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Application of *rpoB* and Zinc Protease Gene for Use in Molecular Discrimination of *Fusobacterium nucleatum* Subspecies▽

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***Fusobacterium nucleatum* is classified into five subspecies that inhabit the human oral cavity (*F. nucleatum* subsp. *nucleatum*, *F. nucleatum* subsp. *polymorphum*, *F. nucleatum* subsp. *fusiforme*, *F. nucleatum* subsp. *vincentii*, and *F. nucleatum* subsp. *animalis*) based on several phenotypic characteristics and DNA-DNA hybridization patterns. However, the methods for detecting or discriminating the clinical isolates of *F. nucleatum* at the subspecies levels are laborious, expensive, and time-consuming. Therefore, in this study, the nucleotide sequences of the RNA polymerase β-subunit gene (*rpoB*) and zinc protease gene were analyzed to discriminate the subspecies of *F. nucleatum*. The partial sequences of *rpoB* (approximately 2,419 bp), the zinc protease gene (878 bp), and 16S rRNA genes (approximately 1,500 bp) of the type strains of five subspecies, 28 clinical isolates of *F. nucleatum*, and 10 strains of *F. periodonticum* (as a control group) were determined and analyzed. The phylogenetic data showed that the *rpoB* and zinc protease gene sequences clearly delineated the subspecies of *F. nucleatum* and provided higher resolution than the 16S rRNA gene sequences in this respect. According to the phylogenetic analysis of *rpoB* and the zinc protease gene, *F. nucleatum* subsp. *vincentii* and *F. nucleatum* subsp. *fusiforme* might be classified into a single subspecies. Five clinical isolates could be delineated as a new subspecies of *F. nucleatum*. The results suggest that *rpoB* and the zinc protease gene are efficient targets for the discrimination and taxonomic analysis of the subspecies of *F. nucleatum*.**

Fusobacterium nucleatum is a Gram-negative spindle-shaped bacteria that may play an important role in periodontal disease (1). *F. nucleatum* has been reported to coaggregate with most oral bacteria, thereby acting as a bridge between the early colonizers (Gram-positive bacteria) and late colonizers (Gram-negative bacteria) (30). *F. nucleatum* can scavenge oxygen and oxidative free radicals from dental plaque, which can maintain or support the conditions needed for the major anaerobic periodontopathogens (4). *F. nucleatum* can modulate the secondary response of T cells to *Aggregatibacter actinomycetemcomitans*, which can help them survive the host immune system (35). *F. nucleatum* can invade the mucosal keratinocytes and induce proinflammatory cytokines and elastase (33). *F. nucleatum* produces butyric acid and metabolic end products that irritate the fibroblast of the gum (15).

F. nucleatum is classified into five subspecies that inhabit the human oral cavity (*F. nucleatum* subsp. *nucleatum*, *F. nucleatum* subsp. *polymorphum*, *F. nucleatum* subsp. *fusiforme*, *F. nucleatum* subsp. *vincentii*, and *F. nucleatum* subsp. *animalis*) based on the polyacrylamide gel electrophoresis pattern of the whole-cell proteins and DNA homology (6), glutamate dehydrogenase and 2-oxoglutarate reductase electrophoretic patterns, and DNA-DNA hybridization patterns (10, 11, 12). However, these methods are laborious, expensive, and time-consuming for use in the detection or discrimination of the clinical isolates of *F. nucleatum* at the subspecies level. Therefore, epidemiological studies of the relationship between the subspecies of *F. nucleatum* and periodontitis are limited.

It was reported that the beta subunit of the DNA-dependent RNA polymerase gene (*rpoB*) of *Escherichia coli* is composed of 1,342 amino acids and has nine variable regions (A to I) between the conserved regions (32). Similarly to the 16S rRNA genes, the nucleotide sequences of *rpoB* are well conserved among bacterial species in evolutionary aspects. Recently, *rpoB* was used to classify bacteria at the species or genus level (5, 20, 21, 22, 24).

A putative *F. nucleatum* subsp. *nucleatum*-specific DNA probe, Fu4, recently was cloned (25). The probe Fu4 (1,268 bp) is composed of the 5′ end of the partial deoxyuridine 5′-triphosphate nucleotidohydrolase (5′dUTPase) gene (273 bp

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TABLE 1. Strains used in this study

