Fusobacterium hwasookii sp. nov., Isolated from a Human Periodontitis Lesion

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Abstract In this study, we classified the five strains (ChDC F128[T], ChDC F145, ChDC F174, ChDC F206, and ChDC F300) as a novel species of genus *Fusobacterium* by DNA–DNA hybridization and multi-locus phylogenetic analysis (MLPA), based on a single sequence (24,715 bp) of 22 concatenated housekeeping genes, with morphological and chemotaxonomic characteristics. DNA–DNA hybridization data showed that the values of genomic relatedness between ChDC F128[T] and each of the other novel strains were ranged from 79.0 to 82.6 %, while those of genomic relatedness between ChDC F128[T] and type strain of each of subspecies of *F. nucleatum* or *Fusobacterium periodonticum* were ranged from 40.9 to 54.4 %. MLPA revealed that the 5 strains were clustered as one group and clearly discriminated with *F. nucleatum* and *F. periodonticum* with 100 % bootstrap value. The DNA G+C content of the five novel strains were ranged from 26.9 to 27.0 mol%. The cellular fatty acid analysis of clinical isolates and type strains revealed $C_{14:0}$, $C_{16:0}$, and cis-9 $C_{16:1}$ as the major fatty acids. The cell wall peptidoglycan of the 5 strains was comprised of meso-lanthionine. These results show that the 5 strains are novel species and belong to the genus *Fusobacterium*. Strain ChDC F128[T] (=KCOM 1249[T] = KCTC 5108[T] = JCM 30218[T]) is suggested to be the type strain of a novel species of genus *Fusobacterium*, for which the name *Fusobacterium hwasookii* sp. nov. is proposed.

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Introduction

Fusobacterium nucleatum is a gram-negative, anaerobic, fusiform-shaped bacterium and is normally isolated from the oral cavity in human [13]. *F. nucleatum* may play an important role in dental plaque formation, acting as a bridge of early colonizers such as gram-positive, facultative anaerobes, and late colonizers such as gram-negative, strict anaerobes [1, 2]. *F. nucleatum* was classified into 5 subspecies (*nucleatum*, *polymorphum*, *vincentii*, *animalis*, and *fusiforme*) based on the polyacrylamide gel electrophoretic pattern of the whole-cell proteins and DNA homology [3] or electrophoretic patterns of glutamate dehydrogenase and 2-oxoglutarate reductase and DNA–DNA hybridization patterns [5, 6]. *F. nucleatum* subsp. *fusiforme* has been reclassified into the *F. nucleatum* subsp. *vincentii* based on phylogenetic analysis using a single sequence (24,715 bp) of concatenated 22 housekeeping genes of 8 *F. nucleatum* strains, including type strains of 5 *F. nucleatum* subspecies [12]. Recently, 5 strains (ChDC F128[T], ChDC F145, ChDC F174, ChDC F206, and ChDC F300) of *F. nucleatum* were isolated from subgingival

plaques of Korean patients and a novel *F. nucleatum* sub-species has been suggested, based on the comparison of nucleotide sequences of RNA polymerase beta subunit gene (*rpoB*) and zinc protease gene [10]. However, these results were not adequate to classify the 5 novel strains as a novel subspecies of *F. nucleatum* or a novel species of the genus *Fusobacterium*. Based on the phylogenetic, bio-chemical, and chemotaxonomic findings presented here, the 5 novel strains were found to represent a novel species of the genus *Fusobacterium*.

