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Application of Gas-Liquid Chromatography to the Routine Identification of Nonfermenting Gram-Negative Bacteria in Clinical Specimens

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A total of 430 strains of glucose-nonfermenting gram-negative bacteria representing 35 species were analyzed for their cellular fatty acid composition by gas-liquid chromatography (GLC). On the basis of qualitative differences in their cellular fatty acid composition, these bacteria could be divided into 19 distinct chromatographic groups. Eight *Pseudomonas* species, *Achromobacter xylosoxidans*, group Vd, and *Agrobacterium radiobacter* were identified from their fatty acid compositions alone. The other glucose-nonfermenting gram-negative bacterial species studied here, classified within nine distinct GLC groups, were easily recognized by using the GLC fatty acid analysis supplemented with a limited number of conventional biochemical tests. The results support the hypothesis that bacterial fatty acid composition is rather specific and that qualitative GLC fatty acid analysis can be adapted in the clinical laboratory either to provide additional criteria for differentiation of closely related groups or to serve as a rapid and highly reproducible method for their routine identification.

As early as 1963, it was suggested that chemical analysis of microorganisms may be used in their classification (1). Subsequently, gas-liquid chromatographic (GLC) analysis of the fatty acid composition of bacteria has been proposed as a valuable test for identification and classification of a variety of bacteria, including nonfermentative gram-negative bacilli (2–15). In the present study, we have evaluated the applicability of GLC bacterial fatty acid analysis to the routine identification procedure for glucose-nonfermenting gram-negative bacteria (NFB) isolated from clinical specimens. Therefore, an extensive number of nonfermenting gram-negative bacteria (430 strains), representing 35 species of currently isolated microorganisms, have been screened. To further extend the potential of the GLC fatty acid analysis, it was of interest to investigate whether a qualitative rather than a quantitative appearance of fatty acid peaks was sufficient for the identification and differentiation of NFB.

MATERIALS AND METHODS

Cultures. A total of 430 strains of NFB were analyzed, including 410 strains selected from clinical isolates and 20 reference strains (Table 1). Identification of the bacteria was essentially based on conventional culturing and biochemical procedures as described by Rubin et al. (16). The commercial identification system API 20E (API System S.A., La Balme les Grottes, Montalieu Vercieu, France) was also used. Culture media were obtained from bioMerieux, Charbonnieres les Bains, France (Kligler agar and Columbia agar), and from BBL Microbiology Systems, Cockeysville, Md. (Mueller-Hinton agar). In general, the material for GLC analysis was obtained after growing the strains for 24 h (or 48 h) at 37°C on a Kligler agar slant.

Samples. A loopful of cells was suspended in 1 ml of saponification mixture (equal volumes of methanol and 2.5 N NaOH) and heated in a waterbath at 100°C for 30 min. After the mixture had cooled to room temperature, 0.5 ml of H_2SO_4 (66%) was added, and the mixture was reheated to 70°C for 60 min. The methylated fatty acid esters were subsequently extracted with 0.3 ml of $CHCl_3$ by shaking for 20 s. Phase separation was promoted by centrifugation, and the upper (aqueous) phase was carefully removed by aspiration. The lower (chloroform) phase, containing the fatty acid methyl esters, was dried over anhydrous sodium sulfate.

GLC. A 2-μl sample of the methyl ester fraction obtained as described above was analyzed on a gas chromatograph (no. 433; Packard Instrument Co., Inc., Rockville, Md.) equipped with a solid injector (Chrompack 8990; Packard). The extracted bacterial fatty acids were separated on a fused-silica capillary column (25 m by 0.23 mm [inner diameter]) coated with CPSil 5 (0.2-μm layer) as the stationary phase; a flame ionization detector was used to monitor the products. The fatty acid methyl esters were identified by comparing retention times with those of a bacterial fatty acid methyl ester reference mixture (no. 4-5436; Supelco, Inc., Bellefonte, Pa.). Operating conditions were as follows: nitrogen inlet pressure, 50 kPa; injector temperature, 250°C; detector temperature, 280°C; column temperature, programmed from 140 to 230°C at 2°C/min. Under these conditions, fatty acid methyl esters ranging from 10 to 20 carbons in length eluted from the column within 25 min.

