Polyphasic Analysis of Strains of the Genus *Capnocytophaga* and Centers for Disease Control Group DF-3

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A polyphasic approach was used to determine the relationships between well-characterized reference strains representing all seven *Capnocytophaga* species. One Centers for Disease Control (CDC) group DF-3 strain, a presumed relative of the genus *Capnocytophaga*, and 15 field isolates were included as well. Fourteen isolates were assigned to named *Capnocytophaga* species, all of which could be differentiated by means of whole-organism protein electrophoresis. A separate position was occupied by the CDC group DF-3 strain and by one field isolate representing a novel *Capnocytophaga* species. The phylogenetic position of each taxon was determined by means of 16S rRNA sequence analysis. A considerable genotypic heterogeneity within the genus *Capnocytophaga* was detected in spite of the minimal phenotypic differences. Comparative 16S rRNA sequence analysis revealed that CDC group DF-3 is not a close relative of the capnocytophagas but constitutes a separate genus that clusters together with *Bacteroides forsythus* and *Bacteroides distasonis*, two generically misclassified *Bacteroides* species. The degree of protein similarity correlated with our and published DNA-DNA binding values. Percentage 16S rRNA similarity values of greater than 97% did not guarantee conspecificity. All *Capnocytophaga* strains had very similar fatty acid contents characterized by significant amounts of 14:0, 15:0 iso (greater than 55%), 16:0, 16:0 3OH, and 17:0 iso 3OH. PCR-mediated DNA fingerprinting allowed discrimination of most species, although some strains could not be classified efficiently because of DNA polymorphisms.

In 1979, Leadbetter and coworkers proposed the genus *Capnocytophaga* for a group of gram-negative, fusiform, micro-aerophilic, fermentative, and gliding bacteria that were isolated from the oral cavities of healthy and diseased humans (27). At that time, the synonymy between *Capnocytophaga* and the Centers for Disease Control (CDC) group DF-1 (disgonic fermenter 1) and between *Capnocytophaga ochracea* and *Bacteroides ochraceus* was established (29, 52). From early on, the delineation of species within the genus *Capnocytophaga* was controversial since two of the three species, i.e., *C. ochracea* and *Capnocytophaga sputigena*, were virtually indistinguishable. Classical phenotypic tests or analysis of enzymatic capacities did not allow a satisfactory species delineation (22, 25–27, 38). However, only low DNA-DNA hybridization values were measured between reference strains of both species (39, 51). The application of various chemotaxonomic markers did not elucidate this problem. Not only *C. ochracea* and *C. sputigena* but also *Capnocytophaga gingivalis* was indistinguishable in analyses of their fatty acid contents (6, 9, 21, 39), polar lipid contents (6), and respiratory quinone contents (6, 39 [all strains contain menaquinone-6 and trace amounts of menaquinone-5]). Protein profiling of 97 field isolates and the type strains of each species allowed distinction of eight distinct clusters, three of them containing one type strain each (24). Recently, Wilson et al. (53) performed restriction fragment length polymorphism analyses of PCR-amplified 16S rDNA genes which also confirmed the separate species status of the three taxa.

Ten years after the description of the first three species, two additional species isolated from dog mouths and from wound infections after dog bites were proposed: *Capnocytophaga canimorsus* (formerly known as CDC group DF-2) and *Capnocytophaga cynodegmi* (formerly known as CDC group DF-2-like) (4). These last two species differed from the others by the presence of catalase and oxidase activities, but their general phenotypic profile conformed to the genus description (4). Furthermore, the fatty acid (8–10, 39) and respiratory quinone profiles (39) of the former CDC group DF-2 were indistinguishable from those of *C. ochracea*, *C. sputigena*, and *C. gingivalis*. Finally, *Capnocytophaga haemolytica* and *Capnocytophaga granulosa* were described by Yamamoto et al. (56). These species were isolated from the human oral cavity and share the same fatty acid and quinone components and general biochemical profile with other human *Capnocytophaga* species (56).

Suprageneric studies by means of DNA-rRNA hybridizations revealed that the genus *Capnocytophaga* constitutes a separate phylogenetic branch within rRNA superfamily V (37). Comparative 16S rRNA sequence analysis situated the genus *Capnocytophaga* within the so-called cytophaga subgroup of the flavobacter-bacteroides phylum of bacteria (13, 18, 46). The capnophilic genera *Capnocytophaga*, *Ornithobacterium*, and *Riemerella* all belong to the same lineage and were included in the emended family *Flavobacteriaceae* (3).

CDC group DF-3 (dysgonic fermenter 3) is a group of unclassified gram-negative rods resembling the other dysgonic fermenters which are now classified as *Capnocytophaga* species. DF-3 strains have been isolated from various human clinical sources and share several biochemical and chemotaxonomic properties with *Capnocytophaga* strains (2, 50). However, their phylogenetic affiliation is unknown.

In the present study, we used a polyphasic approach to determine the relationships between well-characterized refer-

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TABLE 1. Strains used in this study

