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Comparison of the Effects of Acid and Base Hydrolyses on Hydroxy and Cyclopropane Fatty Acids in Bacteria

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The cellular fatty acid compositions of *Legionella oakridgensis*, *Brucella suis*, *Pseudomonas aeruginosa*, and *Francisella tularensis* were compared after base hydrolysis (saponification), acid hydrolysis, and acid methanolysis procedures were used to release the fatty acids. The branched-chain, unsaturated, saturated, and ester-linked hydroxy acids were released as effectively with saponification at 100°C for 30 min as with acid hydrolysis or acid methanolysis at 85°C for 16 h. Although the amide-linked hydroxy acids were released more effectively by acid hydrolysis or acid methanolysis, these methods degraded the cyclopropane fatty acids, producing a number of new peaks or artifacts in the chromatograms. Cyclopropane fatty acids were not degraded by saponification, and at least 50% of the hydroxy acids were released when the cells were saponified with 15% NaOH in 50% aqueous methanol. Thus, the results show that saponification for 30 min at 100°C with 15% NaOH, followed by methylation is an excellent method for routine fatty acid analysis of bacteria and for screening cultures whose identity and fatty acid composition are unknown.

Cellular fatty acid analysis by gas-liquid chromatography is a valuable technique for characterizing many species of bacteria (1–6, 8, 10, 12, 13, 18, 19). It has been successfully used to aid in the identification and classification of a wide variety of microorganisms, including members of the families *Enterobacteriaceae* (5, 8, 18) and *Vibrionaceae* (6, 8) and species of *Pseudomonas* (8, 12, 13, 18), *Legionella* (13, 15), *Brucella* (1, 4), *Yersinia* (5), *Bacteroides* (10), and *Francisella* (2). To analyze bacterial fatty acids by gas-liquid chromatography, the acids must first be released from the cells and then modified chemically (derivatized) to increase their volatility (3, 8, 12, 13, 18). The most common methods used for cellular fatty acid analysis are acid hydrolysis followed by esterification with methanol (8, 10, 18), base hydrolysis (saponification) followed by esterification with methanol (8, 12–14, 18), and acid methanolysis or transesterification (3–5, 8, 12, 19), in which the hydrolysis and esterification steps are combined.

Most of the fatty acids found in bacteria are released equally well by either acid or base hydrolysis (21). However, both methods have disadvantages which can adversely affect the results. Acid hydrolysis is recommended for bacteria whose lipid fractions contain amide-linked fatty acids since these acids are more effectively liberated by acid than by base hydrolysis (21). It has also been reported that the use of basic reagents can produce unsaturated acids as artifacts from substituted 3-hydroxy acids (16, 17), but we have never observed this phenomenon (12, 13). Acid hydrolysis methods also have disadvantages because artifacts can be produced by esterification of hydroxy acids (21) and from the degradation of cyclopropane acids (3–5, 7, 8, 12, 20). These degradation products appear in the chromatograms along with esterified fatty acids and thus interfere with correct interpretation of the fatty acid data (3, 5, 7, 14, 20).

Since base hydrolysis does not degrade cyclopropane acids (1, 3–5, 14, 19), our laboratory has recommended saponification (followed by methylation) as the method of choice for routine analysis of the cellular fatty acids of bacteria (7, 12–14). Since the publication of this method, we have modified our procedures by increasing the saponification time from 15 to 30 min, increasing the methylation time from 5 to 15 min, and incorporating a basic buffer washing step (6, 11).

Recently, other laboratories have reported the fatty acid composition of several genera of bacteria after acid hydrolysis and methylation (10) or acid methanolysis (2, 4, 5, 19). Some species in three of the genera (*Bordetella*, *Brucella*, and *Yersinia*) contain both amide-bound and cyclopropane acids (4, 5); in these reports, the text states that hydroxy acids were optimally released and cyclopropane acids were degraded by

acid hydrolysis, and for these reasons both acid and base hydrolysis methods were used to liberate the fatty acids. However, data comparing the two methods were not shown, and the reader of these and other reports is left with the impression that either acid hydrolysis (10) or acid methanolysis (4, 5) is the preferred technique for analysis of all bacteria, regardless of the presence of cyclopropane acids.