RESULTS AND DISCUSSION

The NFB isolated from clinical specimens are listed in Table 1. This table also shows the 20 reference strains which were included in this study to confirm the chromatographic pattern of a particular NFB group. To analyze the bacterial fatty acid composition of the strains presented, we used a fused-silica capillary column, since iso and anteiso isomers of fatty acids with the same carbon length and also their hydroxy derivates are completely resolved on this column (12). Comparison of the gas chromatograms of the esterified bacterial fatty acids with fatty acid reference elution profiles has established the occurrence and identity of 23 fatty acid peaks (Table 2).

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TABLE 1. NFB strains used in this study

Species	No.[a]	Reference strain[b]
Pseudomonas group		
P. cepacia	10	ATCC 25416
P. stutzeri	22	NCTC 10475
P. mendocina	3	ATCC 25411
P. alcaligenes	4	ATCC 14909
P. pseudoalcaligenes	9	ATCC 17440
P. diminuta	7	ATCC 13184
P. vesicularis	7	ATCC 11426
P. acidovorans	24	ATCC 15668
P. testosteroni	9	ATCC 15666
P. paucimobilis	7	NCTC 11030
P. pickettii	6	ATCC 27511
P. aeruginosa	33	
P. putida	32	
P. fluorescens	21	
P. maltophilia	59	
P. putrefaciens	9	
Pseudomonas group Ve-2	5	
Pseudomonas group Ve-1	1	
Flavobacterium group		
F. meningosepticum	13	NCTC 10016
F. odoratum	11	NCTC 11036
F. breve	2	NCTC 11162
F. indologenes (IIb)	13	ATCC 29897
Sphingobacterium mizutae	1	
Alcaligenes group		
A. faecalis	11	NCIB 8156 + CCEB 554[c]
A. denitrificans	5	ATCC 15173
Achromobacter xylosoxidans	12	
Acinetobacter group		
A. calcoaceticus subsp. *anitratus*	46	ATCC 17903
A. calcoaceticus subsp. *lwoffii*	16	ATCC 17986
Moraxella group		
M. nonliquefaciens	1	
M. osloensis	1	
M. phenylpyruvica	2	
M. urethralis	1	
Others		
Bordetella bronchiseptica	3	
Agrobacterium radiobacter	2	
Group Vd (*Achromobacter* sp.)	2	

[a] Number of distinct strains.
[b] Abbreviations: ATCC, American Type Culture Collection, Rockville, USA; CCEB, Culture Collection of Entomogenous Bacteria, Prague, Czechoslovakia; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, London, England.
[c] *Alcaligenes* (*faecalis*) *odorans*.

TABLE 2. Major fatty acids isolated from NFB[a]

