

Microarray analysis of the microflora of root caries in elderly

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Abstract The present study used a new 16S rRNA-based microarray with probes for over 300 bacterial species to better define the bacterial profiles of healthy root surfaces and root caries (RC) in the elderly. Supragingival plaque was collected from 20 healthy subjects (Controls) and from healthy and carious roots and carious dentin from 21 RC subjects (Patients). Collectively, 179 bacterial species and species groups were detected. A higher bacterial diversity was observed in Controls than in Patients. *Lactobacillus casei/paracasei/rhannosus* and *Pseudoramibacter alactolyticus* were notably associated with most RC samples. *Streptococcus mutans* was detected more frequently in the infected dentin than in the other samples, but the difference

was not significant. *Actinomyces* was found more frequently in Controls. Thus, species other than *Actinomyces* and *S. mutans* may play a role as pathogens of RC. The results from this study were in general agreement with those of our previous study based on 16S rRNA gene sequencing.

Introduction

Root caries (RC) has become a significant oral health issue. Several studies agree that the RC prevalence is much higher in the dentate elderly than in other adult groups [1–3]. Two factors are leading to an increasing number of exposed root surfaces in the elderly: the increased duration of life [4] and the higher number of teeth being maintained in this population [5]. Since RC is clearly a bacterial infection, it is therefore important to identify those bacterial species that are associated with the disease.

Based on culture-dependent studies, *Streptococcus mutans*, lactobacilli and some species of *Actinomyces* have been considered important in the etiology of RC [6, 7]. In contrast, van Houte et al. [8] described the bacterial flora of RC as more diverse, and found that the above mentioned species played less of a role. More recently, culture-independent studies have focused either on single species such as *S. mutans* [9] and *Actinomyces naeslundii* [10], or on the analysis of microbial-derived organic acids [11]. Several other studies have investigated advanced dentinal lesions from coronal caries with molecular methods [12–16], but they were not designed to study RC. Overall, the present understanding of the microflora of RC is limited compared to other infectious oral diseases [8].

Recently, we characterized the bacterial diversity of healthy root surfaces and root surface caries in the elderly based on sequencing of 16S rRNA genes amplified from

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isolated DNA [17]. We demonstrated that other species in addition to *S. mutans*, lactobacilli and *Actinomyces* may be also involved in the RC process [17].

Since only a limited number of subjects were analyzed, our findings only began to assess the bacterial associations with RC. The Human Oral Microbe Identification Microarray (HOMIM) allows for the simultaneous detection of about 300 of the most prevalent oral bacterial species, including those that cannot yet be grown in vitro [18]. Overall, there are about 600 oral bacterial species, of which about 50% have not yet been cultivated [19; www.homim.org]. The arrays are designed specifically for the characterization of bacterial profiles in oral clinical samples. More than 550 bacterial species were first identified by sequence analysis of approximately 20,000 16S rRNA clones derived from human oral samples [19, 20]. In the present study, we used a new 16S rRNA-based microarray method to test the hypothesis that bacteria other than *S. mutans*, lactobacilli and *Actinomyces* are detected from RC lesions in the elderly. Since HOMIM allowed the analysis of a more statistically significant number of subjects, we also tested for the significance of the bacterial association with healthy and carious roots. Moreover, as approximately half the samples had been analysed previously by 16S rRNA gene sequence analysis [17], we also compared the two methods for bacterial detection.

Materials and methods

Subjects

Forty-one elderly subjects aged 70–101 years (36 females and 5 males) were analyzed. Twenty-one of these subjects were used in our previous study [17]. Subjects were selected as previously described. The RC definition and diagnosis were according to the criteria of the World Health Organization [21]. The control subjects (referred to as Controls) ($n=20$) had no RC and the RC group (referred to as Patients) ($n=21$) had at least one RC lesion and one healthy root at the time of examination. Mean age of the Controls was 84.3 years (range 72–92), mean number of teeth 23 (range 16–28), and number of males 3. Mean age in the Patients was 88.7 years (range 70–101), mean number of teeth 15 (range 5–26), and number of males 2.

Samples

The samples ($n=83$) were collected and processed as previously described [17]. Briefly, plaque samples were obtained from the sound roots of Controls. From the Patients, the following samples were analyzed: plaque from one healthy root (plaque healthy), plaque from one carious lesion (plaque carious), and the underlying carious dentin

from the same carious lesion (dental). All the samples were collected by one examiner (D. P.).

