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Carious Dentine Provides a Habitat for a Complex Array of Novel *Prevotella*-Like Bacteria

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Previous analysis of the microbiology of advanced caries by culture and real-time PCR emphasized the high incidence and abundance of gram-negative anaerobic species, particularly *Prevotella*-like bacteria. The diversity of *Prevotella*-like bacteria was further explored by analyzing pooled bacterial DNA from lesions of carious dentine. This was achieved by amplification of a region of the 16S ribosomal DNA with a *Prevotella* genus-specific forward primer and a universal bacterial reverse primer, followed by cloning and sequencing. Cultured *Prevotella* species commonly associated with oral tissues constituted only 12% of the *Prevotella* clones isolated from advanced carious lesions. The remaining 88% consisted of a diverse range of phylotypes. These included five clusters of previously recognized but uncultured oral *Prevotella* spp. and a major cluster containing *Prevotella*-like bacteria most closely related to uncharacterized rumen bacteria. Cluster-specific primers were designed, and the numbers of bacteria within clusters were quantified by real-time PCR, confirming the abundance of these organisms. The data indicated that advanced dental caries provides a unique environment for a complex array of novel and uncultured *Prevotella* and *Prevotella*-like bacteria which, in some cases, may dominate the diverse polymicrobial community associated with the disease.

Extension of an initial enamel carious lesion into the dentine of the tooth occurs as a zone of decalcification preceding a zone of proteolysis with the accumulation of a necrotic zone distal to the advancing front. Decalcification of the dentinal tubules by organic acids released by some species of streptococci and lactobacilli is apparently complemented by proteolytic degradation of the dentinal matrix either by uncharacterized microbial proteinases or by the proteolytic activity of pulpal odontoblasts (14).

Recent analyses using molecular approaches have highlighted the presence of the proteolytic gram-negative anaerobic genus *Prevotella* at some dentinal caries sites (9, 16, 17). The genus *Prevotella* was separated from the *Bacteroidiaceae* in 1990 (23) and presently comprises diverse organisms that constitute the largest group of medically important gram-negative anaerobes. Cultured *Prevotella* organisms have been implicated in a wide range of infections, and some members of the genus express marked proteolytic activity and hemolysins (3–5, 8). Typically, anaerobic culture significantly underestimates the abundance of *Prevotella* and other anaerobes at sites of infection. This was confirmed in our studies of advanced caries (16), where the importance of gram-negative anaerobic species has only recently been emphasized (9, 16, 17). Direct comparison of anaerobic culture and real-time PCR analysis indicated a 40-fold underestimation of *Prevotella*-like organisms by culture analysis (16). In addition, culture and biochemical testing revealed a discrepancy between the number of *Prevotella* organisms and the paucity of the recognized oral forms of the genus. The suggestion of novel species provided the impetus for the present study. Analysis of 16S ribosomal DNA (rDNA) indicated the complexity of the representative phylogenetic groupings of *Prevotella*-like organisms present in advanced dentine carious lesions. Quantitative analysis by real-time PCR confirmed the dominance of novel and uncultured organisms.

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MATERIALS AND METHODS

Source of carious dentine. The source of material for analysis was carious dentine from 65 vital carious teeth collected with informed consent from randomly selected patients of both sexes (29 males and 36 females) aged between 17 and 75 years (average, 37 years) who attended the United Dental Hospital in Sydney, Australia. The patients were a representative cross section of Sydney's ethnically diverse population, including people of European, Asian, Middle Eastern, and Pacific island backgrounds. Patients were excluded from the study if they reported a history of significant medical disease or antimicrobial therapy within the previous 4 months. Unrestored anterior, premolar, and molar teeth with coronal enamel and dentine caries were selected for inclusion in the study on the basis of clinical diagnostic tests that indicated that they were vital, with clinical symptoms of reversible pulpitis (pain and heightened sensitivity to hot and cold stimuli), with no obvious exposure of the pulp tissue, and with a periodontal probing depth of <4 mm. The study was approved by the Central Sydney Area Health Service Ethics Review Committee, Sydney, Australia (reference no. 6/96).

Underlying pulpal tissue was examined for pathological change and categorized for the predominant presentation of essentially normal histology (category 1), hyaline soft tissue degeneration (category 2), extensive calcification (category 3), or infiltration of inflammatory cells (category 4) (16).

