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Bacterial Profiles of Root Caries in Elderly Patients▽†

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Culture-based studies have shown that *Streptococcus mutans* and lactobacilli are associated with root caries (RC). The purpose of the present study was to assess the bacterial diversity of RC in elderly patients by use of culture-independent molecular techniques and to determine the associations of specific bacterial species or bacterial communities with healthy and carious roots. Plaque was collected from root surfaces of 10 control subjects with no RC and from 11 subjects with RC. The bacterial 16S rRNA genes from extracted DNA were PCR amplified, cloned, and sequenced to determine species identity. From a total of 3,544 clones, 245 predominant species or phylotypes were observed, representing eight bacterial phyla. The majority (54%) of the species detected have not yet been cultivated. Species of *Selenomonas* and *Veillonella* were common in all samples. The healthy microbiota included *Fusobacterium nucleatum* subsp. *polymorphum*, *Leptotrichia* spp., *Selenomonas noxia*, *Streptococcus cristatus*, and *Kingella oralis*. Lactobacilli were absent, *S. mutans* was present in one, and *Actinomyces* spp. were present in 50% of the controls. In contrast, the microbiota of the RC subjects was dominated by *Actinomyces* spp., lactobacilli, *S. mutans*, *Enterococcus faecalis*, *Selenomonas* sp. clone CS002, *Atopobium* and *Olsenella* spp., *Prevotella multisaccharivorax*, *Pseudoramibacter alactolyticus*, and *Propionibacterium* sp. strain FMA5. The bacterial profiles of RC showed considerable subject-to-subject variation, indicating that the microbial communities are more complex than previously presumed. The data suggest that putative etiological agents of RC include not only *S. mutans*, lactobacilli, and *Actinomyces* but also species of *Atopobium*, *Olsenella*, *Pseudoramibacter*, *Propionibacterium*, and *Selenomonas*.

The population of the elderly is increasing worldwide. Due to better dental health care, the elderly are experiencing a higher retention of teeth, which implies an increased number of exposed root surfaces susceptible to caries (24, 32). Several studies indicate that the oral microflora can change with advancing age, possibly due to impaired immune function and subsequent colonization with nonoral bacterial species such as staphylococci and enterobacteria (9, 22, 24). Other microbial consequences of aging, e.g., an increase in yeast colonization, are related to long-term medication, reduced salivary flow rate, and denture wearing (18).

Presently not much is known about the microbial etiology of root caries (RC) in the elderly, and there is no consensus as to which microbes might cause the disease (6, 8). In several animal studies, filamentous bacteria, such as species of *Actinomyces*, were implicated in RC etiology (12, 28). Other culture-based cross-sectional human studies have focused on comparing the bacterial floras of plaque associated with sound and carious root surfaces (2, 3, 7, 15, 30). Mutans streptococci alone or in combination with lactobacilli were detected more frequently in plaque overlying carious surfaces than on healthy root surfaces. Other culture-based studies, using nonselective media and anaerobic sampling, suggest that the microflora associated with RC is much more complex than previously assumed (3, 12, 27). Additional species, such as non-mutans streptococci, species of *Bifidobacterium*, *Rothia*, and *Veillonella*, enterococci, anaerobic gram-negative rods, and the yeast *Candida albicans*, have been detected (27).

More recently, the use of culture-independent methods has played a key role in the discovery of previously unrecognized species in the oral cavity as well as in redefining the pathogenesis of the major oral infections (1, 14, 19–21, 23, 31). The outcome of these studies indicates that the major oral infections are polymicrobial (11).

Culture-independent studies agree on the bacterial complexity of advanced coronal caries (4, 20). It has been suggested that the deep coronal lesions comprise similar bacterial species as in the deep layers in RC (10). However, there have not been any culture-independent studies so far to describe the bacterial community of RC in the elderly.

