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Cellular Fatty Acids of *Capnocytophaga* Species

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The cellular fatty acid composition of 18 clinical isolates and 4 reference strains of *Capnocytophaga* species (*Capnocytophaga ochracea, Capnocytophaga gingivalis*, and *Capnocytophaga sputigena*) was determined by gas-liquid chromatography. The fatty acid profiles of the 22 cultures were essentially identical and were characterized by major amounts (60% or greater) of a saturated, iso-branched-chain, 15-carbon acid (13-methyl-tetradecanoate) and the presence of two relatively uncommon saturated, iso-branched, 3-hydroxy acids (13-methyl-3-hydroxy-tetradecanoate and 15-methyl-3-hydroxy-hexadecanoate). The presence and relative amounts of these acids distinguish *Capnocytophaga* spp. from other gliding bacteria.

Approximately 20 years ago, E. O. King described a group of dysgonic gram-negative bacteria which she designated DF-1. These organisms were oxidase negative, fermenting rods which grew best under microaerophilic or anaerobic conditions and were isolated from a variety of human clinical specimens. The DF-1 isolates remained unclassified until 1979, when the results of DNA homology studies revealed their high degree of relatedness with members of a new genus, *Capnocytophaga* (18, 19). The latter group are gram-negative, gliding bacilli which require CO_2 for growth either aerobically or anaerobically and are isolated primarily from human gingival scrapings (6). In the DNA study which compared DF-1 strains to *Capnocytophaga* type strains, seven of eight DF-1 strains showed the greatest degree of homology with the type strain of *Capnocytophaga ochracea*, whereas only one strain was homologous with *Capnocytophaga gingivalis* (19). There was only a low degree of homology between DF-1 strains and *Capnocytophaga sputigena*.

In our laboratory, we have found that cellular fatty acids are useful taxonomic markers for distinguishing various groups of bacteria. Using gas-liquid chromatography (GLC), we developed a rapid method for determining the fatty acid composition of bacteria and applied this method to the identification of reference and clinical isolates of *Pseudomonas* (1, 2, 10), *Alcaligenes* (1), *Flavobacterium* (11), and certain unclassified groups (2, 3). Since *Capnocytophaga* (DF-1) species are being isolated more frequently from clinical specimens, we decided to investigate their cellular fatty acids to ascertain whether they were similar to other gliding bacteria and to determine whether this information was useful as additional criteria for their rapid identification. In this report we compare the cellular fatty acids of clinical isolates of *Capnocytophaga* with reference strains of each of the three species of this genus.

MATERIALS AND METHODS

Cultures. Eighteen clinical isolates of *Capnocytophaga* species, formerly designated group DF-1, were obtained from the stock collection of the Special Bacteriology Laboratory, Centers for Disease Control. These isolates were cultured from a variety of human specimens. The group included strains C4295 and D6182, which were shown in DNA homology studies to be synonymous with *C. ochracea* and *C. gingivalis*, respectively (19). Reference strains *C. gingivalis* 30N-51, *C. ochracea* 25, and *C. sputigena* 4 were supplied by S. C. Holt, University of Massachusetts, Amherst. The latter two strains were also obtained from A. Broadwell, University of Washington, Seattle, along with *C. gingivalis* 27. Detailed descriptions of the morphology, biochemical features, and DNA relationships of *Capnocytophaga* species have been reported previously (6, 8, 15–19).

Culture conditions and derivatization. Bacteria were inoculated onto plates of heart infusion agar (HIA) supplemented with 5% rabbit blood and incubated for 48 h at 35°C in a candle extinction jar. To insure a sufficient amount of growth, we inoculated two plates per strain. Cells for fatty acid analysis were removed from the plates after 48 h and transferred to a 20- by 150-mm glass test tube. Cells were hydrolyzed by adding 4.0 ml of 5% sodium hydroxide in 50% aqueous methanol, sealing the tube tightly with a Teflon-lined cap, and heating for 30 min at 100°C. To form methyl esters of the fatty acids, we added 4.0 ml of 15% HCl