Isolation of bacterial DNA and design of probe and primers for PCR. DNA was isolated from carious dentine as described previously (16, 18). A *Prevotella* genus forward primer, a *Prevotella* genus probe, and a universal reverse primer used for real-time PCR analysis were used as described previously (16, 18), except that the *Prevotella* probe used for the quantification of phylogenetic clusters was in the reverse orientation (Table 1). For cluster-specific reverse primer design, selected *Prevotella* 16S rDNA sequences from GenBank were aligned with the cloned sequences using the Genetics Computer program PileUp (Wisconsin Package version 8) accessed through the Australian National Genomic Information Service (ANGIS) (http://www.angis.org.au). Regions of

TABLE 1. Sequences of oligonucleotide primers and probes used in this study

Bacterium or sequence detected and primer or probe	Sequence (5′ → 3′)	T_m (°C)[a]
Universal[b]		
Reverse	GGACTACCAGGGTATCTAATCCTGTT	58.1
Prevotella[c]		
Forward	CCAGCCAAGTAGCGTGCA	58.1
Probe (anti)	CTGGCACGGAATTAGCCGGTCCTTATT[d]	68.8
Reverse	TGGACCTTCCGTATTACCGC	58.5
Cluster I		
Reverse	CGCACTCAAGCAGGACAGTTT	59.1
Cluster II		
Reverse	CCTACGTTGTGCATACTCAAGTGAA	59.3
Cluster III		
Reverse	CGTTGTGCACACTCAAGTGGA	59.4
Cluster IV		
Reverse	AACGTTGTGCGTACTCAAGGAA	58.4
Cluster V		
Reverse	CGGCGTTGCGCGTACT	59.1
Cluster VI		
Reverse	GTGCGGATGTTGAGCAACC	58.7

[a] The melting temperature (T_m) of DNA was determined using Primer Express software version 1.0 (Applied Biosystems).
[b] See reference 18 for further details.
[c] See reference 16 for further details.
[d] *Prevotella* probe was labeled at the 5′ end with 6-carboxyfluorescein and at the 3′ end with 6-carboxytetramethylrhodomine.

identity were determined visually, and cluster-specific identification was confirmed using the database similarity search program BLAST (1) accessed through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). All cluster-specific reverse primers met the guidelines established by Applied Biosystems (Foster City, Calif.) using their Primer Express software version 1.0.

Amplification of *Prevotella* genus-specific 16S rDNA sequences from multiple carious-dentine samples. From the 65 carious-dentine samples, 56 with *Prevotella* loads between 1.19 and 476 pg of DNA per mg (wet weight) of carious dentine were selected for comprehensive phylogenetic analysis. DNA from each of the 56 carious-dentine samples was diluted to 1 pg of *Prevotella* DNA μl^{-1}, and 1 μl of each diluted sample was pooled. From the pooled DNA samples, 10 pg was used in a 100-μl PCR. PCR fragments, representing a *Prevotella* genus-specific amplicon, were amplified using 400 nM *Prevotella* forward primer and 300 nM universal reverse primer (Table 1), 3.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates (dNTPs), 2.5 U of Amplitaq Gold (Applied Biosystems), and 1× buffer (Applied Biosystems) in three 100-μl reaction mixtures in MicroAmp reaction tubes (Applied Biosystems) using an FTS-320 thermal cycler (Corbett Research, Sydney, Australia). PCR conditions were set at 95°C for 10 min, followed by 30 cycles of 95°C for 15 s and 60°C for 1 min. The PCR product (413 bp) was visualized on a 2% agarose gel, and the size was estimated by comparison with a 100-bp DNA ladder (New England Biolabs, Beverly, Mass.). The PCR product was purified and eluted into 50 μl of H_2O with a Wizard PCR Preps DNA Purification System (Promega Corp., Madison, Wis.) using the manufacturer's protocol.

Cloning of the PCR product. The PCR product was cloned into the plasmid pGEM-T Easy (Promega Corp.) by incubation at 4°C for 21 h using the manufacturer's protocol. The resulting chimeric plasmids were electroporated into competent *Escherichia coli* XL1-Blue cells (Stratagene, West Cedar Creek, Tex.) using a Gene Pulser (200 Ω; 2.45 V; 4 s; 4°C; Bio-Rad Laboratories, Hercules, Calif.). Following incubation on ice for 1 min and then at 37°C for 1 h in Luria-Bertani broth, aliquots of the transformed cells were plated onto Luria-Bertani agar containing 100 μg of ampicillin ml^{-1}, previously surface coated with 100 μl of 24-mg IPTG (isopropyl-β-D-thiogalactopyranoside) ml^{-1} and 40 μl of 20-mg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) ml^{-1}, and incubated at 37°C for 16 h. Representative colonies containing chimeric plasmids were detected by blue-white selection, picked, and subsequently grown in 5 ml of Luria-Bertani broth containing 100 μg of ampicillin ml^{-1} at 37°C for 16 h. Plasmids were extracted from 2 ml of the cultures using the Wizard *Plus* SV Minipreps DNA Purification System (Promega Corp.) according to the manufacturer's instructions. Ten microliters of purified plasmids was visualized on a 1% agarose gel, and the size was estimated by comparison with a 1-kb DNA ladder (New England Biolabs).