Species and strain[a]	KCOM/KCTC no.	Subject no.	Disease state (no. of tooth from which sample was isolated)	GenBank accession no.		
				16S rRNA	*rpoB*	Zinc protease
Fnn ATCC 25586^T			Cervico-facial lesion	GQ301038	GQ274958	GQ275046
Fnp ATCC 10953^T			Inflamed gingiva	GQ301039	GQ274957	GQ275045
Fnv ATCC 49256^T			Periodontal pocket	GQ301040	GQ274959	GQ275047
Fnf ATCC 51190^T			Sinusitis	GQ301041	GQ274960	GQ275048
Fna ATCC 51191^T				GQ301042	GQ274961	GQ275049
Fp ATCC 33693^T			Periodontitis	GQ301043	GQ274990	GQ275078
Fn ChDC F8	1231/5795	YB5	Healthy (35)	FJ471637	GQ274962	GQ275050
Fn ChDC F37	1232/5156	YB4	Gingivitis (35)	FJ471638	GQ274963	GQ275051
Fn ChDC F113	1248/5207	SJH1	Gingivitis (44)	FJ471639	GQ274964	GQ275052
Fn ChDC F119	1001/5459	SJH3	Healthy (36)	FJ471640	GQ274965	GQ275053
Fn ChDC F128	1249/5108	SJH4	Periodontitis (16)	FJ471641	GQ274966	GQ275054
Fn ChDC F130	1250/5165	SJH4	Periodontitis (21)	FJ471642	GQ274967	GQ275055
Fn ChDC F145	1253/5166	SJH5	Gingivitis (41)	FJ471643	GQ274968	GQ275056
Fn ChDC F174	1256/5111	SJH9	Gingivitis (41)	FJ471644	GQ274969	GQ275057
Fn ChDC F175	1002/5551	SJH9	Gingivitis (41)	FJ471645	GQ274970	GQ275058
Fn ChDC F186	1257/5168	SJH10	Gingivitis (36)	FJ471646	GQ274971	GQ275059
Fn ChDC F206	1258/5112	SJH13	Gingivitis (44)	FJ471647	GQ274972	GQ275060
Fn ChDC F218	1260/5113	SJH16	Gingivitis (41)	FJ471648	GQ274973	GQ275061
Fn ChDC F290	1267/5173	SJH25	Healthy (36)	FJ471649	GQ274974	GQ275062
Fn ChDC F300	1268/5174	HS20	Healthy (16)	FJ471650	GQ274975	GQ275063
Fn ChDC F305	1271/5175	HS24	Healthy (24)	FJ471651	GQ274976	GQ275064
Fn ChDC F306	1272/5116	HS26	Healthy (16)	FJ471652	GQ274977	GQ275065
Fn ChDC F309	1274/5688	HS21	Healthy (36)	FJ471653	GQ274978	GQ275066
Fn ChDC F310	1275/5210	YB-P2	Gingivitis (14)	FJ471654	GQ274979	GQ275067
Fn ChDC F311	1276/5176	YB-P2	Gingivitis (36)	FJ471655	GQ274980	GQ275068
Fn ChDC F313	1278/5211	PI9	Periimplantitis (46)	FJ471656	GQ274981	GQ275069
Fn ChDC F315	1279/5198	PI10	Periimplantitis (36)	FJ471657	GQ274982	GQ275070
Fn ChDC F316	1322/5674	PI11	Periimplantitis (38)	FJ471658	GQ274983	GQ275071
Fn ChDC F317	1323/5675	P8	Periodontitis (26)	FJ471659	GQ274984	GQ275072
Fn ChDC F318	1280/5199	P11	Periodontitis (23)	FJ471660	GQ274985	GQ275073
Fn ChDC F319	1281/5177	P11	Periodontitis (23)	FJ471661	GQ274986	GQ275074
Fn ChDC F324	1325/5796	HS35	Healthy (46)	FJ471662	GQ274987	GQ275075
Fn ChDC F330	1330/5477	HS40	Healthy (46)	FJ471663	GQ274988	GQ275076
Fn ChDC F332	1284/5676	SJB1	Periodontitis (36)	FJ471664	GQ274989	GQ275077
Fp ChDC F213	1259/5677	SJH15	Tongue	FJ471665	GQ274991	GQ275079
Fp ChDC F251	1261/5169	SJH20	Tongue	FJ471666	GQ274992	GQ275080
Fp ChDC F260	1262/5170	SJH21	Tongue	FJ471667	GQ274993	GQ275081
Fp ChDC F267	1263/5171	SJH22	Gingivitis (36)	FJ471668	GQ274994	GQ275082
Fp ChDC F312	1277/5197	YB-2	Gingivitis (37)	FJ471669	GQ274995	GQ275083
Fp ChDC F314	1321/5476	PI9	Periimplantitis (46)	FJ471670	GQ274996	GQ275084
Fp ChDC F320	1282/5202	P12	Periodontitis (36)	FJ471671	GQ274997	GQ275085
Fp ChDC F321	1283/5201	P13	Periodontitis (47)	FJ471672	GQ274998	GQ275086
Fp ChDC F334	2305/5678	HS44	Healthy (35)	FJ471673	GQ274999	GQ275087

[a] ATCC, American Type Culture Collection; KCOM, Korean Collection for Oral Microbiology; KCTC, Korean Collection for Type Cultures; Fnn, *F. nucleatum* subsp. *nucleatum*; Fnp, *F. nucleatum* subsp. *polymorphum*; Fnv, *F. nucleatum* subsp. *vincentii*; Fnf, *F. nucleatum* subsp. *fusiforme*; Fna, *F. nucleatum* subsp. *animalis*; Fn, *Fusobacterium nucleatum*; Fp, *Fusobacterium periodonticum*.

out of 441) and the 3′ end of the partial zinc protease gene (878 bp out of 1,227 bp). This probe could identify *F. nucleatum* subsp. *nucleatum* at the subspecies level by restriction fragment length polymorphism (RFLP). *F. nucleatum* subsp. *nucleatum*-specific PCR primers were designed based on the nucleotide sequence of Fu4. However, the PCR primers could amplify the target genes from all of the clinical isolates of *F. polymorphum* as well as *F. nucleatum* tested (unpublished data). These results suggest that the zinc protease and 5′-dUTPase genes are conserved in *F. nucleatum* and *F. polymorphum*.

In this study, an attempt was made to discriminate the subspecies of *F. nucleatum* by comparing the nucleotide sequences of *rpoB* (approximately 2,419 bp out of 3,355 bp) and the zinc protease gene (878 bp). Since the 5′-dUTPase gene in the Fu4 DNA probe is relatively small compared to those of *rpoB* and the zinc protease gene, it was not included in this study.

