JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2005, p. 843–849 Vol. 43, No. 2
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.2.843–849.2005

Molecular Analysis of Microbial Diversity in Advanced Caries

Kim-Ly Chhour,[1] Mangala A. Nadkarni,[1] Roy Byun,[1] F. Elizabeth Martin,[1,2] Nicholas A. Jacques,[1] and Neil Hunter[1]*

Institute of Dental Research, Westmead Millennium Institute and Westmead Centre for Oral Health, Westmead,[1] and Faculty of Dentistry, The University of Sydney, Sydney,[2] New South Wales, Australia

Received 13 May 2004/Returned for modification 15 July 2004/Accepted 24 September 2004

Real-time PCR analysis of the total bacterial load in advanced carious lesions has shown that the total load exceeds the number of cultivable bacteria. This suggests that an unresolved complexity exists in bacteria associated with advanced caries. In this report, the profile of the microflora of carious dentine was explored by using DNA extracted from 10 lesions selected on the basis of comparable total microbial load and on the relative abundance of *Prevotella* spp. Using universal primers for the 16S rRNA gene, PCR amplicons were cloned, and approximately 100 transformants were processed for each lesion. Phylogenetic analysis of 942 edited sequences demonstrated the presence of 75 species or phylotypes in the 10 carious lesions. Up to 31 taxa were represented in each sample. A diverse array of lactobacilli were found to comprise 50% of the species, with prevotellae also abundant, comprising 15% of the species. Other taxa present in a number of lesions or occurring with high abundance included *Selenomonas* spp., *Dialister* spp., *Fusobacterium nucleatum*, *Eubacterium* spp., members of the *Lachnospiraceae* family, *Olsenella* spp., *Bifidobacterium* spp., *Propionibacterium* sp., and *Pseudoramibacter alactolyticus*. The mechanisms by which such diverse patterns of bacteria extend carious lesions, including the aspect of infection of the vital dental pulp, remain unclear.

Dental caries is a progressive disease that expands from the initial focus of enamel degradation and the subsequent exposure of the underlying dentine to a point where microorganisms gain access to the tubular network of dentine that extends to the dental pulp (18). Depending on the response patterns of the odontoblasts that line the pulp chamber, bacterial invasion is sometimes arrested by a layer of so-called secondary or sclerotic dentine. Extension of the lesion can lead to necrosis of the pulp tissue by toxic action, immunopathological response, or frank invasion by bacteria. If left unchecked, the lesion can extend to involve the supporting bone and track to soft tissue, where cellulitis is a potential outcome (17).

Dissolution of the enamel matrix by organic acids, particularly lactic acid produced by mutans streptococci, is considered to be the primary event in caries development (8, 33). Recent analysis has, however, indicated the complexity of the bacterial biofilm associated with dental caries initiation in children (3). Diverse taxa, including a variety of streptococci, bifidobacteria, and organisms belonging to the genera *Actinomyces*, *Selenomonas*, *Leptotrichia*, *Campylobacter*, and *Capnocytophaga* were detected (3). In this context, there is a relative paucity of information concerning the groups of bacteria associated with the ensuing phase of lesion development, namely, invasion and destruction of the dentine matrix. Our previous analysis of the lesion of advanced caries by culture and real-time PCR (19) indicated the presence of many undetected or uncharacterized bacteria. It is evident that incomplete knowledge of the microbial populations that contribute to extension of the carious lesion enforces an empirical approach to therapy rather than specific antimicrobial therapy that might allow more conservative treatment options.

Since many organisms have yet to be isolated and cultured (26), PCR-based techniques are now widely used to identify organisms from an environment. These methods typically use broad-range or universal primers to amplify DNA that has been extracted directly from the environment. This is done based on the premise that DNA is efficiently extracted from most organisms present in the environment and that each species of DNA is amplified proportionately by PCR and cloned without bias (16). The sequence of choice for environmental surveys is the 16S rRNA gene due to its recognized species-specific conservation (35). In the present study, we used population analysis based on 16S rRNA gene(s) to gain insight into the diversity and abundance of the major groups of bacteria within the matrix of 10 advanced caries lesions selected on the basis of comparable total microbial load and high or low abundance of *Prevotella* spp. (19).

MATERIALS AND METHODS

Carious dentine samples and extraction of bacterial DNA. The source of material for analysis was carious dentine obtained in a manner approved by the Human Ethics Committee of Central Sydney Area Health Service (reference no. 6/96) (19, 23). Adult patients elected to have extractions for unrestored anterior, premolar, and molar teeth that presented with large coronal dentine caries lesions that, by macroscopic examination, had not penetrated to the underlying vital pulp tissue. Selected teeth had adjacent periodontal probing depths of <4 mm. After the removal of superficial plaque and debris overlying the lesion, the carious zone of decalcified and partially decalcified dentine was excavated, weighed, and resuspended in reduced transport fluid (31) to a concentration of 10 mg (wet weight) of dentine per ml prior to processing and extraction of bacterial DNA by using the ATL buffer reagent (Qiagen, Clifton Hill, Victoria, Australia) as previously described (19, 23). This method of DNA extraction releases DNA from gram-negative bacteria, as well as from many gram-positive bacteria, including lactobacilli when cultured under anaerobic conditions (4, 19).