Sequencing of 16S rDNA inserts. The 16S rDNA inserts in the purified chimeric plasmids were sequenced using the *Prevotella* forward primer (Table 1). DNA sequencing was carried out on an Applied Biosystems model 373A DNA sequencer by the DNA Sequencing Facility, Westmead Millennium Institute, Westmead, Australia, using Big-Dye terminator (version 3) chemistry. One hundred fourteen inserts were sequenced. The readable length of sequence for the *Prevotella* genus amplicon was 386 bp.

Identification of genera and species using 16S rDNA sequences. The identity of each of the 16S rDNA PCR products was determined by comparing the DNA sequence with those in the nonredundant bacterial nucleic database using the computer program GAP (7) accessed through ANGIS. Identification to the species level was assumed when the sequences possessed >99% identity to the 16S rDNA sequences of known species, and identification to the genus level was assumed when the sequences were >97% identical. Sequences not identical to the 16S rDNA sequences of known bacterial species were called phylotypes. Validation as a species requires comprehensive phenotypic assessment of an organism (21). A subset of the sequences of the 16S rDNA PCR products were analyzed for internal chimeras using the program ChimeraCheck (15). No chimeras were detected.

Phylogenetic tree of *Prevotella* and *Prevotella*-like bacteria. One hundred fourteen sequences of 16S rDNA amplicons from 56 pooled carious-dentine samples were aligned using the application PileUp (7) accessed through ANGIS, along with the 16S rDNA sequence of *Aquifex aeolicus* to root the tree, with *Porphyromonas gingivalis* ATCC33277 (POYRR16SC) (GenBank accession numbers are in parentheses) and *Tanneralla forsythensis* ATCC43037 (formerly *Bacteroides forsythus* AB035460) as outgroups, along with human oral bacterium C65 (AF201972 [12]), *Prevotella* sp. oral clone AA020 (AY005057 [21]), *Prevotella* sp. oral clone AA016 (AY005058 [21]), *Prevotella* sp. oral clone AH125 (AY005060 [21]), *Prevotella* sp. uncultured clone DG059 (AF366269 [2]), *Prevotella* sp. oral clone DO014 (AF385509 [11]), *Prevotella* sp. oral clone DO027 (AF385511 [11]), *Prevotella* sp. oral clone DO033 (AF385512 [11]), *Prevotella* sp. oral clone FM005 (AF432133 [11]), *Prevotella* sp. oral clone AH005 (AY005053 [21]), *Prevotella* sp. oral clone AO036 (AY005054 [21]), *Prevotella* sp. oral clone AO096 (AY005055 [21]), *Prevotella albensis* M384 (PAL011683), *Prevotella bivia* ATCC29303 (PVORR16SK), *Prevotella brevis* GA33 (PBR011682), *Prevotella bryantii* B14 (PBAJ6457), *Prevotella corporis* ATCC33547 (PVORR16SA), *Prevotella dentalis* DSM3688 (PDRRNA16S), *Prevotella denticola* ATCC35308 (PVORR16SC), *Prevotella disiens* ATCC29426 (PVORR16SS), *Prevotella enoeca* ATCC51261 (PEAJ5635), *Prevotella heparinolytica* ATCC35895 (BHRR16SAD), *Prevotella intermedia* ATCC25611 (PVORR16SD), *Prevotella loescheii* ATCC15930 (PVORR16SQ), *Prevotella melaninogenica* ATCC25845 (PVORR16SE), *Prevotella nigrescens* ATCC33563 (PVORR16SG), *Prevotella oralis* ATCC33269 (PVORR16SP), *Prevotella oris* ATCC33573 (PVORR16SJ), *Prevotella oulorum* ATCC43324 (PVORR16SH), *Prevotella pallens* 10371 (PP16S1037), *Prevotella ruminicola* ATCC19189 (PVORR16SR), *Prevotella tannerae* ATCC51259 (EMAJ5634), *Prevotella veroralis* ATCC33779 (PVORR16SI), *Prevotella zoogleoformans* ATCC33285 (BZRR16SAE), and *Prevotella* sp. clone IDR-CEC-0032 (AY550995) as members of the genus *Prevotella* to anchor the tree. The sequences were manually gap edited, resulting in the alignment of DNA sequences of 369 bp. A distance matrix and neighbor-joining tree were constructed from the alignment using the programs DNAdist and Neighbor (6), and the trees were viewed with the program Treeview (20) accessed through ANGIS. The statistical robustness of the trees was calculated by bootstrapping. One hundred random subsets of the alignment were generated with the application Seqboot (6), and distance matrices and neighbor-joining trees were subsequently constructed on each subset. A consensus tree and bootstrap values were calculated using the program Consense (6) accessed through ANGIS.