Materials and Methods

Five clinical strains, ChDC F128^T (=KCOM 1249^T = KCTC 5108^T = JCM 30218^T), ChDC F145 (=KCOM 1253 = KCTC 5166 = JCM 30219), ChDC F174 (=KCOM 1256 = KCTC 5111 = JCM 30220), ChDC F206 (=KCOM 1258 = KCTC 5112 = JCM 30221), and ChDC F300 (=KCOM 1268 = KCTC 5174 = JCM 30222) as well as comparative reference type strains, *F. nucleatum* subsp. *polymorphum* ATCC 10953^T, *F. nucleatum* subsp. *vincentii* ATCC 49256^T, *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586^T, *F. nucleatum* subsp. *animalis* ATCC 51191^T, and *F. periodonticum* ATCC 33693^T were obtained from Korean Collection for Oral Microbiology (KCOM, Korea) or American Type Culture Collection (ATCC, USA). All strains were cultured and maintained in brain heart infusion (BHI, Difco Laboratories, USA) broth or on BHI agar containing sheep blood and incubated at 37 °C in an anaerobic chamber (Model Bactron I, USA) with an atmosphere of 10 % H_2, 10 % CO_2, and 80 % N_2.

Phylogenetic Analysis

Multi-locus phylogenetic analysis (MLPA), based on 22 housekeeping genes (concatenated 24,715 bp), was per-formed, and the nucleotide sequence of each gene was obtained from GenBank (Table 1). Multiple sequences were aligned using the CLUSTAL W algorithm and sequence similarities were calculated using the MegAlign program (DNAStar Lasergene^{TM} 8.0, DNAStar Inc., USA). The evolutionary distance was calculated according to the Kimura 2-parameter model and phylogenetic trees were constructed by maximum-likelihood and neighbor-joining methods in MEGA 6.06 software [17]. The stability of phylogenetic trees was assessed by a bootstrap analysis of 1,000 replicates.

Genomic DNA Isolation

The bacterial cells were harvested and suspended in STES buffer [0.5 M NaCl, 0.01 M EDTA, and 0.2 M Tris–HCl

[pH 8.0] without 1 % sodium dodecyl sulfate (SDS)]. Lysozyme (Sigma, USA) was added at a final concentra-tion of 2 mg/ml and incubated at 55 °C for 1 h. Once the cells were lysed, SDS (final concentration, 1 %) and then proteinase K (Bioneer, Korea) (final concentration 100 μg/ml) were added, and the solution was incubated at 55 °C for 1 h. After preliminary extraction with phenol:chloro-form:isoamyl alcohol (25:24:1), RNase was added to the extract at a final concentration of 50 μg/ml and incubated at 37 °C for 2 h. Genomic DNA was purified using phe-nol:chloroform:isoamyl alcohol (25:24:1). DNA was pre-cipitated with 3 volumes of cold ethanol and centrifuged at 15,000×g for 15 min at 4 °C. The precipitated DNA was air-dried and dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4). DNA concentration was deter-mined by the Epoch^{TM} Microplate Spectrophotometer (BioTek Instruments Inc., USA) at wavelengths of 260 and 280 nm.

DNA–DNA Hybridization

DNA–DNA hybridization was performed as described by Ezaki et al. with modification [4]. Briefly, heat-denatured genomic DNA solution (1 μg of DNA per ml) in phos-phate-buffered saline (PBS) containing 0.1 M $MgCl_2$ (PBSM) was incubated at 37 °C for 2 h in a microplate. After each solution was discarded, the microplate was washed once with PBS and then dried at 45 °C overnight. Photo-biotinylated genomic DNA was prepared as follows: 7 μl photobiotin (Sigma) and 1 μg denatured genomic DNA were mixed in a 1.5-ml tube and then irradiated with UV (100× μJ/cm^2) for 20 min. After irradiation, free photobiotin was removed by 1-butanol. For quantitative detection of biotinylated genomic DNA in the microplate, 200 μl of prehybridization solution (2× SSC, 0.3 M NaCl, 0.03 M sodium citrate, 5× Denhardt solution, and 50 % formamide) containing 100 μg denatured salmon sperm DNA (Sigma) was added to microplate wells that were previously coated with genomic DNA. The microplate was incubated at 37 °C for 1 h. The prehybridization solution was removed from microplate and replaced with hybrid-ization solution (prehybridization solution with 5 % dex-tran sulfate) containing biotinylated genomic DNA. The microplate was covered with sealing tape and incubated at 37 °C overnight. After hybridization, the microplate was washed three times with 200 μl of 2× SSC. Then, 100 μl streptavidin-β-D-galactosidase solution (250 μg/ml in PBS containing 0.5 % bovine serum albumin and 0.1 % Triton X-100) was added to each well and then incubated at 37 °C for 30 min. After incubation, the microplate was washed three times with PBS containing 0.1 % Triton X-100, and 100 μl of 3×10^{-4} μM 4-methylumbelliferyl-β-D-galac-topyranoside in PBS containing 1 mM $MgCl_2$ was added.