Strain ^{a,b}	Other numbers ^{a,b}	Source, year, and place of isolation ^c
Reference strains		
<i>C. canimorsus</i> ATCC 35979 ^T	CDC 7120 ^T	Human blood after dog bite; United States
<i>C. canimorsus</i> LMG 11510	CCUG 12569, CDC A3626	Human blood; 1965; United States
<i>C. canimorsus</i> LMG 11512	CCUG 24741, CDC C8936	Human blood after dog bite; United States
<i>C. canimorsus</i> LMG 11541	MCCM 00060, CDC C8936	Human blood after dog bite; United States
<i>C. cynodegmi</i> LMG 11513 ^T	CCUG 24742 ^T , CDC E6447 ^T , ATCC 49044 ^T	Dog mouth; 1979; United States
<i>C. cynodegmi</i> LMG 11538	MCCM 00262, CDC E679	Hand wound; United States
<i>C. cynodegmi</i> LMG 11539	MCCM 00263, CDC F4039	
<i>C. cynodegmi</i> LMG 11540 ^T	MCCM 00265 ^T , CDC E6447 ^T , ATCC 49044 ^T	Dog mouth; 1979; United States
<i>C. gingivalis</i> LMG 11514 ^T	CCUG 9715 ^T , Holt 27 ^T , ATCC 33624 ^T	Periodontitis; 1978; United States
<i>C. granulosa</i> LMG 16022 ^T	JCM 8566 ^T	Adult supragingival dental plaque; Japan
<i>C. haemolytica</i> LMG 16021 ^T	JCM 8565 ^T	Adult supragingival dental plaque; Japan
<i>C. ochracea</i> ATCC 27872 ^T		Human oral cavity
<i>C. ochracea</i> LMG 11516	CCUG 9972, VPI 13316	Bülöw drain secretion (pulmonary cancer); 1980; Sweden
<i>C. ochracea</i> LMG 11546	CCUG 15407, Holt 25, ATCC 33596	Periodontitis; 1978; United States
<i>C. ochracea</i> LMG 11977	HIM 779-6	Periodontitis; 1982; Germany
<i>C. sputigena</i> LMG 11518 ^T	CCUG 9714 ^T , Holt 4 ^T , ATCC 33612 ^T	Periodontitis; 1978; United States
<i>C. sputigena</i> LMG 11517	CCUG 9277, VPI 13197	Human blood; 1979; Sweden
DF-3 LMG 11519	CCUG 17996, CDC F9047	Cutaneous abscess; 1985; Denmark
Field isolates		
<i>Capnocytophaga</i> sp. LMG 11511 (<i>C. canimorsus</i>)	CCUG 17234, P810	
<i>Capnocytophaga</i> sp. LMG 11551 (<i>C. canimorsus</i>)	MCCM 01373	Human blood; 1990; Switzerland
<i>Capnocytophaga</i> sp. LMG 11505 (<i>C. gingivalis</i>)	CCUG 13095	Periodontitis; 1982; Sweden
<i>Capnocytophaga</i> sp. LMG 12118 (<i>C. gingivalis</i>)	FDC 44	Human subgingival crevice; United States
<i>Capnocytophaga</i> sp. LMG 11544 (<i>C. ochracea</i>)	HIM 1017-2	Sheep
<i>Capnocytophaga</i> sp. LMG 12115 (<i>C. ochracea</i>)	FDC 7	Human subgingival crevice; United States
<i>Capnocytophaga</i> sp. LMG 12117 (<i>C. ochracea</i>)	FDC 43	Human subgingival crevice; United States
<i>Capnocytophaga</i> sp. LMG 11506 (<i>C. granulosa</i>)	CCUG 14446	Human oral cavity; 1983; Sweden
<i>Capnocytophaga</i> sp. LMG 11507 (<i>C. granulosa</i>)	CCUG 14447	Human oral cavity; 1983; Sweden
<i>Capnocytophaga</i> sp. LMG 11515 (<i>C. granulosa</i>)	CCUG 12286, VPI 13779	Gingivum from a healthy individual; 1982; Sweden
<i>Capnocytophaga</i> sp. LMG 12119 (<i>C. granulosa</i>)	FDC 6	Human subgingival crevice; United States
<i>Capnocytophaga</i> sp. LMG 11545 (<i>C. sputigena</i>)	CCUG 17163	Human blood; 1984; Sweden
<i>Capnocytophaga</i> sp. LMG 11509 (<i>C. sputigena</i>)	CCUG 17222	Human blood; 1984; Sweden
<i>Capnocytophaga</i> sp. LMG 11508 (<i>C. sputigena</i>)	CCUG 15264, CDC F1659	Human blood; United States
<i>Capnocytophaga</i> sp. strain LMG 12116 (new species)	FDC 8	Human subgingival crevice; United States

^a Type strains are indicated by superscript T. Names in parentheses indicate how field isolates have been identified.

^b CCUG, Culture Collection of the University of Göteborg, Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden; CDC, Centers for Disease Control, Atlanta, Ga.; FDC, Forsyth Dental Center, Boston, Mass.; HIM, Hygiene-Institut und Medizinisch-Untersuchungsamt, University of Marburg, Marburg, Germany; JCM, Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Saitama, Japan; LMG, Culture Collection Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium; MCCM, Medical Culture Collection of Microorganisms, Marburg, Germany; VPI, Virginia Polytechnic Institute and State University, Blacksburg, Va.

^c If known.

ence strains representing all 7 *Capnocytophaga* species and 15 field isolates. One representative (50) CDC group DF-3 strain was included as well.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains were grown on Trypticase soy agar (catalog no. 11768; BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) and were incubated at 36 to 37°C in a microaerobic atmosphere containing approximately 5% O₂, 3.5% CO₂, 7.5% H₂, and 84% N₂. The strains and their sources are listed in Table 1. Type strains and strains included in previous taxonomic studies are considered reference strains; all others are referred to as field isolates. Bacteriological purity was checked by plating and examining living and gram-stained cells.

Whole-cell protein analysis. All strains were grown for 48 h on one to three petri dishes. Preparation of cellular protein extracts, polyacrylamide gel electrophoresis (PAGE), densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis were performed as described by Pot et al. (34) with the GelCompar 3.1 software package (Applied Maths, Kortrijk, Belgium). The profiles were recorded and stored on a PC computer. The similarity between all pairs of traces was expressed by the Pearson product moment correlation coefficient *r* converted for convenience to a percent value.

Fatty acid methyl ester analysis. After an incubation period of 48 h, a loopful of well-grown cells was harvested, and fatty acid methyl esters were prepared, separated, and identified by using the Microbial Identification System (Microbial ID, Inc., Newark, Del.) as described before (47).

Preparation of high-molecular-weight DNA. Rapid, small-scale DNA extrac-

tions for PCR analysis were performed as described by Pitcher et al. (33). Large-scale, high-molecular-weight native DNA was prepared as described before (47).

DNA fingerprinting. DNA fingerprinting by the PCR technique was performed as described before with Biomed PCR machines and Sphaero-Q *Taq* polymerase (Sphaero-Q, Leiden, The Netherlands) (41, 43). The primers (50 pmol per PCR mixture) listed in Table 2 were employed, either alone or in combination. Approximately 100 ng of template DNA was added, and reaction products were separated on 2% agarose gels in 40 mM Tris acetate (pH 7.8)–1 mM EDTA. Polaroid photographs were taken after ethidium bromide staining. DNA banding patterns were compared by visual inspection.