Real-time PCR. Six *Prevotella*-like clusters were quantified by real-time PCR using the TaqMan PCR core reagent kit (Applied Biosystems). Optimized concentrations of *Prevotella* forward primer, *Prevotella* probe, and cluster-specific reverse primer (Table 1) were used in triplicate 25-μl PCR mixtures containing 3.5 mM $MgCl_2$, 200 μM dNTPs, and template DNA from each of 65 carious-dentine samples. PCR was carried out at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The threshold cycle (C_T) values were compared with a standard curve using *P. melaninogenica* DNA (82.9 fg to 829 pg), 400 nM *Prevotella* forward and reverse primers, and 200 nM *Prevotella* probe (16, 18). The amount of DNA measured for each cluster by real-time PCR was

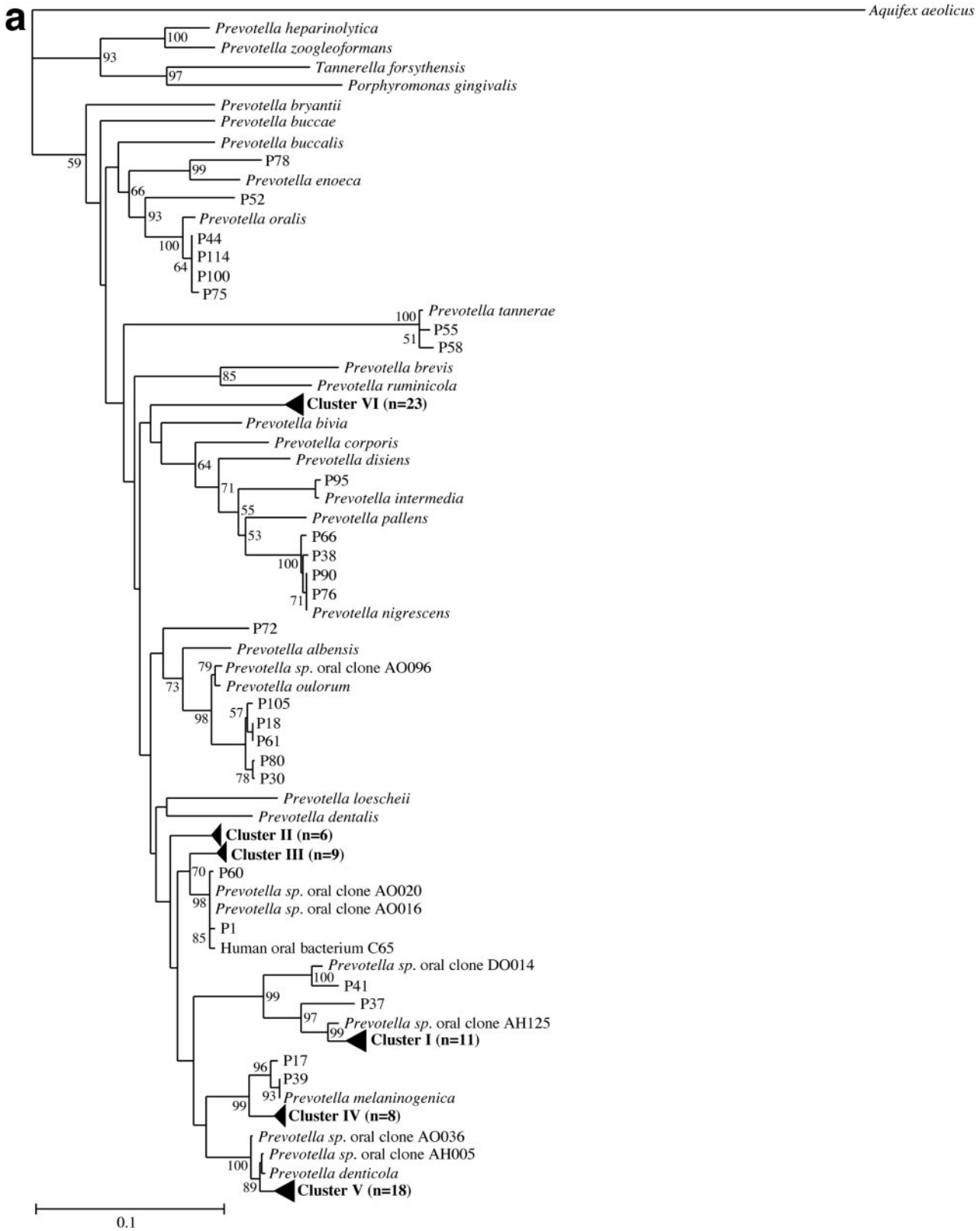

FIG. 1. Phylogenetic tree of *Prevotella*-like amplicons showing relation of clusters I to VI. (a) Distance tree of *Prevotella* clones amplified from pooled carious-dentine samples. The tree includes the sequence of *A. aeolicus* to root the tree, with *P. gingivalis* ATCC33277 (POYRR16SC) and *T. forsythensis* ATCC43037 (formally *B. forsythus* AB035460) as outgroups. The distance tree was constructed using the neighbor-joining method and was performed on an edited alignment of 369 bp from 16S rDNA. The tree was bootstrapped 100 times, and bootstrap values of >50 are shown at the branch nodes. The scale bar indicates the length of a branch that corresponds to 0.1 nucleotide changes per character. n, number of clones in each cluster. (b) Expansion of clusters I to VI shown in panel a. A representative of cluster VI, IDR-CEC-0032, sequenced to near full length (1,438 bp; accession no. AY550995), was included to anchor the cluster VI tree. Note that the scale bar in each panel is 10 times greater than that in panel a and indicates the length of a branch that corresponds to 0.01 nucleotide changes per character.

