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Cellular Fatty Acids in *Fusobacterium* Species as a Tool for Identification

KAJSA TUNÉR,[1]† ELLEN JO BARON,[2,3]* PAULA SUMMANEN,[2] AND SYDNEY M. FINEGOLD[2,3,4]

Huddinge University Hospital, Karolinska Institute, Stockholm, Sweden[1]; Research Service, W151,[2] and Medical Service,[4] Wadsworth VA Medical Center, Wilshire and Sawtelle Boulevards, Los Angeles, California 90073; and Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024[3]*

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Identification of fusobacteria from clinical specimens currently requires analysis of metabolic end products by gas-liquid chromatography in addition to certain biochemical and enzymatic tests because of the relative biochemical inactivity of these bacteria. Even the finding of pointed, thin gram-negative cells on Gram-stained slides can no longer be relied on for identification of *Fusobacterium nucleatum*, since at least four other species of fusobacteria have been seen to exhibit similar morphology. We examined 46 clinical isolates and six American Type Culture Collection type strains of fusobacteria by conventional methods and by the Microbial ID Systems MIDI software package for analyzing cellular fatty acid patterns measured by capillary column gas-liquid chromatography. Distinctive patterns of major fatty acids could be used to reliably identify most clinical isolates to the species level. The MIDI system identified 89% of the isolates correctly and provides an alternative to conventional methods.

The genus *Fusobacterium* consists of anaerobic gram-negative bacilli that are often implicated in infections arising from the oropharyngeal region, the pelvis, and the gastrointestinal tract (7). Different *Fusobacterium* species are recovered from different areas of the human body. The different species also show quite different susceptibilities to antibiotics; *Fusobacterium varium* and *F. mortiferum* are much more resistant to antibiotics than the other species (7). The only *Fusobacterium* species that has been reported to produce a β-lactamase is *F. nucleatum* (18). Thus, a definite and accurate identification of a bacterium serves not only as taxonomic information but also as important information to clinicians for the treatment of infections.

Plaut and Vincent observed and described these bacteria in Vincent's angina and acute necrotizing ulcerative gingivitis in the late 19th century (13, 19). Initially, this genus was identified by morphology and fetid odor, the latter caused primarily by the production of butyric acid. Knorr first proposed the generic name *Fusobacterium* for these bacteria (8). During the last 15 years, the classification of these bacteria has been reevaluated by using nucleic acid analyses, analyses of cellular components (e.g., protein and fatty acids), and enzymatic characteristics (2–4, 6, 10, 14, 15). These methods are too cumbersome to use for the identification of fusobacteria in the nonresearch setting. At present, the genus includes 16 different species, with two subspecies of *F. necrophorum* and three subspecies of *F. nucleatum*. Because these subspecies and the new species *F. pseudonecrophorum* were described after our studies were completed, and because identification is usually dependent on molecular and other sophisticated methods, no attempt was made to separate them for this evaluation. Several classical identification schemes for fusobacteria using colonial and microscopic morphology and biochemical characteristics have been proposed (1, 8, 9, 12).

A preliminary presumptive identification of the genus *Fusobacterium* can be made by special-potency antibiotic discs containing vancomycin (5 μg), kanamycin (1,000 μg), and colistin (10 μg) (17); fusobacteria are susceptible to kanamycin and colistin and resistant to vancomycin. Members of the genus are further characterized by producing butyric acid without any iso acids as their major metabolic end product. Because of the weak metabolic activity of these bacteria, only a few biochemical tools are available to clinical laboratories for separating the species. These are indole and lipase production, esculin hydrolysis, the ability to grow in the presence of 20% bile, fermentation of lactose, production of β-galactosidase, and conversion of threonine and lactate into propionic acid.

In recent years, gas-liquid chromatography (GLC) has been used to study the cellular fatty acid composition of fusobacteria (2, 4, 6). L. V. H. Moore and W. E. C. Moore (Virginia Polytechnic Institute and State University, Blacksburg) have evaluated the cellular fatty acid patterns of *Fusobacterium* species and other anaerobic bacteria by using a standardized extraction protocol (2a). Their results have been incorporated into a constantly expanding computerized data base (MIDI) that is distributed by Microbial ID Software, Inc. (Newark, Del.), and designed to be used in conjunction with a specific Hewlett-Packard GLC for determining the cell wall fatty acid compositions of bacterial extracts. Using an early version of the MIDI VPI Anaerobic Broth Library data base (version 3.2) as a basis for comparison, we analyzed 52 strains of eight different *Fusobacterium* species using both biochemical methods and cellular fatty acid patterns.