In the present study, healthy and carious roots of individuals aged 82 years and older were investigated. The aim of the study was to assess the bacterial diversity of RC in the elderly by use of culture-independent molecular techniques and to determine the associations of specific bacterial species or bacterial communities with healthy and carious roots.

MATERIALS AND METHODS

Subject population. Twenty-one elderly patients from a nursing home in Oslo, Norway (2 males and 19 females) were included in the study. The mean age was 89 years, ranging from 82 to 98 (Table 1). All subjects were examined clinically 1 to 5 days before sampling and were subsequently divided into two groups: a control group ($n = 10$) and an RC group ($n = 11$). The control subjects were RC free and the RC subjects had one or more lesions of RC at the time of the clinical examination. RC definition and diagnosis were based on the criteria of the World Health Organization (33). A lesion on an exposed root surface was classified as carious when it felt soft or leathery on probing. All subjects were instructed not

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TABLE 1. Characteristics of subject groups

Subject group	No. of subjects	Mean (± SD) (range): Age	Mean (± SD) (range): No. of remaining teeth	% Females	No. smoking
Control	10	85.6 ± 3.2 (82–92)	22.9 ± 3.4 (17–28)	90	1
RC	11	91.8 ± 4.2 (86–98)	14.8 ± 6.7 (5–25)	91	2

to clean their teeth the evening and the morning before sampling. Smoking, number of remaining teeth, and tooth-brushing habits were recorded (Table 1). Subjects did not have any clinical signs of mucosal diseases and were not on antibiotic therapy up to 10 days prior to sampling.

Samples. In the control group, supragingival plaque from one healthy root was collected (referred to as control sample), while from each RC subject the following three samples were collected: supragingival plaque from one healthy root in the RC subjects (hrRC sample), plaque from one carious root (carious sample), and the underlying dentin from the same carious root (dentinal sample) (Fig. 1). The sampled healthy root sites were chosen randomly. All samples were taken by one examiner (D. P.). Plaque samples were taken by use of sterile Gracey curettes. The carious root surfaces were cleaned with distilled water and a sterile rubber cup (Turbo Prophy cup; Young Dental, Earth City, MO) after the supragingival plaque (carious sample) had been collected. The outer layer of infected dentin was removed with either a spoon excavator or by a round burr in a low-speed hand piece, and the inner part of the RC lesion was sampled. All samples were immediately suspended in 300 μl of TE buffer (50 mM Tris, 1 mM EDTA, pH 7.6), transported on ice to the laboratory, and stored at −80°C.

DNA extraction. 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.

Amplification of 16S rRNA genes. The 16S rRNA genes were amplified under standard conditions by use of a universal forward primer (5′-GAG AGT TTG ATY MTG GCT CAG-3′) and a universal reverse primer (5′-GAA GGA GGT GWT CCA RCC GCA-3′) (23). PCR was performed in thin-walled tubes with GeneAmp PCR systems 2700 and 9700 (ABI, Foster City, CA). Two microliters of DNA template was added to a reaction mixture (final volume, 50 μl) containing 20 pmol of each primer, 40 nmol of deoxynucleoside triphosphates, 1.5 mmol of Mg^{2+}, and 1 U of Platinum *Taq* polymerase (Invitrogen, San Diego, CA). The samples were preheated at 95°C for 4 min, followed by 30 cycles of amplification under the following conditions: denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and elongation at 72°C for 1.5 min, with an additional 15 s for each cycle. A total of 30 cycles were performed, followed by a final elongation step at 72°C for 15 min. The results of the PCR amplification were examined by electrophoresis in a 1% agarose gel.