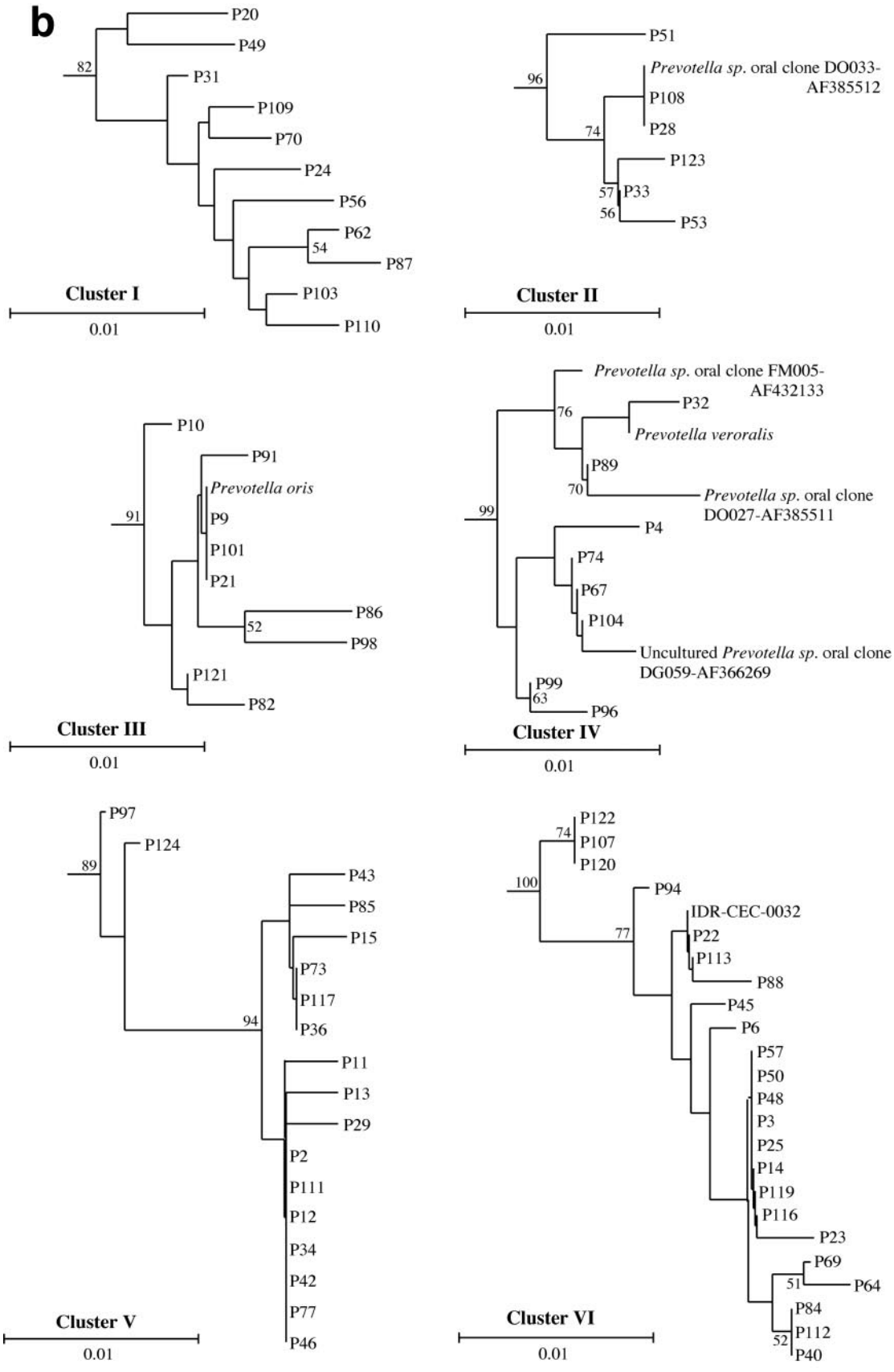

FIG. 1—*Continued*.

converted to theoretical cell numbers, assuming that each cell contains 2.37 fg of DNA (16). The optimized nanomolar concentrations of *Prevotella* forward primer–*Prevotella* probe–cluster-specific reverse primer were 200:200:200 for cluster I and cluster III, 400:100:400 for cluster II, 200:100:200 for cluster IV, 400:200:400 for cluster V, and 100:200:100 for cluster VI. The cluster-specific primers were each checked for specificity using two different chimeric plasmids containing different 16S rDNA inserts. Real-time PCR was performed on duplicate 25-μl samples containing 3.5 mM $MgCl_2$, 200 μM dNTPs, 400 nM *Prevotella* forward primer, 200 nM *Prevotella* probe, and 400 nM cluster-specific reverse primer. ABI 7700 and sequence detection software (version 1.6.3) supplied by Applied Biosystems were used for all real-time PCR analyses.

Nucleotide sequence accession numbers. The DNA sequences of the *Prevotella* species or phylotypes P1 to P124 have been deposited in the GenBank database under accession numbers AY678124 to AY678223, and a representative of cluster VI, IDR-CEC-0032, was sequenced to near full length (1,438 bp) and used to anchor members of cluster VI during phylogenetic analysis (Fig. 1b) under accession number AY550995.

TABLE 2. Specificity of *Prevotella* cluster-specific primers

Cluster-specific primer	Cross-reactivity with cluster-specific 16S rDNA (ΔC_T) for cluster[a]:					
	I	II	III	IV	V	VI
I	0	19	24	24	23	22
II	24	0	1	14	17	11
III	11	8	0	24	19	23
IV	13	12	19	0	9	17