MATERIALS AND METHODS

Eight different species, which included those most likely to be isolated from human clinical specimens, were ana-

* Corresponding author.
† Present address: Roussel Nordiska AB, S-126 12 Stockholm, Sweden.

lyzed. *F. nucleatum* (22 strains), *F. mortiferum* (8 strains), *F. varium* (7 strains), *F. necrophorum* (4 strains), *F. gonidiaformans* (4 strains), and *F. russii* (1 strain) were clinical isolates from the collection of the Wadsworth Clinical Anaerobic Research Laboratory. The following type strains were also included: *F. nucleatum* ATCC 10953, *F. mortiferum* ATCC 9817, *F. varium* ATCC 8501, *F. necrophorum* ATCC 25286, *F. necrogenes* ATCC 25556, and *F. periodonticum* ATCC 33693.

Identification. The bacteria, which had been frozen in skim milk at −70°C, were subcultured on brucella blood agar containing 5% sheep blood supplemented with vitamin K_1 and incubated for 48 h at 35°C in an anaerobic chamber (85% N_2, 10% CO_2, 5% H_2). Colony and microscopic morphologies as viewed under a dissecting microscope were noted. The following biochemical tests were performed to initially separate the different species: indole production, esculin hydrolysis, and lipase production on egg yolk agar. Conventional biochemical inoculations were carried out in prereduced media in the anaerobic chamber. The volatile fatty acid end products of glucose metabolism and the ability of each strain to convert lactate and threonine to propionate were determined with prereduced, anaerobically sterilized media (Carr-Scarborough Laboratories, Stone Mountain, Ga.) by GLC using standard methods outlined in the *Wadsworth Anaerobic Bacteriology Manual*, 4th edition (17), and the VPI *Anaerobe Laboratory Manual*, 4th edition and update (5, 11).

Fatty acid analysis. Ten milliliters of Microbial ID-specific prereduced, anaerobically sterilized peptone-yeast extract-glucose (Carr-Scarborough) in Hungate-stoppered tubes was inoculated on a benchtop with a suspension of a young culture of the test organism in chopped meat broth (Carr-Scarborough) by using a syringe and needle inserted through the rubber stopper to maintain the anaerobic atmosphere inside the tube. After 24 or 48 h of incubation at 35°C, the cultures were centrifuged and the supernatants were removed. The cells were saponified by heating them at 100°C for 30 min after addition of 1 ml of 15% NaOH in 50% aqueous methanol. The samples were cooled to room temperature, and 1 ml of 6 N hydrochloric acid in methanol and 1 ml of 50% aqueous sulfuric acid in methanol were added before the samples were heated at 80°C for 10 min. The samples were rapidly cooled, and the methylated fatty acids were further extracted with methyl-*tert*-butyl ether and hexane (1:1). The samples were mixed by turning them end over end for 10 min. The lower (aqueous) phase was removed, and the upper phase was mixed by turning the samples end over end with 3.0 ml of sodium hydroxide in saturated sodium chloride (5.4 g of NaOH in 450 ml of distilled water saturated with approximately 130 g of NaCl) for 5 min.

Two-thirds of the upper phase was then transferred into vials for analysis by GLC. The GLC analysis was done in an HP 5890 A gas chromatograph (Hewlett-Packard) with an Ultra 2,004-11-09B fused-silica capillary column (25 m by 0.2 mm [inner diameter] with cross-linked 5% phenylmethyl silicon [Hewlett-Packard]) outfitted with an automatic injector. Hydrogen was used as the carrier gas.

The GLC settings, automatically controlled by the computer software, were as follows: injection port temperature, 250°C; detector temperature, 300°C; and initial column temperature, 170°C, increasing by 5°C/min to 270°C and maintained at 270°C for 2 min before recycling back to 170°C. The total analysis time was 25 min, and the sample volume was 1 μl. The peak retention time and peak area values were recorded by an HP 3392 A integrator (Hewlett-Packard 216) and transferred to the Microbial ID Software package for calculation and comparison with the VPI identification library. A fatty acid standard was run for calibration on each occasion.