DNA extraction and amplification of 16S rRNA genes

Bacterial DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen, GmbH, Hilden, Germany) according to the instructions of the manufacturer. The extracts were stored at -20°C until use. The 16S rRNA genes were amplified as previously described [19]. Briefly, two separate PCR reactions were set up with, respectively, forward primer 5'-CCA GAG TTT GAT YMT GGC-3' with reverse primer 5'-GAA GGA GGT GWT CCA RCC GCA -3', and forward primer 5'-GAC TAG AGT TTG ATY MTG GC-3' with reverse primer 5'-GYT ACC TTG TTA CGA CTT-3'. Two μl of genomic DNA template were added to the reaction mixture (final volume 25 μl) containing 20 pmol of each primer, 40 nmol of deoxytriphosphates, 1.5 mM Mg^{2+} and 1 U of Platinum High Fidelity *Taq* polymerase (Invitrogen, San Diego, CA). The samples were preheated at 94°C for 2 min, followed by 32 cycles of amplification under the following conditions: 94°C for 30 s, 55°C for 30 s, and 68°C for 1.5 min, with an additional 1 s for each cycle. A final 10 min elongation step at 68°C was added. The results of the PCR amplification were examined by electrophoresis in a 1% agarose gel. The two parallel PCR reactions were combined and the products purified with QIAquick PCR Purification Kit (Qiagen).

Labeling of PCR products

The labeled nucleotide Cy3-dCTP (GE Healthcare Biosciences, Pittsburgh, PA) was incorporated during a second, nested PCR reaction using the forward primer 5'-GAG TTT GAT YMT GGC TCA G-3' and the reverse primer 5'-GYT ACC TTG TTA CGA CTT-3'. Seven μl of DNA template were added to a reaction mixture (final volume 25 μl) and amplified using the same cycling program (but with 40 cycles) as described above. Cy3-labeled amplicons were purified using a supplementary protocol with the QIAquick PCR Purification Kit (Qiagen). In this protocol, an extra wash was performed using a 35% guanidine hydrochloride aqueous solution, which aids in the removal of excess Cy-dye in the reaction.

Steps prior to hybridization

Printing of arrays 16S rRNA-based oligonucleotide reverse capture probes were custom synthesized with a 5'-(C6)-amine modified base, eight spacer thymidines and 18 to 20 nucleotides of target sequence, and printed (Michigan State University Research Technology Support Facility) on 25×76 mm aldehyde-coated glass slides. The full list of probes for target species and their sequences is found in Table S3 in the supplemental material. Sixty μM oligos were

plated in v-bottom 384 well plates in a 2:1 solution with 2 × spotting buffer giving a final spot concentration of 30 μM. By printing an OmniGrid Arrayer (GeneMachines) was used at 55% humidity. Post-processing of printed slides included immobilization by baking. Probes targeting more than two closely related species appeared as clusters (37 altogether). About 30% of the targeted species were identified using two probe sets. The array was divided in five sections allowing the processing of five samples simultaneously. Each section had two identical probe sets. Array locations were labeled by etching with a diamond pencil, and slides were stored in a slide box under dry conditions. **Blocking.** Immediately before use, the slides were blocked to reduce non-reactive primary alcohols, to remove unreacted aldehyde groups and to minimize fluorescent background after hybridization. This was carried out using the Little Dipper Microarray Processor (SciGene, Sunnyvale, CA). First, the slides were extensively washed to remove unbound DNA molecules and buffer substances by a series of washing steps using 10% SDS and ddH₂O. Then they were placed in a solution of sodium borohydride, 1 × PBS and 99% EtOH for 15 minutes at room temperature. Once complete, another set of washing step was performed, and slides were spun dry for 5 minutes and stored once again in a dessicator cabinet. The blocked slides and the 10×22 mm glass Lifterslips (Thermo Scientific, Hudson, NH) were first cleaned of any residual dust using an air line.

Hybridization A hybridization solution was prepared using the purified labeled DNA, 20 × SSC (2 × final conc.), yeast tRNA (10 μg/μl), H₂O and 0.25 μl 10% SDS (0.1% final). The solution was mixed and spun briefly in a centrifuge, then denatured at 100°C for 5 minutes. One ml of H₂O was added to the Whatman paper inside the hybridization chamber to help prevent the slide from drying out during the incubation. Ten μl of denatured hybridization cocktail was then carefully injected under its respective cover slip using a pipette. The hybridization chamber was sealed, and hybridization was incubated overnight at 55°C. After hybridization the arrays were removed from the hybridization oven and washed at room temperature using the Little Dipper Microarray Processor. The slides were then spun dry in the attached centrifuge on the Little Dipper Microarray Processor and stored in a dark container until scanned as below. Each sample was hybridized in replicate and evaluated after one single hybridization.

