in the sulcusfluid around the abutments compared to that from residual teeth.

Discussion

196 The use of healing abutments to study the microbial diversity of initial biofilms on oral implant
197 abutments using an unspecific molecular biological detection method allows an *in-vivo* analysis of biofilms in
198 supra and subgingival areas along oxic-anoxic gradients. The anaerobic environment enables realistic formation
199 of the same biofilm as during development of mucositis or periimplantitis. The advantage to remove healing
200 abutments atraumatically for sampling adherent bacteria in subgingival areas without contaminating the sample
201 with supragingival microbiota was an important precondition for a realistic comparison of supra- and
202 subgingival biofilm compositions. In the present study a short term investigation of biofilm formation on healing
203 abutments was performed over a period of 14 days. The analysis was performed over this specific period to
204 display the realistic and typical microorganisms in the initial phase of biofilm formation. Furthermore previous
205 investigations approved microbiota around implants to remain unchanged for six months after a comparable
206 period [25]. In recent studies, a similar method directed to the typical periodontal pathogens *Aggregatibacter*
207 *actinomycetemcomitans* or *Porphyromonas gingivalis* in the periimplant crevicular fluid was used [26, 27].
208 However, structural differences between healing abutments and implant retained prostheses have to be
209 considered while interpreting the results. Implant anchored prostheses like crowns or bridges exhibit an anatomic
210 form with typical plaque retentive sites. Furthermore, a gap between the definitive abutments and the dental
211 prostheses can boost the biofilm formation because of their modular configuration. However, an analysis of
212 initial adherent biofilms along oxic-anoxic gradients is not possible with implant retained dental prostheses
213 because sampling of adherent bacteria in subgingival areas without removal of the construction is leading to a
214 contamination with supragingival microbiota in the majority of cases.
215 In the present study several species of the genera *Veillonella*, *Streptococcus*, *Parvimonas*, *Fusobaterium*
216 and *Neisseria* were detected all of them well known from dental plaques. Not all of them fitted to the 16S rRNA
217 gene sequences reported from the type strains of accepted species. However, the identification of the *Veillonella*
218 species is complicated as it has been reported that as least some *Veillonella* species have a rather high intra-
219 chromosomal heterogeneity between their four rRNA gene copies [28]. No *Streptococcus mutans* sequences
220 were found, however, the essential role of *Streptococcus mutans* in the caries process has been questioned [29]
221 as some strains of *Streptococcus oralis* exhibited levels of caries induction in rats that were close to those of
222 *Streptococcus mutans* [30]. *Streptococci* are the principal group of the early colonizing bacteria showing
223 interactions with a number of other bacteria. One example is a metabolic interaction with *Veillonella* species,
224 which coaggregate with *Streptococci* [31]. In this food chain *Veillonella* uses lactic acid produced by the
225 *Streptococci* as an end product of carbohydrate fermentation [32]. *Streptococcus* species were detected in 75%

and *Veillonella* species in 50% of the patients. Only in one patient no *Streptococcus* but *Veillonella parvula* could be found.

Species of the genus *Fusobacterium* are obligate anaerobes and implicated in the pathogenesis of periodontal disease. However, this organism has also been frequently identified in high numbers in healthy adults. Consequently it was found in six out ten patients. The diversity of *Fusobacterium* species between the different patients and also between the different locations was high. Such diversity has also been observed at the clonal level of *F. nucleatum* isolates [33]. Interestingly, in this study also a large dynamic between the clones could be shown. Furthermore, it is known from *F. nucleatum* that is a typically middle colonizer of dental surfaces showing many interactions with the surrounding bacteria cells [34]. Both observations can explain the diversity observed in our study but not the absence of *Fusobacterium* strains in some patients. This finding points to alternative routes to anaerobic communities and plaque formation not involving *Fusobacterium*.

Well known primary colonizers are species of the genus *Neisseria* and found in the majority of the patients but in two out of ten patients they could not be detected. Again it is reasonably to assume that *Neisseria* is not essential for the primary colonization of dental implants and can be substituted by other species. In addition to *Neisseria* many of the bacteria identified in this study including the *Burkholderia* species were also reported from the initial colonization of enamel [35], childhood caries [36] and in periodontal pockets [37]. Interestingly, no *Prevotella* or *Rothia* species was detected which were reported to be rather abundant in a recent study on the oral microbiota of 10 individuals with healthy oral tissues [38]. It is tempting to speculate that these bacteria appear late in the oral biofilm formation and were, therefore, not observed in our two-weeks-study. Such an information can only obtained with methods which are at least “semiquantitative” but are hard to deduce from a metagenomic approach [39]. A further advantage of the culture-independent approach applied here is the detection of novel species, e. g. *Oribacterium* sp. 54-3. According to the gene databases such strain has never been detected before and judging from its remote relatedness to *Oribacterium sinus* it may well belong to a still undescribed genus.