The MIDI program analyzes cellular fatty acid methyl ester (FAME) patterns on the basis of major peaks or clusters. A GLC system configured in this way detects and names fatty acid methyl esters, aldehydes, and dimethylacetyls and detects other peaks or clusters, called summed features, that are not named specifically. For each organism tested, a numerical value is assigned to the percentage of the area under the curve of the largest principal component (peak or cluster of peaks) in relation to the total area of all peaks measured, and a similar value is assigned to the next largest component. These values are plotted on the x and y axes of a graph. Similar organisms will map to the same general area of the two-dimensional plot generated by fatty acid methyl ester patterns. The relative distance in two-dimensional space between two strains (Euclidean distance) is a measure of their relatedness; shorter distances indicate closer relationships.

Once a series of strains that are similar (as determined by biochemical, genetic, or other characteristics) has been analyzed (or, alternatively, one strain has been analyzed several different times with slightly different incubation and growth parameters), the entire area occupied by their plotted values can be encircled and labeled. Strains with patterns similar enough to fall within the geographic group are thought to be similar (usually within the same genus, species, or biovar, etc.). The location thus identified by pattern analysis, if it contains all strains of a known species, is called a library entry. Analyses of many strains or smaller numbers of strains repeated many times become the library data base against which new strains are compared. The pattern of a new strain being analyzed can then be plotted, and the Euclidean distance from the point representing the percentage of the total area occupied by its principal components to a point in the center of the area occupied by the previously analyzed strains (a statistically generated mean) can be measured. The similarity index represents a calculation based on the distance of the new strain from the established group of strains. Index values range from 0.0 (no similarity) to 1.0 (the new strain falls directly in the center of the comparative group). Obviously, the correctness of the identification of the strains used for original library generation determines the accuracy of subsequent identifications based on similarity. For testing of anaerobic bacteria with this system, an acceptable match requires an index value of 0.300 or greater.

RESULTS

Identification. Conventional biochemical results were consistent with expected reactions. All strains of *F. mortiferum*, *F. necrogenes*, and *F. varium* tested were resistant to 20% bile; 25% of the *F. nucleatum* strains were also resistant. None of the *F. necrophorum* or *F. gonidiaformans* strains or the single American Type Culture Collection strains of *F. russii* and *F. periodonticum* were able to grow in 20% bile-containing media. One-half of the *F. varium* strains produced indole. *F. gonidiaformans*, *F. nucleatum*, *F. necrophorum*, and *F. periodonticum* were always indole positive. The lipase-positive reaction for the two lipase-positive strains of *F. necrophorum* tested could be observed after 2 days of incubation. Two other strains thought to be *F.*

TABLE 1. Mean areas of key FAMEs of clinical isolates of six species of *Fusobacterium* correctly identified by the MIDI system

Fatty acid	Mean area[a]					
	F. nucleatum (21/20)[b]	*F. gonidiaformans* (3/3)	*F. varium* (12/7)	*F. necrophorum* (8/2)	*F. mortiferum* (13/8)	*F. russii* (3/1)
12:0 FAME	1.3 (0.25)[c]	5.1 (1.94)	ND[d]	6.0 (2.08)	0.8 (0.19)	5.0 (1.14)
14:0 FAME	26.1 (3.69)	31.2 (5.58)	17.8 (2.68)	16.7 (3.14)	23.0 (1.7)	11.7 (2.25)
16:1 *cis* 7 FAME	0.7 (0.17)	2.9 (1.17)	2.4 (0.53)	5.3 (0.53)	ND	8.1 (0.63)
16:1 *cis* 9 FAME	20.3 (4.29)	6.3 (6.0)	18.1 (2.38)	19.7 (1.53)	17.1 (3.14)	15.7 (1.20)
16:0 FAME	21.8 (4.50)	32.7 (2.69)	14.5 (2.92)	16.4 (1.88)	23.6 (5.16)	35.7 (0.93)
16:0 DMA[e]	5.1 (3.29)	1.3 (0.89)	14.9 (1.59)	5.5 (1.76)	9.4 (2.59)	ND
16:0 3-OH FAME	4.1 (0.56)	0.9 (0.17)	ND	ND	ND	ND
18:1 *cis* 9 FAME	0.5 (0.22)	0.8 (0.49)	0.9 (0.46)	1.8 (1.13)	0.8 (0.58)	7.1 (1.37)
Summed feature 10[f]	7.9 (2.33)	0.9 (0.66)	1.6 (0.43)	3.0 (0.71)	2.0 (0.64)	4.1 (1.66)
Summed feature 5[g]	4.9 (0.7)	5.4 (4.51)	7.7 (0.84)	9.2 (1.85)	7.2 (1.24)	6.6 (0.5)