Peak	Identity	
	Abbreviation	Name
1	3-OH $C_{10:0}$	3-Hydroxydecanoate
2	$C_{12:0}$	Dodecanoate
3	$C_{13:0}$	Tridecanoate
4	2-OH $C_{12:0}$	2-Hydroxydodecanoate
5	3-OH $C_{12:0H}$	3-Hydroxydodecanoate
6	$C_{14:0}$	Tetradecanoate
7	i-$C_{15:0}$ (13-Me)	13-Methyltetradecanoate
8	a-$C_{15:0}$ (12-Me)	12-Methyltetradecanoate
9	$C_{15:0}$	Pentadecanoate
10	2-OH $C_{14:0}$	2-Hydroxytetradecanoate
11	3-OH $C_{14:0}$	3-Hydroxytetradecanoate
12	$C_{16:1}$ (^{9}*cis*)	Hexadecenoate (^{9}*cis*)
13	$C_{16:0}$	Hexadecanoate
14	a-$C_{17:0}$ (14-Me)	14-Methylhexadecanoate
15	$C_{17:0}$ Δ (cyclic 9,10)	Δ-*cis*-9,10-methylenehexadecanoate
16	$C_{17:0}$	Heptadecanoate
17	2-OH $C_{16:0}$	2-Hydroxyhexadecanoate
18	3-OH $C_{16:0}$	3-Hydroxyhexadecanoate
19	$C_{18:2}$	Octadecadienoate
20	$C_{18:1}$ (^{9}*cis*)	Octadecenoate (^{9}*cis*)
	$C_{18:1}$ (^{9}*trans*)	Octadecanoate (^{9}*trans*)
21	$C_{18:0}$	Octadecanoate
22	i-3-OH $C_{17:0}$	15-Methyl-3-hydroxyhexadecanoate
23	$C_{19:0}$ Δ (cyclic 11,12)	Δ-*cis*-11,12-methyleneoctadecanoate

[a] The fatty acids are numbered according to their elution order from the GLC column.

To evaluate the GLC fatty acid analysis as a routine procedure for NFB identification, we evaluated the elution profiles in a qualitative rather than a quantitative manner. In this way, GLC standardization problems are not encountered and the interpretation of the GLC elution profiles involves minimal time and errors.

Table 3 summarizes the cellular fatty acid composition of the investigated NFB, as determined by the chromatographic procedure. The results were obtained by repetitive analysis of NFB strains (at least five determinations). It should be noted that in addition to the 23 acids, trace amounts of other fatty acids could be detected. However, because their relative peak heights were less than 1% and because their appearance was not essential for identification of the bacterial source, these peaks are omitted from Table 3. Some fatty acids have no diagnostic value, since they appear in most of the NFB species (e.g., peaks 6, 12, 13, 20, and 21). In contrast, other peaks are more or less specific and can be detected only in a relatively small number of NFB species. Selecting the minimum combination of fatty acid peaks essential for identification of each GLC group, only 12 fatty acids are recognized as important markers (indicated by the solid circles in Table 3). Identical chromatographic patterns were obtained when colonies from Mueller-Hinton or Columbia agar were used as a source for the GLC bacterial identification technique.

Pseudomonads in GLC groups 4 to 11, *Achromobacter xylosoxidans*, group Vd, and *Agrobacterium radiobacter* can be identified from the fatty acid data alone. However, for the final identification of the other NFB groups, conventional tests are also required (Table 4).

Group 1 consists of the fluorescent species *P. aeruginosa*, *P. putida*, and *P. fluorescens* and the CDC Ve group (spp. Ve-1 and Ve-2). The major fatty acids observed for this group are summarized in Table 3. The simultaneous presence of three hydroxy acids, 3-hydroxydecanoate (3-OH $C_{10:0}$), 2-hydroxydodecanoate (2-OH $C_{12:0}$), and 3-hydroxydodecanoate (3-OH $C_{12:0}$), was found to be characteristic of strains classified within this group. This group is identical to the GLC group 1 described by Moss and Dees (10), and the present data are in agreement with their observations, except with respect to both cyclopropane acids ($C_{17:0}$ and $C_{19:0}$), which were always virtually absent. Ve-1 and Ve-2 were placed in this group because the chromatographic pattern of their fatty acids did not reveal any qualitative difference from that of the species discussed above. Recently, the fatty acid compositions of *P. luteola* (CDC group Ve-1) and *P. oryzihabitans* (CDC group Ve-2) have been reported by Freney et al. (5), confirming the qualitative