For these reasons, the following study was done to compare the effects of acid and base hydrolysis on the fatty acid composition of *Legionella oakridgensis* (15), *Brucella suis* (1), *Pseudomonas aeruginosa* (12), and *Francisella tularensis* (2). These four species were chosen because they contain representative types of most of the fatty acids commonly found in bacteria.

MATERIALS AND METHODS

Cultures were obtained from the stock cultures at the Centers for Disease Control, Atlanta, Ga., and included *L. oakridgensis* 10 (ATCC 33761), *B. suis* B6537, *P. aeruginosa* E-8039, and *F. tularensis* KC1458. *L. oakridgensis* was grown on plates of charcoal-yeast extract agar (15); *B. suis* and *P. aeruginosa* were grown on plates of heart infusion agar with 5% rabbit blood (Nolan Biological Laboratories, Inc., Atlanta, Ga.); and *F. tularensis* was grown on plates of chocolate heart infusion agar (Nolan Biological Laboratories, Inc.). Agar plates (20 by 100 mm) were inoculated with each of the four cultures (10 plates for each culture) and were incubated at 35°C; *B. suis* cultures were incubated in a candle jar. After 24 or 48 h, the growth from each set of plates was removed with sterile distilled water and placed in a small flask. The cells were killed by exposure to flowing steam for 20 min. The cell suspensions were diluted with sterile distilled water to a density of a no. 3 McFarland standard; 0.5 ml of each suspension was dispensed into screw-capped tubes (16 by 125 mm or 20 by 150 mm) which were fitted with Teflon-lined caps. All suspensions were frozen at −20°C.

The cell suspensions were thawed, and the methyl esters of the cellular fatty acids of each species were prepared by the following four methods.

Method A. Four milliliters of 5% NaOH in 50% aqueous methanol (50 g of NaOH, 500 ml of methanol, 500 ml of distilled water; N = 1.2) was added to the thawed cells, and the mixture was heated in a 100°C water bath for 30 min. The sample was cooled to ambient temperature, 5 ml of 15% HCl-methanol reagent (150 ml of concentrated HCl, 850 ml of methanol; N = 1.8) was added, and the mixture was heated at 100°C for 15 min.

Method B. Four milliliters of 15% NaOH in 50% aqueous methanol (150 g of NaOH, 500 ml of methanol, 500 ml of distilled water; N = 3.3) was added to the cells, and the mixture was heated at 100°C for 30 min. After cooling to ambient temperature, 5 ml of 25% hydrochloride-methanol reagent (250 ml of concentrated HCl, 750 ml of methanol; N = 3) was added, and the mixture was heated at 85 or 100°C for 15 min.

Method C. Method C was a modification of the acid methanolysis method described by Jantzen et al. (2–5). The thawed cells were mixed with 3 to 4 ml of 3 N HCl in methanol (250 ml of concentrated HCl, 750 ml of methanol). The contents of the tube were flushed with nitrogen gas, and the sample was sealed and heated in an 85°C oil bath for 16 h.

Method D. Method D was a modification of the hydrolysis method described by Mayberry et al. (10). The thawed cells were mixed with 3 to 4 ml of 3 N HCl (250 ml of concentrated HCl, 750 ml of distilled water). The sample was flushed with nitrogen gas, the tube was sealed, and the sample was heated in an 85°C oil bath for 16 h. After it was cooled to ambient temperature, 4 ml of the 3 N hydrochloride-methanol reagent (method C) was added, and the sample was heated at 85°C for 15 min.