Cloning and sequencing. Cloning of PCR products was performed using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Briefly, transformation was done with competent *Escherichia coli* TOP10 cells. The transformed cells were plated onto Luria-Bertani agar plates supplemented with kanamycin (50 μg/ml) and incubated overnight at 37°C. Colonies were transferred to 70 μl of 10 mM Tris-HCl. Amplification of inserts was performed with (M13) primers (forward, 5′-GTAAAACGACGGCCAG-3′; reverse, 5′-CAGGAAACAGCTATGAC-3). PCR products of the correct size (containing 16S rRNA genes) were purified with the QIAquick PCR purification kit (Qiagen) and sequenced with an ABI Prism cycle sequencing kit (BigDye Terminator cycle sequencing kit with AmpliTaq DNA polymerase FS and GeneAmp PCR systems 2700 and 9700; ABI). The primers used for sequencing have been described previously (23). Quarter-dye chemistry was used with 80 μM primers and 1.5 μl of PCR product in a final volume of 20 μl. Cycle sequencing was performed with a GeneAmp PCR system 9700 (ABI), with 25 cycles of denaturation at 96°C for 10 s, annealing at 55°C for 5 s, and extension at 60°C for 4 min. The sequencing reactions were run on an ABI 3730 DNA sequencer (ABI). The single-read sequences of 29 clones were obtained by Qiagen Genomic Services by use of the same M13 primers.

Sequence analysis. A total of 3,544 clones each with an insert of approximately 1,500 bases were analyzed. The number of sequenced clones per sample ranged from 60 to 94. A sequence of approximately 500 bases was first obtained to determine identity or approximate phylogenetic position. For the identification of closest relatives, the sequences of the inserts were compared to the 16S rRNA gene sequences of over 10,000 microorganisms in our database and over 400,000 sequences in the Ribosomal Database Project (5), EMBL (http://www.ebi.ac.uk/embl/), GenBank (http://www.ncbi.nlm.nih.gov/GenBank/), and DDBJ (http://www.ddbj.nig.ac.jp/) nucleotide sequence databases. Corrections for similarity matrices (13) and chimeric sequences (Chimera Check program in The Ribosomal Database Project [RDP-II] [http://rdp.cme.msu.edu/]) and construction (25) and drawing (29) of phylogenetic trees were done according to the method described by Paster et al. (23).

Nucleotide sequence accession numbers. The complete 16S rRNA gene sequences of clones representing novel phylotypes defined in this study (OCG019 [EU669563], OCG080 [EU669564], OCH033 [EU669565], OCN091 [EU669566], OCT046 [EU669567], and OCV103 [EU669568]), sequences of species not previously reported, and published sequences are available for electronic retrieval from the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers shown in Fig. 2 and in Tables S6 and S7 in the supplemental material.

RESULTS

General results. In the present study, bacterial profiles of healthy and diseased roots from 21 elderly subjects were investigated. A remarkably high diversity, 245 bacterial species

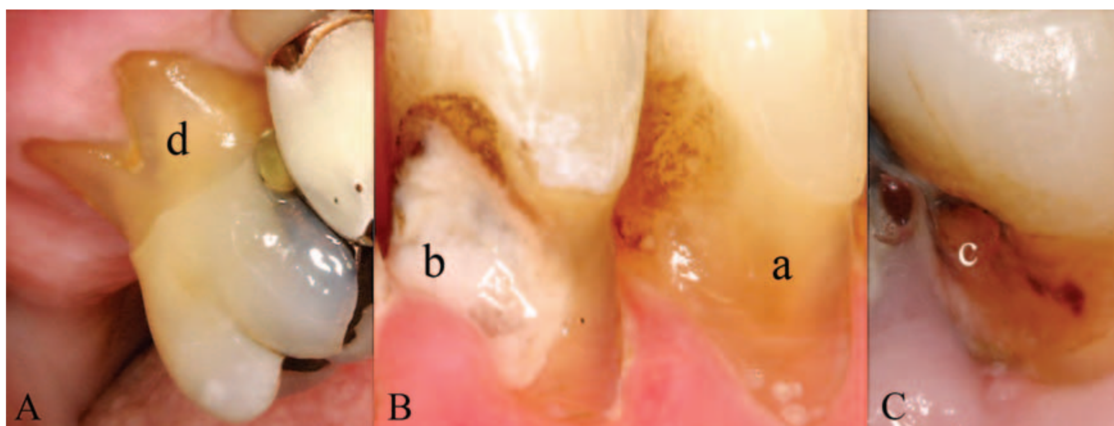

FIG. 1. (A) Control subject. d, plaque, healthy root. (B and C) RC subject. (B) a, plaque, healthy root; b, plaque, carious root. (C) c, dentin, same carious root.