Ten dentinal caries samples were chosen for comprehensive bacterial analyses from a pool of 65 (19) on the basis of comparable total microbial load (range,

* Corresponding author. Mailing address: Mailing address: Institute of Dental Research, Westmead Centre for Oral Health, PO Box 533, Wentworthville, NSW 2145, Australia. Phone: 612-9845-8762. Fax: 612-9845-7599. E-mail: nhunter@dental.wsahs.nsw.gov.au.

1.4×10^8 to 1.1×10^9 bacteria per mg [wet weight] of dentine) and variable characteristics with respect to high or low abundance of *Prevotella* spp. as previously determined by real-time PCR enumeration (19). When we chose the 10 samples, patient age and sex were cross matched within the limitations of the sample pool, and a mixed presentation of pulpal histopathology (19) was chosen to maximize the extent of the species and/or phylotypes detected (Table 1).

DNA amplification and cloning. Using the universal primers previously described by Nadkarni et al. (23), each of the 10 samples were subjected to PCR amplification with a FTS-320 thermal cycler (Corbett Research, Sydney, Australia). PCR was performed in triplicate in a total volume of 100 μl by using the Ampli*Taq* Gold PCR core reagent kit (Applied Biosystems) containing 300 nM concentrations of each of the universal forward and reverse primers. An initial denaturation step of 95°C for 10 min was followed by 25 cycles of 95°C for 15 s and 60°C for 1 min, with a final resting temperature of 4°C. The triplicate PCR samples were combined and purified by using the Wizard PCR Preps DNA purification system (Promega, Sydney, New South Wales, Australia) before being cloned into linearized plasmids by using the pGEM-T Easy vector system (Promega) according to the manufacturer's instructions. Ligated plasmids were transformed into competent *Escherichia coli* XL1-Blue cells as described previously (4). Between 105 and 110 white colonies were randomly selected from each of the 10 samples and inoculated into 5 ml of Luria-Bertani broth supplemented with 100 μg of ampicillin ml^{-1} and incubated at 37°C with shaking for 18 h.

Plasmid isolation. A 2-ml aliquot of each of the overnight cultures was used to extract plasmid DNA by using the Wizard Plus MiniPreps DNA purification system (Promega) according to the manufacturer's instructions. The purified plasmids were separated through a 1% agarose gel in 40 mM Tris-acetate buffer (pH 8.0) containing 1 mM EDTA (TAE buffer), stained with 500 ng of ethidium bromide ml^{-1}, and visualized under short-wavelength UV light. Chimeric plasmids containing cloned 16S rRNA gene inserts were selected for sequencing.

Sequencing and phylogenetic analyses. Purified plasmids were sequenced at Westmead DNA Sequencing Facility, Westmead Hospital, Wentworthville, New South Wales, Australia, by using the universal forward primer (23). Approximately 410 to 430 bp of each 16S rRNA gene sequence were obtained by this approach.

Chimeric 16S rRNA sequences were identified by the program Chimera_Check, accessed through the Ribosomal Database Project (5), and discarded. All other sequences were compared to 16S rRNA gene in the Ribosomal Database Project (5) and in GenBank. Sequences sharing >99% nucleotide identity to a known species or to an uncultured bacterium were grouped, and a representative sequence was selected for further analysis. The representative sequences were aligned by using the program CLUSTAL W (32) and a distance matrix was calculated by using Dnadist with the Jukes-Cantor model (13). A phylogenetic tree was constructed by the neighbor-joining method of Saitou and Nei (28) using the program Neighbor. Phylogenetic data were subjected to bootstrap analysis of 1,000 replicates by using Seqboot and Consense accessed through the Australian National Genomic Information Service (http://www.angis.org.au).

