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Note

Use of pentafluorobenzyl and pentafluoropropionyl-pentafluorobenzyl esters of bacterial fatty acids for gas chromatographic analysis with electron-capture detection

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Gas chromatographic (GC) analysis of cellular fatty acids is a useful technique in bacterial classification and identification [1]. Typically, the fatty acids are analysed as methyl ester derivatives using flame-ionization detection (FID). However, electron-capture detection (ECD) of fatty acids derivatized to halogenated derivatives, such as pentafluorobenzyl (PFB) [2–6] and trichloroethyl (TCE) [7] esters, provides superior sensitivity. Both the hydroxyl and carboxyl groups of hydroxy acids can be halogenated [8]. Various methods for removing the halogen-containing reagents from the samples prior to GC–ECD analysis have been employed: extraction [2,7], decreasing the amounts of the reagents and increasing the reaction temperature [3], and the use of solid support reagents [6,9]. An alternative approach is to use polar disposable extraction columns, which are easy to use and allow several samples to be run simultaneously [10].

In the present paper a method is described for esterification of bacterial non-hydroxy and hydroxy fatty acids to PFB and pentafluoropropionyl-pentafluorobenzyl (PFPO-PFB) esters, respectively, followed by removal of excess reagents using disposable silica columns. The PFB and PFPO-PFB ester derivatives of several typical bacterial fatty acids present in a reference mixture could be detected in the low femtomole range using ECD and were well separated on a 30-m fused-silica capillary column coated with SE-54.

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EXPERIMENTAL

Organism

Escherichia coli, isolated from human urine, was used.

Chemicals and glassware

The solvents were of glass-distilled reagent grade and not redistilled before use. Heptafluorobutyric anhydride (HFBA), p.a. grade, and pentafluoropropionic anhydride (PFPA), p.a. grade, were from Pierce (Rockford, IL, U.S.A.), 2,3,4,5,6-pentafluorobenzyl bromide (PFB-Br), purity >99%, and 2,3,4,5,6-pentafluorobenzyl alcohol (PFB-OH), purity >96%, from Jansen Chimica (Beerse, Belgium) and trichloroethanol (TCE-OH), purity >96%, and methyl nonadecanoate, GC grade, from Merck (Darmstadt, F.R.G.). The bacterial fatty acid reference mixture (Part No. 4-5436) was purchased from Supelco (Bellefonte, PA, U.S.A.) whereas 3-hydroxymyristic acid (3-OH 14:0) was synthesized [5]. 2-Hydroxymyristic acid (2-OH 14:0) and myristic acid (14:0), purity >98%, were purchased from Fluka (Buchs, Switzerland) and the disposable silica columns (1 ml, Bond Elut) were from Analytichem International (Harbour City, CA, U.S.A.).

All glassware was washed with 5% Deconex, rinsed several times both with tap water (hot) and distilled water, soaked overnight in 5 *M* hydrochloric acid and rinsed with water and ethanol (95%) before being heated for 10 h at 400°C. The test-tubes used were equipped with PTFE-lined screw caps.

Derivatization procedures

The fatty acids in the reference mixture and the *E. coli* cells were liberated by saponification. Typically, samples were heated at 90°C for 1 h in 1 ml of methanol–water solution (1:1, v/v) containing sodium hydroxide (15%, w/w) [5]. After cooling, 2 ml of water and 1 ml of hexane were added, and the tubes were shaken and centrifuged (ca. 1000 *g*). The hexane phase was removed. Dilute aqueous hydrochloric acid was added to the aqueous phase until the pH fell below 2, and the free acids were extracted twice with 1 ml of methylene chloride. The combined organic phases were evaporated just to dryness under dry nitrogen. The acids were redissolved in 30 μl of triethylamine-containing (1% v/v) acetonitrile before the addition of 10 μl of PFB-Br (35% v/v, in acetonitrile). After 15 min at room temperature, 20 μl of PFPA were added; after another 15 min, 0.5 ml of hexane and 2 ml of 1 *M* phosphate buffer solution (pH 7.0) were added. After extraction and centrifugation (ca. 1000 *g*), the hexane phase was applied to a disposable silica column, which had previously been rinsed twice with 3 ml of methylene chloride, and the PFB and PFPO-PFB esters were eluted using 3 ml of the same solvent. After evaporation, the product was redissolved in heptane and subjected to the GC–ECD analysis.