[a] Percentage of total area of named peaks.
[b] Number of strains analyzed/number of strains with similarity index of >0.3. In some cases, two or more different subcultures of a single strain were extracted to produce more information.
[c] Data in parentheses are standard deviations.
[d] ND, not detected.
[e] DMA, dimethylacetyl.
[f] Summed feature 10 includes peak 18:1 c11/t9/t6 FAME and unknown peak UN 17.834, which coelute or are not reliably separated.
[g] Summed feature 5 includes peaks 15:0 DMA and 14:0 3-OH FAME, which coelute or are not reliably separated.

necrophorum were lipase negative. Although half of the *F. varium* strains produced lipase, this reaction did not appear until the fifth to seventh day of incubation.

All strains produced butyric acid as the major volatile fatty acid, without iso acids; esculin was hydrolyzed by *F. necrogenes* and *F. mortiferum*. All strains except for *F. russii* produced propionate from threonine, but only *F. necrophorum* produced propionate from lactate.

Colony morphology on brucella blood agar was not a consistent parameter. *F. gonidiaformans* colonies were small, pinpoint, and translucent; *F. nucleatum* colonies were either bread crumb-like or flat and speckled. *F. necrophorum* colonies were either bread crumb-like or flat and umbonate, like fried eggs. *F. necrogenes* type strain colonies were small, smooth, and translucent; *F. mortiferum* colonies were large umbonate colonies; and colonies of *F. varium* were either small and smooth or large and umbonate. The Gram stain morphology (from colonies on brucella blood agar) was also variable within the same species. *F. varium* formed either big, polymorphic gram-negative rods or small *Bacteroides*-like gram-negative rods. *F. nucleatum* sometimes formed long straw-like gram-negative rods and sometimes formed short rods with pointed ends resembling *F. necrophorum*. *F. necrophorum* occasionally formed large, polymorphic rods resembling cells of *F. varium* and *F. mortiferum*. The most common morphology seen for *F. mortiferum* was that of large, pleomorphic, swollen, bizarre-looking rods.

Cellular fatty acid analysis. The mean percentages of the total peak area occupied by each named fatty acid ester, dimethylacetyl, aldehyde, or other component detected in the analyses of our fusobacteria in comparison with the values of the strains already present in the software library, version 3.2, are shown in Table 1. Only clinical isolates yielding similarity indices of 0.300 or greater were included in the analysis. Incorrectly identified strain data were not included in the table.

Strains were identified to the species level by the MIDI program as indicated in Table 2. However, six fusobacterial strains were misidentified. Two of four clinical *F. necrophorum* strains, which yielded results similar to those for *F. nucleatum* in the biochemical tests but converted lactate to propionate, were identified as *F. nucleatum* by cellular fatty acid patterns. Two strains of *F. nucleatum* were identified as *F. perfoetens* by the MIDI system, although the other *F. nucleatum* strains were correctly identified. The type strain of *F. necrogenes* was identified as *F. gonidiaformans*, and one strain of *F. gonidiaformans* was identified as *F. nucleatum* by the MIDI system.

DISCUSSION

In earlier studies, *Fusobacterium* species seemed to separate into two distinct groups; one group described by Jantzen and Hofstad was characterized by the absence of 14:0 3-OH FAME and contained the species *F. prausnitzii*, *F. russii*, and *F. naviforme* (6). This fatty acid was not well separated by our GLC and is included within summed

TABLE 2. Performance of MIDI FAME analysis system (version 3.2) for identification of fusobacteria

Species	No. of clinical isolates tested	Type strain tested	No. (%) of isolates identified correctly	Misidentifications
F. gonidiaformans	4	None	3 (75)	1 isolate identified as *F. nucleatum*
F. mortiferum	8	ATCC 9817	9 (100)	
F. necrogenes	0	ATCC 25556	0 (0)	Type strain identified as *F. gonidiaformans*
F. necrophorum	4	ATCC 25286	3 (60)	2 clinical isolates identified as *F. nucleatum*
F. nucleatum	22	ATCC 10953	21 (91)	2 clinical isolates identified as *F. perfoetens*
F. periodonticum	0	ATCC 33693	1 (100)	
F. russii	1	None	1 (100)	
F. varium	7	ATCC 8501	8 (100)	