MATERIALS AND METHODS

Bacterial isolation and strains. A total of 6 *Fusobacterium* type strains and 37 *Fusobacterium* clinical isolates were used in this study (Table 1). The type strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA). All clinical strains were isolated from the subgingival plaque or tongue of 32 subjects using a *F. nucleatum* selective medium (36) and were identified at the species level using the comparison method of 16S rRNA gene sequences. They were cultivated in Schaedler broth (Difco Laboratories, Detroit, MI) at 37°C for 48 h in an anaerobic chamber (Bactron I; Sheldon Manufacturing, Cornelius, OR) in a 10% H_2, 10% CO_2, and 80% N_2 atmosphere. The protocols of this study were approved by the Chosun University Institutional Research Board.

Bacterial genomic DNA preparation. The bacterial genome was prepared using a G-spin genomic DNA extraction kit (iNtRON Co., Seoul, Korea) according to the manufacturer's instructions. The DNA concentrations were de-

termined by UV spectrophotometry (Ultrospec 2000; Pharmacia Biotech, Cambridge, United Kingdom) at wavelengths of 260 and 280 nm.

PCR amplification of 16S rRNA genes, *rpoB*, and zinc protease genes. 16S rRNA genes or the zinc protease gene from the bacteria were amplified by PCR using the primers 27F and 1492R (27) or Fu4-F38 (5′-TTC TCC TCT ATA ATC ACT GTC AAC-3′) and Fu4-R1189 (5′-GTA TAG AAA AAG AAA GAA ATG TGA-3′), respectively. The primers for amplifying the 2,440 bp of the *rpoB* sequence was designed based on the nucleotide sequences of *rpoB* of *F. nucleatum* ATCC 25586^T (GenBank accession number AE009951) and *F. nucleatum* ATCC 10953^T (GenBank accession number NW-002062357). To reduce errors in nucleotide fidelity during the PCR amplification of *rpoB*, three sets of PCR primers were designed and amplified as three fragments. The 5′ end and 3′ end of the amplicon of the second PCR primer set was overlapped with the 3′ end of the first PCR amplicon and the 5′ end of the third one, respectively. The primer names and sequences were (i) Fn-RpoB-F1 (5′-CTK GAT GAA GAA ACA GGA GAR T-3′) and Fn-RpoB-R1 (5′-AGT AGC AAG YGA YCC AAT AAG T-3′), (ii) Fn-RpoB-F2 (5′-AAC ACC AGA AGG ACC AAA YAT T-3′) and Fn-RpoB-R2 (5′-ATA TCY CCY GGT CCT ACT TCT G-3′), and (iii) Fn-RpoB-F3 (5′-ATA TGA RAT WGA TGC AAG AAC TAC A-3′) and Fn-RpoB-R3 (5′-AGC TTC YAA TGC CCA AAC T-3′). PCR was carried out using an AccuPower PCR PreMix (Bioneer Corp., Korea), which contained 5 nmol each deoxynucleoside triphosphate, 0.8 μmol KCl, 0.2 μmol Tris-HCl (pH 9.0), 0.03 μmol $MgCl_2$, and 1 U of *Taq* DNA polymerase. The bacterial genomic DNA and 20 pmol each primer then were added to a PCR PreMix tube. PCR was carried out in a final volume of 20 μl. The PCR was run for 30 cycles on a Peltier thermal cycler (Model PTC-200 DNA Engine; MJ Research Inc.). The PCR conditions for amplifying the 16S rRNA gene were the same as those described elsewhere (2). The PCR conditions for amplifying the *rpoB* and zinc protease genes were as follows: denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72°C for 1 min. The final cycle included an additional extension time of 10 min at 72°C. A 2-μl aliquot of the reaction mixture was analyzed by 1.5% agarose gel electrophoresis in a Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.0]) at 100 V for 30 min. The amplification products were stained with ethidium bromide and visualized by UV transillumination.

Cloning and sequencing of the genes. The PCR products were purified using an AccuPrep PCR purification kit (Bioneer Co., Daejeon, Korea) and ligated directly using a pGEM-T easy vector (Promega Corp., Madison, WI). Nucleotide sequencing was carried out using the dideoxy chain termination method with a Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The primers ChDC-GEM-F (5′-TTC CCA GTC ACG ACG TTG TAA AA-3′) and ChDC-GEM-R (5′-GTG TGG AAT TGT GAG CGG ATA AC-3′) (37) were used for the nucleotide sequencing of the 16S rRNA genes, *rpoB*, and zinc protease gene. The additional sequencing primers for 16S rRNA genes were Seq-F1 (5′-CCT ACG GGA GGC AGC AG-3′), Seq-R2 (5′-GAC TAC CAG GGT ATC TAA TCC-3′) (23), and F16 (5′-TAG ATA CCC YGG TAG TCC-3′) (29). The additional sequencing primer for the zinc protease genes was All-Fu4-Seq1 (5′-TAT CTC CTA CTA TTG TTT GTG A-3′). All sequences were compared to a similar sequence of a reference organism using the BLAST program (a genome database of the National Center for Biotechnology Information).

Phylogenetic analysis. Multiple sequences were aligned using the CLUSTAL W algorithm in the MegAlign program (Lasergene 8.0; DNAStar, Inc., Madison, WI). The alignments were refined against sequences of a representative of the genus *Fusobacterium* retrieved from the GenBank database by a visual inspection (2). The sequence similarities were calculated using the MegAlign program (Lasergene 8.0; DNAStar, Inc.). Phylogenetic analyses were performed by applying the distance matrix, Fitch-Margoliash, maximum-parsimony, and neighbor-joining methods using the PHYLIP program package (8). Evolutionary distances were calculated according to the Jukes and Cantor model (16). The phylogenetic trees were constructed by the maximum-parsimony (9) and neighbor-joining (31) methods. The stability of the resulting trees was assessed by the bootstrap analysis (7) of the neighbor-joining method based on 1,000 resamplings.