TABLE 3. Cellular fatty acid composition of the investigated NFB

Group no.	NFB group members	Presence of peak no.[a]:																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	*P. aeruginosa*, *P. putida*, *P. fluorescens*, group Ve-1, group Ve-2	●	○		●	●	○						○	○							○	○		
2	*P. cepacia*, *P. mallei*, *P. pseudomallei*											●	○	○				●	●		○	○		
3	*P. stutzeri*, *P. mendocina*, *P. alcaligenes*, *P. pseudoalcaligenes*	●	○			●	○			○			○	○	○	○	○				○	○		
4	*P. diminuta*					●	○			○			○	○	○		○				○	○		V
5	*P. vesicularis*					●	○	○	○	○			○	○	○	●	○				○	○		
6	*P. maltophilia*		○			●		●	●				○	○							○	○		
7	*P. putrefasciens*		○			●	○	●		○			○	○	○		○				○	○		
8	*P. acidovorans*	●	○				○			○			○	○		○	○							
9	*P. testosteroni*	●	○				○			○			○	○	○	○	○	●			○	○		
10	*P. paucimobilis*						○			○	●		○	○		○	○				○	○		
11	*P. picketii*						○			○	●	●	○	○		○	○	○			○	○		
12	*Flavobacterium* spp., *Sphyngobacterium* spp.						○	●	○	○			○	○					●				●	
13	*Alcaligenes* spp.		○		●		○					●	○	○		○	○				○	○		
14	*Achromobacter xylosoxidans*		○		●		○			○	●	●	○	○		○	○				○	○		
15	Group Vd (*Achromobacter* sp.)						○	○	○	○			○	○			○				○	○		●
16	*Acinetobacter* spp.		○		V	●	○	○	○	○		●	○	○	○		○				○	○		
17	*Bordetella* spp.		○		●		○			○		●	○	○		○	○		●		○	○	●	
18	*Agrobacterium radiobacter*		○				○	○	○	○		●	○	○					●		○	○		
19	*Moraxella* spp.		○			●	○			○		○	○	○	○					●	○	○		

[a] The peak numbers refer to the corresponding peaks listed in Table 2. Major fatty acids for each GLC group are indicated (○, ●). Peaks indicated by ● are characteristic for each group. Peaks indicated by V (variable) are not always apparent.

identity in fatty acid composition of both Ve species and the GLC data presented in Table 3. Nevertheless, Ve-1 and Ve-2 can be easily distinguished from members of the fluorescent group by a single oxidase test, and further differentiation between the fluorescent *Pseudomonas* species can be obtained by using a limited series of simple biochemical tests.

Group 2 consists of *P. cepacia*, *P. mallei*, and *P. pseudomallei* and is identical to the GLC group 2 described by Moss and Dees (10). The chromatogram of this group is unique in that it contains 3-hydroxytetradecanoate (3-OH $C_{14:0}$) and 2- and 3-hydroxyhexadecanoate (2-OH $C_{16:0}$ and 3-OH $C_{16:0}$, respectively). The presence of 2-OH $C_{16:0}$ has a high diagnostic value, because it represents a key characteristic for distinguishing the GLC group 2 species from *Agrobacterium radiobacter*.

In agreement with the observations made by Moss and Dees (10), *P. stutzeri*, *P. mendocina*, *P. alcaligenes*, and *P. pseudoalcaligenes* were placed in a single GLC group (group 3). Strains in this group were differentiated from other organisms by the presence of both 3-OH $C_{10:0}$ and 3-OH $C_{12:0}$ and the absence of 2-OH $C_{12:0}$. 2-OH $C_{12:0}$ provides an easy criterion for distinguishing GLC group 3 strains from group 1 strains. Qualitatively identical chromatograms were obtained with *P. stutzeri*, *P. mendocina*, *P. alcaligenes*, and *P. pseudoalcaligenes*.

TABLE 4. Additional tests for the identification of GLC groups

GLC group	Test
1	Indophenol oxidase, growth at 42°C, gelatin hydrolysis
2	Arginine dihydrolase, gelatin hydrolysis
3	Gas from nitrate, arginine dihydrolase, fructose oxidation
12	Indole, ONPG,[a] amylase, esculin hydrolysis
13	Gas from nitrate, nitrite reduction
16	Glucose oxidation
17	Urea
19	Except for *M. urethralis* (GLC) and *M. phenylpyruvica* (urea), complete biochemical characterization is required to differentiate between each species

[a] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.