Extraction of FAME. One milliliter of a saturated aqueous solution of NaCl was added to all the samples, and the fatty acid methyl esters (FAME) were extracted with 5 to 10 ml of a 1:1 mixture of diethyl ether and normal hexane (1:1 E-H vol/vol). The extraction step was repeated, and the organic phases containing the FAME were combined in a 50- or 100-ml beaker. The FAME samples were concentrated with nitrogen gas to approximately 0.3 ml and transferred to a screw-capped tube (13 by 100 mm). The beaker was rinsed with 0.3 ml of 1:1 E-H which was combined with that in the tube. Samples prepared by either method A or method B were mixed with 0.5 ml of 0.1 M phosphate buffer (14.2 g of Na_2HPO_4 and 4.0 g of NaOH per liter), and samples prepared by either method C or method D were mixed with 0.5 ml of 0.4 M phosphate buffer (56.8 g of Na_2HPO_4 and 16.0 g of NaOH per liter). After 5 to 10 min at ambient temperature, the E-H layer was transferred to a screw-capped tube (13 by 100 mm). The aqueous layer was reextracted with 0.3 to 0.5 ml of 1:1 E-H. If the organic phase did not completely separate from the aqueous phase, 0.05 to 0.1 ml of methanol was added, the mixture was centrifuged at 1,000 × *g* for 3 to 5 min, and the organic phase was removed. The organic phases were combined and concentrated to 0.3 ml. Approximately 0.5 g of anhydrous Na_2SO_4 was mixed with the FAME sample, and the mixture was centrifuged at 1,000 × *g* for 3 to 5 min. The E-H was decanted into a clean (13 by 100 mm), and 0.3 ml of E-H was used to rinse the Na_2SO_4 and was combined with that in the tube. The FAME samples were evaporated with a gentle flow of nitrogen to 0.3 to 0.4 ml and were stored at −20°C until the analyses by gas-liquid chomatography were done.

The FAME samples were analyzed on a fused-silica capillary column (50 m by 0.2 mm [inside diameter]) with cross-linked 5% phenyl methyl silicone (SE-54) as the stationary phase (Hewlett-Packard, Avondale, Pa.). The column was installed in a 5790 gas chromatograph (Hewlett-Packard) equipped with a flame ionization detector. For analysis of the FAME of *L. oakridgensis*, *B. suis*, and *P. aeruginosa*, the column was temperature programmed from 180 to 260°C at 8°C/min and was maintained at 260°C for 8 min; for analysis of *F. tularensis*, the column was temperature programmed from 195 to 275°C at 8°C/min and was maintained at 275°C for 21 min. The injector temperature was 250°C, and the detector temperature was 280°C. The carrier gas was hydrogen at a flow rate of approximately 0.7 ml/min; the sample size was 1 μl with a split ratio of approximately 50:1.

TABLE 1. Comparison of the effects of four hydolysis procedures on the cellular fatty acid composition of *L. oakridgensis*

Hydrolysis procedure[a]	Fatty acids (% of total fatty acids)[b]															
	Un	i14:0	a15:0	15:1	15:0	Un-OH	i16:1	i16:0	16:1	Un-16:1	16:0	Ar-1	Ar-2	a17:1	Ar-3	a17:0
A	—	tr	2	2	1	tr	2	26	10	1	10	tr	—	1	—	4
B	tr	tr	2	2	1	tr	2	25	9	1	10	tr	tr	1	—	4
C	—	tr	2	1	tr	4	1	25	7	—	9	3	2	tr	2	4
D	3	tr	2	1	1	3	1	26	7	1	10	3	1	tr	3	4

[a] A, 5% NaOH in 50% aqueous methanol at 100°C for 30 min; B, 15% NaOH in 50% aqueous methanol at 100°C for 30 min; C, 3 N hydrochloride-methanol at 85°C for 16 h; D, 3 N hydrochloride-water at 85°C for 16 h.
[b] Numbers to the left of the colon refer to the number of carbon atoms; numbers to the right refer to the number of double bonds; i indicates a methyl branch at the *iso* carbon atom; a indicates a methyl branch at the *anteiso* carbon atom; cyc refers to a cyclopropane acid; OH refers to an hydroxyl group; Ar refers to an artifact; Un means unidentified. Values are means of at least two determinations. —, Not detected; tr, <0.5%.