FIG. 2. Phylogenetic tree and distribution between samples of the dominant species. The marker bar represents a 10% difference in nucleotide sequences. The samples are divided in the columns with those from the control subjects first, followed by the three different samples from subjects with RC. The prevalence of the different species is color coded. ss., subsp.

representing eight bacterial phyla, was observed for the 3,544 clones analyzed (Table 2; also see Table 4 below). The overall bacterial profile had 112 (46%) cultivable species within known genera and 133 (54%) sequences from not-yet-cultivated phylotypes or species that are currently unrecognized (Table 4 below; also see Table S6 in the supplemental material). The number of species detected in control samples ranged from 16 to 41 per sample and from 8 to 35 in those from the RC patients (Table 3).

Firmicutes was the predominant phylogenetic group fol-

TABLE 2. Number of clones per sample group

Sample group	Total no. of clones	Mean	Range
Control	834	83	70–94
RC subject			
hrRC	910	83	60–94
Carious	942	86	68–94
Dentinal	858	78	68–90
Total	3,544	82	60–94

TABLE 3. Number of different species per sample

Subject group	Sample	No. of species per sample	
		Range	Mean
Control	Control	16–41	28
RC	hrRC	9–35	25
	Carious	8–31	24
	Dentinal	10–34	21

lowed, in all sample groups, except for the dentinal samples, by *Bacteroidetes*. In dentin, *Actinomyces* was the second most dominant genus (Tables 4 and 5). Species of *Veillonella* (20%), S*elenomonas* (20%), and *Streptococcus* (12%) were the most dominant bacterial species in terms of the total number of clones. It was noteworthy that recognized caries-associated taxa, such as species of *Actinomyces* (4.2%) and lactobacilli (2%), constituted only a minor part of the number of bacterial species detected (see Table S6 in the supplemental material). *Selenomonas sputigena*, *Veillonella parvula*/*Veillonella dispar*, and *Veillonella* sp. clone AA050 were the most commonly detected. The most prevalent species among the streptococci were *Streptococcus mutans*, *S. gordonii*, and *Streptococcus intermedius* (see Table S6 in the supplemental material). *Streptococcus* spp. were detected at similarly high levels in all categories, while *Veillonella* and *Prevotella* spp. were less prevalent in the dentinal samples. The levels of *Selenomonas* spp. in both plaque samples of the RC subjects were twice as high as in the control and dentinal samples.

Microflora of control subjects. On the sound root surfaces of the control subjects, most bacteria were present in low or moderate numbers (Fig. 2). Only *Veillonella* spp., *Selenomonas noxia*, and *S. gordonii* were detected at high clone levels in a few healthy subjects (between 16 and 40% of the clones). Health-associated bacterial species, such as *Kingella oralis*, *Fusobacterium nucleatum* subsp. *polymorphum*, *Leptotrichia* spp., *Streptococcus cristatus*, *Campylobacter curvus*, *Corynebacterium matruchotii*, and *Selenomonas noxia*, were rare in the majority of samples from RC subjects (Fig. 2; Table 5). *K. oralis*, *Prevotella conceptionensis*, *Streptococcus mitis* bv. 2, and *Streptococcus anginosus* were absent in the dentinal samples. Lactobacilli were not detected, *S. mutans* was present in only 1, and *Actinomyces* was present in 5 of the 10 healthy subjects, with clone levels of less than 5% (except for healthy subject no. 3) (Fig. 2). Plaque from the control subjects consisted of a diverse bacterial flora with the predominance of a few species and an average number of 28 species per sample (Table 3).