TABLE 2. Primers used for PCR fingerprinting of *Capnocytophaga* species

Code	Base sequence	Reference
ERIC1	CACTTAGGGGTCTCTGAATGTA	49
ERIC2	AATAAGTGACTGGGGTGAGCG	49
REP1	IIICGICGICATCTGGC	49
REP2	ICGICTTATCTGGCCTAC	49
D14307	CCTTGGGTGAGAATTGCACG	1
D9355	CCGGATCCGTGATGCGGTGCG	1
D11344	AGTGAATTCGCGGTGAGATGCCA	1

TABLE 3. Similarity matrix for *Capnocytophaga* strains based on 16S rRNA sequence comparisons^a

Species	Sequence accession no. ^b	Reference	Similarity				
			<i>C. ochracea</i> ATCC 27872 ^T	<i>C. ochracea</i> LMG 12115	<i>C. ochracea</i> LMG 12117	<i>C. ochracea</i> ATCC 33596	<i>C. sputigena</i> ATCC 33612 ^T
<i>C. ochracea</i> ATCC 27872 ^T	U41350	This study		98.7	98.1	97.4	95.8
<i>C. ochracea</i> LMG 12115	U41353, U41354	This study	1.3		98.3	98.0	96.3
<i>C. ochracea</i> LMG 12117	U41351	This study	1.9	1.7		97.4	95.8
<i>C. ochracea</i> ATCC 33596	L14635	46	2.7	2.0			96.4
<i>C. sputigena</i> ATCC 33612 ^T	L14636	46	4.3	3.8	4.4	3.7	
<i>C. haemolytica</i> JCM 8565 ^T	U41349	This study	8.0	8.6	8.4	9.1	9.0
<i>C. cynodegmi</i> ATCC 49044 ^T	L14638	46	6.9	6.8	7.0	6.9	6.9
<i>C. canimorsus</i> ATCC 35979 ^T	L14637	46	7.3	7.3	7.7	7.6	7.7
<i>Capnocytophaga</i> sp. strain LMG 12116	U41352	This study	9.9	10.1	9.8	10.0	10.5
<i>C. granulosa</i> LMG 12119	U41348	This study	9.0	8.7	9.1	8.8	8.9
<i>C. granulosa</i> JCM 8566 ^T	U41347	This study	9.2	9.0	9.4	9.0	9.1
<i>C. gingivalis</i> LMG 12118	U41346	This study	9.0	8.7	8.6	8.8	9.4
<i>C. gingivalis</i> ATCC 33624 ^T	L14639	This study	8.6	8.6	8.6	8.8	8.7
<i>Flavobacterium aquatile</i> ATCC 11947 ^T	M28236	55	13.3	12.7	13.2	13.1	13.5
<i>Cytophaga uliginosa</i> ATCC 14397 ^T	M28238	55	14.9	14.1	14.5	14.6	15.3

^a Numbers above the diagonal are uncorrected percentages of similarity, and those below the diagonal are percentages of difference corrected for multiple base changes by the method described by Jukes and Cantor.

^b 16S rRNA sequences for these strains are available for electronic retrieval from GenBank and EMBL under the indicated accession numbers.

Isolation and labeling of species-specific DNA probes. Species-specific DNA fragments were selected by comparison of PCR fingerprints. Fragments that appeared to be present in all banding patterns of a given species and apparently absent in all other species were excised from the agarose gels. DNA was purified by the GeneClean assay (Bio 101, La Jolla, Calif.), ethanol precipitated, and subsequently radioactively labeled by random-primed incorporation of [α -³²P]dATP (Amersham; 3,000 Ci/mmol) (15). The probes were hybridized at 42°C to Southern blots containing fingerprint DNA as described previously (20). The blots were washed twice in 15 mM sodium citrate (pH 7.0)–150 mM NaCl–0.1% sodium dodecyl sulfate at 60°C. Autoradiography was performed at –70°C for at least 16 h.

DNA base compositions. All of the mean mole percentage guanine-plus-cytosine (mol% G+C) values were determined by thermal denaturation and calculated by using the equation described by Marmur and Doty (28), as modified by De Ley (11).

DNA-DNA hybridization experiments. Degrees of DNA-DNA binding, expressed in percentages, were determined spectrophotometrically by the initial renaturation rate method described by De Ley et al. (12). Each value is the average of at least two hybridization experiments. Values of 30% DNA binding and less do not represent significant DNA homology. The total DNA concentration was about 60 µg/ml, and the optimal renaturation temperature in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was 60.9°C.

16S rRNA sequencing. With the exception of the type strains of *C. haemolytica*, *C. granulosa*, and *C. ochracea*, 16S rRNA sequencing was performed with purified rRNA as previously described (31). In addition to the standard sequencing primers (primers 2 to 5, 9, and 10 in Table 2 in Eaton et al. [14]), the following two primers were employed: X54 (5'-GGG GTT GCG GTC GTT ATA GGA CTT-3' [1092 to 1115 reverse]) and A44 (5'-ACT AGC GAT TCC AGC TTC-3' [1331 to 1348 reverse]).

The type strains of *C. haemolytica*, *C. granulosa*, and *C. ochracea* were sequenced from PCR-amplified 16S rDNA by using cycle sequencing as previously described (17).

Phylogenetic analysis of 16S rRNA sequence information. The sequences were entered into RNA, a program for analysis of 16S rRNA data written in Microsoft QuickBASIC for use on IBM-PC-compatible computers, and were aligned as previously described (31). The data base contains approximately 500 sequences determined in our laboratory and 400 sequences obtained from GenBank or from other researchers. Similarity matrices were constructed from aligned sequences by using only base positions for which 90% of the strains had data. The similarity matrices were corrected for multiple base changes by the method described by Jukes and Cantor (23). Phylogenetic trees were constructed by the neighbor-joining method (35).

Nucleotide sequence accession numbers. The GenBank and culture collection accession number for DF-3 strain LMG 11519 is U41355; all others are given in Table 3.