TABLE 2. Comparison of the effects of four hydrolysis procedures on the cellular fatty acid composition of *B. suis*

Hydrolysis procedure[a]	Fatty acids (% total fatty acids)[b]										
	Un	16:0	17:0	Ar-7	18:1	18:0	Ar-10	Ar-11	Ar-12	Ar-13	Ar-14	Ar-15
A	—	6	3	—	38	7	2	—	—	—	—	—
B	—	6	3	—	37	6	1	—	—	—	—	—
C	1	6	3	2	27	6	9	2	1	5	3	3
D	tr	6	3	2	37	7	8	tr	—	—	2	2

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*, for abbreviations.

TABLE 3. Comparison of the effects of four hydrolysis procedures on the cellular fatty acid composition of *P. aeruginosa*

Hydrolysis procedure[a]	Fatty acids (% of total fatty acids)[b]															
	10:0	3-OH-10:0	12:0	Un	2-OH-12:0	3-OH-12:0	14:0	15:0	16:1	16:0	Ar-1	Ar-3	17:1	17-cyc	17:0	Ar-6
A	tr	4	3.5	—	6	1.5	1	1	10	32	—	—	tr	2	tr	—
B	tr	4	3.5	—	6	5	1	1	9	33	—	—	tr	2	tr	—
C	—	5	3	1	6	9	0.5	1	7	29	tr	1	tr	—	0.5	1
D	tr	4	3	1.5	6	7.5	1	1	8	31	0.5	—	0.5	—	0.5	—

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*, for abbreviations.

TABLE 4. Comparison of the effects of four hydrolysis procedures on the cellular fatty acid composition of *F. tularensis*

Hydrolysis procedure[a]	Fatty acids (% of total fatty acids)[b]									
	10:0	2-OH-10:0	12:0	14:0	15:0	16:0	3-OH-16:0	18:1	18:1	18:0
A	13	0.5	tr	6	0.5	10	3	7	tr	5
B	12	0.5	tr	6	0.5	10	2.5	6.5	0.5	4.5
C	12	0.5	tr	6	1	10	2	5	—	4.5
D	15	1	tr	6.5	0.5	12	2	7	—	5.5

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*, for abbreviations.

TABLE 1—*Continued*

Fatty acids (% of total fatty acids)[b]															
Ar-4	Ar-5	17-cyc	17:0	Ar-6	i18:0	Ar-8	18:0	Ar-10	Ar-12	Un-OH	Ar-15	19:0	20:0	Ar-26	Total Ar after 16:0
—	—	17	4	—	2	tr	12	—	—	tr	tr	3	3	—	tr
—	—	17	4	—	2	tr	13	—	—	tr	tr	4	3	—	tr
2	tr	—	3	1	2	3	13	2	1	2	1	4	3	2	19
2	2	—	4	tr	2	—	13	4	tr	—	—	4	2	1	16

TABLE 2—*Continued*

Fatty acids (% of total fatty acids)[b]												
19-cyc	Ar-16	Ar-17	Ar-18	Ar-19	Ar-20	Ar-21	Ar-22	Ar-23	Ar-24	Ar-25	Ar-26	Total Ar after 16:0
42	—	2	tr	—	—	—	—	—	—	—	—	4
45	—	1	tr	1	—	—	—	—	—	—	—	3
—	3	9	7	2	4	1	1	3	2	tr	—	57
8	1	5	3	1	2	—	—	5	4	2	2	39

TABLE 3—*Continued*

Fatty acids (% of total fatty acids)[b]															
18:1	18:0	Ar-10	Ar-12	Ar-13	Ar-15	19-cyc	Ar-16	Ar-17	Ar-18	Ar-19	Ar-20	Ar-23	Ar-24	Ar-25	Total Ar after 16:0
31	1	—	—	—	—	7	—	—	tr	—	—	—	—	—	tr
27	1	—	—	0.5	—	7	—	—	tr	—	—	—	—	—	0.5
22	2	1.5	0.5	1	0.5	—	1.5	1	—	3	2	0.5	—	0.5	14
28	1.5	1	tr	0.5	tr	1	—	0.5	—	0.5	tr	1	1	0.5	5.5