V	8	6	13	8	0	11
VI	23	16	17	20	16	0

[a] The average ΔC_T values obtained by real-time PCR following the detection of plasmid-encoded 16S rDNA from clones P24 and P87 (cluster I), P51 and P33 (cluster II), P10 and P98 (cluster III), P4 and P89 (cluster IV), P15 and P34 (cluster V), and P94 and P69 (cluster VI) (cf. Fig. 2b) by each pair of cluster-specific primers varied by ≤1. The percent likelihood that a specific set of cluster-specific primers will detect the 16S rDNA is given by $100/2^{\Delta C_T}$.

RESULTS AND DISCUSSION

Phylogenetic analysis of *Prevotella* phylotypes identified in pooled dentine caries samples. One hundred fourteen amplicons generated from the pooled carious-dentine samples using the *Prevotella* forward and universal bacterial reverse primers were sequenced. Analysis of these 114 sequences showed that 14 were amplified from species of genera other than *Prevotella*. The distribution of these sequences included *Capnocytophaga sputigena* (one sequence; 98% similarity), *Capnocytophaga* sp. oral clone (one sequence; 95% similarity), uncultured β *Proteobacterium* (one sequence; 99% similarity), *Bacteroidetes* sp. oral clone FX069 (one sequence; 98% similarity), *Acidovorax* sp. (two sequences; 99% similarity), uncultured candidate division TM7 (one sequence; 99% similarity), *T. forsythensis* (formally *B. forsythus*; one sequence; 99% similarity), uncultured *Porphyromonas* sp. clone (one sequence; 98% similarity) and *Lactococcus lactis* (one sequence; 99% similarity). The remaining four sequences could not be identified.