Having assessed the microbial diversity of young biofilms in the human mouth it is interesting to compare the findings with detrimental biofilms reported in the literature [40]. Socransky and coworkers assessed the microbial diversity of a large number of plaque samples and correlated it with the clinical assessment [15]. Multivariate analyses revealed five groups of bacteria increasingly related to periodontitis. We compared this finding with our results and found that none of the species in the group strikingly related to periodontal disease was detected in our study. This indicates no forms of gingivitis or periodontitis. Beside pathogens related to dental diseases other pathogens were found in a few samples, e. g. *Neisseria mucosa, Actinomyces israelii* or

Haemophilus parainfluenzae. The detection of *Haemophilus parainfluenzae* fits to the suggestion that typical respiratory pathogens may colonize the teeth of hospitalized intensive care and nursing home patients [41].

The occurrences of some bacteria were found to be discriminative for some sites. *Streptococcus mitis* and *Lactobacillus delbrueckii* contributed together with *Parvimonas* sp. 72-1 to the discrimination of subgingival biofilm communties while *Neisseria mucosa* and *Parvimonas micra* where mainly contributing to the discrimination of supragingival biofilms. This also confirms their role as primary colonizers. Our results confirmed earlier findings [42] but extended the number of site specific bacteria considerably. We also showed that *Prevotella* and *Rothia* species, abundant in the oral cavity of healthy individuals, are not members of the young biofilm communities. Concerning the experimental set-up used our results show as well that healing abutments can be used as a convenient model to study initial colonization of implants under natural conditions in humans.

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Figure legends:

Figure 1: Clinical signs of periimplantitis with an inflammation of the peri-implantary mucosa (arrows) (a) and a subsequent destruction of the peri-implantary bone with the typical signs of radiographic bone loss around the dental implant (arrows) (b).

Figure 2: New sterile Healing abutment (regio 12) *in situ*.

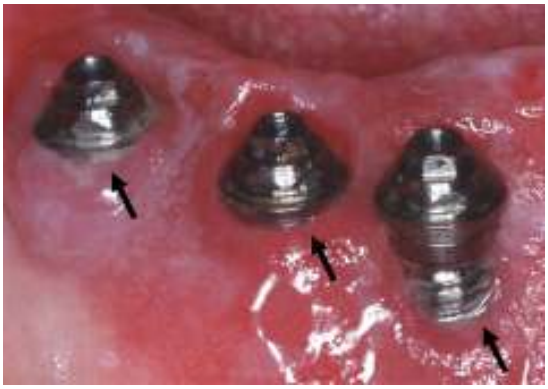
Figure 3: Unrooted phylogentic tree based on 16S rRNA gene sequences obtained by sequencing SSCP bands and the nearest type strain using neighbour joining. Bootstraps values are shown at the branching points; only values above 50% are shown. Only sequences with more than 350 unambiguously determined base pairs were included. Numbers in bold stand for sequences of SSCP bands deposited at GenBank. Scale bar represents 5 nucleotide substitutions per 100 bases.

Figure 4: SSCP samples from 5 patients; sp = supragingival, sb = subgingival, ab = sulcusfluid from abutment, rt = sulcusfluid from residual teeth. Numbered bands are 1 = *Neisseria mucosa*, 2 = *Veillonella parvula*, 3 = *Burkholderia* sp. 113-1, 4 = *Marinobacter santoriniensis*, 5 = *Parvimonas micra*, 6 = *Corynebacterium* sp. 51-2, 7 = *Leptotrichia* sp. 51-3, 8 = *Klingella* sp. 112-2, 9 = *Campylobacter gracilis*, 10 = *Centipeda periodontii*, 11 = *Oribacterium* sp. 54-3, 12 = *Fusobacterium nucleatum*, 13 = *Fusobacterium* sp. 63-3, 14 = *Parvimonas* sp. 72-1, 15 = *Parvimonas* sp. 72-2, 16 = *Fusobacterium naviforme*, 17 = *Lautropia mirabilis*, 18 = *Neisseria* sp. 71-2, 19 = *Streptococcus mitis*, 20 = *Lactobacillus delbrueckii*, 21 = *Streptococcus intermedius*, 22 = *Capnocytophaga sputigena*.

Figure 5: Discriminant analysis between the four sampling sites and the presence/absence of their bacteria. Only roots 1 and 2 are shown. **5a** (left): Plot of the four different sample types; circle: supragingival biofilms (solid ellipse), square: subgingival biofilms (dashed ellipse), rhomb: sulcusfluids of the abutments (mixed dashed ellipse), triangle: sulcusfluids of the residual teeth (solid ellipse). **5b** (right): factor loadings of the bacteria discriminating the four groups.