TABLE 4—*Continued*

Fatty acids (% of total fatty acids)[b]											
Un	Un	3-OH-18:0	20:1	20:0	22:1	22:0	Un	24:1	24:0	26:1	26:0
tr	tr	11	1	3.5	2	10	tr	12	13	1	1.5
tr	tr	14	1	3.5	2	10	tr	11	13	1	1.5
1	tr	19	1	3.5	2	10	—	9	12	0.5	1
1.5	—	14	1	4	2	9	tr	9.5	8.5	tr	1

The fatty acids were identified by retention time comparisons to standards (Supelco, Inc., Bellefonte, Pa.; Applied Science Division, Milton Roy Company, State College, Pa.; Regis Chemical Co., Morton Grove, Ill.). Their identity was confirmed after trifluoroacylation, hydrogenation, and mass spectrometry (6, 12, 13). Quantitation of peak areas was done with a model 3390 Hewlett-Packard reporting integrator.

RESULTS AND DISCUSSION

The effects of the four hydrolysis procedures on the cellular fatty acids of *L. oakridgensis*, *B. suis*, *P. aeruginosa*, and *F. tularensis* are compared in Tables 1, 2, 3, and 4, respectively. The fatty acids in these four species included branched-chain, unsaturated and saturated straight-chain, two- and three-hydroxy, and cyclopropane fatty acids. Both amide- and ester-linked fatty acids were represented in these species (8, 12, 18).

Examination of the data in Tables 1 through 3 shows that cyclopropane fatty acids were found in *L. oakridgensis* (Table 1; 17-carbon cyclopropane [17-cyc]), *B. suis* (Table 2; 19-cyc), and *P. aeruginosa* (Table 3; 17-cyc and 19-cyc) when the cells were hydrolyzed with sodium hydroxide (method A or B). However, these cyclopropane acids were absent or found in greatly reduced concentrations when cells of these three species were hydrolyzed with acid (method C or D). In each of the acid-hydrolyzed samples, a number of new peaks which eluted after palmitic acid (16:0) appeared in the chromatograms. These peaks were designated as artifacts (Ar) and are listed in Tables 1 through 3 as Ar-1 through Ar-26.

The destructive effect of acid hydrolysis on the 17-cyc acid with production of artifacts is clearly illustrated by comparing the chromatograms of *L. oakridgensis* cells processed by methods B and C (Fig. 1). In the base-treated cells (method B), the 17-cyc acid was the second largest peak in the chromatogram (Fig. 1A) and comprised 17% of the total acids; however, this peak was completely absent in acid-treated cells (Fig. 1B). Moreover, the quantitative data in Table 1 indicate that the 17-cyc acid was completely converted to artifacts since these totaled at least 16% in acid-treated cells (methods C and D, Table 1) but were essentially absent in base-treated cells. (methods A and B). The relative concentrations of the branched- and straight-chain fatty acids were almost identical, regardless of the method used to process the cells. The unidentified hydroxy acids which eluted after pentadecanoic acid (15:0) and Ar-12 (Table 1) disappeared when these samples were acylated, and new peaks which eluted approximately 1 min earlier appeared on the chromatograms. These two peaks may be dihydroxy or monohydroxy acids similar to those reported in *Legionella pneumophila* (9); however, because of their low concentrations, no other attempts were made to identify them.