Microarray validation

Reproducibility One sample was hybridized on two different sections on the same slide to test section-to-section reproducibility. Further, one sample was hybridized on two different slides to test the slide-to-slide reproducibility.

Duplicate probe sets for each clinical sample under the same cover slip tested the reproducibility.

Specificity. Pools of genomic DNA from 10 target bacterial species or phlotypes (e.g., from cloned inserts) were processed on one array to confirm the specificity of the bacterial probes and to identify any possible cross-reacting probes. After these experiments, those probes that were determined to cross-react or not specifically detect the target bacterial species were deleted from the probe panel. Results from another two probes found to give dubious results, were removed from the analysis.

Controls. Negative controls (buffer only and blank spots, probes for bacteria not found in humans) were included in both the original and duplicate probe set. They were used to identify high background and non-specific binding.

Scanning and analysis

The arrays were manually inspected, bad spots, including spots with obvious disagreements between the two parallels, were discharged. An Axon 4000B microarray scanner (Molecular Devices, Sunnyvale, CA) was used for scanning the slides. They were scanned using a wavelength of 532 nm to visualize probes hybridized with Cy3-labeled amplicons. The PMT (photomultiplier tube) value was adjusted accordingly, with Power at 100%. Median pixel intensities for each individual spot could be calculated using the analysis function of the GenePix Pro (Molecular Devices) accessory software. The median background intensity for each individual feature was subtracted from the median feature intensity yielding a normalized “median intensity score” for each individual feature. The generated gpr files were exported to the HOMIM tool website for further analysis (<http://bioinformatics.forsyth.org/homim/>). This analysis allowed the determination of the presence or absence of a particular microorganism based on specific criteria set for that individual spot, and thus microbial profile maps for each sample, and subsequent cluster analyses. The cluster method used was UPGMA with a correlation distance function. The similarity measurement was calculated with the Person's Correlation Coefficient with value from -1 to +1, when identical: 1, and completely opposition: -1. The signal intensities were categorized from 0 to 5.

Statistical analysis

When comparing the prevalence of subjects with a specific species in the two subject groups, a chi-square test was used to calculate p-values. When comparing p-values of

Table 1 Number of species belonging to various bacterial phyla identified in the different sample categories, and comparison of data from microarray and sequencing ^a

| Phylogenetic group | Total | | | Control samples | | | Patient samples | | | | | | | | |
|--------------------|-------|-----|-----|-----------------|----|-----|---------------------|----|-----|---------------------|----|-----|----------|----|-----|
| | | | | | | | Plaque healthy root | | | Plaque carious root | | | Dentinal | | |
| | MT | MS | SS | MT | MS | SS | MT | MS | SS | MT | MS | SS | MT | MS | SS |
| Firmicutes | 76 | 63 | 102 | 49 | 38 | 49 | 63 | 39 | 67 | 50 | 38 | 73 | 55 | 44 | 55 |
| Bacteroidetes | 25 | 19 | 50 | 19 | 11 | 24 | 15 | 14 | 24 | 12 | 7 | 27 | 15 | 12 | 17 |
| Actinobacteria | 22 | 15 | 44 | 7 | 3 | 16 | 8 | 5 | 13 | 16 | 5 | 16 | 17 | 14 | 30 |
| Proteobacteria | 20 | 16 | 19 | 14 | 9 | 13 | 15 | 13 | 10 | 14 | 12 | 10 | 13 | 9 | 3 |
| Fusobacteria | 6 | 5 | 21 | 6 | 5 | 15 | 4 | 3 | 11 | 3 | 3 | 7 | 6 | 4 | 6 |
| Spirochetes | 7 | 6 | 4 | 5 | 4 | 2 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 3 | 3 |
| TM7 | 4 | 4 | 3 | | | | 2 | 2 | 2 | 4 | 1 | 1 | 2 | 2 | 1 |
| Synergistes | 4 | 3 | 2 | 1 | 1 | 1 | 4 | 3 | | 1 | 1 | 1 | 3 | 1 | 2 |
| OP11 | 1 | | | | | | 1 | | | | | | 1 | | |
| Total | 165 | 131 | 245 | 101 | 71 | 123 | 114 | 80 | 129 | 101 | 67 | 137 | 113 | 89 | 117 |

^aMT = Microarray total (includes samples from 20 Controls and 21 root caries subjects). MS and SS = microarray and sequencing subset, respectively (includes analysis of the same samples from 10 Controls and 11 root caries subjects), with sequencing data from Preza et al. [17].

bacterial species in two different samples in one group, a sign test was used. H_0 was defined as “the true prevalence in the two groups is identical”.