Nucleotide sequence accession numbers. The 16S rRNA gene, *rpoB*, and zinc protease gene sequences determined in the course of this work were deposited in GenBank and are listed in Table 1.

RESULTS

Identification of the clinical isolates of *F. nucleatum* and *F. periodonticum* by 16S rRNA gene sequence analysis. All *Fusobacterium* isolates were obtained from a single colony grown on an *F. nucleatum* selective medium at 37°C for 48 h under anaerobic conditions. A total of 37 isolates were identified as being *F. nucleatum* (28 strains) or *F. periodonticum* (9 strains) using 16S rRNA gene sequence analysis (Table 1). The similarities of the 16S rRNA gene sequences between the clinical isolates and five subspecies type strains of *F. nucleatum* were 98.5 to 99.8%. From two of the subjects (YB-P2 and PI9), both *F. nucleatum* and *F. periodonticum* strains were isolated; both strains from the same subgingival dental plaque of one subject (PI9) and from different subgingival dental plaques from another subject (YB-P2) (Table 1).

The 16S rRNA gene-based tree yielded two groups of clusters with a bootstrap value of 92%; group 1 contained *F. nucleatum* subsp. *polymorphum* (C1), the nonclassified cluster (C5), and *F. periodonticum* (C6), and group 2 contained *F. nucleatum* subsp. *nucleatum* (C2), *F. nucleatum* subsp. *fusiforme*/*F. nucleatum* subsp. *vincentii* (C3), and *F. nucleatum* subsp. *animalis* (C4) (Fig. 1A). All *Fusobacterium* strains among these two cluster groups showed more than 98.1% 16S rRNA gene similarity. Although this tree could not delineate the *F. nucleatum* groups from *F. periodonticum*, all of the clinical strains were grouped as one of the known *F. nucleatum* subspecies or as *F. periodonticum*, except for cluster C5.

A comparison of the 16S rRNA gene variable sequence regions showed that the strains in clusters C1, C5, and C6 had an 18-nucleotide deletion between bases 70 and 87, and the strains in cluster C2 have a 4-nucleotide deletion between bases 79 and 83 corresponding to the nucleotide sequences of *Escherichia coli* 16S rRNA genes (GenBank accession no. 3DG5_A) (Table 2). The clusters of the C3 and C4 strains had a single-nucleotide (T) deletion compared to the sequence of C2 (Table 2).

Phylogenetic analysis of *F. nucleatum* subspecies based on the nucleotide sequences of *rpoB* and the zinc protease gene. The nucleotide sequences of *rpoB* and the zinc protease gene were determined and analyzed to discriminate the subspecies of *F. nucleatum*. The sequence similarities of the *rpoB* and zinc protease genes between the six types or representative strains of the *F. nucleatum* groups were 92.0 to 99.1% (mean, 94.4%) and 89.7 to 99.7% (mean, 92.0%), respectively (Fig. 1B and C). Their similarities were significantly lower than that of the 16S rRNA gene sequence (98.1 to 99.6%; mean, 98.7%). It was clear that the base substitution rates for both genes were much faster than that of 16S rRNA genes. Additionally, these marker genes showed significantly higher genetic variations than the 16S rRNA genes according to comparative sequence analyses. The two gene sequences showed remarkable discrimination in this group. At the interspecies level, *F. nucleatum* and *F. periodonticum*, the sequence divergences of *rpoB* and the zinc protease gene were 10.2 to 11.7% and 12.4 to 13.2%, respectively (Fig. 1). At the subspecies level of the *F. nucleatum* group, the sequence divergences of *rpoB* and the zinc protease gene were 4.3 to 8.5% (mean, 6.3%) and 6.7 to 10.9% (mean, 9.0%), respectively (Fig. 1). Their substitution rates were significantly higher than that of 16S rRNA genes (0.6 to 1.9%; mean, 1.3%) (Fig. 1). Only cluster C5 harbored no type strain. *F. nucleatum* subsp. *vincentii* and *F. nucleatum* subsp. *fusiforme* were not distinguished from one another by the sequence analysis of *rpoB* and the zinc protease gene sequences due to their