The destructive effects of acid on cyclopropane fatty acids is further demonstrated by comparing the results obtained with *B. suis* (Table 2). When *B. suis* cells were saponified (methods A and B), 19-cyc acid was the major peak in the chromatogram (Fig. 2A) and accounted for at least 42% of the total cellular fatty acids; when cells were processed by acid methanolysis (method C), no cyclopropane acid was detected, and the artifacts accounted for over 50% of the total area of peaks in the chromatogram (Fig. 2B). In acid-hydrolyzed cells (method D), the total percentages of the 19-cyc acid (8%) and the artifacts (39%) were approximately the same as that found for 19-cyc acid in the saponified cells. The small percentage of artifacts found in cells processed by methods A and B (4 and 3%, respectively) was not produced during the saponification step but occurred during the heating step with the methanolic HCl reagents. If the samples were heated for more than 15 min or with more concentrated acid, the percentage of the artifacts increased with a concomitant decrease in the amount of 19-cyc acid. These results agree with those of Vuillet et al. (20), who reported that artifact formation from cyclopropane acids was a function of the acid concentration and the amount of cyclopropane acids present. Our results showed that less degradation occurred in saponified samples of *B. suis* when methylation was done for 10 min instead of 15 min or at 85°C instead of 100°C.

The cyclopropane fatty acids in *P. aeruginosa* (Table 3) were also degraded by methods C and D, but the degradation was not as pronounced because this species contained considerably less cyclopropane acid than *L. oakridgensis* or *B. suis*. There appeared to be more extensive degradation of the cyclopropane acids in both *B. suis* and *P. aeruginosa* when acid methanolysis (method C) was used than when acid hydrolysis followed by methylation (method D) was used. Other than Ar-8 and Ar-19, the artifacts found in the samples did not match the retention times of any of the standard acids available to us. The retention times of Ar-8 (Table 1) and Ar-19 (Tables 2 and 3) were the same as oleic acid (18:1) and eicosenoic acid (20:1), respectively. However, Ar-8 and Ar-19 were not found in greater than trace amounts in saponified cells, and these two peaks were not affected by acylation or hydrogenation. Thus, they are probably artifacts rather than amide-linked or unsaturated fatty acids.

Except for the cyclopropane acids, the only other fatty acids whose concentrations were