Results

General findings

In the present study, bacterial profiles of healthy and diseased roots from 41 elderly subjects were investigated. Overall, nine bacterial phyla comprising 164 bacterial species and 15 clusters (species groups detected by probes targeting more than two closely related species) were detected (Table 1, S1). The number of bacterial species per sample ranged from 5 to 45 (Table 2). Some species (such as *Fusobacterium nucleatum* subsp. *polymorphum* oral taxon 202, *Capnocytophaga sputigena* oral taxon 775 and

Lactobacillus casei/paracasei/rhamnosus oral taxon 749/716/568) were dominant while others appeared to be present in low prevalence. A higher bacterial diversity was observed in the Controls as compared to the Patients; while the number of species within the various samples from each patient was reasonably stable (Table 2).

Species distribution within the four sample categories

Several bacterial species were commonly found in all categories, including species of *Streptococcus* (present in 95% of the samples), *Campylobacter* (90%), *Veillonella* and *Selenomonas* (83%), *Capnocytophaga* (65%), *Prevotella* (63%) and *Eubacterium* and *Actinomyces* (59%). The specific species present in the highest number of samples were as follows: *Campylobacter gracilis* oral taxon 623 (84%), *F. nucleatum* subsp. *polymorphum* oral taxon 202 (70%), *Veillonella atypica* oral taxon 524 (53%), *Seleno-*

Table 2 Species diversity in the different sample categories, and a comparison between microarray and sequencing results ^a

| Samples | Comparison | | | | | |
|---------------------|------------|------|-------|------|-------|------|
| | MT | | MS | | SS | |
| | Range | Mean | Range | Mean | Range | Mean |
| Controls | | | | | | |
| Control | 15–45 | 26 | 21–33 | 26 | 16–41 | 28 |
| Patients | | | | | | |
| Plaque healthy root | 8–43 | 22 | 12–33 | 20 | 9–35 | 25 |
| Plaque carious root | 8–35 | 19 | 8–30 | 16 | 8–31 | 24 |
| Dentinal | 5–38 | 22 | 15–38 | 22 | 10–34 | 21 |

^aMT = Microarray total (includes samples from 20 Controls and 21 root caries subjects). MS and SS = microarray and sequencing subset, respectively (includes analysis of the same samples from 10 Controls and 11 RC subjects), with sequencing data from Preza et al. [17].

monas infelix oral taxon 126 and *Parvimonas micra* oral taxon 111 (52%), *Dialister invisus* oral taxon 118 (47%), *Eubacterium saburreum* oral taxon 494 and *Synergistes* sp. oral taxon 363, clone W090 (46%) (Table S1). Twenty-two species were found only in the plaque, dentin, or plaque and dentin of the carious roots; however, their prevalence was low (e.g., present in only one or two Patients) (Table S1).

For bacterial species previously associated with RC, such as *Streptococcus*, *Lactobacillus* and *Actinomyces*, the species with the highest prevalence were *Streptococcus parasanguinis* oral taxon 721/411 (45%), *L. casei/paracasei/rhamnosus* oral taxon 749/716/568 (47%) and *Acti-*

nomyces gerencseriae oral taxon 618 (45%). *S. mutans* oral taxon 686 was found in only 19% (e.g., in three control samples, three plaque samples from healthy and carious roots, and seven dentinal samples). In the Control group, *S. mutans* oral taxon 686 was found together with *L. casei/paracasei/rhamnosus* oral taxon 749/716/568 (one subject) or *Actinomyces* (two subjects). In the Patient group, the combination of *S. mutans* oral taxon 686, lactobacilli and *Actinomyces* was as follows: *S. mutans* oral taxon 686, lactobacilli and *Actinomyces* were found together in one plaque sample from carious roots and two plaque samples from healthy roots and dentinal samples. *S. mutans* oral