Distance analysis showed that the remaining 100 *Prevotella* sequences grouped into multiple phylotypes (Fig. 1). Nine were identified as known species (>99% identity), and two were identical to *Prevotella* sp. oral clone DO033-AF385512 (Fig. 1b). Twenty-seven of the remaining 89 *Prevotella* clones were grouped into seven phylotypes, the members of which possessed identical DNA sequences. Five of these phylotypes (22 clones), along with 53 other clones, could be segregated into six major clusters containing closely related phylotypes (clusters I to VI) (Fig. 1). The six clusters were statistically significant, as the bootstrap values of the nodes leading to each of the clusters were >80% (Fig. 1b). The majority of the members of each of the first five of these clusters possibly represented a single species, as evidenced by their similarity to known oral clones. Thus, the members of cluster I showed 98% similarity to *Prevotella* sp. oral clone AH125; cluster II showed 99 to 100% similarity to *Prevotella* sp. oral clone DO033; cluster III showed 99% similarity to unidentified *Eubacterium* sp. clone 3.3 (data not shown; accession number UEU43698); cluster IV showed 99% similarity to *Prevotella* sp. oral clone BE073 and uncultured *Prevotella* sp. clone DG059; and cluster V showed 99 to 100% similarity to *Prevotella* sp. oral clone AH005 and *Prevotella* sp. oral clone AO036. In contrast, cluster VI was a numerically important and distinct group of closely related uncharacterized *Prevotella*-like species most closely related to an unidentified rumen bacterium (*Prevotella*-like; 93 to 94% similarity). The numerical dominance of this cluster in some carious-dentine lesions (see below) was particularly noteworthy, considering that the genus *Prevotella* is the most prominent and genetically diverse population in the bovine rumen and that these rumen *Prevotella*-like bacteria were considered to be unrelated to any known cultured *Prevotella* species found in the oral cavity prior to this study (22). A similar level of genetic diversity within the genus *Prevotella* has also been reported in the pig gastrointestinal tract (13), and environmental stress has been suggested to increase genetic diversity in some species (19).

Design and specificity of primers for quantifying *Prevotella* clusters using real-time PCR. To facilitate the quantitative analysis of the six major clusters of novel *Prevotella*-like bacteria, primers were designed for real-time PCR analysis of each cluster (Table 1). Using these primers, the size of each cluster-specific amplicon, as predicted from the sequence information (~270 bp), was confirmed by agarose gel electrophoresis (data not shown). The specificity of each of the cluster-specific primers was further validated using two chimeric plasmids containing the 16S rDNA from representative clones of each cluster as a template for real-time PCR.

Although the cluster-specific primers showed some cross-reactivity with DNA from other clusters, the interference was insignificant for quantitative analysis in all but one instance. Cluster II primers showed very strong cross-reactivity with cluster III, as only a twofold difference in the level of detection was obtained (a 1-C_T difference). However, cluster III primers were specific for cluster III (Table 2). No explanation for this observation was apparent. Altering the PCR variables, including Mg^{2+} and/or the annealing temperature, did not affect the results. As a consequence, the number (x) of *Prevotella* organisms belonging to cluster II was determined using the formula $x = z - 0.5y$, where z is the number of *Prevotella* organisms detected using cluster II primers and y is the number of *Prevotella* organisms detected using cluster III primers.

Quantification and distribution of *Prevotella*-like bacteria in carious dentine. All six *Prevotella*-like clusters were quantified in the 65 carious-dentine samples. For clarity, the 20 samples with mean quantities of *Prevotella*-like clusters below 0.1 cell ng of dentine^{-1} (range, 0.02 to 0.1 cell ng of dentine^{-1}) were deleted from the data displayed in Fig. 2. All six *Prevotella*-like clusters were represented in most carious-dentine samples.