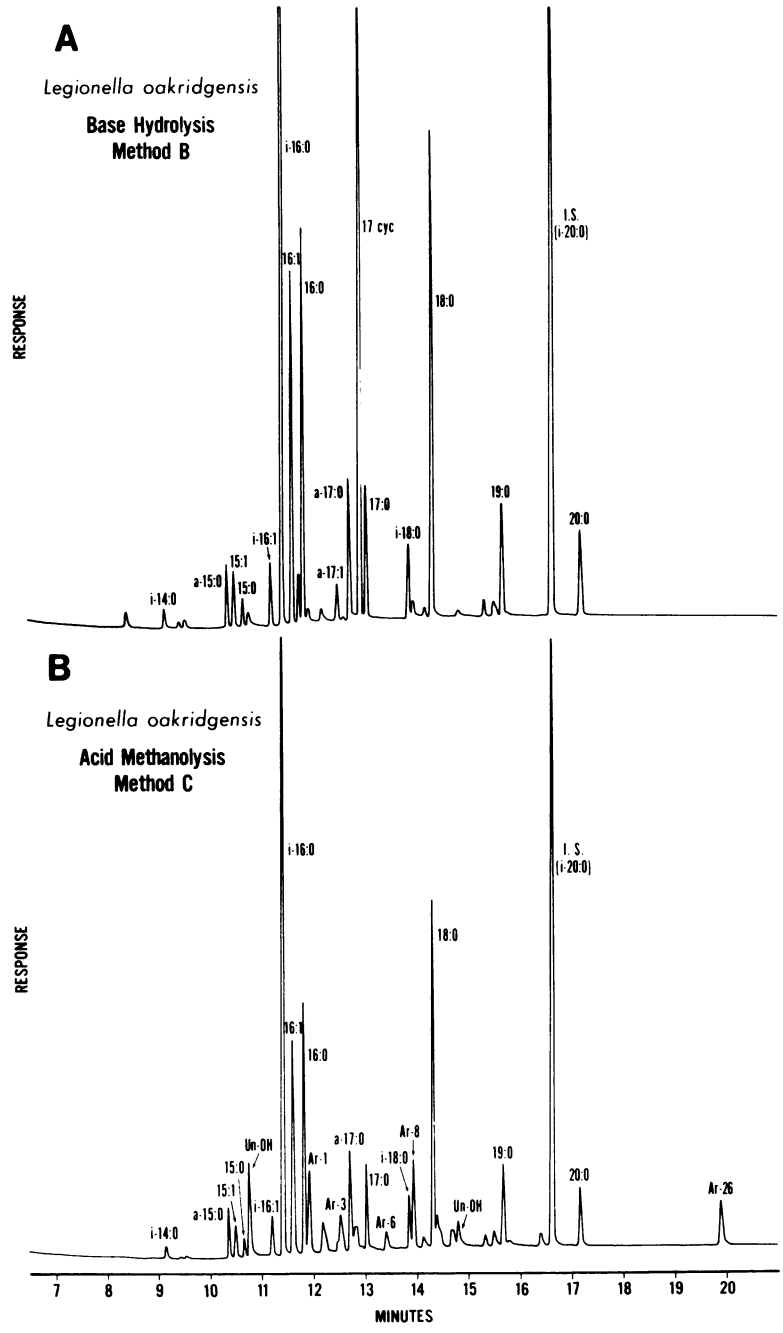

FIG. 1. Gas chromatograms of fatty acids (as methyl esters) from base-hydrolyzed cells (A) and acid-methanolyzed cells (B) of *L. oakridgensis* analyzed on a cross-linked SE-54 fused-silica capillary column (50 m by 0.2 mm). I.S., Internal standard.

affected by the hydrolysis procedure were *cis*-vaccenic (18:1) acid in *B. suis* and *P. aeruginosa*, 3-hydroxy-lauric (3-OH-12:0) acid in *P. aeruginosa*, and 3-hydroxy-octadecanoic (3-OH-18:0) acid in *F. tularensis* (Table 4). The concentration of 18:1 in *B. suis* and *P. aerugionosa* was lower in cells processed by method C than by any of the other three methods. However, this difference may not be directly related to the hydrolysis procedure because cells processed by method C consistently had higher concentrations of artifacts than cells processed by the