RESULTS

Analysis of 16S rRNA amplicons. Ninety-eight percent of the 942 amplicons of the 16S rRNA from 10 different carious lesions displayed a high level of sequence identity (>99%) to either the 16S rRNA sequences of known species or to those of uncultured bacteria lodged in GenBank. The exceptions were three sequences most closely related to *Lactobacillus pontis*, nine to *Clostridium leptum*, three to the *Lachnospiraceae* oral clone MCE9-31, one to *Prevotella ruminicola*, and one to *Denitrobacterium detoxificans*. The region of the 16S rRNA genes that were sequenced did not allow differentiation of the closely related oral streptococcal species, *Streptococcus sanguinis*, *Streptococcus oralis*, and *Streptococcus mitis*, which collectively represented 10 amplicons. Similarly, the lack of sequence divergence in the 16S rRNA amplicons did not allow *Lactobacillus casei* and *Lactobacillus rhamnosus* or *Lactobacillus panis* and *Lactobacillus reuteri* to be differentiated with confidence. Overall, 75 different taxa were identified from the 942 16S rRNA amplicons. In each of the contributing samples, the number of taxa ranged from 7 in sample 3 to 31 in sample 7. In lesions where lactobacilli were the predominant bacterial flora, an average of nine different species or phylotypes were found to predominate in the lesions.

TABLE 1. Characteristics of the carious dentine samples used for microbial analysis

Sample no.	Patient gender[a]	Patient age (yr)	Total load (cells ng of dentine^{-1})[b]			Histopathologic category[c]
			Microbial	*Prevotella*	*Lactobacillus*	
1	M	30	1,100	39	106	I
2	F	30	490	1	44	IV
3	F	32	360	0	46	I
4	M	55	310	0	10	II
5	M	65	320	140	19	I
6	M	40	140	86	4	II
7	M	55	210	16	1	III
8	F	39	450	165	11	IV
9	F	32	670	180	3	II
10	F	33	400	7	1	I

[a] M, male; F, female.
[b] Data obtained by real-time PCR analysis (4, 19).
[c] Underlying pulpal tissue was examined for pathologic change and categorized for the predominant presentation of essentially normal histology (category I), hyaline soft tissue degeneration (category II), extensive calcification (category III), or infiltration of inflammatory cells (category IV) (19).

Phylogenetic analysis of predominant bacteria. To provide an overview of the diversity of species associated with advanced caries, a phylogenetic tree was constructed from 75 16S rRNA sequences representing the different species and phylotypes in the ten carious lesions (Fig. 1). Of the 75 different bacterial lineages identified, the major families were the *Lactobacteriaceae* and the *Prevotellaceae*, with 15 and 13 distinct phylogenetic lineages, respectively. Other diverse families included the *Acidaminococcaceae* (seven lineages), *Eubacteriaceae* (six lineages), and *Lachnospiraceae* (six lineages). Less diverse were the *Streptococcaceae*, *Coriobacteriaceae*, and *Bifidobacteriaceae*, each comprising three distinct lineages. The remaining species were found to represent various divergent bacterial lineages which, with the exception of *Pseudoramibacter alactolyticus*, *Fusobacterium nucleatum*, and *Propionibacterium* spp., were generally found to comprise minor proportions in the 10 carious lesions.

Lesion-dependent species diversity. Species belonging to the family *Lactobacillacae* were abundant and represented 50% of total amplicons (Table 2). Since this family was dominant in four of the eight lesions in which they were detected, comprising 97, 85, 98, and 95% of the bacteria in samples 1, 2, 3, and 4, respectively, they were designated high-*Lactobacillus* samples (Table 2).

The family *Prevotellaceae* were also diverse and abundant, representing 15% of total species in the 10 carious dentine samples. The *Prevotellaceae* were detected in six lesions. They were dominant in samples 8 and 9, comprising 39 and 46%, respectively, of the species present (Table 2). These carious dentine samples were referred to as high-*Prevotella* and were found to be populated by a range of other species or phylotypes unlike the high-*Lactobacillus* samples. In particular, *Olsenella* spp. and *Pseudoramibacter alactolyticus* were found in significant proportions in these lesions. For instance, in sample 8, *Olsenella* spp. comprised 23% of the amplicons, whereas in sample 9, *Olsenella* spp. and *Pseudoramibacter alactolyticus* comprised 14 and 26% of amplicons, respectively.