Figures:

Figure 1 a/b:



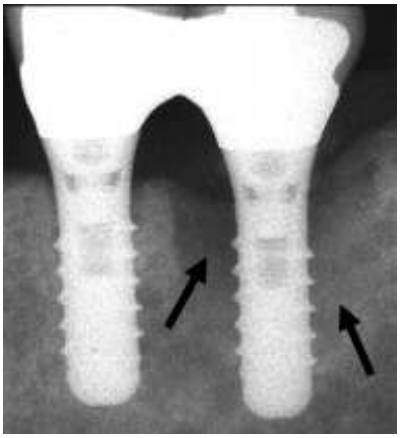
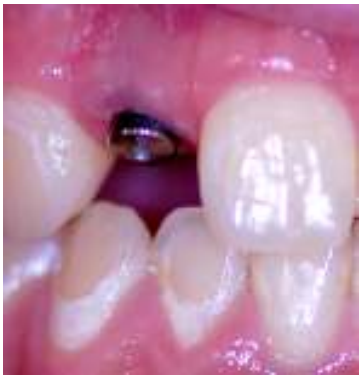


Figure 2:



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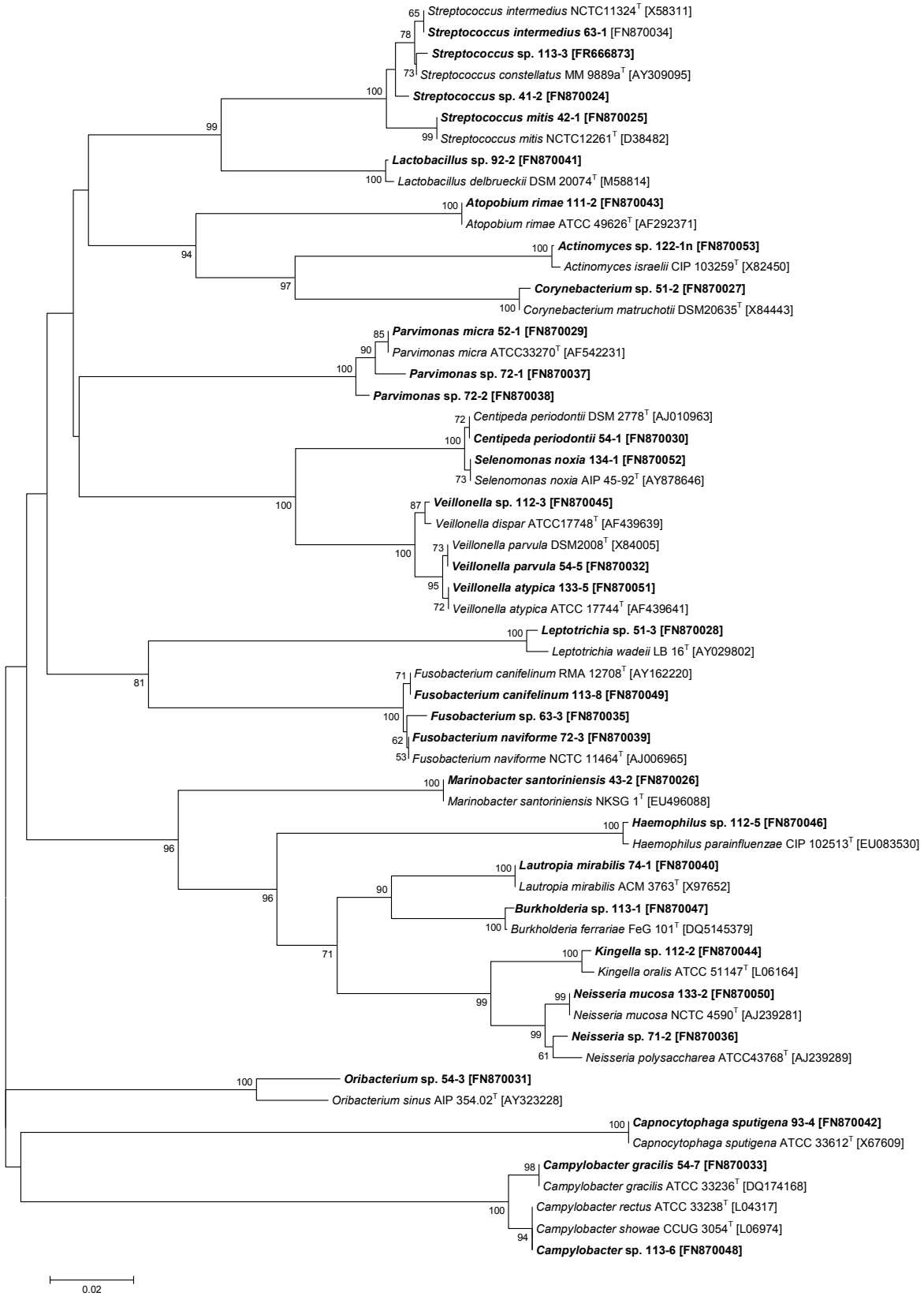