Table 3 Species with significant differences in their association with healthy and diseased roots

| Bacteria | Samples | | | | Sample comparison | | | | | |
|---|-------------------|-----------------------------|-----------------------------|--------------------|------------------------------------|------------------------------------|---------------------------|--|-------------------------------------|-------------------------------------|
| | Control (n=20) | Plaque healthy (n=21) | Plaque carious (n=21) | Dentinal (n=21) | Control vs Patient | | | Within Patients | | |
| | | | | | Control vs Plaque healthy | Control vs Plaque carious | Control vs Dentinal | Plaque healthy vs Plaque carious | Plaque healthy vs Dentinal | Plaque carious vs Dentinal |
| <i>Lactobacillus</i> spp. ^a | 5 | 10 | 10 | 15 | ns | ns | .005 | ns | ns | ns |
| <i>Lactobacillus casei/paracasei/rhamnosus</i> ^a | 5 | 10 | 10 | 14 | ns | ns | .012 | ns | ns | ns |
| <i>Pseudoramibacter alactolyticus</i> ^a | 2 | 8 | 5 | 9 | ns | ns | .032 | ns | ns | ns |
| <i>Streptococcus oralis</i> | 9 | 5 | 14 | 11 | ns | ns | ns | .012 | ns | ns |
| <i>Streptococcus parasanguinis</i> | 9 | 4 | 13 | 11 | ns | ns | ns | .022 | ns | ns |
| <i>Dialister pneumosintes</i> | 0 | 5 | 0 | 0 | .048 | ns | ns | ns | ns | ns |
| TM7 sp. clone AH040/BS003 | 0 | 5 | 2 | 0 | .048 | ns | ns | ns | ns | ns |
| <i>H. parainfluenzae/A. aphrophilus</i> | 8 | 2 | 5 | 6 | .032 | ns | ns | ns | ns | ns |
| <i>Actinomyces</i> cluster ^b | 7 | 1 | 5 | 5 | .02 | ns | ns | ns | ns | ns |
| <i>Campylobacter</i> spp. | 20 | 20 | 16 | 19 | ns | .048 | ns | ns | ns | ns |
| <i>Campylobacter showae</i> | 11 | 6 | 4 | 6 | ns | .025 | ns | ns | ns | ns |
| <i>Propionobacterium</i> spp. | 4 | 3 | 1 | 0 | ns | ns | .048 | ns | ns | ns |
| <i>Streptococcus cristatus</i> / <i>S.</i> sp. clone BM053 ^c | 4 | 0 | 0 | 3 | .048 | .048 | ns | ns | ns | ns |
| <i>Cardiobacterium hominis</i> ^e | 9 | 1 | 3 | 4 | .004 | .043 | ns | ns | ns | ns |
| <i>Capnocytophaga</i> cluster ^{ce} | 17 | 11 | 10 | 13 | .043 | .02 | ns | ns | ns | ns |
| <i>Eubacterium saburreum</i> ^e | 15 | 7 | 9 | 7 | .012 | ns | .012 | ns | ns | ns |
| <i>Eubacterium</i> spp. ^e | 17 | 10 | 12 | 10 | .02 | ns | .02 | ns | ns | ns |
| <i>Leptotrichia</i> cluster ^{de} | 17 | 14 | 10 | 9 | ns | .02 | .009 | ns | ns | ns |
| <i>Capnocytophaga sputigena</i> ^c | 12 | 2 | 4 | 5 | .001 | .011 | .028 | ns | ns | ns |
| <i>Campylobacter concisus</i> ^e | 13 | 4 | 4 | 5 | .004 | .004 | .012 | ns | ns | ns |
| <i>Gemella morbillorum</i> ^e | 14 | 7 | 5 | 7 | .029 | .005 | .029 | ns | ns | ns |
| <i>Fusobacterium nucleatum</i> ss <i>polymorphum</i> ^e | 20 | 15 | 12 | 11 | .021 | .001 | 0.00 | ns | ns | ns |
| Total no. of samples | 20 | 21 | 21 | 21 | | | | | | |

^a Species associated with RC subjects.

^b *Actinomyces* cluster= All species.