In dentine samples 5, 6, and 7, lower proportions of lacto-

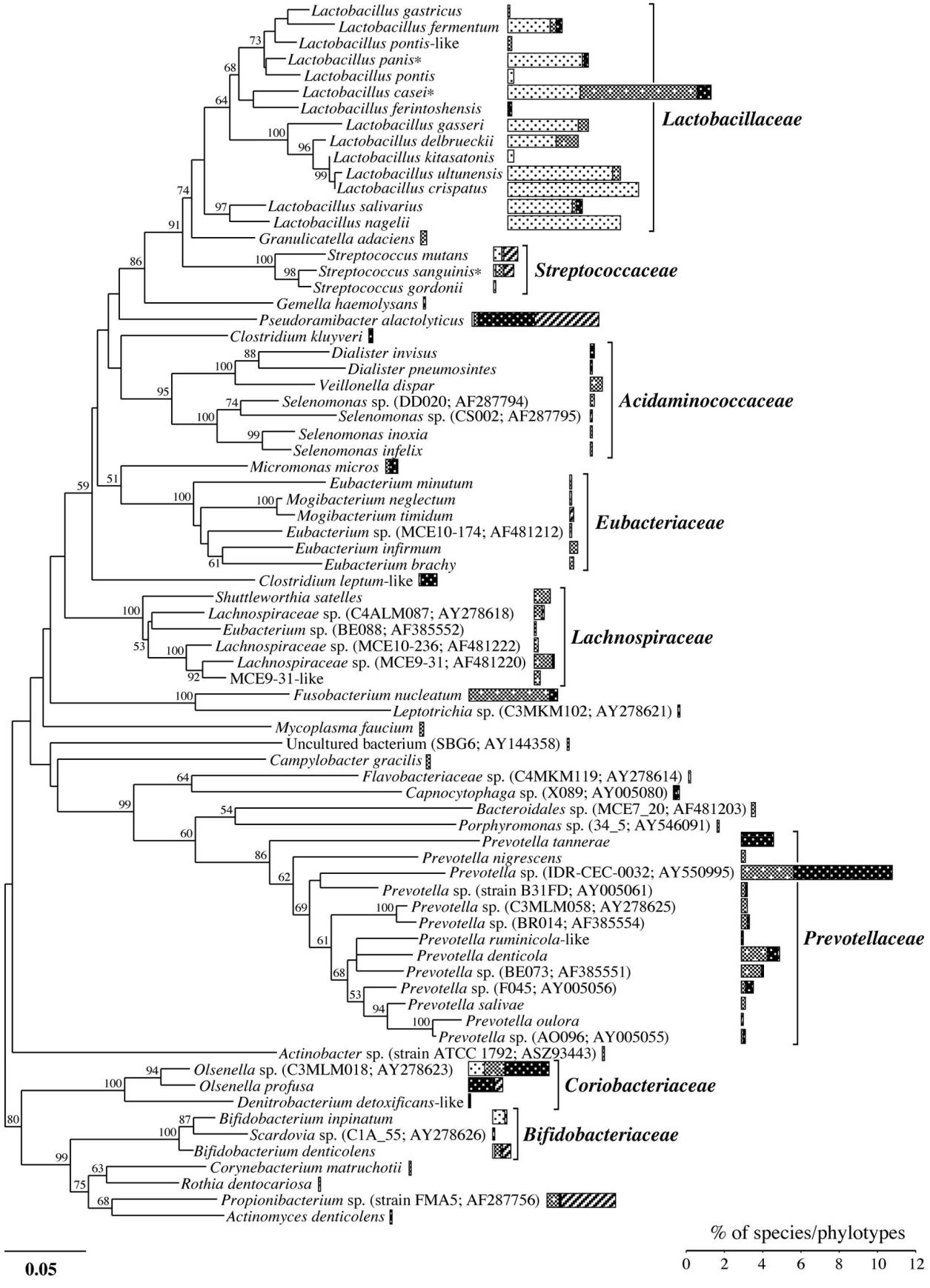

TABLE 2. Major families, species, and phylotypes identified in 10 carious dentine lesions

Family and/or species	No. of 16S rRNA amplicons detected in a given carious dentine sample[a]									
	High-*Lactobacillus*				Mid-*Lactobacillus*/*Prevotella*			High-*Prevotella*		Low-*Lactobacillus*/*Prevotella*
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
Lactobacillaceae	92	77	99	96	67	9	10	17	0	0
Lactobacillus fermentum	3	5	9	4	–	–	3	3	–	–
Lactobacillus panis and *Lactobacillus reuteri*	3	9	21	4	–	1	–	2	–	–
Lactobacillus casei and *Lactobacillus rhamnosus*	14	–	3	19	50	2	5	7	–	–
Lactobacillus gasseri	6	19	6	4	–	5	–	–	–	–
Lactobacillus delbrueckii	24	–	–	–	11	–	–	–	–	–
Lactobacillus ultunensis	33	19	–	–	2	1	1	–	–	–
Lactobacillus crispatus	–	5	60	–	–	–	–	–	–	–
Lactobacillus salivarius	6	17	–	9	2	–	–	3	–	–
Lactobacillus nagelii	–	–	–	56	–	–	–	–	–	–
Lactobacillus spp.	3	3	–	–	2	–	1	2	–	–
Streptococcaceae	3	0	0	3	1	0	4	0	0	12
Streptococcus mutans	3	–	–	1	–	–	1	–	–	7
Streptococcus sanguinis, *Streptococcus mitis*, and *Streptococcus oralis*	–	–	–	1	1	–	3	–	–	5
Streptococcus gordonii	–	–	–	1	–	–	–	–	–	–
Acidaminococcaceae	0	0	0	0	1	0	9	3	0	1
Eubacteriaceae	0	0	0	0	3	6	0	0	0	2
Lachnospiraceae	0	0	0	0	1	11	15	1	0	1
Prevotellaceae	0	0	0	0	24	17	23	38	41	2
Prevotella tannerae	–	–	–	–	–	–	–	16	–	–
Prevotella sp. oral clone IDR-CEC-0032	–	–	–	–	18	8	–	9	40	–
Prevotella denticola	–	–	–	–	6	4	3	5	–	1
Prevotella sp. oral clone BE073	–	–	–	–	–	–	10	1	–	–
Prevotella sp. oral clone F045	–	–	–	–	–	1	1	4	–	–
Prevotella spp.	–	–	–	–	–	4	9	3	1	1
Coriobacteriaceae	0	8	0	0	0	1	9	22	14	4
Olsenella sp. clone C3MLM018	–	8	–	–	–	1	9	22	–	–
Olsenella profusa	–	–	–	–	–	–	–	–	13	4
Denitrobacterium detoxificans-like	–	–	–	–	–	–	–	–	1	–
Bifidobacteriaceae	0	6	0	1	0	0	3	1	0	6
Other										
Fusobacterium nucleatum	–	–	–	–	2	31	7	4	–	–
Pseudoramibacter alactolyticus	–	–	–	1	–	2	–	5	23	32
Propionibacterium sp. oral strain FMA5	–	–	–	–	–	–	6	–	1	27
Other bacteria	0	0	2	0	1	7	8	6	11	3
Total	95	91	101	101	100	84	94	97	90	90