Table 1 List of genes used for multi-locus phylogenetic analysis

Genes (nts)	GenBank accession no./location				
	ChDC F128^T	ChDC F145	ChDC F174	ChDC F206	ChDC F300
rpoB (2,402)	GQ274966/18..2419	KF149873/17..2419	KF149874/18..2419	KF149875/18..2419	KF149876/18..2419
atpC (849)	NZ_ALVD01000005/192221..193069	KF149877/1..849	KF149878/1..849	KF149879/1..849	KF149880/1..849
exoA (762)	NZ_ALVD01000004/89946..90707	KF149881/1..762	KF149882/1..762	KF149883/1..762	KF149884/1..762
pfkA (969)	NZ_ALVD01000009/21497..22465	KF149885/1..969	KF149886/1..969	KF149887/1..969	KF149888/1..969
pyrB (891)	NZ_ALVD01000009/14828..15718	KF149889/1..891	KF149890/1..891	KF149891/1..891	KF149892/1..891
gapA (1,008)	NZ_ALVD01000006/54187..55194	KF149893/1..1008	KF149894/1..1008	KF149895/1..1008	KF149896/1..1008
pgk (532)	NZ_ALVD01000006/55931..56462	KF149897/1..532	KF149898/1..532	KF149899/1..532	KF149900/1..532
gpmA (687)	NZ_ALVD01000001/194030..194716	KF149901/1..687	KF149902/1..687	KF149903/1..687	KF149904/1..687
pckA (1,584)	NZ_ALVD01000003/299580..301163	KF149905/1..1584	KF149906/1..1584	KF149907/1..1584	KF149908/1..1584
ldh (957)	NZ_ALVD01000003/213011..213967	KF149909/1..957	KF149910/1..957	KF149911/1..957	KF149912/1..957
ung (678)	NZ_ALVD01000006/89583..90260	KF149913/1..678	KF149914/1..678	KF149915/1..678	KF149916/1..678
tpi (756)	NZ_ALVD01000009/60911..61666	KF149917/1..756	KF149918/1..756	KF149919/1..756	KF149920/1..756
oadA (1,347)	NZ_ALVD01000009/68250..69596	KF149921/1..1.1,347	KF149922/1..1.1,347	KF149923/1..1,347	KF149924/1..1.1,347
ddl (864)	NZ_ALVD01000008/11731..12594	KF149925/1..864	KF149926/1..864	KF149927/1..864	KF149928/1..864
murA (1,272)	NZ_ALVD01000008/79608..80879	KF149929/1..1,272	KF149930/1..1,272	KF149931/1..1,272	KF149932/1..1,272
eno (1,302)	NZ_ALVD01000007/32052..33353	KF149933/1..1,302	KF149934/1..1.1,302	KF149935/1..1,302	KF149936/1..1,302
dnaB (1,341)	NZ_ALVD01000002/129345..130685	KF149937/1..1341	KF149938/1..1341	KF149939/1..1341	KF149940/1..1341
pgi (1,122)	NZ_ALVD01000004/215839..216960	KF149941/1..1,122	KF149942/1..1,122	KF149943/1..1,122	KF149944/1..1,122
recA (1,134)	NZ_ALVD01000001/356244..357377	KF149945/1..1,134	KF149946/1..1,134	KF149947/1..1,134	KF149948/1..1,134
gyrA (1,452)	NZ_ALVD01000004/168251..169702	KF149949/1..1,452	KF149950/1..1,452	KF149951/1..1,452	KF149952/1..1,452
gyrB (1,634)	NZ_ALVD01000004/164503..166136	KF149953/146..1779	KF149954/146..1779	KF149955/146..1779	KF149956/146..1779
groEL (1172)	NZ_ALVD01000001/247525..248696	KF149957/1..1172	KF149958/1..1172	KF149959/1..1172	KF149960/1..1172