Microflora of hrRC samples. The microflora of plaque overlying the healthy root surfaces in the RC subjects (hrRC samples) had lower diversity than the control samples, i.e., 9 to 35 versus 16 to 41 species detected per sample (Table 3). Compared to the predominant species in control subjects, the occurrence of *Fusobacterium nucleatum* subsp. *polymorphum*, *S. cristatus*, *S. gordonii*, *C. curvus*, *C. matruchotii*, and *S. noxia* was reduced, and other bacterial species like *Campylobacter gracilis*, *Selenomonas* sp. clone FT050, *S. sputigena*, *P. melaninogenica*, and *S. mutans* (Fig. 2; Table 5) were predominant. The most prevalent species described above differed from subject to subject in this category (Fig. 2). Lactobacilli were detected for three subjects. *S. mutans* levels increased, while the presence of *Actinomyces* was at the same level in healthy and control samples (five subjects). The levels of *Actinomyces* presence in the control and hrRC samples were similar.

Microflora of carious samples. The microflora of plaque covering RC lesions (carious samples) had even lower diversity than the microflora of the sound root surfaces in this subject group (Table 3). Seven of the 11 subjects (subjects 1, 4, and 7 to 11) had species with clone levels of 16 to over 40% (Fig. 2). The levels of *V. parvula*/*V. dispar* and *Selenomonas* sp. clone CS002 were more than 40% in these two subjects. In only two subjects (no. 1 and 8), the same microorganisms (*V. parvula*/*V. dispar* and *Veillonella* sp. clone AA050) exhibited the same levels in plaque samples from both healthy and diseased root surfaces of the same subject. *Selenomonas* sp. clone CS002 (no. 7 and 10), *C. matruchotii* (no. 9), and *F. nucleatum* subsp. *polymorphum* (no. 11) were other dominant bacterial taxa in this category (Fig. 2). *Selenomonas* sp. clone CS002 and *C. matruchotii* were rare in the hrRC and healthy subjects. *S. mutans* was detected in three carious samples. The phylotype *Selenomonas* sp. clone CS002 showed a strikingly high prevalence and the highest level among the bacterial species detected in the plaque of the diseased root surfaces.

Only one subject had *S. mutans* both in the hrRC and in the carious sample. *Actinomyces* was found in 4 and lactobacilli in 6 of the 11 carious samples (Fig. 2; Table 5). The lactobacilli showed unchanged levels but prevalence higher (6 of 11 subjects) than in the hrRC samples.

Microflora of dentinal samples. The bacterial profiles of the carious dentin (dentinal samples) differed in diversity as well as in bacterial dominance from the other categories. The number of species per sample ranged from 10 to 34 (Table 3). Nine of the 11 dentinal samples (no. 1 and 4 to 11) had at least one species with clone levels of 16 to 40% or higher (Fig. 2). In three subjects (no. 4, 7, and 8), *Enterococcus faecalis*, *S. mutans*, and *Pseudoramibacter alactolyticus* were found with levels higher than 40%. *V. parvula*/*V. dispar* (no. 1), *Lactobacillus casei*/*Lactobacillus paracasei*/*Lactobacillus rhamnosus* (no. 4), *Propionibacterium* sp. strain FMA5 (no. 5), *Selenomonas* sp. clone CS002 (no. 5 and 7), *P. alactolyticus* (no. 6), *Actinomyces* sp. clone IP073 (no. 9), *Atopobium* and *Olsenella* spp. (no. 10), *C. matruchotii*, and *Leptotrichia* spp. (no. 11) had clone levels of 16 to 40% (Fig. 2). *V. parvula*/*V. dispar*, *Selenomonas* sp. clone CS002, and *C. matruchotii* were also prevalent in the plaque overlying the carious lesion.

