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A Simple Procedure for Detecting the Presence of Cyclopropane Fatty Acids in Bacterial Lipids

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Four gram-negative bacterial species, including *Escherichia coli* strain B, *Serratia marcescens*, *Pseudomonas fluorescens*, and *Vibrio cholerae* (*comma*) strain NIH 41, were investigated for fatty acid content by gas-liquid chromatography involving a preparatory technique which facilitated detection of cyclopropane fatty acids. Methyl esters of fatty acids were subjected to mild catalytic hydrogenation to eliminate unsaturates. Hydrogenation was followed by bromination which removed cyclopropane acids from chromatographic profile patterns. Lactobacillic acid (*cis*-11,12-methyleneoctanoate) and *cis*-9,10-methylenehexadecanoate, previously reported in lipids of *E. coli* and *S. marcescens*, were found in small amounts in *P. fluorescens* but were not detected in *V. cholerae*.

Reports of lactobacillic acid (*cis*-11,12-methyleneoctanoate) in lipids of *Lactobacillus arabinosus* (9), *Lactobacillus casei* (10), *Agrobacterium tumefaciens* (11), *Escherichia coli* (18), and *Clostridium butyricum* (8) were followed by detection of both lactobacillic and *cis*-9,10-methylenehexadecanoic acids in lipids of *E. coli* (14, 21), *A. tumefaciens* (15), *Serratia marcescens* (2, 16, 17), and *Aerobacter aerogenes* (19).

The methods usually employed for identification of cyclopropane fatty acids include mild hydrogenation for selective removal of unsaturated acids, infrared spectroscopy of isolated acids, and strong catalytic hydrogenation of the ring structure, followed by analyses of resulting branched chain products (2, 9, 14). Hydrogenation of the cyclopropane ring is difficult and results in additional peaks when mixed fatty acid methyl esters are analyzed by gas-liquid chromatography.

The present study involves an investigation of a new and simple procedure for detection of bacterial cyclopropane acids. Two organisms known to contain these acids, *E. coli* and *S. marcescens*, were compared with two organisms, *Vibrio cholerae* (*comma*) and *Pseudomonas fluorescens*, in which such acids have not been reported. Cultural conditions which favored production of cyclopropane acids were used.

MATERIALS AND METHODS

Cultural conditions. Cultures of *E. coli* strain B, *S. marcescens*, *P. fluorescens*, and *V. cholerae* strain NIH 41 were maintained by monthly transfer on Trypticase Soy Agar (BBL) slants. A 1-ml amount of an 18-hr (37 C) broth culture (*p*H 7.3) was inoculated into each of two Roux flasks containing 100 ml of Trypticase Soy broth and 3% agar. Flasks were incubated for 24 hr at 37 C. Cells harvested with 10 ml of 0.85% NaCl were centrifuged for 30 min at 4,500 × *g*. All tests were conducted in duplicate.

Lipid extraction and esterification of acids. Wet bacteria were suspended in 20 ml of $CHCl_3$-CH_3OH (2:1, v/v) and allowed to stand overnight. The suspension was filtered, and the solvent was evaporated in a stream of nitrogen. Fatty acids were esterified with 5 ml of BCl_3-CH_3OH (4). $CHCl_3$ (10 ml) and water (3 ml) were added, the mixture was shaken in a separatory funnel, and the lower phase was evaporated with nitrogen.

Elimination procedures. After gas-liquid chromatography of methyl esters from extracted lipids, unsaturated components were hydrogenated for 15 min in 20 ml of $CHCl_3$-CH_3OH (2:1, v/v) with 200 mg of 5% Pt on charcoal (5). This procedure is selective in that unsaturated acids are converted to saturated ones, although cyclopropane acids are not affected (9, 14, 16).

After gas-liquid chromatography of hydrogenated samples, remaining methyl esters (approximately 10 mg) were diluted in 2 ml of diethyl ether (anhydrous, reagent grade) and cooled to 0 C. A 1-ml amount of bromine (reagent grade) in ether (1:5, v/v) was added to each sample. Ether and excess bromine were evaporated at 50 C with N_2.