Group 4 contains *P. diminuta* and corresponds to the GLC group 4 described by Moss and Dees (10). The group is characterized by the presence of 3-OH $C_{12:0}$ and Δ-*cis*-11,12-methyleneoctadecanoate ($C_{19:0}\Delta$). Moss and Dees (10) have reported that the $C_{19:0}\Delta$ acid accounts for approximately 30% of the total fatty acids in each of their nine distinct isolates of *P. diminuta*. Subsequently, they proposed the presence of this fatty acid as the major difference between *P. diminuta* and *P. vesicularis*. However, in contrast to their results, we observed that the presence of the $C_{19:0}\Delta$ acid was variable. Two of our seven strains of *P. diminuta* had only trace amounts of this acid (<2%) and were identified as *P. diminuta* from conventional biochemical identification tests.

Group 5 contains *P. vesicularis*. The fatty acid profile of *P. vesicularis* is characterized by the presence of 3-OH $C_{12:0}$ and Δ-*cis*-9,10 methylenehexadecanoate ($C_{17:0}\Delta$), and by the absence of 3-OH $C_{10:0}$. Moss and Dees (10) have designated the presence of the $C_{19:0}\Delta$ acid in *P. diminuta* as the major difference between *P. vesicularis* and *P. diminuta*. Nevertheless, a screening for the 3-hydroxy fatty acids reported here is adequate to differentiate *P. vesicularis* from all other NFB, including *P. diminuta*.

Group 6 contains *P. maltophilia* (*Xanthomonas maltophilia*), which is characterized by the presence of 3-OH $C_{12:0}$ and two 15-carbon branched-chain fatty acids: 13-methyltetradecanoate (i-$C_{15:0}$) and 12-methyltetradecanoate (a-$C_{15:0}$). The presence of these acids is in agreement with the observations made by Moss and Dees (10).

Group 7 contains *P. putrefasciens*, which can also be identified from its bacterial fatty acid profile alone, i.e., the presence of both 3-OH $C_{12:0}$ and i-$C_{15:0}$ with the character-