[a] –, Not detected.

bacilli and prevotellae were identified, with lactobacilli accounting for 67, 11, and 11% of the amplicons and prevotellae accounting for 24, 24 and 20%, respectively (Table 2). These carious dentine samples were referred to as mid-*Lactobacillus*/*Prevotella*. Although sample 5 was composed primarily of lactobacilli and prevotellae, samples 6 and 7 contained relatively high proportions of species belonging to the *Lachnospiraceae*, with 16 and 13% of the species, respectively, belonging to this

FIG. 1. Unrooted neighbor-joining tree of 942 16S rRNA amplicon sequences obtained from 10 carious dentine samples. Sequences were grouped by identity and are represented by the most closely related bacterial species (>99% identity in ~400-bp sequence of 16S rRNA). Species marked by an asterisk represent more than one species (see the text). For sequences which are most closely related to uncharacterized phylotypes, the clone or strain number and the GenBank accession number is shown in parentheses. The percent prevalence of the species or phylotype among the samples in each of the four categories displayed in Table 2 is represented by horizontal bars with the scale bar shown on the right as follows: ▨, high-*Lactobacillus*; ▩, mid-*Lactobacillus*/*Prevotella*; ■, high-*Prevotella*; ▨, low-*Lactobacillus*/*Prevotella*. Bootstrap values (>50) near the nodes represent percentages of 1,000 replicates. The scale bar on the left represents the genetic distance.

family. In addition, *F. nucleatum* was the predominant species in sample 6, representing 37% of the amplicons.

Since no lactobacilli were detected in the carious dentine sample 10 and only two *Prevotella* spp. were found, this sample was designated low-*Lactobacillus*/*Prevotella* (Table 2). In this sample, *Pseudoramibacter alactolyticus* and *Propionibacterium* spp. were predominant, with 36 and 30%, respectively, of the species belonging to these two genera.

Of the 15 major lineages in the family *Lactobacillaceae*, nine species/phylotypes were identified with higher frequency (Fig. 1). These were generally identified in the high-*Lactobacillus* samples. Of note was the observation that *Lactobacillus casei* and/or *Lactobacillus rhamnosus* were identified in samples where significant proportions of *Prevotella* were also found. Among the *Prevotella*, 5 of the 13 species or phylotypes were identified with higher frequency. In particular, the uncharacterized *Prevotella* spp., corresponding to oral phylotype IDR-CEC-0032 (GenBank accession number AY550995), were found to comprise the highest proportion of these *Prevotella*.

DISCUSSION

Phylogenetic analysis based on 16S rRNA gene(s) has demonstrated the extreme complexity of microbial populations in a variety of habitats within the oral cavity, with the current estimate of the total oral flora to be ca. 700 species (14). This complexity is particularly evident for the gingival crevice (26) and dorsum of the tongue (14) but also for the dental plaque biofilm associated with the initiation of the carious enamel lesion (3). It is believed that only 50% of bacteria in the oral cavity are cultivable (30), requiring a molecular approach to elucidate the diversity of microflora associated with disease progression.