RESULTS

PAGE of whole-cell proteins. Duplicate protein extracts of several strains were prepared to check the reproducibility of

growth conditions and extractions. The correlation level between duplicate protein patterns was $r \geq 0.93$. After visual comparison of the protein patterns with the dendrogram obtained after numerical comparison and clustering of the profiles, we delineated seven stable clusters and 3 separate strains at a similarity level of 85% (Fig. 1). Cluster I consists of three *C. canimorsus* reference strains and two field isolates. Cluster II consists of the four reference strains of *C. cynodegmi* clustering at higher than 88%. Cluster III contains one *C. ochracea* reference strain and three field isolates. Cluster IV contains two *C. ochracea* reference strains grouping at 89%. Cluster V contains two *C. sputigena* reference strains and three field isolates grouping at 87%. Cluster VI contains the *C. granulosa* type strain and four field isolates grouping at 87%. Finally, cluster VII contains the type strain of *C. gingivalis* and two field isolates grouping at a similarity level of greater than 87%. *C. haemolytica* LMG 16021^T, strain LMG 12116, and DF-3 strain LMG 11519 have distinct protein profiles and occupy separate positions in the dendrogram (Fig. 1).

Fatty acid methyl ester composition. The average fatty acid methyl ester compositions of each protein electrophoretic cluster of strains were calculated and are indicated in Table 4. Except for strain DF-3 LMG 11519, the general fatty acid patterns of the strains examined were very similar, with 14:0, 15:0 iso, 16:0, 16:0 3OH, and 17:0 iso 3OH as predominant components. 15:0 iso accounts for more than 55% of the total fatty acid contents of these strains. DF-3 strain LMG 11519 has a different fatty acid profile which is characterized by a large amount of 15:0 anteiso (37%) and significant amounts (5 to 12%) of 15:0 iso, 16:0 3OH, and 17:0 iso 3OH. Furthermore, unique features for this strain were 14:0 iso, 16:1 w7c, 16:0 iso 3OH, and 17:0 2OH.

DNA fingerprinting. DNA of all strains (except LMG 11513, LMG 11519, LMG 16021, and LMG 16022) was subjected to nine amplification assays. It appeared that especially the combination of ERIC1 and ERIC2 primers enabled the generation of complex DNA fingerprints for all isolates (Fig. 2). The banding patterns reveal a relatively high degree of heterogeneity; all isolates except *C. canimorsus* LMG 11512 and 11541, which are subcultures of the same strain, and *C. sputigena* LMG 11508 and LMG 11509 display unique fingerprints.

TABLE 3—Continued

Similarity									
<i>C. haemolytica</i> JCM 8565 ^T	<i>C. cynodegmi</i> ATCC 49044 ^T	<i>C. canimorsus</i> ATCC 35979 ^T	<i>Capnocytophaga</i> sp. strain LMG 12116	<i>C. granulosa</i> LMG 12119	<i>C. granulosa</i> JCM 8566 ^T	<i>C. gingivalis</i> LMG 12118	<i>C. gingivalis</i> ATCC 33624 ^T	<i>Flavobacterium</i> <i>aquatile</i> ATCC 11947 ^T	<i>Cytophaga uliginosa</i> ATCC 14397 ^T
92.4	93.4	93.0	90.7	91.6	91.4	91.6	91.8	87.8	86.5
91.9	93.5	93.0	90.6	91.8	91.5	91.7	91.8	88.3	87.2
92.0	93.3	92.6	90.8	91.5	91.2	91.8	91.9	87.9	86.8
91.4	93.4	92.8	90.7	91.7	91.5	91.7	91.7	88.0	86.7
91.5	93.4	92.7	90.2	91.6	91.4	91.1	91.8	87.6	86.2
	91.9	91.8	89.4	89.3	89.2	89.7	89.7	85.6	85.3
8.6		98.7	91.0	92.5	91.7	91.7	92.2	88.5	87.0
8.6	1.3		90.6	92.0	91.2	91.6	91.8	88.9	87.2
11.4	9.6	10.0		96.1	96.0	95.4	95.7	86.8	84.8
11.5	7.9	8.5	4.0		99.4	98.1	98.6	88.2	86.3
11.7	8.8	9.4	4.1	0.6		97.6	98.4	87.7	85.9
11.0	8.8	8.9	4.7	1.9	2.5		98.5	87.1	85.9
11.1	8.3	8.7	4.4	1.4	1.6	1.5		87.8	86.2
16.0	12.4	12.0	14.5	12.8	13.5	14.1	13.3		87.7
16.4	14.3	14.0	17.0	15.1	15.6	15.7	15.2	13.5	

When other primers were used, DNA was not amplified for all *C. sputigena* strains. Several conserved elements within these amplification patterns were found. For instance, all *C. sputigena* strains share several DNA amplification fragments when ERIC1 and ERIC2 sequences were used as primers (Fig. 2). One of these common fragments with an approximate molecular length of 400 bp was used as a species-specific DNA probe (19). Application of either REP1 or D11344 sequences as primers generated species specific DNA fragments for *C. canimorsus*. In the case of REP1-primed PCR, a species-specific DNA fragment of approximately 700 bp was found, while in the case of D11344-primed PCR, at least four common fragments occurred (data not shown). DNA fragments specific for protein electrophoretic cluster VI (*C. granulosa*) and cluster VII (*C. gingivalis*) strains were generated in several PCR assays. This was most apparent when the primers REP1 and REP2 were combined in one assay (Fig. 3A). The application of these DNA fragments as probes is illustrated in Fig. 3B and C. Two fragments (of approximately 500 bp in length for *C. granulosa* and approximately 400 bp in length for *C. gingivalis*) in Fig. 3A were excised, purified, and hybridized with a Southern blot made from the gel displayed in Fig. 3A. Clearly, both probes hybridize with all strains of the same cluster but not with other strains. We did not find DNA probes specific for *C. ochracea* or *C. cynodegmi*. Four of six *C. ochracea* strains form a genetically homogeneous group, whereas strains LMG 12115 and LMG 12117 behave aberrantly with most of the primers (Fig. 2; data not shown). The three *C. cynodegmi* strains examined also appear to be very different (Fig. 2; data not shown).

DNA base compositions. The DNA base ratios of representative strains per electrophoretic cluster were determined and are listed in Table 5.