feature 5 of the MIDI data analysis (Table 1). *F. nucleatum* and *F. periodonticum* formed a homogenous subgroup with 16:0 3-OH FAME as a distinct characteristic, in disagreement with Calhoon and coworkers (2) but in agreement with findings for *F. nucleatum* and other strains reported by Jantzen and Hofstad (6) and with the description of *F. periodonticum* in the VPI *Anaerobe Manual Update* of February 1991 (11). *F. periodonticum* differs from *F. nucleatum* somewhat in its FAME pattern; this may be the only way to distinguish these species from each other. According to Slots et al., *F. periodonticum* ATCC 33693 ferments fructose, galactose, and glucose (16). We tested this type strain several times but could not detect any fermentation of those sugars, even with very good growth. The remaining species showed grossly similar FAME patterns (Table 1) and could only be distinguished on the basis of minor quantitative differences (2, 6). We only tested half of the species now included in the genus, but those tested were representative of those species likely to be encountered in a routine clinical microbiology laboratory.

For analysis with the MIDI system, we used the recommended modified prereduced, anaerobically sterilized peptone-yeast extract-glucose medium. All strains tested had been transferred on blood agar plates at least five times (and usually many more times) after being reconstituted from frozen stock before the growth rate appeared to be comparable to that of fresh isolates. Even after numerous transfers, some of the fusobacteria tested required extension of the incubation time to 48 h to obtain sufficient growth. This appears to be too long for some of the strains; degeneration of the FAMEs was the most common reason for the errors and is usually due to harvesting cells beyond the logarithmic growth phase. When bacteria were harvested after 24 h, the FAME patterns gave an excellent match to patterns in the MIDI library. Another reason for poor or absent matching was that too little cell mass was extracted. In such cases, we did not attempt to concentrate the extract but retested the strain from a fresh culture.

Each analysis was repeated from two to four times with different peptone-yeast extract-glucose broth cultures. All phenotypic tests were performed twice. When discrepant results were obtained with the MIDI system, similarity values were not consistently different from those seen with correct results. The correct species was occasionally listed at a lower similarity level.

Six strains were misidentified by the MIDI system in this study. Two strains similar to *F. nucleatum* in colony morphology, Gram stain, and biochemical reactions converted lactate to propionate and were identified by conventional methods as *F. necrophorum*; however, the MIDI system identified them as *F. nucleatum*. Without nucleic acid analysis, it is not possible to determine which species designation is correct. Two *F. nucleatum* strains were identified as *F. perfoetens* by cellular fatty acid analysis. In this case, the Gram stain morphology was the most important differentiating parameter (*F. perfoetens* consists of coccoid rods). Two strains identified by conventional methods as *F. necrogenes* (the American Type Culture Collection type strain) and *F. gonidiaformans* were misidentified as *F. gonidiaformans* and *F. nucleatum*, respectively, by the MIDI system. Although the *F. necrogenes* analysis was repeated three times, the system failed to identify the strain. Either this strain had lost some key characteristics during storage, or the early version of the library was insufficient to recognize it. Subsequent versions have not had this difficulty. Overall, the number of misidentified strains was small and the specificity of the analyses was very good. Improved and expanded versions of the data base are available yearly; several have been published since this evaluation. As the data base becomes better with the addition of diverse and accurately identified strains, the differential ability of the computer-generated cellular fatty acid analysis improves.

For those laboratories that require definitive identification of anaerobic bacteria, detection of short-chain volatile fatty acid end products of metabolism by GLC is a standard procedure. Analysis of whole-cell fatty acids requires a different GLC configuration (including a capillary column), special software, and an extraction process more complex than that used to prepare samples for volatile fatty acid analysis. Even microbiologists unfamiliar with the use of conventional GLC, however, will have no trouble learning to use the MIDI system. For most anaerobic isolates, conventional GLC could be bypassed after initial simple tests have been used to establish the genus or presumptive identification. With the system described here, all components of which are available commercially, computer-assisted data base analysis and species matching are now within the capability of any laboratory.

ADDENDUM IN PROOF

A new penicillinase produced by a highly penicillin-resistant (MIC, 1,024 mg/liter) strain of *Fusobacterium mortiferum* has recently been described (C. E. Nord and L. Lindqvist, Abstr. 32nd Intersci. Conf. Antimicrob. Agents Chemother., p. 323, abstr. 1274).

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