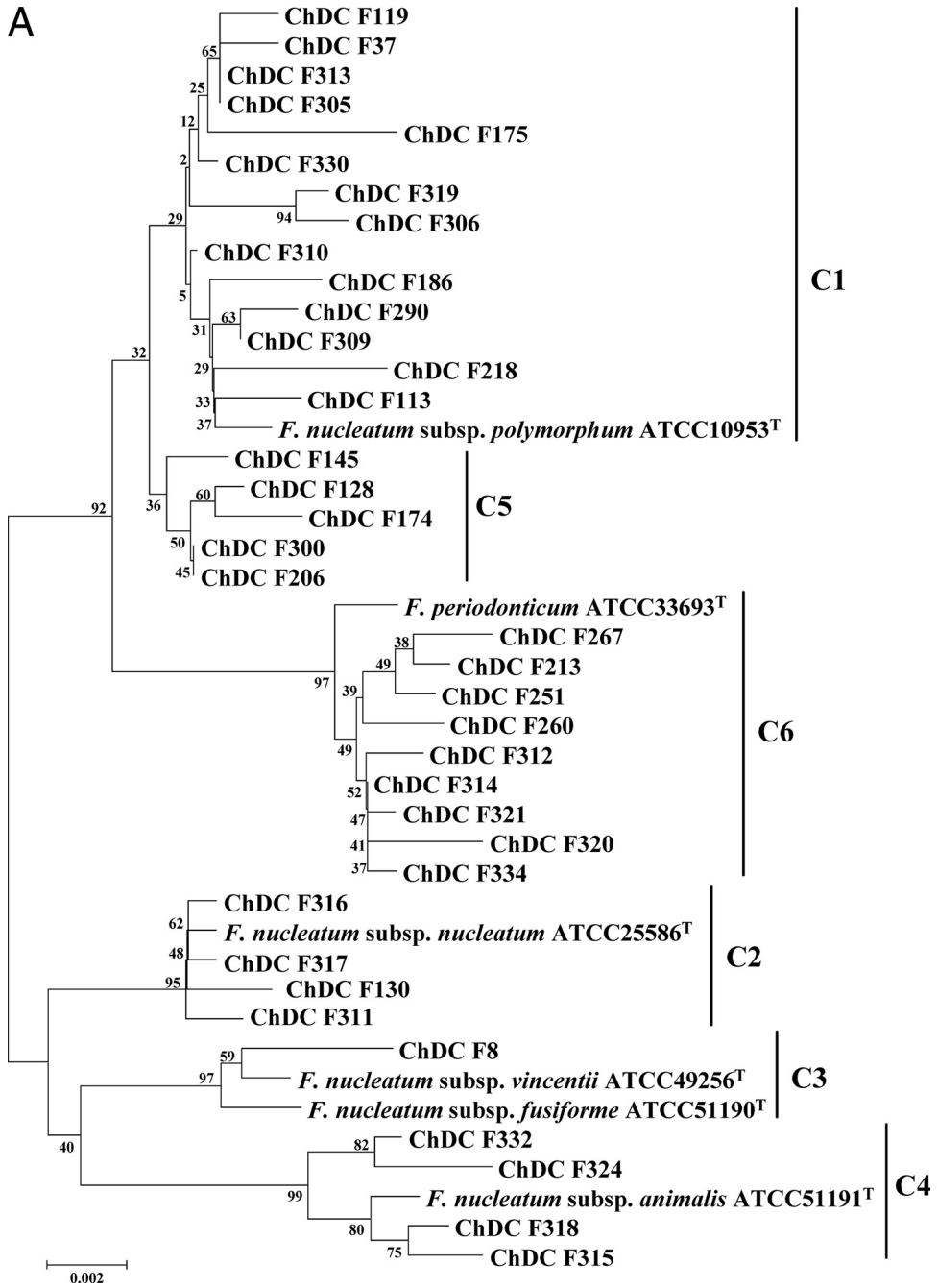

FIG. 1. Phylogenetic trees based on the partial nucleotide sequences of (A) 16S rRNA genes (about 1.5 kb), (B) *rpoB* (about 2,419 bp out of 3,555 bp), and (C) the zinc protease gene (878 bp out of 1,227 bp) of type strains and clinical isolates of *Fusobacterium nucleatum* and *Fusobacterium periodonticum*. The resulting tree topology was evaluated by bootstrap analyses of the neighbor-joining tree based on 1,000 resamplings.

high similarity (>99.0%). From this study, pairwise analyses indicated that the two gene sequences are more discriminatory than those of the other genes for species or subspecies differentiation in the group.

The phylogenetic tree compiled from the *rpoB* sequences showed that all of the *Fusobacterium* strains could be separated clearly into six distinct clusters with high bootstrap values (100%) (Fig. 1B). The zinc protease gene sequence-based phylogenetic analysis was highly consistent (bootstrap values, >98%) with results obtained with *rpoB* sequences (Fig. 1C). The phylogenetic trees also delineated the *F. nucleatum* groups (C1, C2, C3, C4, and C5) from *F. periodonticum* (C6) by deep branches (Fig. 1B and C). Except for C5, all clusters appeared to correspond to one of the known *F. nucleatum* subspecies or to *F. periodonticum*. Within the *F. nucleatum* group, the gene sequences also were clearly separated into five distinct clusters