^c *Capnocytophaga* cluster= *C. gingivalis* oral taxon 337, *C. granulosa* oral taxon 325, *C.* sp. clone AH105, *C.* sp. clone S3, *C.* sp. oral taxon 325, clone BB167, *C.* sp. clone TFI Cap08

^d *Leptotrichia* cluster= *L. buccalis* oral taxon 563, *L.* sp. oral taxon 498/462/463/417, clone IK040/GT018/GT020/ C3MK102

^e Species associated with control subjects.

taxon 686 and lactobacilli (*L. casei/paracasei/rhamnosus* oral taxon 749/716/568) were detected together in only three dentinal samples. *S. mutans* and *Actinomyces* were found together in one plaque sample from carious roots and one dentinal sample. Lactobacilli and *Actinomyces* together were similarly prevalent in the three Patient sample categories (20–25% of the samples). *S. mutans* oral taxon 686 alone was found in only one plaque from carious roots.

Cluster analysis

A cluster analysis was performed in order to group samples with similar bacterial profiles. The cluster analysis was based on both the presence of species and their relative quantity measured as spot intensities (level 1–5). No specific clustering was observed, and no distinct patterns of bacterial species characterized the different sample categories.

Statistical analysis

Although the species detected did not form distinct clusters, when looking at the four sample categories, there were significant differences between these categories (Table 3). The prevalences of species in the four sample categories are shown in Table 3 and Fig. 1. A total of 182 bacterial species, clusters and species groups (genus level) were included. Each sample category was compared pairwise with the three other categories. Significant differences of prevalence in at least one of the comparisons were observed for 22 target species and clusters (Table 3).

Altogether, 546 significance tests were performed. Based on random distribution, 5%, i.e., 27 of the comparisons would be expected to be significantly different at a $p=0.05$

level. The results showed, however, 34 significant results; suggesting that seven out of the 34 significant results (approximately 21%) listed in Table 3 are true positives. Lactobacilli were present in a significantly higher number of dentinal samples. The most dominant species of this group was *L. casei/paracasei/rhamnosus* oral taxon 749/716/568. Besides lactobacilli, *Pseudoramibacter alactolyticus* oral taxon 538 was significantly associated with the dentinal samples. The putative cariogenic pathogens, *S. mutans* oral taxon 686 and *Actinomyces*, showed no significant associations. The *Capnocytophaga* cluster (*C. gingivalis* oral taxon 337, *C. granulosa* oral taxon 325, *C. sp.* clone AH105, *C. sp.* clone S3, *C. sp.* oral taxon 325, clone BB167, *C. sp.* clone TFI Cap08) showed a significant association with the Control samples (Table S1). *C. sputigena* oral taxon 775, *Campylobacter concisus* oral taxon 575, *Gemella morbillorum* oral taxon 046 and *F. nucleatum* subsp. *polymorphum* oral taxon 202 were significantly associated with Controls in all three comparisons. The probability of getting three significant results for one species, if the true prevalence of the two groups was identical, is approximately 0.0001. Thus the association of these species with healthy subjects is reasonably substantiated.

Comparison of microarray analysis with results from sequencing

The microarray results were in general agreement with the results of our previous study on bacterial diversity based on cloning and sequencing of 16S rRNA genes (Table 4) [17]. Of the species determined by clonal analysis, 72% were also detected by HOMIM. More specifically, of the 164 species detected by microarray, 89 were detected by both methods. The microarray and gene sequencing data were