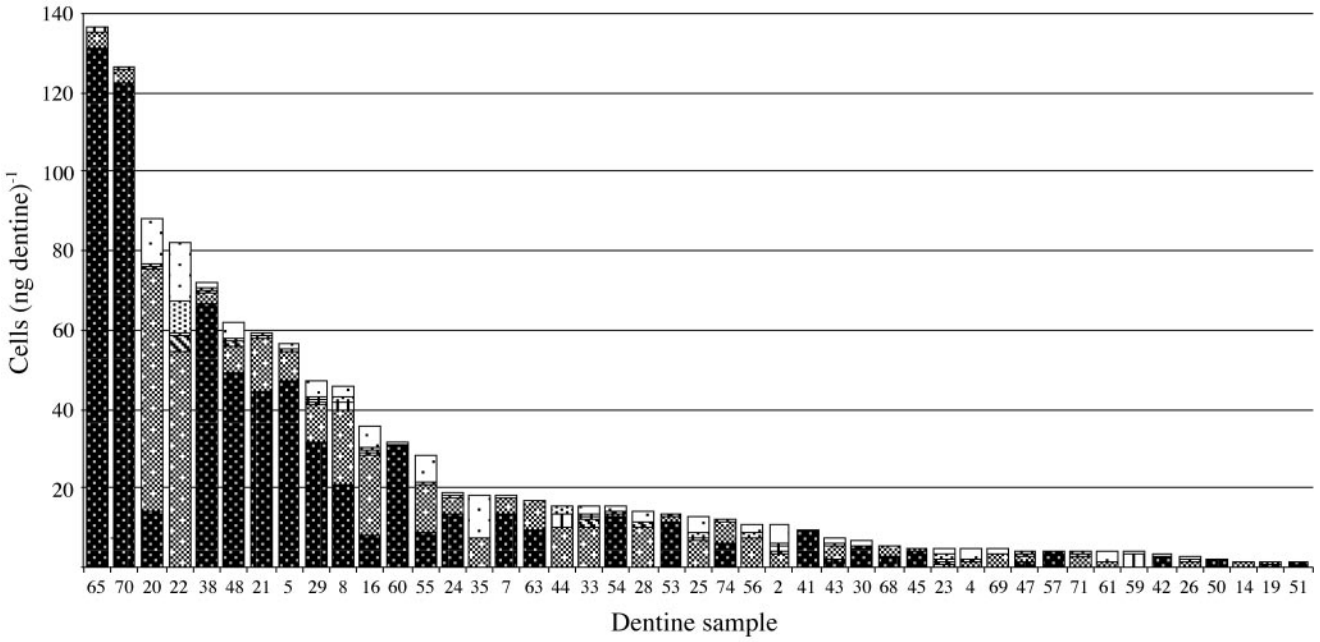

FIG. 2. Quantitative analysis of *Prevotella*-like clusters I to VI in carious-dentine samples. , cluster I; , cluster II; , cluster III; , cluster IV; , cluster V; and , cluster VI.

Cluster I (mean value, 1.5×10^6 cells mg [wet weight] of dentine^{-1}) and cluster IV (mean value, 2.9×10^5 cells mg [wet weight] of dentine^{-1}) were found in 96% (43 of 45) of the samples, cluster II (mean value, 3.8×10^5 cells mg [wet weight] of dentine^{-1}) was found in 82% (37 of 45) of the samples, cluster III (mean value, 2.9×10^5 cells mg [wet weight] of dentine^{-1}) was found in 91% (41 of 45) of the samples, cluster V (mean value, 4.9×10^6 cells mg [wet weight] of dentine^{-1}) was found in 100% (45 of 45) of the samples, and cluster VI (mean value, 1.1×10^7 cells mg [wet weight] of dentine^{-1}) was found in 76% (34 of 45) of the samples. Cluster I and cluster V were generally found in higher numbers than cluster II and III. However, even though cluster VI was detected in only 76% of the carious-dentine lesions, it was dominant in 53% (24 of 45) of the samples (Fig. 2).

Relationship to tissue response patterns. It was previously reported that the pooled *Prevotella* load in carious dentine did not correlate with tissue response patterns (16). The quantitative relation of the clusters of *Prevotella*-like bacteria was examined with respect to the predominant histological presentation in the pulp tissue. The results indicated that there were no strong trends for the association of clusters with the tissue response category (data not shown). It is probable that future refined cross-sectional analyses will yield complex patterns of association between the polymicrobial flora and the predominant pathological changes in the proximal underlying pulp tissue.

Concluding remarks. Although the six clusters appeared to represent the numerically dominant forms of *Prevotella*, a number of carious-dentine samples contained additional phylotypes (Fig. 1a). It is unclear why this environment is so favorable for colonization by diverse forms of this genus and whether these different phylotypes compete for the same environmental habitat. It is possible that the matrix of partially degraded dentine provides unique sites of attachment as a prerequisite for colonization by the bacteria. As *Prevotella* strains are only part of the complex polymicrobial flora present in carious dentine (10, 14, 16), it is also likely that both physical and metabolic interactions between different species promote this diverse range of *Prevotella* organisms. An unexpected finding was the numerical dominance of uncharacterized organisms that segregated primarily into the six clusters. By accepted criteria of relatedness, the numerically dominant cluster VI represents a novel genus, most closely related to uncharacterized *Prevotella*-like rumen bacteria (93 to 94% similarity). Within pooled carious-dentine samples, cluster VI segregated by distance analysis into at least six identifiable subclusters, suggesting a range of species (Fig. 1b). By implication, there is a closer association among *Prevotella*-like bacteria colonizing different mammalian hosts and different parts of the gastrointestinal tract than had previously been recognized.

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