The microplate was incubated at 37 °C for several periods of time. The fluorescence intensity was measured with a Fluoroskan Ascent Microplate Fluorometer (Thermo Scientific, USA) at an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

Determination of DNA G+C Content

The G+C content of the genomic DNA was determined by the modified method described by Tamaoka and Komagata [16]. Briefly, genomic DNA was hydrolyzed into nucleosides using nuclease P1 and alkaline phosphatase. The resultant nucleosides were analyzed by high-performance liquid chromatography (HPLC; Shimadzu, Japan) equipped with a reversed-phase column (YMC pack ODS-A, 150×4.6 mm, Japan). Elution of the nucleosides was carried out with 0.55 M $NH_4H_2PO_4$/acetonitrile (20:1, v/v), at a flow rate of 1 ml/min at 30 °C. Each nucleotide was detected by UV absorbance at 270 nm. The relative G+C contents were calculated by using DNA isolated from *Escherichia coli* KCTC 2441[T].

Characterization of Morphology and Phenotype

Cell shape and size of the bacterial strains were determined by scanning electron microscopy. The cultured cells were fixed in 2.5 % glutaraldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4 °C. After pre-fixation, the specimens were washed in the same buffer solution and then post-fixed in 1.5 % osmium tetroxide solution in 0.1 M phosphate buffer solution for 3 h at room temperature. Specimens were dehydrated in increasing concentrations of ethanol, and freeze-dried cells were mounted directly on stubs by using double-sided adhesive tape and coated with platinum by using a sputter coater. The cells were observed under a scanning electron microscope (S-4800, Hitachi, Japan) at 5 kV.

API 20A and API ZYM test strips (bioMerieux, France) were used to analyze the biochemical and physiological traits, enzyme activities, and sugar fermentation patterns of the strains, as per the manufacturer's instructions. All tests were performed using fresh cultures of the strains under the same experimental conditions.

## Analysis of Fatty Acids, Peptidoglycan, and Polar Lipids

Cellular fatty acids were saponified, methylated, and extracted using the MIDI/Hewlett Packard Microbial Identification System (MIDI Microbial ID, USA), according to the manufacturer's instructions. The fatty acids were analyzed using gas chromatography (model 6890N and Auto-sampler 7683; Agilent) and identified using the

Microbial Identification Sherlock software package (classical method, MIDI Sherlock system 4.1, TSBA library version 4.0, USA). The results were validated by NMR spectra, recorded on a UNITY Inova 400 (Varian Inc., USA) with methanol-d4. For peptidoglycan analysis, 5 mg of freeze-dried cells were hydrolyzed with 1 ml of 6 N HCl in a screw-capped tube at 100 °C for 18 h. The hydrolysate was filtered and concentrated by drying in a rotary evaporator at 65 °C. The residue was dissolved in distilled water. Each sample was analyzed by two-dimensional thin-layer chromatography on cellulose plates. Polar lipid analysis was carried out by the Identification Service of the DSMZ (Braunschweig, Germany).