Gas-liquid chromatography. Gas-liquid chromatography was performed with an aerograph (Varian Associates, Palo Alto, Calif.; model 204-1B) gas chromatograph with flame ionization detectors. Columns (10 ft × 0.125 inch or 3 m × 3 mm) containing 20% diethylene glycol succinate (DEGS) polyester on Chromosorb W (60/80 mesh) were operated at 180 C. Detector and injector temperatures were 210 C, and the N_2 flow was 20 ml/min.

TABLE 1. *Per cent fatty acids before and after hydrogenation*

Fatty acid[a]	Relative retention time[b]	*Escherichia coli*		*Serratia marcescens*		*Pseudomonas fluorescens*		*Vibrio cholerae*	
		Before	After	Before	After	Before	After	Before	After
14:0	0.56	3.8	4.2	1.2	1.2	1.3	1.3	3.9	4.6
14:1	0.65	—	—	—	—	—	—	tr[c]	—
15:0	0.74	1.2	1.3	tr	tr	1.0	tr	tr	tr
iso 16:0[d]	0.87	—	—	—	—	—	—	2.0	2.3
16:0	1.00	43.8	46.2	43.2	48.2	29.3	40.3	29.5	62.8
16:1	1.15	2.1	—	5.2	—	12.3	—	34.2	—
17:0	1.34	1.4	tr	1.0	1.2	1.5	2.3	1.2	1.9
cyc 17:0	1.54	20.9	22.0	31.4	31.2	3.1	3.3	—	—
17:1 / iso 18:0[d]	1.56	—	—	—	—	—	—	1.8	tr
18:0	1.82	tr	7.6	1.2	12.5	1.5	44.9	3.1	26.8
18:1	2.08	7.2	—	11.0	—	42.3	—	22.9	—
cyc 19:0	2.82	18.8	17.8	5.4	5.1	7.7	6.9	—	—

[a] Number preceding colon indicates number of carbons and number following designates degree of unsaturation.

[b] Retention time relative to 16:0 (palmitic acid).

[c] Trace, peak less than 1%.

[d] Tentative identification.

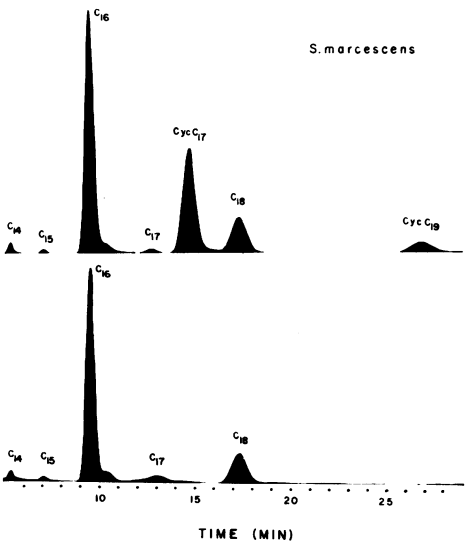

FIG. 1. *Gas-liquid chromatograms of fatty acid methyl esters of Serratia marcescens hydrogenated (upper) and brominated (lower).*

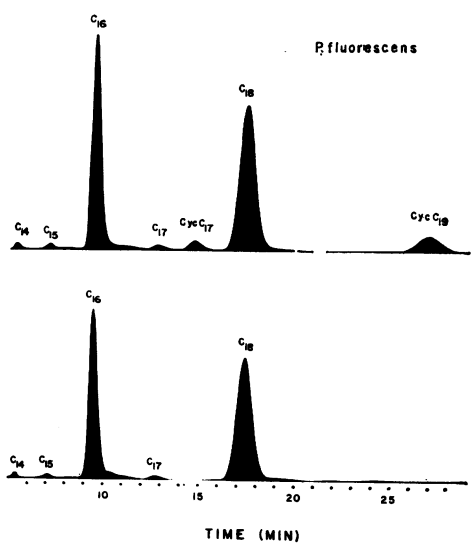

FIG. 2. *Gas-liquid chromatograms of fatty acid methyl esters of Pseudomonas fluorescens hydrogenated (upper) and brominated (lower).*

Samples (1 μliter) of dilutions of esters (1 to 10 g/100 ml) were injected onto the column with electrometer input attenuation – 10 and output attenuation –8. Methyl esters were tentatively identified by comparison with standards (Applied Science Laboratories, College Station, Pa.). Peaks below C_{14} were in trace amounts in lipid extracts and were not considered in this study. Quantitative data were obtained with a disc integrator (Disc Instruments, Inc., Santa Ana, Calif.). Accuracy of the analytical system was within the limits suggested by Horning et al. (12).