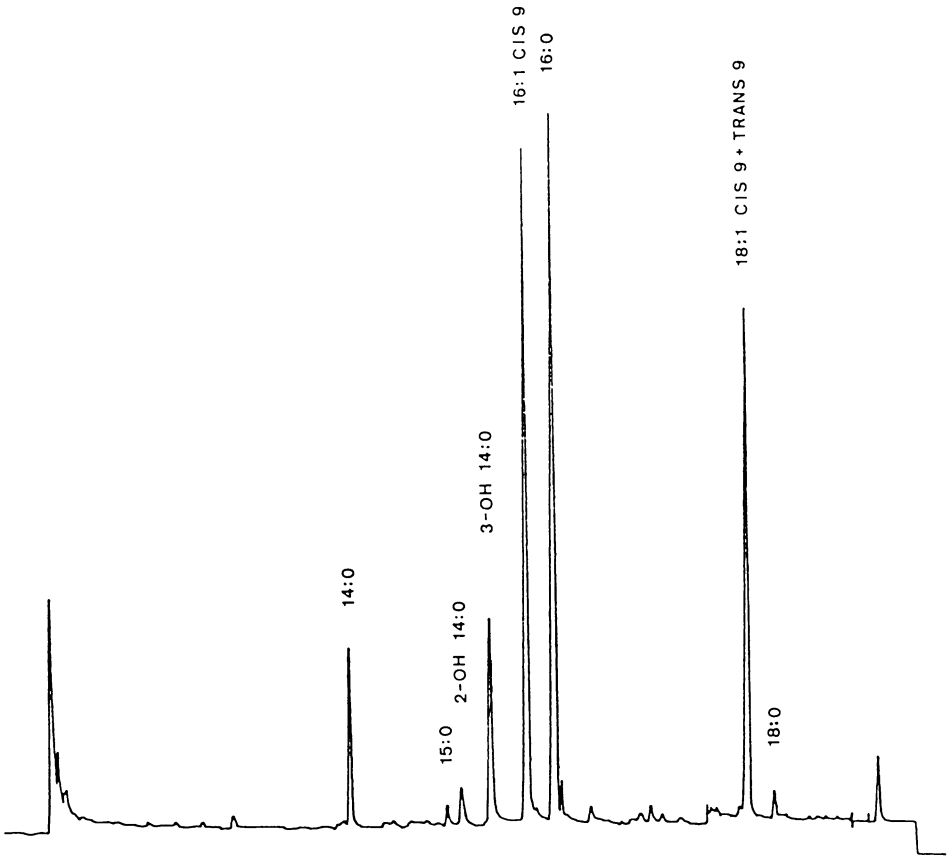

FIG. 1. Gas chromatogram of esterified fatty acids derived from *P. picketii*. Separation was performed on a fused-silica capillary column. Conditions were as described in Materials and Methods.

istic absence of a-$C_{15:0}$. These results also agree with the previous work of Moss and Dees (10).

Group 8 contains *P. acidovorans*. At variance with the observations made by Moss and Dees (10), *P. acidovorans* represents another *Pseudomonas* species which can be identified from its fatty acid profile alone. The combination of the presence of 3-OH $C_{10:0}$ and the absence of 2-OH $C_{16:0}$ is characteristic of this NFB.

Group 9 contains *P. testosteroni*. The fatty acid profile of *P. testosteroni* can be confused with that of *P. acidovorans*. Moss and Dees (10) placed both species in the same GLC group. However, besides the characteristic presence of 3-OH $C_{10:0}$, 2-OH $C_{16:0}$ was also present in all *P. testosteroni* strains.

Group 10 contains *P. paucimobilis*. *P. paucimobilis* is identified by the presence of a 2-hydroxy 14-carbon saturated acid (2-OH $C_{14:0}$) and the absence of its 3-hydroxy form (3-OH $C_{14:0}$). These results are in concordance with the data reported by Dees et al. (4).

Group 11 contains *P. picketii*. The fatty acid profile of *P. picketii* has not been previously published; it is characterized by the presence of both 2-OH $C_{14:0}$ and 3-OH $C_{14:0}$. A representative chromatographic pattern is shown in Fig. 1.

Group 12 contains *Flavobacterium* and *Sphingobacterium* species. In the genus *Flavobacterium*, clinically important members include *Flavobacterium* group IIb, which has recently been renamed *Flavobacterium indologenes* (17), *F. meningosepticum*, *F. odoratum*, and *F. breve*. The cellular fatty acid composition of each subgroup has been investigated (total number of subgroups = 40). In agreement with the work of Moss and Dees (11), the GLC profiles show the characteristic presence of $C_{14:0}$ and 3-OH $C_{16:0}$. From their fatty acid compositions, no distinction can be made between the different subgroups of the genus *Flavobacterium*. *Sphingobacterium* strains, which are similar to *Flavobacterium* strains except for their lack of indole production, have also been included in this study. Not surprisingly, the similarity of the two genera was further confirmed by their fatty acid analysis, which did not reveal any qualitative difference. It was found that biochemical characterization is not necessary to distinguish flavobacteria and sphingobacteria from similar pigmented *Pseudomonas* or *Pseudomonas*-like bacteria (Table 3).