Results and Discussion

The phylogenetic tree constructed with concatenated 22 housekeeping genes showed that all the *F. nucleatum* strains were clearly separated into 5 distinct lineages with 100 % bootstrap value (Fig. 1 and supplementary Fig. S1). The group distance between 5 novel strains and *F. nucleatum* subsp. *nucleatum*, *F. nucleatum* subsp. *polymorphum*, *F. nucleatum* subsp. *vincentii*, *F. nucleatum* subsp. *animalis*, or *F. periodonticum* was 6.5, 4.8, 7.2, 8.0, and 10.2 %, respectively. Similarity of the concatenated 22 housekeeping genes between ChDC F128[T] and *F. nucleatum* subsp. *nucleatum* ATCC25586[T], *F. nucleatum* subsp. *polymorphum* ATCC10953[T], *F. nucleatum* subsp. *vincentii* ATCC51190[T], *F. nucleatum* subsp. *animalis* ATCC51191[T], or *F. periodonticum* ATCC33693[T] was 93.6, 94.5, 92.5, 91.8, and 90.6 %, respectively. In a previous study, the phylogenetic tree complied from the 16S rDNA sequences could not delineate the *F. nucleatum* subspecies from *F. periodonticum*, but the novel *F. nucleatum* subspecies strains (ChDC F128[T], ChDC F145, ChDC F174, ChDC F206, and ChDC F300) were clustered as one group and were clearly separated from the other *F. nucleatum* subspecies and *F. periodonticum* [10]. The values of genomic relatedness between ChDC F128[T] and ChDC F206, ChDC F145, ChDC F174, or ChDC F300 were 82.9, 79.0, 82.6, and 86.1 %, respectively, which indicated that these 5 strains represented the same species. The values of genomic relatedness between ChDC F128[T] and *F. nucleatum* subsp. *nucleatum* ATCC25586[T], *F. nucleatum* subsp. *polymorphum* ATCC10953[T], *F. nucleatum* subsp. *vincentii* ATCC51190[T], *F. nucleatum* subsp. *animalis* ATCC51191[T], or *F. periodonticum* ATCC33693[T] were 45.6, 53.7, 54.4, 49.6, and 40.9 %, respectively. It was recommended that values of 70 % and DNA–DNA relatedness with ΔTm of 5 °C or less are reasonable borders for species circumscription [19]. The 5 clinical strains of *F. nucleatum* used in this study were suggested to be a novel subspecies of *F. nucleatum* by

Fig. 1 Maximum-likelihood phylogenetic tree based on a single sequence (24,715 bp) by concatenating 22 housekeeping genes. The stability of phylogenetic trees was assessed by a bootstrap analysis of 1,000 replicates by using MEGA version 6.06 [17]. *Bar* indicates 0.01 changes per nucleotide position
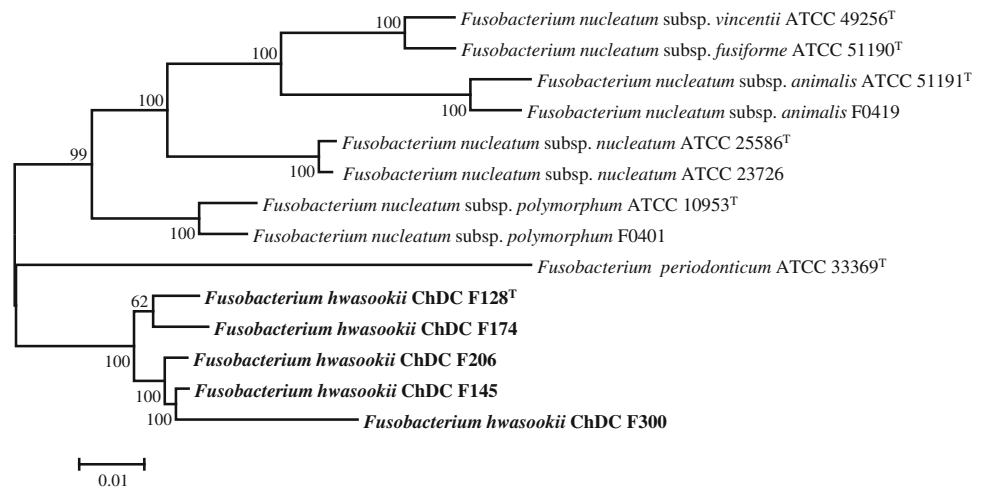
Table 2 Cellular fatty acid compositions of clinical isolates and closely related type strains