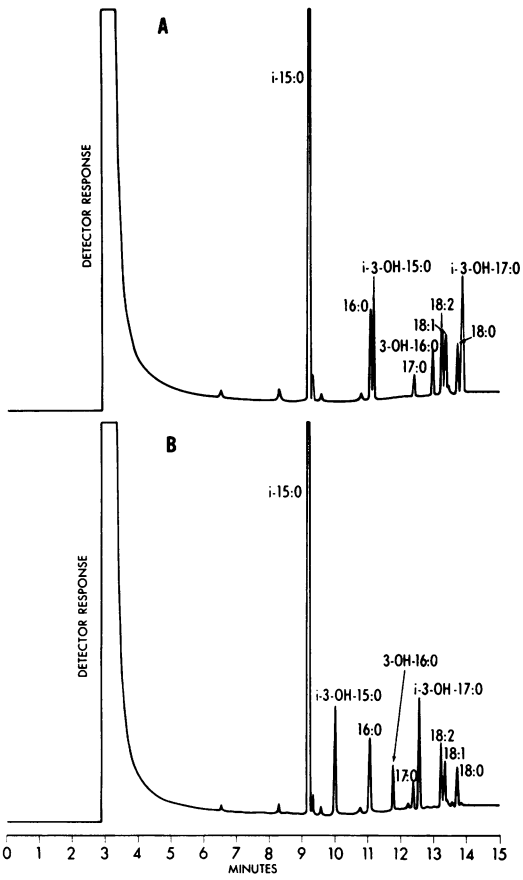

FIG. 1. Gas chromatograms of esterified fatty acids of *Capnocytophaga ochracea* C4295 (DF-1). Hydroxy acids in chromatogram B have been acetylated with TFAA. Analysis was made on a 50-m by 0.20-mm OV-101 fused-silica glass capillary column.

in methanol to the cooled hydrolysate, and the contents were heated at 100°C for 15 min. After being heated, the sample was allowed to cool, and the fatty acid methyl esters were extracted with 4.0 ml of a 1:1 mixture of diethyl ether and hexane (nanograde; Mallinckrodt, Paris, Ky.). After the contents of the tube were mixed several times, the organic layer was removed and the extraction step was repeated. Organic layers from both extractions were combined in a beaker and reduced to a small volume (0.5 ml) by evaporation under a gentle stream of nitrogen. The sample was then mixed with an equal volume of Na_2HPO_4 buffer, pH 11.0, by agitating the tube manually or by blending with a Vortex mixer. This step removed any traces of unesterified fatty acids by converting them to sodium salts. The organic (top) layer was then removed to a clean 13- by 100-mm test tube, and approximately 0.5 μl of the sample was injected into the gas chromatographic column.

GLC. Samples were analyzed on a fused-silica glass capillary column (50 m by 0.2 mm inner diameter) with OV-101 as the stationary phase (Quadrex Corp., New Haven, Conn.). The thickness of the stationary phase was 0.2 μm. The column was installed in a 3700 Varian gas chromatograph equipped with an all-glass capillary system (Varian Instruments, Palo Alto, Calif.). The sample size was 0.5 μl, which was split 50:1 to give a 0.01 μl sample on the column. Conditions for the GLC analysis were described in an earlier report (12). Bacterial fatty acids were tentatively identified by comparing GLC retention times to those of authentic standards (Applied Science, State College, Pa.; Analabs, North Haven, Conn.), and their identities were confirmed by GLC-mass spectrometry (10) and by acetylation and hydrogenation procedures (1). Quantitative data were obtained with an electronic integrator (Hewlett-Packard, model 3390A).

RESULTS AND DISCUSSION

The cellular fatty acid composition of the 18 clinical isolates of *Capnocytophaga* spp. were essentially identical and indistinguishable from reference strains of *C. ochracea, C. gingivalis*, and *C. sputigena*. A representative chromatogram of the fatty acids (as methyl esters) of *C. ochracea* C4295 run on a fused-silica capillary column is shown in Fig. 1. With this column, essentially base-line resolution was obtained for all peaks in the chromatogram. This degree of separation is not possible with packed columns (12). For example, with a packed column containing a nonpolar stationary phase (i.e., SE-30, OV-1, OV-101), i-15:0 coelutes with a-15:0, i-3-OH-15:0 with 16:0, 18:2 with 18:1, and i-3-OH-17:0 with 18:0 (chemical names have been abbreviated as follows: a, anteiso; i, iso; OH, hydroxy; c-v, *cis*-vaccenic; left of colon, number of carbon atoms; right of colon, number of double bonds). Also shown is the chromatogram obtained after treating the methyl ester sample with trifluoroacetic anhydride (TFAA) (Fig. 1B). This reagent reacts with free hydroxyl groups to form diester derivatives, which upon subsequent analysis will elute from the GLC column earlier than will the corresponding methyl ester. It is clear that each of the designated hydroxy fatty acids in Fig. 1A reacted with TFAA, as noted by the decrease in retention time for each of these three components (Fig. 1B). This reaction with TFAA confirmed mass spectral data which indicated a free hydroxyl group at C-3, as evidenced by the characteristic $m/e = 103$ and $M^+ - 50$ ions for each of the three hydroxy acid methyl esters (10).