For the dentinal samples, more taxa with high levels were detected than for the other sample categories. Most dentinal samples had one or few predominant species in their bacterial profiles, but the species differed from subject to subject. Inter-

TABLE 4. Bacterial phyla identified in sample groups

Phylogenetic group	Total[a]		Control		RC subject sample					
					hrRC		Carious		Dentinal	
	Taxa (n = 245)	% Total clones	Taxa (n = 123)	% Total clones	Taxa (n = 129)	% Total clones	Taxa (n = 137)	% Total clones	Taxa (n = 117)	% Total clones
Firmicutes	102	64.1	49	58.3	67	65.3	73	68.8	55	63.2
Bacteroidetes	50	9.8	24	9.7	24	10.6	27	12.7	17	5.7
Actinobacteria	44	11.4	16	10.7	13	5.0	16	7.7	30	22.8
Fusobacteria	21	8.7	15	13.4	11	9.1	7	6.7	6	6.1
Proteobacteria	19	5.2	13	7.3	10	8.8	10	3.5	3	1.2
Spirochaetales	4	0.5	2	0.2	2	0.8	2	0.3	3	0.7
TM7	3	0.1	0	0	2	0.3	1	0.1	1	0.1
Synergistes	2	0.1	1	1	0	0	1	0.1	2	0.2

[a] The total number of clones was 3,544.

estingly, *L. casei*/*L. paracasei*/*L. rhamnosus* and *Atopobium* and *Olsenella* spp. were absent from the control subjects but increased their presence in hrRC and carious samples (Table 5). The uncharacterized *Propionibacterium* sp. strain FMA5 was the only dominant species found exclusively in the carious dentin.

S. mutans was present in 5, lactobacilli in 7, and *Actinomyces* spp. in 9 of the 11 dentinal samples (Table 5). *S. mutans* (no. 7), lactobacilli (*L. casei*/*L. paracasei*/*L. rhamnosus* [no. 4]), and *Actinomyces* spp. (*Actinomyces* sp. clone IP073 [no. 9]) were each present in only one dentinal sample (Fig. 2). One dentinal sample (no. 6) had *S. mutans*, lactobacilli, and *Actinomyces* spp. together. Six dentinal samples showed no signs of *S. mutans* (no. 1, 2, 4, 8, 10, and 11).

In subject no. 11, no lactobacilli and only moderate levels of *Actinomyces* spp. were detected. Subject no. 2 did not have dominant species or any lactobacilli. The bacterial profile of this dentinal sample showed a combination of moderate levels (5 to 16% of clones) of *S. intermedius*, *Anaeroglobus* and *Megasphaera* spp., *Fusobacterium nucleatum* subsp. *animalis*, and many other bacterial species at low levels, including *Actinomyces* spp. (1 to 5% of the clones) (Fig. 2).

Three dentinal samples (2, 8, and 11) had low or moderate *Actinomyces* levels and no *S. mutans* or lactobacilli. Two dentinal samples showed *Actinomyces* (no. 6 and 7); one of these samples had high levels of *S. mutans* (no. 7), while the other sample had only low levels of *S. mutans* and lactobacilli. *Actinomyces* spp. were detected in 9 of 11 dentinal samples. The phylotype *Actinomyces* sp. clone IP073 was found in four dentinal samples at high levels (Table 5).

DISCUSSION

The present study offers the first description of the microflora associated with root surfaces in elderly subjects based on culture-independent methods. Among the 21 subjects examined, different bacterial profiles were observed. The overall bacterial diversity of RC was considerable; 245 species or phylotypes were identified, of which 54% have not yet been cultivated. This breadth of diversity was also observed in another study of advanced carious dentinal lesions (20).