DNA-DNA hybridization results. DNAs of *C. gingivalis* LMG 11514^T and of strains LMG 11515 and LMG 12116 were cross-hybridized. Only nonsignificant DNA-binding values of less than 30% were measured (results not shown).

16S rRNA sequence analysis. Approximately 1,450 bases of sequence information were determined for each strain listed in Table 3. Preliminary sequence comparisons demonstrated that all of the *Capnocytophaga* strains fell into a single monophyletic cluster. The DF-3 strain LMG 11519 was not part of the *Capnocytophaga* cluster but fell in the *Bacteroides* subgroup of the flavobacter-bacteroides phylum (18). A similarity matrix (Table 6) was determined for the aligned sequences of DF-3,

C. ochracea, *C. gingivalis*, and reference species from the flavobacter-bacteroides phylum. This matrix is based on comparisons at 1,422 base positions, for which more than 90% of the strains had data. The lower half of the matrix is expressed as percent differences corrected for multiple base changes by the method described by Jukes and Cantor (23). The neighbor-joining tree for these organisms is shown in Fig. 4. A similarity matrix was also determined for the aligned sequences of the *Capnocytophaga* strains (Table 3). The tree for these strains is shown in Fig. 5.

DISCUSSION

Whole-cell protein analysis. We used a polyphasic approach to examine the taxonomic relationships among well-characterized strains representing all *Capnocytophaga* species and a number of field isolates. As a first step in the analysis, we compared the cellular protein patterns of all strains. Indeed, it has been established that by this technique, clusters of highly related strains, i.e., strains belonging to the same species, can be delineated (7, 30, 44, 45, 47, 48). Furthermore, this technique was successfully applied to subdivide a large number of *Capnocytophaga* field isolates (24). The results of the present study confirm these findings; except for *C. ochracea*, the reference strains of each species belong to a single electrophoretic cluster (Fig. 1). One reference strain of *C. ochracea* (LMG 11977) does not cluster with the other references of the same species, although Speck et al. (39) found high DNA homology values among the three strains. However, its protein profile, like those of the field isolates grouping in the same cluster (cluster III [Fig. 1]), contains a dense protein band with a somewhat lower molecular weight than that of the corresponding bands in the patterns of other *C. ochracea* reference strains. Such a variability in the molecular weight of dense protein bands among strains of a single species has been described for several genera (reviewed by Costas [7]). Often this variable region can be used for subspecific typing purposes, while it can be omitted from the numerical analysis in order to identify strains to the species level (7). The cluster III and IV strains are therefore to be considered as different protein electrophoretic types of *C. ochracea*.

Given the high similarity in protein content among the reference strains and the field isolates in each of these clusters, we conclude that strain LMG 11551 is *C. canimorsus* (cluster I);

FIG. 1. Protein profiles of *Capnocytophaga* and *Capnocytophaga*-like strains and corresponding dendrogram derived from the unweighted pair group average linkage of correlation coefficients expressed for convenience as percentages. Roman numerals are cluster numbers as discussed in the text.

strains LMG 11544, LMG 12115, and LMG 12117 are *C. ochracea* (cluster III); strains LMG 11545, LMG 11508, and LMG 11509 are *C. sputigena* (cluster V); strains LMG 11515, LMG 12119, LMG 11507, and LMG 11506 are *C. granulosa* (cluster VI); and strains LMG 12118 and LMG 11505 are *C. gingivalis* (cluster VII). Only a single *Capnocytophaga* field isolate, LMG 12116, remained unidentified after protein anal-

ysis. These identification results were further substantiated by the results of various other approaches described below.

16S rRNA sequence analysis. The 16S rRNA sequences of the type strains of *C. canimorsus*, *C. cynodegmi*, *C. gingivalis*, *C. ochracea*, and *C. sputigena* and the taxonomic structure and neighborhood of the genus *Capnocytophaga* have been reported previously (46). Analysis of the 16S rRNA sequences of

FIG. 2. PCR fingerprinting patterns of *Capnocytophaga* species with a combination of primers ERIC1 and ERIC2. The molecular weight markers 1 and 2 are 1,600 and 500 bp in length, respectively.

the type strains of two newly described species, *C. granulosa* and *C. haemolytica*, revealed that the former is a close relative of *C. gingivalis*, whereas the latter occupies a quite distinct position within the genus *Capnocytophaga* (Fig. 5). In general, rRNA sequence analyses of *Capnocytophaga* strains demonstrated substantial genotypic heterogeneity as differences in 16S rRNA sequences of more than 11% were calculated (Table 3). This genotypic heterogeneity becomes even more remarkable considering the difficulty in finding phenotypic traits which reliably differentiate these species.

The results of identification of the field isolates by means of whole-cell protein analysis were further substantiated by 16S rRNA sequence comparison for five field isolates (LMG 12115 through LMG 12119). At present, 16S rRNA sequence analysis is the gold standard to reveal the position of an unknown isolate in a phylogenetic framework (54), but it has been demonstrated that high similarity (97% or more) in 16S rRNA sequence does not necessarily guarantee species identity (5, 16, 40). Strains LMG 12115 and LMG 12117 were identified as *C. ochracea* by means of protein analysis and show the highest 16S rRNA sequence similarity values (between 97.4 and 98.7%) with the type strain and with a second reference strain of this species (Table 3). Similarity values of 95.8 and 96.3% were calculated with *C. sputigena*, the closest neighbor of *C. ochra-*

TABLE 5. DNA base ratios of strains examined

Organism used for DNA isolation	mol% (G+C)	PAGE cluster
<i>C. canimorsus</i> LMG 11510	37	I
<i>C. canimorsus</i> LMG 11512	38	I
<i>C. cynodegmi</i> LMG 11513 ^T	36	II
<i>C. cynodegmi</i> LMG 11538	35	II
<i>C. cynodegmi</i> LMG 11539	35	II
<i>C. ochracea</i> LMG 11977	37	III
<i>C. ochracea</i> LMG 11516	38	IV
<i>C. ochracea</i> LMG 11546	39 ^a	IV
<i>C. sputigena</i> LMG 11517	39	V
<i>C. sputigena</i> LMG 11518 ^T	38 ^a	V
<i>C. sputigena</i> LMG 11508	40	V
<i>C. granulosa</i> LMG 16022 ^T	42 ^b	VI
<i>C. gingivalis</i> LMG 11514 ^T	40 ^c	VII
<i>C. haemolytica</i> LMG 16021 ^T	44 ^b	— ^c
<i>Capnocytophaga</i> sp. strain LMG 12116	34	—
DF-3 strain LMG 11519	38	—

^a Data from reference 37.