The major fatty acid in *Capnocytophaga* was a saturated, iso-branched, 15-carbon acid which we identified as 13-methyl-tetradecanoate (i-15:0) (Fig. 1). This acid comprised up to 78% of the total acids present. On the capillary column i-15:0 was readily separated from its anteiso isomer (a-15:0), which can be observed as the

small peak eluting just after i-15:0 at approximately 9.3 min. Mass spectrometry confirmed the GLC retention time data indicating the presence of an i-15:0 acid. Mass spectra showed a molecular ion at $M^+ = 256$ and intense M^+-31 and M^+-29 ions, which appeared consistently in a ratio of 2:1 or greater and indicated that the methyl branch was at the penultimate carbon of the acid (iso branch). When the methyl branch occurs at the antepenultimate carbon (anteiso branch), the ratio of M^+-31 and M^+-29 is 1:1 (14). Another characteristic feature of *Capnocytophaga* spp. was the presence of iso-branched, 3-hydroxy 15- and 17-carbon acids (i-3-OH-15:0, i-3-OH-17:0), which, as noted above, are readily identified by their reaction with TFAA (Fig. 1B) and by mass spectra. Although iso-branched hydroxy acids are relatively rare in bacteria, we have reported their presence in strains of *Flavobacterium* spp. (2, 11), *Pseudomonas maltophilia* (10), and in the unclassified groups IIj and DF-2, which were isolated from the gingival scrapings of dogs (3). However, the presence of an i-2-OH-15:0 and an unsaturated, iso-branched, 17-carbon acid (i-17:1) in flavobacteria and group IIj readily distinguishes these organisms from *Capnocytophaga* spp. Moreover, *P. maltophilia* is characterized by i-2-OH-11:0, i-3-OH-11:0, and i-3-OH-13:0, none of which were detected in the *Capnocytophaga* isolates. The cellular fatty acid composition of DF-2 strains is essentially identical to that of *Capnocytophaga* (DF-1) strains, and therefore conventional criteria must be used. Primarily, acid production from sucrose and demonstration of oxidase and catalase activity can be used to distinguish DF-2 from *Capnocytophaga* isolates. The clinical source of these bacteria also provides a major difference, in that DF-2 strains are isolated from the mouths of dogs and from disease associated with animal bites (3), whereas *Capnocytophaga* strains have been isolated only from humans (19). A valid taxonomic disposition of DF-2 cannot be made until the DNA relationships of these organisms with already described species have been established. These studies are now in progress.

Other fatty acids detected in minor amounts in the *Capnocytophaga* strains were 14:0, 15:0, $16:1^{\Delta 9}$, 16:0, 17:0, 3-OH-16:0, 18:2, 18:1 (oleic), 18:1 (c-v), and 18:0. Quantitative data on the fatty acid composition of selected strains are presented in Table 1. The data show that the reference and clinical isolates of *Capnocytophaga* were essentially identical. The majority of fatty acids characterizing the three species of *Capnocytophaga* examined were saturated and iso branched, and these comprised more than 80% of the total acids in some of the strains. The major acid, i-15:0, ranged from 61 to 78% of the

TABLE 1. Cellular fatty acid composition of *Capnocytophaga* spp.

Strain	Fatty acid (% of total)[a]												
	i-13:0	14:0	i-15:0	a-15:0	15:0	$16:1^{\Delta 9}$	16:0	3-OH-i-15:0	17:0	3-OH-16:0	18:2	Oleic, 18:1	C-v, 18:1	18:0	3-OH-i-17:0
C. ochracea C4295	—	T	77	T	T	T	4	5	T	2	3	2	T	2	5
C. gingivalis D6182	T	T	76	T	T	T	5	3	T	2	4	3	T	2	5
C. ochracea 25[b]	—	T	61	T	T	T	12	3	T	2	10	6	T	4	2
C. gingivalis 27[b]	3	T	75	T	T	T	3	2	T	4	3	2	T	T	8
C. sputigena 4[b]	T	T	78	T	T	T	4	3	T	4	2	T	T	2	7

[a] T, Less than 2%; —, not detected.
[b] Type strain.