Characteristic	1	2	3	4	5	6	7	8	9	10
12:0	1.27	0.68	2.04	0.86	0.85	2.57	1.25	1.15	0.53	1.03
14:0	29.37	24.41	27.19	26.68	27	29.97	29.22	25	21.56	20.17
14:0 DMA	–	–	0.96	–	–	2.11	1.33	2.5	4.9	1.15
15:0	0.58	0.36	0.5	–	0.43	–	0.4	0.98	0.42	–
16:0 ALDE	–	–	1.66	–	–	1.84	1.71	2.37	2.57	1.68
16:1 cis 7	–	–	–	–	–	–	–	–	0.38	0.75
16:1 cis 9	15.08	15.82	13.05	15.43	14.95	13.02	13.55	5.94	9.55	12.86
16:1 cis 9 DMA	–	–	1.07	–	–	1.2	0.73	1.44	1.77	1.73
16:1 cis 11	0.5	0.52	–	0.48	0.44	–	0.36	–	–	1.08
16:0	28.33	29.97	19.64	28.76	29.53	15.91	21.52	20.05	21.75	18.61
16:0 DMA	1.12	1.36	8.55	1.8	1.51	7.37	8.62	12.4	11.42	9.68
16:0 3OH	4.9	5.41	6.54	5.54	5.22	8.23	5.78	5.91	6.22	5.43
18:2 cis 9,12	1.03	1.38	0.94	1.27	1.43	–	1.16	2.21	1.2	1.17
18:1 cis 9	3.81	4.88	3.05	4.72	4.97	1.93	4.04	8.53	4.84	4.69
18:0	2.66	3.11	1.48	3.06	2.92	1.32	1.58	1.45	1.96	3.79
18:1 CIS 11 DMA	–	–	0.72	–	–	–	–	0.9	–	1.18
19:0 ANTEISO	–	–	–	–	–	2.93	–	–	–	–
Summed feature 1	–	–	0.44	–	–	1.19	0.46	0.69	1.33	–
Summed feature 4	–	–	–	–	–	–	–	–	0.46	–
Summed feature 5	4.55	4.49	6.38	4.69	4.41	6.14	5.21	5.4	4.69	5.03
Summed feature 10	6.8	7.6	5.43	6.72	6.34	4.27	3.06	3.08	4.46	9.97

Strains: *1* strain ChDC F128[T], *2* strain ChDC F145, *3* strain ChDC F174, *4* strain ChDC F206, *5* strain ChDC F300, *6 F. nucleatum* subsp. *nucleatum* ATCC25586[T], *7 F. nucleatum* subsp. *polymorphum* ATCC10953[T], *8 F. nucleatum* subsp. *vincentii* ATCC49256[T], *9 F. nucleatum* subsp. *animalis* ATCC51191[T], *10 F. periodonticum* ATCC33693[T]. Values are expressed as percentages of fatty acids. Summed feature 1 contains 13:1 CIS 12 and/or 14:0 ALDE; Summed feature 4 contains 15:2; Summed feature 5 contains 15:0 DMA and/or 14:0 3OH; and Summed feature 10 contains 18:1c11/t9/t6. Symbol: –, not detected

comparing nucleotide sequences of *rpoB* and zinc protease gene [10]. However, DNA–DNA hybridization values were lower than the suggested threshold for species delineation [19]. These results reveal that the 5 strains should be classified as a novel *Fusobacterium* species.

The DNA G+C content of strains ChDC F128[T], ChDC F206, ChDC F145, ChDC F174, and ChDC F300 were 26.9, 27.0, 27.0, 26.9, and 27.0 mol%, respectively. These results are similar to the values reported previously for *F. nucleatum* subspecies [6, 8, 15].