^b Data from reference 55.

^c —, separate position in the protein electrophoretic dendrogram.

TABLE 4. Fatty acid compositions of the strains studied^a

Strain (n) ^b	Composition (%)														
	13:0 iso	ECL 13:56 ^c	14:0 iso	14:0	15:0 iso	15:0 anteiso	15:0	16:1 w/c	16:0	15:0 iso 3OH	15:0 3OH	ECL 16:580	16:0 iso 3OH	16:0 3OH	17:0 iso 3OH
<i>C. canimorsus</i> (5)	1.3 ± 0.5	2.2 ± 0.8	ND	1.7 ± 0.1	66.7 ± 1.7	1.4 ± 0.5	ND	ND	2.3 ± 0.5	6.6 ± 1.6	ND	TR	ND	4.3 ± 0.7	11.5 ± 0.4
<i>C. cynodegmi</i> (4)	1.0 ± 0.4	1.9 ± 0.7	ND	2.2 ± 0.7	66.5 ± 4.7	1.3 ± 0.5	ND	ND	2.8 ± 0.4	3.9 ± 0.6	ND	1.0 ± 0.2	ND	4.6 ± 2.5	12.9 ± 3.6
<i>C. gingivalis</i> (3)	3.9 ± 0.7	1.7 ± 0.3	ND	1.0 ± 0.1	63.5 ± 4.9	1.8 ± 0.2	1.6 ± 0.9	ND	1.9 ± 1.1	2.6 ± 1.1	TR	1.2 ± 0.3	ND	5.7 ± 1.5	14.0 ± 2.2
<i>C. ochracea</i> (6)	2.1 ± 0.5	1.3 ± 0.4	ND	1.5 ± 0.8	63.5 ± 9.3	1.6 ± 1.0	TR	ND	3.6 ± 2.3	1.2 ± 0.4	ND	TR	ND	8.1 ± 6.6	13.8 ± 2.8
<i>C. sputigena</i> (5)	2.9 ± 1.0	1.2 ± 0.9	ND	2.2 ± 1.0	68.0 ± 2.1	1.9 ± 0.3	TR	ND	4.2 ± 1.5	1.2 ± 0.4	ND	TR	ND	5.9 ± 1.2	9.7 ± 2.9
<i>C. granulosa</i> (5)	2.1 ± 1.0	TR	ND	1.9 ± 1.2	75.5 ± 6.8	1.3 ± 0.3	TR	ND	4.2 ± 4.0	1.6 ± 0.5	ND	TR	ND	5.0 ± 2.7	6.5 ± 1.7
<i>C. haemolytica</i> (1)	TR	TR	ND	2.2	60.7	1.8	TR	ND	9.8	TR	ND	TR	ND	8.4	9.4
Strain LMG 12116	1.5	1.7	ND	1.8	55.9	TR	TR	ND	4.2	3.4	TR	ND	ND	8.6	14.7
DF-3 strain LMG 11519	ND	TR	3.1	3.1	5.7	37.0	TR	3.9	4.9	TR	1.7	ND	ND	10.3	12.0
															5.8

^a Results for fatty acids for which the average amounts for all taxa were less than 1% are not given. Therefore, the percentages for each group do not total 100%. Mean percentages ± standard deviations are given. TR, trace amount (less than 1%); ND, not detected.

^b n, number of strains studied.

^c ECL, equivalent chain length value.

TABLE 6. Similarity matrix for species in the flavobacter-bacteroides phylum based on 16S rRNA sequence comparisons^a

Species	Similarity									
	<i>Prevotella melaninogenica</i>	<i>Prevotella loeschii</i>	<i>Bacteroides fragilis</i>	<i>Bacteroides vulgatus</i>	<i>Porphyromonas gingivalis</i>	<i>Porphyromonas endodontalis</i>	[<i>Bacteroides</i>] <i>forsythus</i>	[<i>Bacteroides</i>] <i>distasonis</i>	CDC group DF-3	[<i>Bacteroides</i>] <i>splanchnicus</i>
<i>Prevotella melaninogenica</i>		89.8	84.5	84.3	81.0	80.8	82.7	80.8	81.7	81.8
<i>Prevotella loeschii</i>	10.9		83.1	83.9	81.3	80.7	81.6	81.4	80.4	80.8
<i>Bacteroides fragilis</i>	17.4	19.1		91.4	84.5	83.4	86.0	85.9	85.2	82.7
<i>Bacteroides vulgatus</i>	17.7	18.1	9.1		85.1	83.7	86.1	84.6	85.5	82.9
<i>Porphyromonas gingivalis</i>	21.9	21.5	17.3	16.6		88.3	87.5	84.3	84.6	82.0
<i>Porphyromonas endodontalis</i>	22.1	22.4	18.8	18.4	12.7		86.3	83.4	83.9	81.0
[<i>Bacteroides</i>] <i>forsythus</i>	19.7	21.1	15.5	15.3	13.7	15.2		89.0	88.1	84.9
[<i>Bacteroides</i>] <i>distasonis</i>	22.2	21.3	15.7	17.3	17.6	18.7	11.9		87.2	84.0
CDC group DF-3	21.0	22.7	16.4	16.2	17.2	18.1	12.9	14.0		83.0
[<i>Bacteroides</i>] <i>splanchnicus</i>	20.9	22.2	19.6	19.4	20.5	21.9	16.9	18.0	19.3	
[<i>Cytophaga</i>] <i>fermentans</i>	22.0	23.1	18.3	19.4	19.5	18.1	17.2	18.8	17.4	17.3
[<i>Cytophaga</i>] <i>salmonicolor</i>	21.1	23.6	17.8	17.9	18.8	18.4	17.6	18.7	16.8	15.2
[<i>Bacteroides</i>] <i>putredinis</i>	22.5	22.6	21.6	22.7	19.9	20.0	18.0	20.3	19.0	18.5
<i>Rikenella microfus</i>	22.8	21.7	19.6	22.0	19.7	19.8	18.6	19.9	20.1	20.6
<i>Sphingobacter mizutae</i>	24.4	24.7	23.9	23.5	21.8	21.9	21.7	22.0	22.7	23.3
[<i>Flavobacterium</i>] <i>heparinum</i>	24.8	25.8	21.9	23.9	21.7	22.7	21.7	22.3	21.7	23.3
<i>Capnocytophaga ochracea</i>	27.0	27.8	24.6	26.8	25.0	24.7	22.6	24.2	23.2	22.4
<i>Capnocytophaga gingivalis</i>	24.7	26.9	24.2	25.1	24.1	23.2	22.4	23.7	21.8	21.9
<i>Flavobacterium aquatile</i>	25.3	26.4	24.1	25.2	24.0	23.7	24.1	25.0	22.6	21.5
<i>Cytophaga lytica</i>	24.9	25.9	22.3	23.6	22.8	21.7	22.4	24.3	22.4	22.7
<i>Chryseobacterium meningosepticum</i>	22.8	24.6	22.3	23.6	21.4	22.5	20.1	22.7	20.2	19.3
<i>Chryseobacterium gleum</i>	25.0	27.2	23.8	24.7	24.3	24.7	23.6	26.3	23.6	22.7
<i>Saprospira grandis</i>	29.0	28.8	29.5	30.0	27.4	27.8	28.7	30.7	30.2	25.9
[<i>Flavobacterium</i>] <i>ferrugineum</i>	29.2	31.3	25.4	27.4	28.9	29.3	26.5	26.0	27.3	26.4