RESULTS

Quantitative data from the chromatographic patterns for the four bacterial species before and after mild hydrogenation are shown in Table 1. Tentative identifications of the unsaturates (C_{16} and C_{18} monoenes) were confirmed by the quantitative increase in the saturates (palmitic and stearic acids) after hydrogenation of extracts from all four test species. For example, in *E. coli*, 2.1% of hexadecenoate and 43.8% of palmitate were

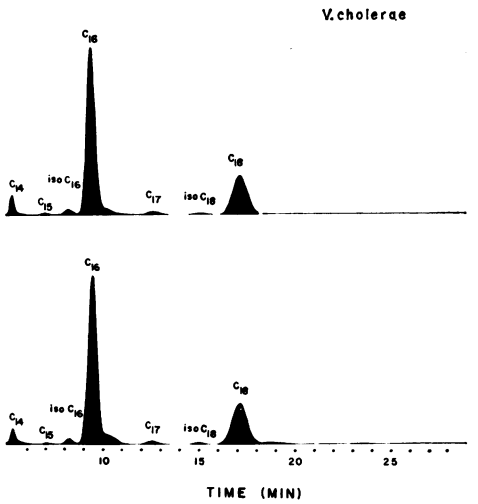

FIG. 3. *Gas-liquid chromatograms of fatty acid methyl esters of Vibrio cholerae strain NIH 41 hydrogenated (upper) and brominated (lower).*

detected in the unhydrogenated material. The hydrogenated sample yielded 46.2% of palmitate, which closely reflects the quantitative sum (45.9%) for palmitic and hexadecenoic acids before hydrogenation.

Mild hydrogenation did not significantly alter the quantity of those fatty acids tentatively identified as cyclic C_{17} and cyclic C_{19} acids, as suggested by the data (Table 1) for the three strains in which they were found (*E. coli*, *S. marcescens*, *P. fluorescens*). Figures 1 and 2 illustrate the results of bromination of the previously hydrogenated methyl esters from *S. marcescens* and *P. fluorescens*. Two peaks (C_{17} and C_{19} cyclopropane acids) were completely removed by bromination. Brominated components moved more slowly through the column and were not apparent with isothermal operation. The chromatographic patterns before and after bromination of the *V. cholerae* sample appeared similar (Fig. 3), and confirmed the earlier data (Table 1) which failed to reveal cyclic fatty acids.

DISCUSSION

Abel, de Schmertzing, and Peterson (1) presented early evidence for the feasibility of classifying microorganisms by fatty acid content. This taxonomic concept was later supported by other reports (3, 13, 20). In early studies, identifications of fatty acids were based solely on retention time data. This practice is sufficient for initial studies, but should be followed by other confirmatory tests for demonstration of various reactive groups associated with the compounds in question. Procedures such as hydrogenation and bromination are simple, rapid, and add to the value of fatty acid data obtained in survey work.

A variety of cultural conditions, such as temperature, media constituents, and age of culture, are known to affect fatty acid content. It would, therefore, be advisable for workers to include reference species, such as *E. coli* or *S. marcescens* cultured under specified conditions, in their studies to facilitate analysis of fatty acid data.

Our relative retention data agree closely with those of Kaneshiro and Marr (14), who used a column containing 25% DEGS at a temperature of 187 C. Although the percentage of cyclic C_{17} in *E. coli* appears to agree closely with previously published results, we found a much larger amount (18 to 19%) of lactobacillic acid than that reported by other workers (6, 7, 14, 21). Our cyclopropane acid content for *S. marcescens* agrees closely with Kates and Hagen (17), Kates, Adams, and Martin (16), and Bishop and Still (2). Cyclopropane acids do not appear to be present in lipids of typical *V. cholerae* but are present in *P. fluorescens* in small amounts.

By use of mild hydrogenation of unsaturates followed by bromination, we have confirmed the identification of unsaturated and cyclopropane fatty acids in *E. coli* and *S. marcescens* and have detected cyclopropane fatty acids in *P. fluorescens*. We recommend that these procedures be used in routine survey work.

ACKNOWLEDGMENT

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