The present molecular study was undertaken to define the profile of bacteria associated with carious dentine after 25 rounds of PCR, with 30 cycles being the most commonly used in other studies (16, 20, 26). Only the most prevalent bacteria were detected by the methods used since after 25 rounds of PCR the chance of detecting a given bacterium with 95% confidence requires the bacterium to be present as ≥3% of the population when 100 clones are sequenced. This implies that less-abundant species or phylotypes were not disclosed. Although the method used to extract and protect DNA from degradation readily lyses gram-negative bacteria, as well as many gram-positive bacteria such as lactobacilli growing under anaerobic conditions, the method does not allow for the quantitative extraction of DNA from all gram-positive bacteria whether or not they are growing anaerobically (4, 23). Thus, streptococci, including the mutans streptococci which are associated with the initiation of dental caries (8, 33), would be underestimated by this method. However, in the 10 samples analyzed, the relative levels of cultivable streptococci were known in relation to the colony counts for *Prevotellaceae* and lactobacilli (and other species [19]). Based on the chance of finding streptococci in the 10 samples analyzed, the data for 3 of the samples were at odds with the cultivable data. These were samples 1 and 6, in which more streptococci would be expected to be detected, and sample 4, in which no streptococci would be expected after 25 rounds of PCR amplification despite streptococci being known to be present in the sample. Interestingly, a recent study of five carious dentine samples reported that the numbers of cariogenic *Streptococcus mutans* were very low in three of the five samples analyzed (20). The same study also reported very low levels of anaerobic gram-negative bacteria, suggesting a bias toward extraction of DNA from gram-positive bacteria consistent with the DNA extraction protocol used (6).

Although there is a risk of overinterpretation of the dynamics of the microbial populations during progression of the carious lesion within the dentine matrix, the results of the current study of 10 selected carious lesions, chosen on the basis of comparable total microbial load and variable *Prevotella* load (19), show a high degree of variability. Up to 31 different taxa were detected in each of the 10 carious dentine samples, similar to the diversity reported to be associated with endodontic infections (21) and to the diversity in carious dentine seen by using a combination of culture-based and cloning techniques (20), but greater than the level of diversity determined in previous carious dentine studies with only culture-based methods (1, 7, 11).

Lesions 1, 2, 3, and 4 were dominated by lactobacilli in various combinations, a finding compatible with real-time PCR analysis of these samples that indicated the paucity of *Fusobacterium* spp. and *Micromonas* (formally *Peptostreptococcus*) *micros*, *Porphyromonas endodontalis*, and *Porphyromonas gingivalis* (19). Rather, high levels of *Lactobacillus delbrueckii*, *Lactobacillus ultunensis*, *Lactobacillus crispatus*, and *Lactobacillus salivarius* (Table 2) were associated with these lesions in agreement with the finding by Munson et al. (20) that taxa of the genus *Lactobacillus* dominate carious dentine. It is possible that successful early colonization of the dentinal matrix by lactobacilli is favored by abundant dietary carbohydrate that, once fermented, creates an acidic environment which excludes subsequent colonization by other species. Exclusion may also depend on the fermentation by-products of some of these lactobacilli, since those of *Lactobacillus delbrueckii*, *Lactobacillus ultunensis*, and *Lactobacillus crispatus* (a group of organisms more frequently associated with other regions of the gastrointestinal tract [4] are not well characterized. It is known, however, that the proportions of lactic, acetic, and propionic acids vary considerably between lesions (9). Lactate-dependent *Veillonella* spp. were infrequently detected, and even though other members of the family *Acidaminococcaceae*, such as *Selenomonas* spp., as well as species such as *Pseudoramibacter alactolyticus*, are capable of metabolizing lactate (12), none of these bacteria were associated with high-*Lactobacillus* lesions. This suggests that lactate is not the major fermentation by-product in these lesions. However, these lactate-utilizing bacteria were found in other lesions, particularly those containing *Prevotella* (Table 2). Interestingly, *Pseudoramibacter alactolyticus*, which can also ferment both carbohydrates and lactate, is found in 56% of endodontic infections (29) and was present at high levels in samples 9 and 10 where no lactobacilli were detected.

In contrast to the high-*Lactobacillus* lesions, five of the six carious dentine samples with a low abundance of lactobacilli were associated with high levels of *Prevotellaceae*, as well as members of either the family *Lachnospiraceae*, or *Olsenella* spp. (Table 2). Numerically abundant *Prevotella* organisms were mainly allocated to novel, uncultured forms of unknown

biology with a major group represented by phylotype IDR-CEC-0032, which is most closely related to a *Prevotella*-like rumen bacterium (22).