^a Numbers above the diagonal are uncorrected percentages of similarity, and those below the diagonal are percentages of difference corrected for multiple base changes by the method described by Jukes and Cantor.

cea (Table 3; Fig. 5). Likewise, strain LMG 12118 shares the highest similarity value (98.5%) with the *C. gingivalis* type strain (LMG 11514^T), and strain LMG 12119 shares the highest similarity value (99.4%) with the *C. granulosa* type strain (LMG 16022^T). It is striking that these last two type strains share about 98.4% of their rRNA sequence, while only low-level or nonsignificant DNA-DNA binding was detected between these strains (this study; data not shown) (56). An even lower level of 16S rRNA similarity (97.4%) was detected between two different reference strains of *C. ochracea* (ATCC 27872^T and ATCC 33596 [LMG 11546]). However, these two strains share approximately 80% DNA-DNA homology (52, 56). As explained above, the percentage similarity in whole-cell protein content confirmed the DNA-DNA hybridization value in both cases. These findings corroborate that, also in the genus *Capnocytophaga*, 16S rRNA sequence analysis is an excellent tool to allocate strains into a phylogenetic tree but not to identify strains at the species level (40). They also confirm that high similarity in whole-cell protein content is a parameter for high genomic relatedness, as revealed by DNA-DNA hybridization studies confirming the general applicability of this technique (7).

As discussed above, strain LMG 12116 remained unidentified after whole-cell protein analysis. 16S rRNA sequence analysis demonstrated that this strain belongs to the genus *Capnocytophaga* but revealed a rather isolated position in the dendrogram, with *C. gingivalis* and *C. granulosa* as its closest neighbors (95.4 to 96.1% 16S rRNA similarity [Table 3]). The general conclusion of Stackebrandt and Goebel (40) that strains sharing less than 97% 16S rRNA similarity do not belong to the same species is confirmed here, since DNA-DNA hybridization experiments between strain LMG 12116 and the type strains of *C. gingivalis* and *C. granulosa* yielded only nonsignificant DNA-binding values (data not shown). This indicates that strain LMG 12116 represents a new species within

the genus *Capnocytophaga*. The DNA base ratio of this species is rather low (34%) was calculated for strain LMG 12116 [Table 5]), which further expands the range within the genus *Capnocytophaga* from 34 to 44 mol% G+C.

Comparative analysis of the 16S rRNA sequence of DF-3 strain LMG 11519 revealed that this organism does not belong to the genus *Capnocytophaga*, since similarity values of approximately 80% were calculated (Table 6). An extensive comparison with representative organisms belonging to different lineages within the gram-negative bacteria showed that the DF-3 isolate, which is a typical representative of its group (50), fell into a cluster (Fig. 4) with [*Bacteroides*] *forsythus* and [*Bacteroides*] *distasonis* (names in brackets refer to generically misclassified species). This result is consistent with cellular fatty acid analysis which had shown that the DF-3 group had a fatty acid profile that was distinctly different from those of *Capnocytophaga* species (Table 4) (50). The biochemical similarities and the superficial similarities in fatty acid composition did not necessarily indicate a close genotypic relatedness between capnocytophagas and DF-3 strains, as assumed by several investigators (8, 50). *Capnocytophaga* species possess high-percentages 13-methyltetradecanoate (15:0 iso) (50) (Table 4), whereas the DF-3 group strains, [*B.*] *forsythus* and [*B.*] *distasonis*, are characterized by significant levels (24 to 38%) of 12-methyltetradecanoate (15:0 anteiso) and only low to moderate levels (1 to 6%) of 15:0 iso (36) (Table 4). The DF-3 group strains, [*B.*] *forsythus*, and [*B.*] *distasonis*, form a new genus which branches next to the genus *Porphyromonas* (32). Formal description of this new genus will be made elsewhere.