Overall, the bacterial profiles exhibited reduced diversity when moving from healthy to diseased subjects. A similar observation was made for childhood caries (1, 16) and for saliva samples from caries-free and caries-active individuals (17). In the control subjects, the bacterial profiles showed high bacterial diversity and no dominance of particular bacterial species. In the RC subjects, the plaque of the sound root surfaces exhibited a lower bacterial diversity, and the diversity decreased further when moving to plaque from affected teeth. The lowest diversity was observed for the dentinal samples, which might be expected, as this is a more remote and specialized environment.

Another interesting characteristic of the profiles was the considerable subject-to-subject variation. The bacterial profiles differed as to the presence of dominant bacterial species and in the shift of dominance from one category to the other. Certain bacterial species appeared to be strongly associated with health, as they were rarely detected for (e.g., *Leptotrichia* spp. and *S. noxia*) or were absent from (*K. oralis*, *P. conceptionensis*, *S. mitis* bv. 2 and *S. anginosus*) the RC subjects but were commonly found in the control subjects. *F. nucleatum* subsp. *polymorphum* was found in all 10 control subjects, but it was also present at high concentrations in certain plaque samples from carious roots. In the hrRC samples, several species, such as *Veillonella* sp. clone AA050, *V. parvula*/*V. dispar*, *S. noxia*, *C. gracilis*, *S. mutans*, *S. sputigena*, *S. infelix*, and *F. nucleatum* subsp. *polymorphum*, were found at high levels. The high prevalence and levels of phylotype *Veillonella* sp. clone AA050 and *Actinomyces* sp. clone IP073 were notable. The phylotype *Selenomonas* sp. clone CS002 showed a strikingly high prevalence and the highest concentration in the carious samples. Lactobacilli (*L. casei*/*L. paracasei*/*L. rhamnosus*), *E. faecalis*, *P. alactolyticus*, and *Propionibacterium* sp. strain FMA5 appeared to be associated with disease, as they were common in dentinal samples while rare or absent in other categories. Hoshino (10) described the dominance of *Propionibacterium* spp. in deep dentin layers, but the study was culture based, which may explain the absence of the other bacterial species mentioned above.

The most predominant bacterial species in terms of health or disease association were as follows: *F. nucleatum* subsp. *polymorphum* (healthy root in control subject), *S. sputigena* (healthy root in RC subject), *Selenomonas* sp. clone CS002 (plaque on carious root), and *Propionibacterium* sp. strain FMA5 (dentin from carious root).