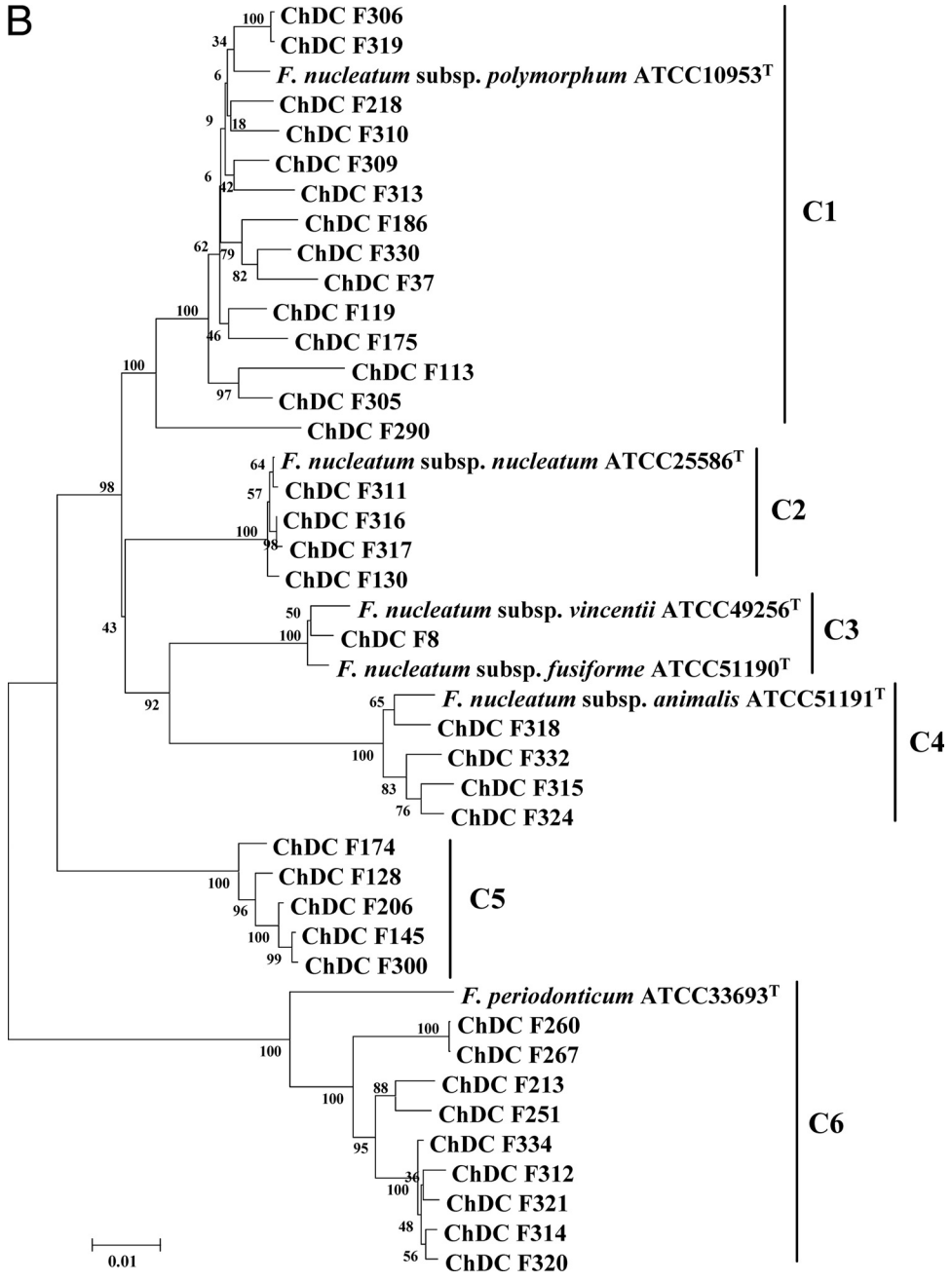

FIG. 1—*Continued*.

with high bootstrap values (>98%). All five clusters harbored identical members that were grouped by the two gene sequences. Of the *F. nucleatum* groups, all strains of clusters C2, C3, and C5 formed a monophyletic clade with 100% bootstrap support. Only cluster C5 harbored no type strain and formed a monophyletic clade with 100% bootstrap support; *rpoB* and the zinc protease gene similarities among them were 98.9 to 99.7% (mean, 99.3%) and 97.9 to 100% (mean, 99.1%), respectively (Table 3). This suggests a new subspecies candidate in this group. The stability of the resulting trees was confirmed by the maximum-parsimony algorithms and supported by a 97% bootstrap value, except for C1, which had a bootstrap value of 83%.

DISCUSSION

F. nucleatum, an inhabitant of the human oral cavity, is classified into five subspecies based on several phenotypic characteristics and DNA-DNA hybridization patterns. The methods for detecting or discriminating the clinical isolates of *F. nucleatum* at the subspecies level are laborious, expensive, and time-consuming. Therefore, a rapid and reliable method is needed to examine the relationships between subspecies and periodontal diseases. Therefore, in this study, the nucleotide sequences of *rpoB* and the zinc protease gene were analyzed to discriminate the subspecies of *F. nucleatum*.