Random- or repetitive element-primed PCR fingerprinting has been successfully used to type individual strains within a variety of microorganisms (42), and amplified fragments have been excised and used as species-specific probes in *Campylobacter*, *Naegleria*, *Chlamydia*, and *Yersinia* species (19). *C. gin-*

TABLE 6—Continued

Similarity													
[<i>Cytophaga</i>] <i>fermentans</i>	[<i>Cytophaga</i>] <i>salmonicolor</i>	[<i>Bacteroides</i>] <i>putredinis</i>	<i>Rikenella</i> <i>microfus</i>	<i>Sphingobacter</i> <i>mizutae</i>	[<i>Flavobacterium</i>] <i>heparinum</i>	<i>Capnocytophaga</i> <i>ochracea</i>	<i>Capnocytophaga</i> <i>gingivalis</i>	<i>Flavobacterium</i> <i>aquatile</i>	<i>Cytophaga</i> <i>lytica</i>	<i>Chryseobacterium</i> <i>meningosepticum</i>	<i>Chryseobacterium</i> <i>gleum</i>	<i>Saprospira</i> <i>grandis</i>	[<i>Flavobacterium</i>] <i>ferrugineum</i>
81.0	81.6	80.6	80.3	79.1	78.9	77.3	78.9	78.5	78.8	80.3	78.8	75.9	75.8
80.1	79.7	80.5	81.2	79.0	78.1	76.8	77.4	77.8	78.1	79.1	77.2	76.1	74.4
83.8	84.1	81.3	82.7	79.5	81.0	79.0	79.4	79.4	80.7	80.7	79.6	75.6	78.5
82.9	84.1	80.4	80.9	79.8	79.5	77.5	78.7	78.6	79.8	79.7	79.0	75.3	77.1
82.8	83.3	82.5	82.6	81.1	81.2	78.8	79.4	79.4	80.4	81.4	79.3	77.1	76.0
83.9	83.7	82.4	82.6	81.0	80.4	79.0	80.0	79.7	81.2	80.6	78.9	76.7	75.7
84.6	84.3	84.0	83.5	81.2	81.1	80.5	80.6	79.4	80.6	82.4	79.7	76.1	77.7
83.4	83.5	82.2	82.5	80.9	80.7	79.3	79.6	78.7	79.3	80.4	77.8	74.8	78.0
84.5	85.0	83.2	82.4	80.4	81.1	80.1	81.0	80.5	80.6	82.3	79.7	75.1	77.1
84.5	86.2	83.6	82.0	80.0	80.0	80.6	81.0	81.3	80.4	83.0	80.4	78.1	77.7
	90.6	84.7	86.9	83.8	83.4	84.3	85.5	85.9	85.9	85.7	82.7	78.7	78.9
10.0		85.1	86.3	83.7	83.3	81.9	84.0	83.0	83.4	85.6	83.4	78.9	79.0
17.1	16.6		88.8	81.9	82.2	80.6	81.3	81.7	81.2	84.1	82.3	79.6	78.1
14.3	15.1	12.2		83.7	83.1	81.1	81.6	81.1	82.7	83.8	82.2	77.4	76.6
18.2	18.4	20.8	18.4		89.6	79.8	81.1	79.6	81.4	83.8	81.9	79.1	76.3
18.7	18.9	20.3	19.2	11.2		79.5	82.0	81.0	82.1	82.8	79.1	79.1	78.6
17.6	20.7	22.4	21.8	23.5	24.0		92.0	88.1	87.9	85.4	83.1	78.7	78.7
16.1	18.0	21.5	21.1	21.7	20.6	8.4		88.4	88.5	85.8	84.1	78.7	79.7
15.7	19.2	20.9	21.8	23.8	21.9	13.0	12.6		90.0	86.5	83.8	79.5	80.2
15.6	18.7	21.7	19.6	21.4	20.5	13.2	12.5	10.8		85.7	82.3	78.8	78.4
15.9	16.0	17.9	18.3	18.3	19.6	16.3	15.7	14.9	15.8		94.4	82.4	79.8
19.6	18.7	20.1	20.4	20.8	24.5	19.1	17.9	18.3	20.1	5.8		80.8	78.6
25.1	24.7	23.8	26.8	24.5	24.5	25.1	25.1	23.9	24.9	20.0	22.1		79.5
24.8	24.6	26.0	28.0	28.6	25.2	25.1	23.7	23.0	25.5	23.5	25.2	23.9	

FIG. 3. PCR fingerprinting patterns and DNA probing of *Capnocytophaga* strains with a combination of REP1 and REP2 as PCR primers and species-specific DNA fragments as molecular probes. (A) Results of PCR analysis. The molecular weight markers 1 and 2 are 1,600 and 500 bp in length, respectively. (B and C) Probe hybridizations of Southern blots made from gels as displayed in panel A.

FIG. 4. Phylogenetic tree for CDC group DF-3, allied bacteria, and reference species based on 16S rRNA sequence similarity. The scale bar represents 5% difference in nucleotide sequence as determined by measuring the lengths of the horizontal lines connecting any two species.

givalis, *C. sputigena*, *C. canimorsus*, and *C. granulosa* strains each give rise to similar DNA-banding patterns, underscoring the homogeneity of the species. For these species, DNA probes could be derived as a byproduct of the genotyping studies. In contrast, *C. ochracea* and *C. cynodegmi* appear to be more heterogeneous.

Finally, the data from fatty acid analysis confirm and extend the published data: all *Capnocytophaga* species, including the new species represented by strain LMG 12116, have very similar fatty acid components which are characterized by large amounts of branched-chain fatty acids, some of which are hy-

droxylated (Table 2). Under our growth conditions, *C. canimorsus* strains are characterized by percentages of 15:0 iso 3OH that were higher than the others, whereas *C. granulosa* contains a very high percentage of 15:0 iso. Strain LMG 12116 differs from other *Capnocytophaga* species by the presence of 5% 15:0, while this fatty acid is present in trace amounts in only some of the other species. Considering the standard deviations shown in Table 4, no other significant differences were detected.

In summary, we found a considerable genotypic heterogeneity within the genus *Capnocytophaga*, in spite of the minimal numbers of differentiating phenotypic features. These results showed that the degree of whole-cell protein similarity correlated with DNA-DNA homology and that percentage 16S rRNA similarity values of greater than 97% did not guarantee conspecificity, which is in overall agreement with the general data from these techniques (7, 40). We also showed that CDC group DF-3 is not a close relative of the *Capnocytophaga* species but constitutes a separate genus together with [*B.*] *forsythus* and [*B.*] *distasonis*, two generically misclassified *Bacteroides* species.

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FIG. 5. Phylogenetic tree for *Capnocytophaga* and reference species based on 16S rRNA sequence similarity. The scale bar represents 5% difference in nucleotide sequence as determined by measuring the lengths of the horizontal lines connecting any two species.

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