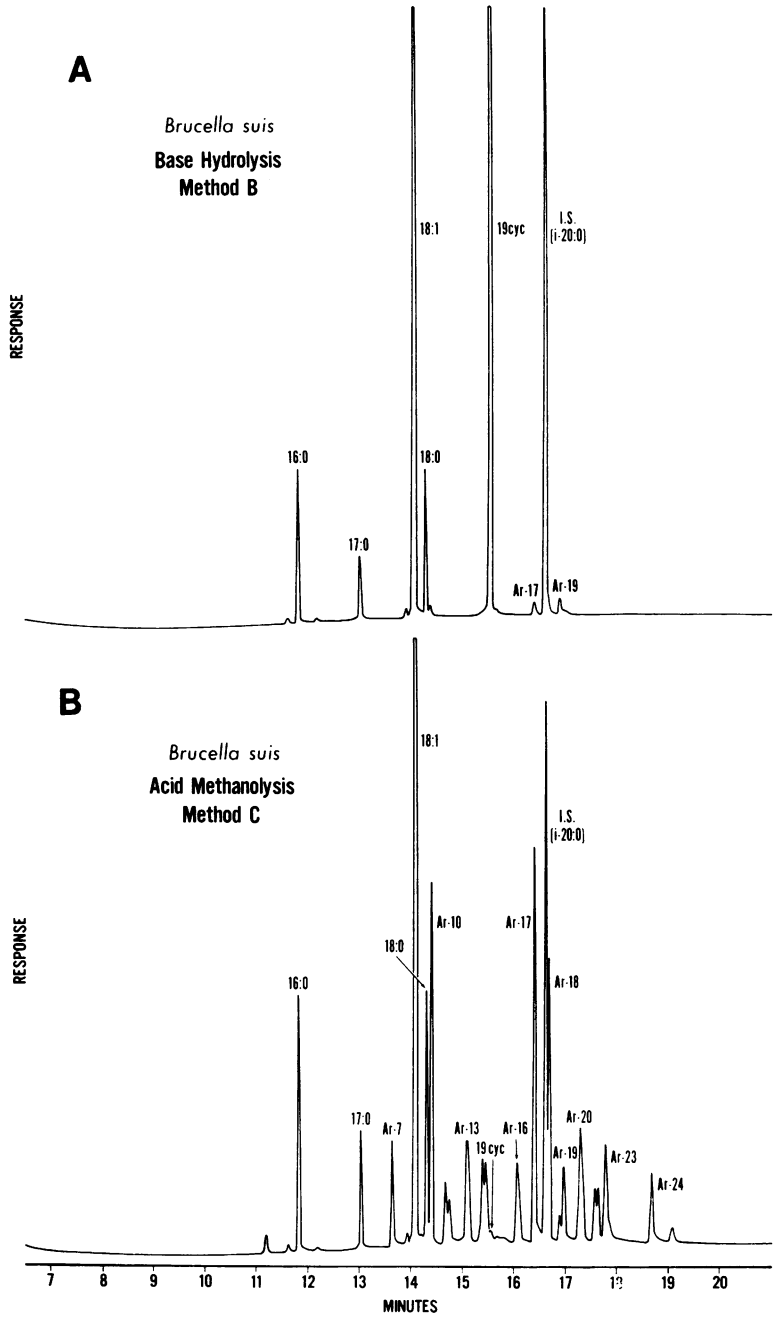

FIG. 2. Gas chromatograms of fatty acids (as methyl esters) from base-hydrolyzed cells (A) and acid-methanolyzed cells (B) of *B. suis* analyzed on a cross-linked SE-54 fused-silica capillary column (50 m by 0.2 mm). I.S., Internal standard.

other methods. The differences in concentration of the 3-hydroxy acids in *P. aeruginosa* and *F. tularensis* are directly related to the hydrolysis procedures because these are amide-linked fatty acids which are not quantitatively released by alkaline hydrolysis (21). Results for *P. aeruginosa* (Table 3) and for *F. tularensis* (Table 4) show that the highest concentrations of three-hydroxy acids are found in cells processed by method C (acid methanolysis). Mild saponification with 5% NaOH (method A) released 17% of the 3-OH-12:0 in *P. aeruginosa* and 58% of the 3-OH-18:0 in *F. tularensis*. When the concentration of the base was increased to 15% (method

B), 56% of the 3-OH-12:0 and 75% of the 3-OH-18:0 were released. Acid hydrolysis (method D) released 83% of the hydroxy acid in *P. aeruginosa* and 75% of that in *F. tularensis*. Saponification with 10% NaOH in 50% aqueous methanol released 28% of the 3-OH-12:0 in *P. aeruginosa* and 63% of the 3-OH-18:0 in *F. tularensis*; saponification with 20% NaOH did not release the hydroxy acids any more effectively than 15% NaOH did.

From these results, it appears that saponification with 15% NaOH (method B) offers an excellent compromise for routine fatty acid analysis or for screening cultures whose identity and fatty acid composition are unknown. At least 50% of the bound hydroxy acids were released, and the cyclopropane fatty acids were not degraded. Except for some amide-linked hydroxy fatty acids, all other acids were released as effectively with 30 min of saponification at 100°C as with acid hydrolysis or acid methanolysis at 85°C for 16 h. If a complete analysis of all amide- and ester-linked fatty acids present in the cells is needed, the residual aqueous phase from saponification (which is usually discarded after the FAME are extracted) can be reheated with additional acid to release and methylate any remaining bound fatty acids. These FAME can then be extracted, concentrated, and combined with the original FAME samples from saponification for analysis by gas-liquid chromatography.

Saponification and methylation of bacterial fatty acids by method B does not require anhydrous or inert conditions, and cells do not have to be lyophilized (3–5, 19). Rather, the cells can be removed directly from an agar slant or plate or from a centrifuged broth culture and processed immediately for fatty acids or frozen for analysis at a later time. The chemicals, reagents, and glassware required are common laboratory supplies, and fatty acid analysis with method B can easily be used by clinical and research laboratories with experience in gas chromatography.

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