Group 13 contains *Alcaligenes* species. The occurrence of 3-OH $C_{12:0}$ and 3-OH $C_{14:0}$ was found to be indicative of *Alcaligenes* species. Similar observations have been made by Dees and Moss (2). The GLC profiles of this genus resemble those of *Acinetobacter* (group 16) and *Bordetella* (group 17) species but can be distinguished from both of these groups by the absence of 3-OH $C_{12:0}$ and 3-OH $C_{16:0}$. However, qualitative interpretation of the fatty acid composition provides insufficient criteria to differentiate between the distinct *Alcaligenes* species themselves. Only *Achromobacter xylosoxidans*, which has been classified by some investigators as *Alcaligenes denitrificans* (6), can be readily distinguished (see below).

Group 14 contains *Achromobacter xylosoxidans*. As mentioned above, *Achromobacter xylosoxidans* represents a separate GLC group, since it shows a characteristic GLC

profile. Identification is based upon the simultaneous occurrence of 3-OH $C_{12:0}$, 2-OH $C_{14:0}$, and 3-OH $C_{16:0}$.

Group 15 contains *Achromobacter* group Vd. The fatty acid composition of *Achromobacter* group Vd is markedly different from those of all other NFB described in this study. The nonidentity of group Vd and *Achromobacter xylosoxidans* has been demonstrated previously (3). Group Vd was the only species tested in this study which demonstrated a total absence of hydroxy acids. This group also shows the presence of a 19-carbon cyclopropanoic acid $C_{19:0}\Delta$, a rather unusual fatty acid, which has also been found in variable amounts in *P. diminuta* (GLC group 4).

Group 16 contains the *Acinetobacter* species. The genus *Acinetobacter* is characterized by the presence of 3-OH $C_{12:0}$ and 3-OH $C_{14:0}$. In agreement with the observations made by Moss et al. (15), the fatty acid analysis appears to be only genus specific. Moss et al. (13) have reported that the genus *Acinetobacter* was essentially identified by the presence of 2-OH $C_{12:0}$. However, we found the appearance of this fatty acid to be variable, and it certainly could not be regarded as a strict criterion for the identification of *Acinetobacter* species.

Group 17 contains *Bordetella* species. The three clinical isolates of *Bordetella bronchiseptica* show a characteristic GLC profile. They all show a simultaneous occurrence of 2-OH $C_{12:0}$, 3-OH $C_{14:0}$, 3-OH $C_{16:0}$, and 15-methyl-3-hydroxyhexadecanoate (i-3-OH $C_{17:0}$). Although these fatty acids clearly distinguish the genus *Bordetella* from other genera included in this study, it still remains unknown whether their appearance is specific for the species *Bordetella bronchiseptica* rather than for the genus *Bordetella* itself.

Group 18 contains *Agrobacterium radiobacter*. For the genus *Agrobacterium*, four species have been proposed: *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *Agrobacterium rubi*, and *Agrobacterium radiobacter*. However, only the last species has ever been isolated from clinical specimens. Besides its potential pathogenicity, a proper identification of *Agrobacterium radiobacter* is important because it may be confused with *Pseudomonas* or *Achromobacter* species. *Agrobacterium radiobacter* can be identified by the characteristic presence of 3-OH $C_{14:0}$ and 3-OH $C_{16:0}$ (Table 3).

Group 19 contains the *Moraxella* species. The *Moraxella* strains that have been investigated showed the characteristic presence of an 18-carbon unsaturated acid $C_{18:2}$, a fatty acid which was not found in any of the other NFB reported here. However, since the number of *Moraxella* strains used in this study was limited, and since this genus displays a complex series of species, it is not clear whether the presence of this $C_{18:2}$ acid represents a genus-specific property or not. The qualitative fatty acid data for different species classified within this genus were strikingly similar, with the exception of one species: *Moraxella urethralis*. The distinct GLC elution pattern of *Moraxella urethralis* further supports its reclassification as *Oligella urethralis*. These data confirm the recent observations by Moss et al. (15).

The GLC data presented in this study confirm and extend the pioneer work of Moss and co-workers (2–4, 10–15) and Jantzen et al. (7, 8). From the data presented here, it appears that this GLC technique is a powerful method for rapid and accurate routine identification of NFB.

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