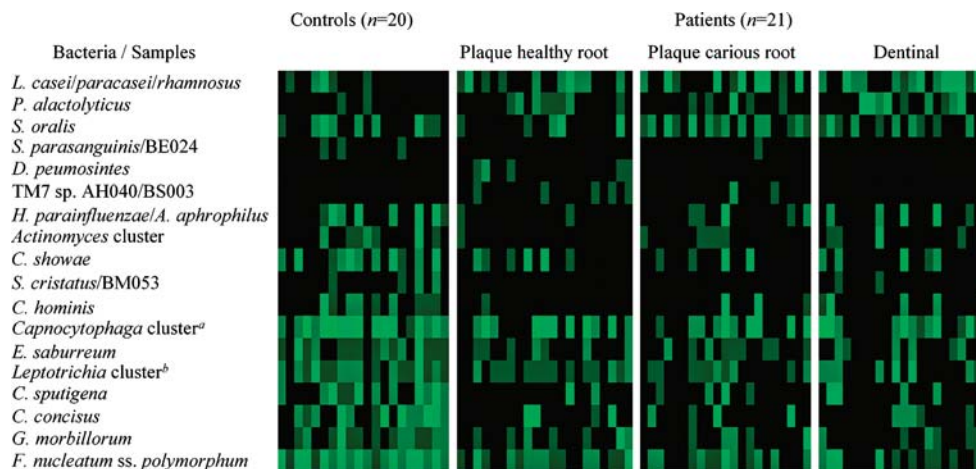


Fig. 1 Distribution of species with significant differences within the sample categories. Each column represents one sample. Five different intensities of green correspond to signal intensities of the arrays.^a *Capnocytophaga* cluster= *C. gingivalis* oral taxon 337, *C. granulosa* oral taxon 325, *C. sp.* clone AH105, *C. sp.* clone S3, *C. sp.* oral taxon 325, clone BB167, *C. sp.* clone TFI Cap08 ^b *Leptotrichia* cluster= *L. buccalis* oral taxon 563, *L. sp.* oral taxon 498/462/463/417, clone IK040/GT018/GT020/ C3MK102

Table 4 Comparison between microarray and previously published 16S rRNA gene sequencing data [17]

| Bacteria | Control sample | | Patient samples | | | | | |
|---|----------------|----|---------------------|----|---------------------|----|--------------|----|
| | | | Plaque healthy root | | Plaque carious root | | Dental | |
| | MS (n=10) | SS | MS (n=11) | SS | MS (n=11) | SS | MS (n=11) | SS |
| <i>Propionibacterium</i> sp. strain FMA5 ^a | | | | | | | | 7 |
| <i>Pseudoramibacter alactolyticus</i> ^a | | | 1 | | 3 | 1 | 5 | 3 |
| <i>Prevotella multisaccharivorax</i> ^a | | | | | | 1 | 1 | 1 |
| <i>Enterococcus faecalis</i> ^a | | | | | | 1 | 1 | 1 |
| <i>Lactobacillus</i> spp. ^a | 2 | | 5 | 3 | 4 | 6 | 7 | 5 |
| <i>Lactobacillus casei/paracasei/rhamnosus</i> ^a | 2 | | 5 | 1 | 4 | 2 | 6 | 5 |
| <i>Actinomyces</i> spp. | 8 | 5 | 10 | 5 | 5 | 4 | 6 | 8 |
| <i>Selenomonas</i> sp. clone CS002 | 3 | 2 | 1 | 1 | 5 | 7 | 4 | 6 |
| <i>Streptococcus mutans</i> | 3 | 1 | 2 | 3 | 1 | 3 | 4 | 5 |
| <i>Actinomyces</i> sp. clone IP073 | 1 | 1 | 2 | 5 | 2 | 3 | 1 | 4 |
| <i>Dialister invisus</i> | 5 | 3 | 5 | 4 | 7 | 5 | 8 | 5 |
| <i>Selenomonas</i> sp. strain GAA14 | 3 | 1 | 3 | 3 | 6 | 5 | 7 | 4 |
| <i>Prevotella denticola</i> | 2 | 1 | 2 | 5 | 5 | 5 | 3 | 3 |
| <i>Prevotella melaninogenica</i> | 1 | 2 | 2 | 5 | 1 | 5 | 1 | 2 |
| <i>Selenomonas sputigena</i> | 5 | 4 | 6 | 10 | 5 | 7 | 3 | 4 |
| <i>Corynebacterium matruchotii</i> | 1 | 8 | | 3 | | 4 | 2 | 4 |
| <i>Campylobacter gracilis</i> | 9 | 6 | 10 | 9 | 9 | 6 | 10 | 3 |
| <i>Prevotella nigrescens</i> | 3 | 5 | 4 | 6 | 2 | 3 | 2 | 2 |
| <i>Selenomonas infelix</i> | 7 | 5 | 6 | 4 | 4 | 3 | 3 | 2 |
| <i>Streptococcus gordonii/anginosus</i> | 5 | 6 | 3 | 3 | 3 | 5 | 6 | 3 |
| <i>Leptotrichia</i> spp. | 9 | 9 | 7 | 6 | 4 | 3 | 4 | 2 |
| <i>Granulicatella adiacens</i> | 1 | 4 | | 2 | | 3 | | 1 |
| <i>Streptococcus sanguinis</i> | 1 | 5 | | 1 | | 2 | 2 | 1 |
| <i>Selenomonas noxia</i> ^b | 6 | 7 | 5 | 3 | 3 | 2 | 6 | 1 |
| <i>Fusobacterium nucleatum ss polymorphum</i> ^b | 10 | 10 | 6 | 3 | 6 | 4 | 6 | 1 |
| <i>Streptococcus anginosus</i> ^b | 1 | 3 | 2 | 3 | 1 | 3 | 1 | |
| <i>Streptococcus mitis</i> bv2 ^b | 2 | 5 | 1 | 3 | 1 | 2 | | |
| <i>Kingella oralis</i> ^b | 7 | 5 | 2 | | 3 | 1 | 3 | |

The numbers refer to the numbers of samples with that particular species present.