Invasion of bacteria into the dentinal tubules implies that properties such as adhesion and motility are involved. Members of the families *Acidaminococcaceae*, such as *Selenomonas* spp., and the *Lachnospiraceae* possess flagellae and hence are motile, although the large size of some selenomonads (2 by 10 μm) could limit their penetration into tubules which at the dentine-enamel junction are ca. 1 to 2 μm in diameter (15). On the other hand, nonmotile proteolytic bacteria such as *Porphyromonas gingivalis* have been shown to invade dentinal tubules in vitro by coaggregation to chains of *Streptococcus gordonii* since these chains extend into the tubules as the *Streptococcus* organisms multiply (18). Our data suggest, however, that neither the *Streptococcaceae* nor the porphyromonads are found in high numbers in samples containing high proportions of *Lactobacillus* and/or *Prevotella* spp. (Table 2). This finding supports our previous analysis of 65 carious dentine lesions wherein the abundance of highly proteolytic organisms, such as *Porphyromonas gingivalis* and *Porphyromonas endodontalis* (27), was found to be uniformly low (19) despite the expectation that the exposed decalcified and denatured collagenous framework of the dentinal tubules would provide a rich nutrient source (2). This implies that an essential nutrient, such as hemin (25, 36), may be limiting or that some other unspecified environmental factor restricts the dominance of the porphyromonads. However, other bacteria, including *Micromonas micros*, certain *Prevotella* spp. and the *Propionibacterium* sp. oral strain FMA5, which accounted for 30% of the bacterial population in sample 10, have been shown to degrade proteins such as gelatin and albumin (10, 24, 34). Such bacteria may therefore provide peptides and amino acids required by other bacteria within the polymicrobial population. Interestingly, *Propionibacterium* sp. oral strain FMA5 was one of only three taxa found in all five carious dentine samples recently analyzed by Munson et al. (20).

Since no particular consistent pattern of dominance was observed in the 10 selected carious dentine samples, it is probable that dominance by individual taxa was achieved after early invasion of the exposed dentine matrix. This would suggest that the composition of the dominant groups could depend on early events, including invasion by species present in supragingival plaque on the tooth surface and/or random contamination of the open lesion from salivary and dietary sources. Rates of lesion progression would then depend on the nature and abundance of acidogenic and aciduric organisms and, at least initially, on the continued availability of dietary fermentable carbohydrates. Migration of proteolytic bacteria to the proximity of the pulp to exploit additional nutrient resources could drive the final phase of the carious lesion and result in pulpal death and translocation of the bacteria into the pulp chamber. Accordingly, the composition and pathogenic potential of bacterial populations subsequently associated with endodontic infections might be determined by relatively early events occurring in the establishment of the carious dentine lesion.