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418 Figure 4:

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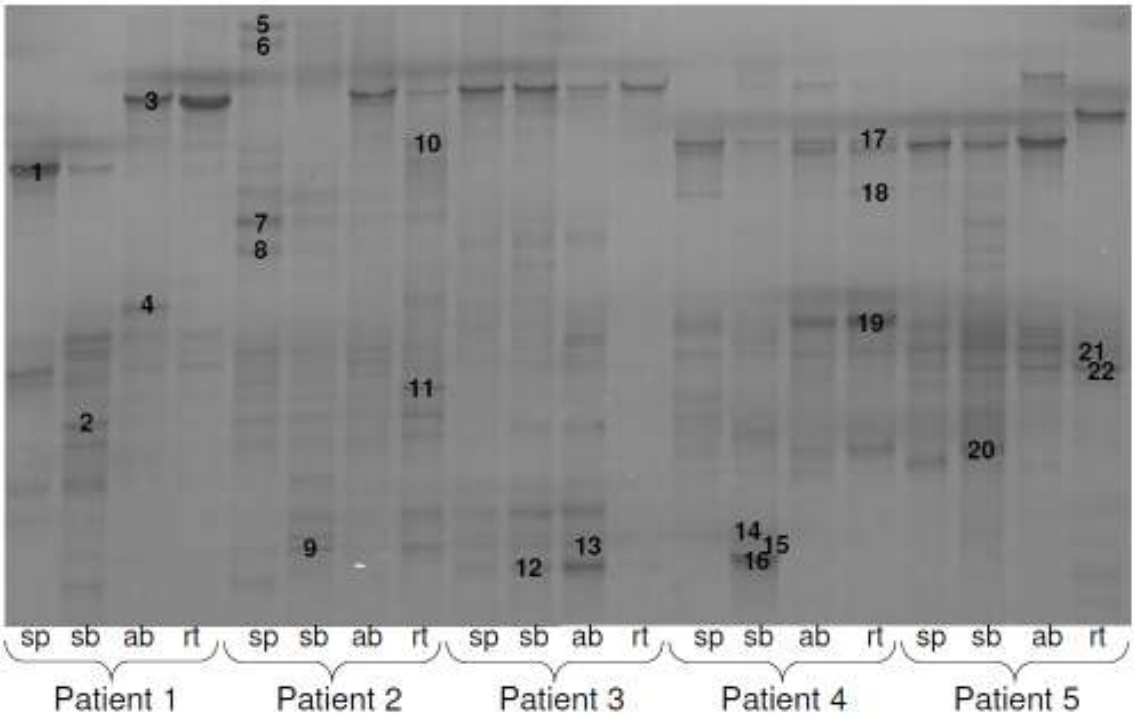

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422 Figure 5:

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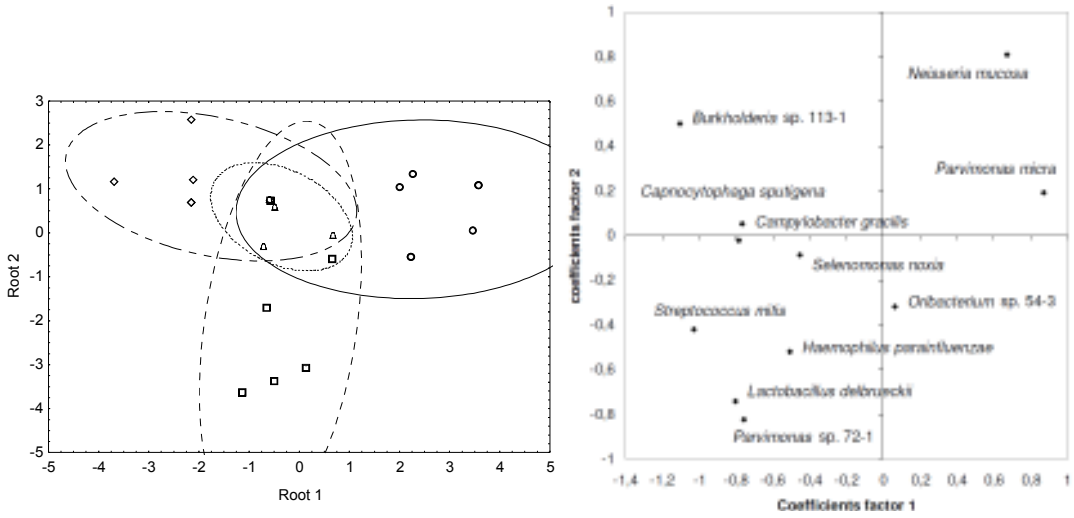

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