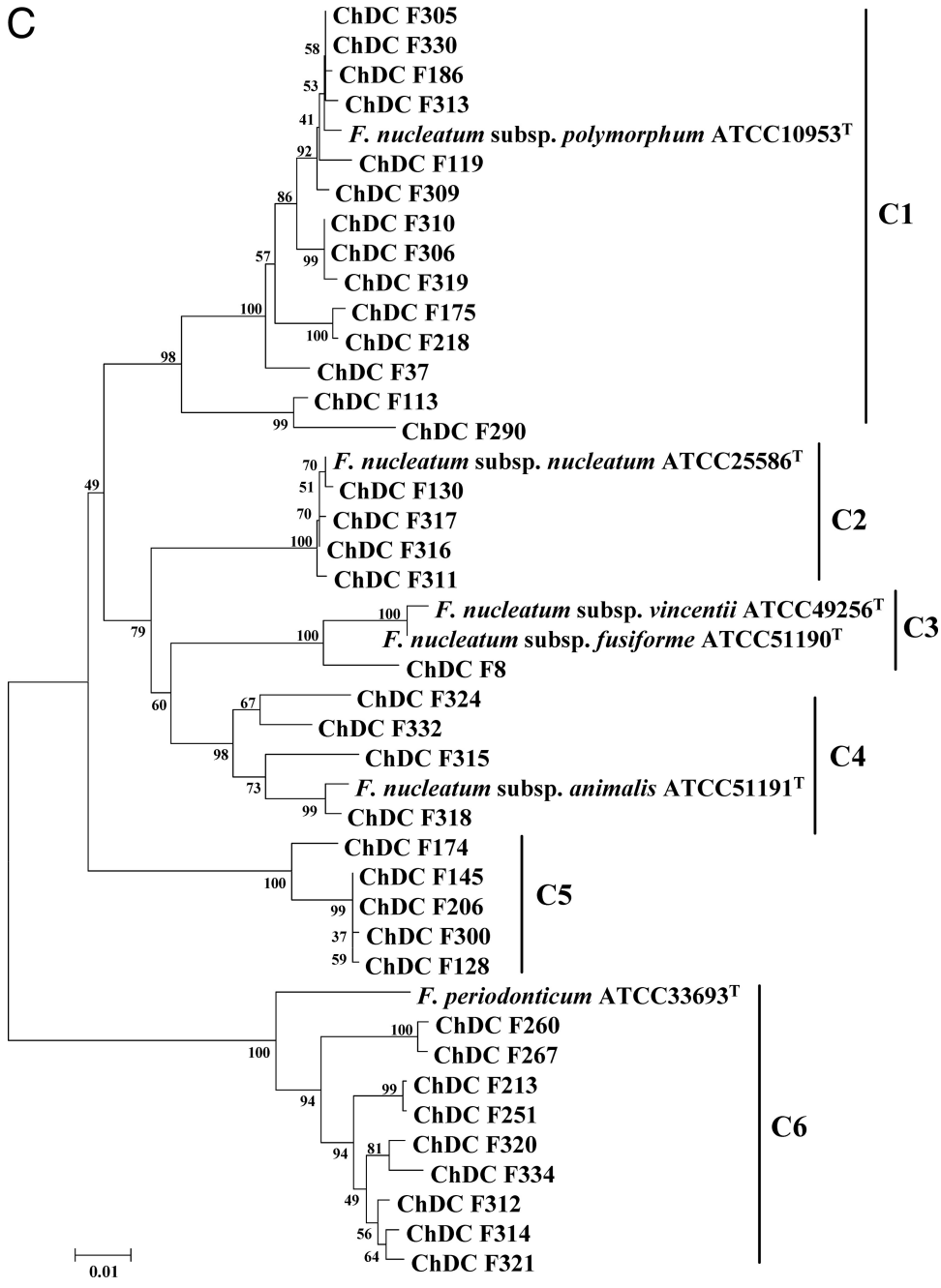

FIG. 1—*Continued*.

These results showed that the topology of the two trees made by analyzing both *rpoB* and the zinc protease gene was identical and clearly delineated the subspecies of *F. nucleatum* (Fig. 1). The clades of the resulting trees also were confirmed by other treeing algorithms and supported by the high bootstrap value (>97%). The sequence diversity between the subspecies of the cluster of zinc protease was 1.5 times higher than that of *rpoB*. In addition, the size of the zinc protease gene was 2.7 times smaller than that of *rpoB*. These findings suggest that *rpoB* and the zinc protease gene are useful markers in the phylogenetic discrimination of *F. nucleatum* at the subspecies level. Furthermore, the zinc protease gene could be a better molecular marker than *rpoB* for a study of taxonomic relationships at the subspecies level in *F. nucleatum*.

Strauss et al. (34) used 16S rRNA genes (bp 52 to 868; 817 bp) and the *rpoB* sequence (bp 2757 to 3257; 501 bp) to classify the clinical isolates of *F. nucleatum* and *F. periodonticum* from the human gut. In their study, the 16S rRNA gene-based tree and *rpoB*-based tree had the same topology. Our data showed that all five clusters harbored identical members of *F. nucleatum* strains grouped by 16S rRNA genes and *rpoB*, but the topology of the phylogenetic tress based on the 16S rRNA

TABLE 2. Alignment of the selected 16S rRNA gene regions of type strains and clinical isolates of *F. nucleatum* and *F. periodonticum*

Species or cluster	Nucleotide at position[a]:																					
	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Escherichia coli	G	T	A	A	C	A	G	G	A	A	G	A	A	G	C	T	T	G	C	T	T	C
C2	G	A	A	T	T	T	G	G	G	T					T	T	T	T	T	A	A	C
C3	G	A	A	T	T	T	G	G	G	T					T	T	T	T		A	A	C
C4	G	A	A	T	T	T	G	G	G	T					T	T	T	T		A	A	C
C1	G																			A	A	C
C5	G																			A	A	C
C6	G																			A	A	C

[a] Corresponding to positions in the *E. coli* 16S rRNA gene (GenBank accession no. 3DG5_A).

genes and *rpoB* was different (Fig. 1). This was attributed to the longer sizes of the nucleotide sequences of 16S rRNA genes (approximately 1,440 bp) and *rpoB* (2,419 bp) than those used by Strauss et al. (34). Because the first 500 bp of the 16S rRNA gene region is the most variable among bacteria, this region can be used to identify bacterial species. However, in our experience, the BLAST search data sometimes showed that the results obtained using the total 16S rRNA gene sequence (approximately 1,500 bp) are different from those obtained using the first 500 bp of 16S rRNA genes. Since the 16S rRNA genes have several conserved and variable regions, it appears that all variable regions should be included for more precise analysis.

In this study, the 16S rRNA gene analysis results showed a variable region between the clusters (Table 2). It was reported that 16S rRNA gene variability occurred mainly within the five regions of the gene in *F. nucleatum* (14). One of them, the region between base 77 and 92, was different compared to the data in this study, even though the regions are quite similar. The main reason for this discrepancy may be due to the difference in the analysis tools used.

DNA-DNA hybridization and a comparison of the 16S rRNA gene sequences are the gold standard methods for classifying bacteria at the species level (26, 2001). Two strains are considered to be the same species if they have 70% or higher relatedness by DNA-DNA hybridization and >97% region homology of the nucleotides of 16S rRNA genes. This is because the DNA-DNA hybridization technique is difficult to apply to identify all of the clinical strains at the species level. Therefore, 16S rRNA genes generally are used for this purpose. The results showed that *F. nucleatum* was not delineated from *F. periodonticum* by the 16S rRNA gene sequence analysis, even though the clinical isolates and type strains of *F. nucleatum* were separated clearly from the *F. periodonticum* group (Fig. 1). According to the analysis of *rpoB* and the zinc protease gene, the *F. nucleatum* group was clearly discriminated from the *F. periodonticum* group. These results suggest that the *rpoB* and zinc protease gene rather than 16S rRNA genes can be useful in the identification of *F. nucleatum* at the species level.