For the control subjects *S. mutans* was rare and lactobacilli

TABLE 5. Distribution of major species in the sample groups

Species	Control subjects ($n = 10$)		RC subjects ($n = 11$)					
			hrRC samples[e]		Carious samples		Dentinal samples	
	No. of clones	No. of subjects with species	No. of clones	No. of subjects with species	No. of clones	No. of subjects with species	No. of clones	No. of subjects with species
Propionibacterium sp. strain FMA5[a]							43	7
Pseudoramibacter alactolyticus[a]					1	1	76	3
Prevotella multisaccharivorax AB200414[a]					5	1	6	1
Enterococcus faecalis[a]					5	1	49	1
Lactobacillus spp.[a]			4	3	14	6	57	5
Atopobium spp. *Olsenella* spp.[a]			7	3	16	7	32	7
Lactobacillus casei/*Lactobacillus paracasei*/*Lactobacillus rhamnosus*[a]			2	1	7	2	31	5
Actinomyces spp.[a]	25	5	30	5	22	4	70	8
Selenomonas sp. clone CS002[a]	4	2	2	1	92	7	48	6
Streptococcus mutans[a]	3	1	25	3	17	3	52	5
Actinomyces sp. clone IP073[a]	7	1	12	5	10	3	36	4
Dialister invisus[a]	7	3	7	4	24	5	16	5
Selenomonas sp. strain GAA14[a]	1	1	8	3	10	5	11	4
Prevotella denticola[b]	3	1	9	5	33	5	8	3
Prevotella melaninogenica[b]	2	2	14	5	20	5	4	2
Selenomonas sp. clone FT050[b]	3	2	19	6	2	2	2	2
Anaeroglobus spp./*Megasphaera* spp.[b]	9	3	17	3	26	6	13	5
Selenomonas sputigena[b]	5	4	61	10	12	7	8	4
Veillonella parvula/*Veillonella dispar*[b]	58	9	103	9	126	9	42	8
Veillonella sp. clone AA050[b]	82	9	74	7	78	7	23	6
Corynebacterium matruchotii[c]	41	8	7	3	25	4	20	4
Campylobacter gracilis[c]	13	6	63	9	22	6	7	3
Selenomonas sp. clone IQ048[c]	9	4	16	4	8	5	4	2
Prevotella nigrescens[c]	13	5	16	6	6	3	4	2
Selenomonas infelix[c]	12	5	24	4	13	3	2	2
Selenomonas sp. clone AA024/DS051[c]	14	5	3	2	1	1	6	2
Streptococcus gordonii[c]	36	6	14	3	9	5	5	3
Campylobacter curvus[c]	10	7	4	2	3	2	2	2
Leptotrichia spp.[c]	33	9	24	6	7	3	16	2
Granulicatella adiacens[d]	26	4	5	2	16	3	1	1
Streptococcus sanguinis[d]	6	5	1	1	2	2	1	1
Streptococcus cristatus[d]	22	6	6	2	2	1	1	1
Selenomonas noxia[d]	45	7	25	3	15	2	5	1
Fusobacterium nucleatum subsp. *polymorphum*[d]	50	10	22	3	34	4	1	1
Streptococcus anginosus[d]	7	3	9	3	8	3		
Streptococcus mitis bv. 2[d]	11	5	4	3	11	2		
Prevotella conceptionensis[d]	19	5	8	3	7	1		
Kingella oralis[d]	11	5			1	1		

[a] Species associated with RC subjects.
[b] Species associated with both control and RC subjects.
[c] Species associated more with control than with RC subjects.
[d] Species associated with control subjects.

were absent, while for the hrRC and carious samples the prevalence and levels of *S. mutans* and lactobacilli increased. *Actinomyces* prevalence and levels in the hrRC and carious samples were similar to what was seen for the control subjects. The most prevalent *Actinomyces* species, phylotype *Actinomyces* sp. clone IP073, was an exception, as it was detected in the plaque of only one control subject but for four RC subjects. The numbers of *S. mutans* and *Actinomyces* decreased slightly, while lactobacilli increased both in prevalence and levels from hrRC to carious samples. *S. mutans* was present in only 50% of the dentinal samples, which causes the role of *S. mutans* in the development of RC to be questioned (7) and contradicts culture studies implicating *S. mutans* as the major putative agent causing RC (26). The prevalences of *S. mutans* alone or in combination with lactobacilli were similar in hrRC and carious samples (i.e., present in half the samples), which is in line with previous observations (17, 26). Lactobacilli were absent in the healthy subjects but highly represented in carious dentin, supporting the suggestion that lactobacilli might not play a significant role in the initiation of RC but could be important in its progression or cause (12). Similarly, the observation that *Actinomyces* was found in half of the control, hrRC, and carious samples at similar low levels and in nine dentinal samples at high levels might indicate that they are not involved in the initiation but in the progression of the RC process. Overall, the present results suggest that not all the three classical bacterial species associated with RC need to be present for the disease to develop.

The microbial flora associated with RC was far more complex than previously assumed. Bacterial species typically associated with RC were detected, such as *S. mutans*, lactobacilli, and *Actinomyces*; however, additional species, such as *Atopobium* spp., *Olsenella* spp., *Pseudoramibacter alactolyticus*, and *Propionibacterium* sp. strain FMA5, were also commonly found. The data suggest that these last species may also be involved in the development of RC.

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