Table 3 Differential characteristics of clinical isolates and closely related type strains

Characteristic	1	2	3	4	5	6	7	8	9	10
Indole production	+	+	+	+	+	+	+	+	+	+
Catalase	–	–	–	–	–	–	–	–	–	–
Enzyme activity										
Esterase	–	–	–	–	–	–	–	–	–	–
Esterase lipase	–	–	–	–	–	–	–	–	–	–
Acid phosphatase	–	–	–	–	–	–	–	–	–	–
Naphthol-AS-BI-phosphohydrolase	w	w	w	+	w	w	w	w	w	–
DNA G+C content (mol%)	26.9	27.0	26.9	27.0	27.0	27.2	26.8	27.6	26–28	28.0
Genomic relatedness (%) with 1	100	79.0	82.6	82.9	86.1	45.6	53.7	54.4	49.6	40.9

Strains: *1* strain ChDC F128[T], *2* strain ChDC F145, *3* strain ChDC F174, *4* strain ChDC F206, *5* strain ChDC F300, *6 F. nucleatum* subsp. *nucleatum* ATCC25586[T], *7 F. nucleatum* subsp. *polymorphum* ATCC10953[T], *8 F. nucleatum* subsp. *vincentii* ATCC49256[T], *9 F. nucleatum* subsp. *animalis* ATCC51191[T], *10 F. periodonticum* ATCC33693[T]. All the data presented were obtained in this study except the data of DNA G+C content from 6 to 10 [6, 8, 15]. *Symbol* +, positive; *w*, weakly positive; –, negative

The cell lengths of ChDC F128[T], ChDC F145, ChDC F174, ChDC F206, and ChDC F300 were 28.9 (7.3–65.5) × 4.9 (4.5–5.0), 10.3 (5.5–40.7) × 4.4 (4.3–4.6), 65.7 (11.2–165.5) × 3.7 (3.4–4.4), 12.7 (5.2–50.7) × 4.6 (4.3–5.0), and 14.9 (4.9–35.8) × 4.6 (4.3–5.0) μm, respectively (Supplementary Fig. S2).

The cellular fatty acid analysis of the clinical isolates and type strains revealed $C_{14:0}$, $C_{16:0}$, and cis-9 $C_{16:1}$ as being the major fatty acids. Among the analyzed strains, the major fatty acids of clinical isolates were similar to those of *F. nucleatum* subsp. *nucleatum* ATCC25586[T], *F. nucleatum* subsp. *polymorphum* ATCC10953[T], and *F. periodonticum* ATCC33693[T], whereas cis-9 $C_{16:1}$ was found to be relatively low in *F. nucleatum* subsp. *vincentii* ATCC49256[T] and *F. nucleatum* subsp. *animalis* ATCC51191[T]. The major fatty acids detected in the strains, $C_{14:0}$, $C_{16:0}$, and cis-9 $C_{16:1}$, were consistent with those found in *F. nucleatum* [7, 11, 18]. A comparison between the major fatty acids of the clinical isolates and those of the 5 *Fusobacterium* type strains is shown in Table 2.

In the API ZYM panel, tests for naphthol-AS-BI-phosphohydrolase were positive in only strain ChDC F206 and weakly positive in the other strains (ChDC F128[T], ChDC F145, ChDC F174, and ChDC F300), while most reactions were negative. API 20A test for indole production was positive, while the remaining 18 tests, including the test for catalase, were negative in the 5 strains (Table 3). The biochemical test results for the 5 strains were almost the same as those of *F. nucleatum* and *F. periodonticum*, except for the results of the test for naphthol-AS-BI-phosphohydrolase.

Lanthionine has been reported as an essential component of *F. nucleatum* peptidoglycan [9]. Lanthionine has also been found to exist in the cell wall peptidoglycan of clinical isolates, *F. nucleatum* subspecies, and *F. periodonticum* ATCC33693[T] (Supplementary Fig. S3). The

data indicate that the clinical isolates belong to the genus *Fusobacterium*.