REFERENCES

1. **Ando, N., and E. Hoshino.** 1990. Predominant obligate anaerobes invading the deep layers of root canal dentin. Int. Endod. J. **23:**20–27.
2. **Armstrong, W. G.** 1958. Further studies on the action of collagenase on sound and carious human dentin. J. Dent. Res. **37:**1001–1015.
3. **Becker, M. R., B. J. Paster, E. J. Leys, M. L. Moeschberger, S. G. Kenyon, J. L. Galvin, S. K. Boches, F. E. Dewhirst, and A. L. Griffen.** 2002. Molecular analysis of bacterial species associated with childhood caries. J. Clin. Microbiol. **40:**1001–1009.
4. **Byun, R., M. A. Nadkarni, K.-L. Chhour, F. E. Martin, N. A. Jacques, and N. Hunter.** 2004. Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. J. Clin. Microbiol. **42:**3128–3136.
5. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje.** 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. **31:**442–443.
6. **Grimont, F., and P. A. D. Grimont.** 1991. DNA fingerprinting, p. 249–276. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Ltd., Chichester, England.
7. **Hahn, C. L., W. A. Falkler, Jr., and G. E. Minah.** 1991. Microbiological studies of carious dentine from human teeth with irreversible pulpitis. Arch. Oral Biol. **36:**147–153.
8. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44:**331–384.
9. **Hojo, S., N. Takahashi, and T. Yamada.** 1991. Acid profile in carious dentin. J. Dent. Res. **70:**182–186.
10. **Holdeman, L. V., E. P. Cate, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg, Va.
11. **Hoshino, E.** 1985. Predominant obligate anaerobes in human carious dentin. J. Dent. Res. **64:**1195–1198.
12. **Hoshino, E., M. Sato, T. Tamura, K. Kota, H. Uematsu, N. Sato, and F. Nakazawa.** 2003. Glucose and lactate metabolisms of *Pseudoramibacter alactolyticus*, abstr. 1675. Proceedings of the 81st General Session of the International Association for Dental Research. International Association for Dental Research, Goteborg, Sweden.
13. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, Inc., New York, N.Y.
14. **Kazor, C. E., P. M. Mitchell, A. M. Lee, L. N. Stokes, W. J. Loesche, F. E. Dewhirst, and B. J. Paster.** 2003. Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. J. Clin. Microbiol. **41:**558–563.
15. **Kingsley, V. V., and J. F. M. Hoeniger.** 1973. Growth, structure and classification of *Selenomonas.* Bacteriol. Rev. **37:**479–521.
16. **Leser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Moller.** 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl. Environ. Microbiol. **68:**673–690.
17. **Love, R. M., and H. F. Jenkinson.** 2002. Invasion of dentinal tubules by oral bacteria. Crit. Rev. Oral Biol. Med. **13:**171–183.
18. **Love, R. M., M. D. McMillan, Y. Park, and H. F. Jenkinson.** 2000. Coinvasion of dentinal tubules by *Porphyromonas gingivalis* and *Streptococcus gordonii* depends upon binding specificity of streptococcal antigen I/II adhesin. Infect. Immun. **68:**1359–1365.
19. **Martin, F. E., M. A. Nadkarni, N. A. Jacques, and N. Hunter.** 2002. Quantitative microbiological study of human carious dentine by culture and real-time PCR: association of anaerobes with histopathologic changes in chronic pulpitis. J. Clin. Microbiol. **40:**1698–1704.
20. **Munson, M. A., T. F. Banerjee, T. F. Watson, and W. G. Wade.** 2004. Molecular analysis of the microflora associated with dental caries. J. Clin. Microbiol. **42:**3023–3029.
21. **Munson, M. A., T. Pitt-Ford, B. Chong, A. Weightman, and W. G. Wade.** 2002. Molecular and cultural analysis of the microflora associated with endodontic infections. J. Dent. Res. **81:**761–766.
22. **Nadkarni, M. A., C. E. Caldon, K.-L. Chhour, I. P. Fisher, F. E. Martin, N. A. Jacques, and N. Hunter.** 2004. Carious dentine provides a habitat for a complex array of novel *Prevotella*-like bacteria. J. Clin. Microbiol. **42:**5238–5244.
23. **Nadkarni, M. A., F. E. Martin, N. A. Jacques, and N. Hunter.** 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology **148:**257–266.
24. **Ng, J., L. K. Ng, D. Mayrand, and J. A. R. Dillon.** 1998. Aminopeptidase activities in *Peptostreptococcus* spp. are statistically correlated to gelatin hydrolysis. Can. J. Microbiol. **44:**303–306.
25. **Paramaesvaran, M., K. A. Nguyen, E. Caldon, J. A. McDonald, S. Najdi, G. Gonzaga, D. B. Langley, A. DeCarlo, M. J. Crossley, N. Hunter, and C. A. Collyer.** 2003. Porphyrin-mediated cell surface heme capture from hemoglobin by *Porphyromonas gingivalis*. J. Bacteriol. **185:**2528–2537.
26. **Paster, B. J., S. K. Boches, J. L. Galvin, R. E. Ericson, C. N. Lau, V. A. Levanos, A. Sahasrabudhe, and F. E. Dewhirst.** 2001. Bacterial diversity in human subgingival plaque. J. Bacteriol. **183:**3770–3783.
27. **Rosen, G., M. Shoshani, R. Naor, and M. N. Sela.** 2001. The purification and characterization of an 88-kDa *Porphyromonas endodontalis* 35406 protease. Oral Microbiol. Immunol. **16:**326–331.

28. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
29. **Siqueira, J. F., I. N. Rocas, M. De Uzeda, A. P. Colombo, and K. R. N. Santos.** 2002. Comparison of 16S rDNA-based PCR and checkerboard DNA-DNA hybridisation for detection of selected endodontic pathogens. J. Med. Microbiol. **51:**1090–1096.
30. **Socransky, S. S., R. J. Gibbons, A. C. Dale, L. Bortnick, E. Rosenthal, and J. B. Macdonald.** 1963. The microbiota of the gingival crevice area of man. I. Total microscopic and viable counts of specific organisms. Arch. Oral Biol. **8:**275–280.
31. **Syed, S. A., and W. J. Loesche.** 1972. Survival of human dental plaque flora in various transport media. Appl. Microbiol. **26:**459–465.
32. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
33. **van Ruyven, F. O. J., P. Lingstrom, J. van Houte, and R. Kent.** 2000. Relationship among mutans streptococci, "low-pH" bacteria, and iodophilic polysaccharide-producing bacteria in dental plaque and early enamel caries in humans. J. Dent. Res. **79:**778–784.
34. **Wei, G. X., J. S. van der Hoeven, J. W. Smalley, F. H. M. Mikx, and M. W. Fan.** 1999. Proteolysis and utilization of albumin by enrichment cultures of subgingival microbiota. Oral Microbiol. Immunol. **14:**348–351.
35. **Woese, C. R.** 2002. On the evolution of cells. Proc. Natl. Acad. Sci. USA **99:** 8742–8747.
36. **Zerr, M. A., C. D. Cox, W. T. Johnson, and D. R. Drake.** 2000. Growth stimulation of *Porphyromonas endodontalis* by hemoglobin and protoporphyrin IX. Oral Microbiol. Immunol. **15:**365–370.