TABLE 3. Percent identity between strains of *F. nucleatum* in the same cluster

Cluster	% Identity[a] (means)		
	16S rRNA gene	*rpoB*	Zinc protease gene
C1	98.9–100 (99.5)	95.4–99.9 (97.9)	93.2–100 (97.5)
C2	99.7–99.9 (99.8)	99.6–99.9 (99.8)	99.5–99.9 (99.8)
C3	99.2–99.4 (99.3)	99.1–99.3 (99.2)	96.8–99.7 (97.9)
C4	99.2–99.7 (99.5)	98.3–99.2 (98.7)	95.8–99.3 (96.8)
C5	99.5–100 (99.8)	98.9–99.7 (99.3)	97.9–100 (99.1)

[a] Sequence similarities were calculated using MegAlign (Lasergene 8.0; DNAStar, Inc.).

Recently, the genomes of the type strains corresponding to three subspecies (*F. nucleatum* subsp. *nucleatum*, *F. nucleatum* subsp. *vincentii*, and *F. nucleatum* subsp. *polymorphum*) of *F. nucleatum* were sequenced completely or incompletely and compared to the data for *F. nucleatum* subsp. *polymorphum* (17–19). According to the analysis, 919 genes account for the differences between the three strains. It appears that the differences between the three type strains are relatively high. A species can be divided into two or more subspecies based on consistent phenotypic variations or genetically determined clusters of strains within the species (26). Currently, there are no guidelines for the establishment of a subspecies; the subspecies usually are determined according to the investigator's choice (26). Therefore, this study attempted to genetically discriminate the subspecies of *F. nucleatum* by analyzing the nucleotide sequences of the *rpoB* and zinc protease gene.

rpoB of *F. nucleatum* is composed of 3,555 nucleotides. The *rpoB* regions of *F. nucleatum* used in this study contain seven variable regions (C to I) corresponding to those of *E. coli*. According to this data, region C of *F. nucleatum* is the most variable compared to that of *E. coli* (data not shown). Therefore, this region can be used to identify bacteria at the species or subspecies level.

The zinc protease gene used to identify *F. nucleatum* at the subspecies level was the gene encoding the DNA probe Fu4, originating from the genomic DNA of *F. nucleatum* subsp. *nucleatum* ATCC 25586^T (25); the genomic DNA nucleotide sequence of this strain shows that it can produce only three amino acids (glutamate, aspartate, and asparagine) and several amino acids or peptide transporter proteins (18). Considering this, it appears that the zinc protease of *F. nucleatum* plays a role in dissociating proteins or peptides within the dental plaque matrix, making them available in their cytoplasm.

In this study, *F. nucleatum* subsp. *vincentii* and *F. nucleatum* subsp. *fusiforme* could not be distinguished from one another. This is in agreement with previous reports, in that *F. nucleatum* subsp. *vincentii* is genetically similar to *F. nucleatum* subsp. *fusiforme* (3, 13, 14, 34). Considering these findings, these two subspecies can be classified into a single subspecies.

These results showed that *F. nucleatum* subsp. *polymorphum*

is also the most frequently isolated subspecies in the Korean oral cavity. It was reported that the isolation ratio of *F. nucleatum* subsp. *vincentii*, *F. nucleatum* subsp. *nucleatum*, and *F. nucleatum* subsp. *polymorphum* in the gingival crevice was 7:3:2 (28). It also was reported that *F. nucleatum* subsp. *nucleatum* is isolated mostly in the periodontal disease sites and is the subspecies isolated most frequently, whereas *F. nucleatum* subsp. *polymorphum* and *F. nucleatum* subsp. *fusiforme* are isolated from healthy sites but rarely in the oral cavity (13). The difference in the isolation frequency of the subspecies of *F. nucleatum* from these studies may result from either host foods being consumed in various geographical ethnic groups, the detection methods for the subspecies, or the small sampling sizes employed. Therefore, rapid and concise detection methods need to be developed for the epidemiological studies of the periodontal diseases associated with the subspecies of *F. nucleatum*.

F. nucleatum subsp. *animalis* originally was isolated from animal colons (10). It was reported that *F. nucleatum* subsp. *animalis* is isolated more frequently from the gut than the oral cavity in humans (34). In the present study, *F. nucleatum* subsp. *animalis* also was isolated at a frequency similar to that of *F. nucleatum* subsp. *nucleatum* in Korean oral cavities. In a future study, the isolation frequency of *F. nucleatum* subsp. *animalis* from the gut and oral cavity from Koreans will be determined and compared to data from that previous report (34).

In this study, two different subspecies of *F. nucleatum* were isolated from the same subjects, SJH4, SJH9, YB-P2, and P11 (Table 1). Strauss et al. (34) also reported the isolation of two subspecies of *F. nucleatum* from the mouth of the same patient. The reason for the appearance of multiple subspecies is unclear, but it may be due to the horizontal transfer of the different subspecies between people, such as between husband and wife or between parent and child. Further studies will be needed to confirm this hypothesis.

In conclusion, the 16S rRNA gene sequence is extremely limited in discriminating both the species and subspecies in the *F. nucleatum* group. Comparative sequence analysis showed that *rpoB* and the zinc protease gene of *F. nucleatum* showed significantly higher genetic variations than did 16S rRNA genes. These results strongly suggest that *rpoB* and the zinc protease gene can be a useful alternative to DNA-DNA hybridization, SDS-PAGE protein profiles, and limited allozyme analysis for the identification and phylogenetic analysis of *F. nucleatum* at the subspecies level.

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