TABLE 2. Cellular fatty acid composition of *Capnocytophaga* spp. cultured on HIA and HIA supplemented with 10 mmol of leucine or isoleucine

Strain	Growth medium	Fatty acid (% of total)[a]				
		i-13:0	14:0	i-15:0	a-15:0	15:0
C. ochracea 25	HIA	—	T	61	T	T
	HIA + leucine	3	T	55	T	T
	HIA + isoleucine	2	T	62	2	T
C. gingivalis 27	HIA	3	T	75	T	T
	HIA + leucine	T	T	74	T	T
	HIA + isoleucine	T	T	71	5	T
C. sputigena 4	HIA	T	T	78	T	T
	HIA + leucine	3	T	76	T	T
	HIA + isoleucine	T	T	70	5	T

total, with a mean percentage (data not shown) of 67% for the 24 strains.

Reports on the fatty acid composition of other gliding bacteria (*Cytophaga-Flexibacter* group, myxobacteria) show that these organisms, like *Capnocytophaga*, contain significant amounts of saturated and branched-chain as well as iso-branched chain hydroxy acids (4). However, myxobacteria (*Myxococcus fulvus, Stigmatella aurantiaca*) are readily distinguished by the presence of iso-branched, 2-hydroxy acids (i-2-OH-15:0, i-2-OH-17:0) and i-17:0, which are not found in *Capnocytophaga*. The *Cytophaga-Flexibacter* group can also be readily distinguished from *Capnocytophaga* by the presence of i-2-OH-15:0 and 15:1 acids. In addition, there are significant differences in the relative amounts of i-3-OH-17:0, i-15:0, and 15:0 acids between *Capnocytophaga* spp. and the *Cytophaga-Flexibacter* group. In one report (5), a-15:0 was identified as the major acid of *Capnocytophaga*. However, these workers used a packed GLC column and did not report any mass spectral data to confirm their identification. In the present paper, the i-15:0 acid which was the major component of all the *Capnocytophaga* strains was identified by retention-time comparison on the capillary column and by mass spectrometry. As noted previously, i-15:0 (as methyl ester) is resolved from a-15:0 on the capillary column (Fig. 1; references 9, 12). Also, the mass spectrum was consistent in all respects with i-15:0, including the ratio of M^+-31 and M^+-29 ions (14).

Studies with various bacterial species have shown that the relative abundance of cellular branched-chain acids may be affected by the availability in the growth medium of precursors of the terminal portion of the acids (7, 13). The amino acids L-leucine and L-isoleucine are two common precursors; L-leucine serves as the precursor for iso-acids, and L-isoleucine for anteiso-acids. In the present study, 10-mmol amounts of each amino acid were added separately to HIA supplemented with rabbit blood (Norland Biological Laboratories, Tucker, Ga.); plates were prepared with this agar and then inoculated with reference strains 4, 25, and 27. After 48 h of incubation, cells of each strain were removed and processed for cellular fatty acids. Neither amino acid significantly changed the relative amounts of iso- or anteiso-acids from those observed in the unsupplemented HIA medium (Table 2). In each of the three strains the i-15:0 isomer was the single major acid even when isoleucine was added to the medium. Essentially identical fatty acid profiles were also observed for three strains after growth for 48 h on Trypticase soy agar (BBL Microbiology Systems) plates supplemented with sheep blood, whether incubated under anaerobic conditions or in candle extinction jars. These data are similar to those of others, who found little differences in the fatty acid composition of capnobacteria grown in broth or agar medium for various periods of time (5).

In summary, *C. ochracea, C. sputigena*, and *C. gingivalis* were essentially identical in fatty acid composition to each other and to clinical isolates which were formerly designated DF-1. Although the data clearly show that no distinction among species can be made by cellular fatty acid composition, the GLC procedure sharply distinguished *Capnocytophaga* from other gliding bacteria and, with the exception of DF-2, from related non-gliding bacteria, which have some common cellular fatty acids. At present there are no absolute criteria for identifying isolates of *Capnocytophaga* to species level (16). Nevertheless, the speed and reliability of the GLC fatty acid procedure make it highly adaptable for use as a rapid screening procedure for the presence of capnobacteria in clinical specimens.

TABLE 2—*Continued*

Fatty acid (% of total)[a]									
$16:1^{\Delta 9}$	16:0	3-OH-i-15:0	17:0	3-OH-16:0	18:2	Oleic, 18:1	C-v, 18:1	18:0	3-OH-i-17:0
T	12	3	T	2	10	6	T	4	2
T	9	4	T	3	9	4	T	2	11
T	5	4	T	3	5	2	T	2	13
T	3	2	T	4	3	2	T	T	8
T	4	4	T	4	3	T	T	T	11
T	3	4	T	5	3	T	T	T	9
T	4	3	T	4	2	T	T	2	7
T	3	3	T	4	3	T	T	T	8
T	4	4	T	5	3	T	T	T	9

[a] T, Less than 2%; —, not detected.

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