SS=Sequencing subset. Previously published sequencing results [17].

MS=Microarray subset. Species detected in the same sequenced samples [17].

^a Species associated with Patients.

^b Species associated with Controls.

directly compared as to species associated with either healthy or carious roots according to the previous study (Table 4). Some of the disease-associated species, e.g., *Propionibacterium* sp. oral taxon 191, strain FMA5 and *Actinomyces* sp. oral taxon 448, clone IP073, were either not, or only rarely, detected by the microarrays. Probes to these species are therefore being reevaluated. *Actinomyces* species were previously described as increased in their prevalence from the Controls to the dental samples, while the present study did not show such a correlation. Both *S. mutans* oral taxon 686 and *L. casei/paracasei/rhamnosus* oral taxon 749/716/568 were associated with the dental samples with both methods. *S. mutans* oral taxon 686 was moderately prevalent, and lactobacilli, especially *L. casei/*

paracasei/rhamnosus oral taxon 749/716/568, showed a clear association with the dental samples in both studies.

Discussion

The present study investigated the bacterial flora of RC using HOMIM, a new rRNA-based microarray technique, which allows the detection of both cultivable and not yet-cultivated species. As we previously demonstrated by sequence analysis of 16S rRNA genes amplified from clinical samples [17], the bacterial diversity of root surfaces of healthy and diseased teeth was extensive. In the present study, 164 species and 15 clusters were detected in the 83 samples examined.

The dominance hierarchy of phyla in the different sample categories was reasonably similar, the more prominent exception being Actinobacteria. This phylum was the second most dominant in dentinal samples, but less common in the other categories. Several species were clearly associated with Control (healthy) subjects, including *F. nucleatum* subsp. *polymorphum* oral taxon 202, *G. morbillorum* oral taxon 046, *C. sputigena* oral taxon 775 and *C. concisus* oral taxon 575 (Table 3 and Fig. 1). The differences between Patients and Controls were more distinct than the differences between samples from healthy or diseased sites within the Patients. Species such as *F. nucleatum* subsp. *polymorphum* oral taxon 202, *P. alactolyticus* oral taxon 538 and *L. casei/paracasei/rhamnosus* oral taxon 749/716/568 that showed significant results in the statistical comparison between the Patient and Control group, have also previously been notably associated with either healthy or carious roots [17].

No obvious patterns were found when cluster analyses were used in an attempt to distinguish the four categories of samples, suggesting that there are no distinct groups of bacterial species that are associated with either RC, in comparison with other samples from Patients, or with Patients as compared to Controls, i.e. no particular bacteria appear to be consistently involved in the pathogenesis of RC. Notably, we did not see an association between *Actinomyces* and RC, which is in contrast to conclusions from earlier studies [6]. Similarly, while certain reports have associated *S. mutans* oral taxon 686 with RC, our previous [17] and present results indicate that it may play only a limited role. Conversely, *Lactobacillus* spp. and *P. alactolyticus* oral taxon 538 were notably associated with most RC samples. Consequently, it is clear that there is subject-to-subject variation concerning the microbial etiology of RC— it is not just *S. mutans* oral taxon 686.

Half of the samples were included in a comparison between the present results and those of previous analyses using cloning and sequencing (Table 4). Combining the two methods, a total of 320 species were detected in the RC profiles. These species were divided between nine of the 12 phylogenetic groups observed in oral samples (Table 1). In most cases the bacteria were detected by both methods, but some species observed in the sequencing study were not detected (Table S2). The species observed when sequencing, yet not detected by the microarrays, were either not represented on the array, or typically present at low concentrations. The discrepancies may be partly ascribed to stochastic effects when it comes to finding bacteria with relatively low copy number. In some cases, species such as *Propionibacterium* species oral taxon 191, strain FMA5, observed in a large number of clones were not found by the arrays. These probes are presently being re-evaluated. Nevertheless, our data indicate that HOMIM represents a rapid and reliable means of identifying

microbial profiles directly from oral clinical samples, such as plaques samples on root surfaces.

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