Polar lipids consisted of phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, 3 unknown phospholipids, and an unknown aminolipid, with one unknown aminophospholipid in ChDC F128[T]. The major polar lipids of *F. nucleatum* using fast-atom-bombardment mass spectrometry (FAB-MS) were phosphatidylethanolamine and phosphatidylglycerol [14]. However, the major polar lipid in ChDC F128[T] was diphosphatidylglycerol (Supplementary Fig. S4).

On the basis of the molecular, chemical, and phenotypic evidence presented in this study, we propose that the strains ChDC F128[T], ChDC F145, ChDC F174, ChDC F206, and ChDC F300 should be assigned to a novel species of *Fusobacterium*, which we propose should be named *Fusobacterium hwasookii* sp. nov.

Description of *Fusobacterium hwasookii* sp. nov.

Fusobacterium hwasookii [hwa.so.ok'i.i. N.L. gen. masc. n. hwasookii named after Hwa-sook Kim, a Korean microbiologist who isolated these strains and has contributed to the description of this species]

Fusobacterium hwasookii is a gram-negative, anaerobic, and fusiform-shaped bacterium with variable size. The cell length of ChDC F128[T] was 28.9 (7.3–65.5) × 4.9 (4.5–5.0). The API ZYM system showed negative reaction for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-lucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase, whereas naphthol-AS-BI-phosphohydrolase was weakly positive for strain ChDC F128[T]. In the API 20A system, strain ChDC F128[T] showed indole

production but were negative for acid production from D-glucose, D-mannitol, D-lactose, D-saccharose, D-maltose, salicin, D-xylose, L-arabinose, glycerol, D-cellobiose, D-mannose, D-melezitose, D-raffinose, D-sorbitol, D-rhamnose, and D-trehalose. For strain ChDC F128^T, esculin was not hydrolyzed, gelatin was not digested, catalase reaction was negative, and the cellular fatty acids were mainly composed of $C_{14:0}$, $C_{16:0}$, and cis-9 $C_{16:1}$. The G+C content of ChDC F128^T was 26.9 mol%. DNA–DNA relatedness between ChDC F128^T and ChDC F145, ChDC F174, ChDC F206, or ChDC F300 was approximately 79.0, 82.6, 82.9, and 86.1 %, respectively. DNA–DNA relatedness between ChDC F128^T and *F. nucleatum* subsp. *nucleatum* ATCC25586^T, *F. nucleatum* subsp. *polymorphum* ATCC10 953^T, *F. nucleatum* subsp. *vincentii* ATCC51190^T, *F. nucleatum* subsp. *animalis* ATCC51191^T, or *F. periodonticum* ATCC33693^T was 45.6, 53.7, 54.4, 49.6, and 40.9 %, respectively. The cell-wall diamino acid of strain ChDC F128^T was found to be meso-lanthionine. The major polar lipids of ChDC F128^T were phosphatidylethanolamine, diphosphatidylglycerol, and phosphatidylglycerol.

The cell lengths of ChDC F145, ChDC F174, ChDC F206, and ChDC F300 were 10.3 (5.5–40.7) × 4.4 (4.3–4.6), 65.7 (11.2–165.5) × 3.7 (3.4–4.4), 12.7 (5.2–50.7) × 4.6 (4.3–5.0), and 14.9 (4.9–35.8) × 4.6 (4.3–5.0) μm, respectively. In API ZYM system, naphthol-AS-BI-phosphohydrolase was positive for only strain ChDC F206 and weakly positive for ChDC F145, ChDC F174, and ChDC F300 strains. The G+C content of ChDC F145, ChDC F174, ChDC F206, and ChDC F300 ranged between 26.9 and 27.0 mol%.

These results show that the 5 strains are novel species that belong to genus *Fusobacterium*. The type strain, ChDC F128^T (=KCOM 1249^T = KCTC 5108^T = JCM 30218^T), was isolated from subgingival plaques of Korean patient.

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