Standard solutions (5 μg each) of non-hydroxylated and hydroxylated (2-OH and 3-OH) 14:0 were used for quantitative measurement. The yields of the PFB and PFPO-PFB derivatives were calculated with GC–FID using 50 ng/μl methyl nonadecanoate as an internal standard.

For comparison, various other reaction conditions for preparation of the PFB

and PFPO-PFB esters were also studied. The use of HFBA instead of PFPA was evaluated. In addition, the fatty acids were subjected to derivatization with TCE and PFPA, extractive alkylation with PFB-Br followed by PFPA acylation, and esterfication with PFB-OH and PFPA, as previously reported [8].

Gas chromatography

A Carlo Erba instrument, Model 4160 (Rodano, Italy), equipped with a ^{63}Ni (10 mCi) electron-capture detector operating in the frequency-pulsed mode, an on-column injector and a fused-silica capillary column (30 m×0.32 mm I.D.) with cross-linked DB-5 (equivalent to SE-54) (J&W, Folsom, CA, U.S.A.), was used. The helium carrier gas and the argon–methane (95:5) make-up gas flow-rates were 1.7 and 50 ml/min, respectively. The temperature of the detector was 300°C. The temperature of the column was initially 110°C, increased directly after injection to 140°C and then programmed to 270°C at a rate of 5°C/min.

Reaction yields were evaluated with the same type of instrument equipped with a flame-ionization detector, an all-glass splitless injection system and a fused-silica capillary column (25 m×0.2 mm I.D.) with cross-linked BP-5 (equivalent to SE-54) (SGE, Ringwood, Victoria, Australia). Hydrogen, at a flow-rate of 2.0 ml/min, served as the carrier gas. The temperature of the injector was 280°C and that of the detector 290°C; the temperature of the column was programmed (starting 1 min after injection) from 140 to 260°C at 10°C/min. The split valve was opened 1 min after injection.

A Hewlett-Packard Model 3390A (Avondale, PA, U.S.A.) electronic integrator was used for peak evaluations.

RESULTS AND DISCUSSION

The use of GC–ECD for bacterial fatty acid analysis has been suggested previously [6,11]. The high sensitivity of the electron-capture detector is a very attractive feature since it renders it possible to analyse the cellular acids of bacteria even when they are present in minute amounts.

The present communication describes a simple, rapid and reproducible method for preparing halogenated derivatives of bacterial cellular fatty acids. All the PFB- and PFPO-PFB-derivatized fatty acids in the reference mixture were completely separated (Fig. 1A). The hydroxyl group of the hydroxy acids was derivatized in order to prevent peak tailing (which may increase the detection limit and render quantitative measurements difficult) and to avoid selective adsorption in the disposable silica columns. PFPO acylation of the PFB-derivatized hydroxy acids also resulted in shorter retention times and increased sensitivities: the ECD response to 2-O-PFPO-PFB 14:0 and 3-O-PFPO-PFB 14:0 was 1.3 and 1.6 times higher, respectively, than that to the non-hydroxylated PFB 14:0. The detection limit for 3-O-PFPO-PFB 14:0 was estimated to be ca. $1 \cdot 10^{-15}$ mol at a signal-to-noise ratio of 3.

Besides its use as a reagent for the hydroxyl groups, PFPA also acts as a catalyst in the PFB esterification of carboxyl groups [8]. The PFB esters were formed quantitively, and the PFPO-PFB derivatives virtually quantitatively (mean ± S.D., 93 ± 3%), under the prevailing reaction conditions. Shorter duration (less than 15 min) of the PFB and PFPO-PFB reactions resulted in lower

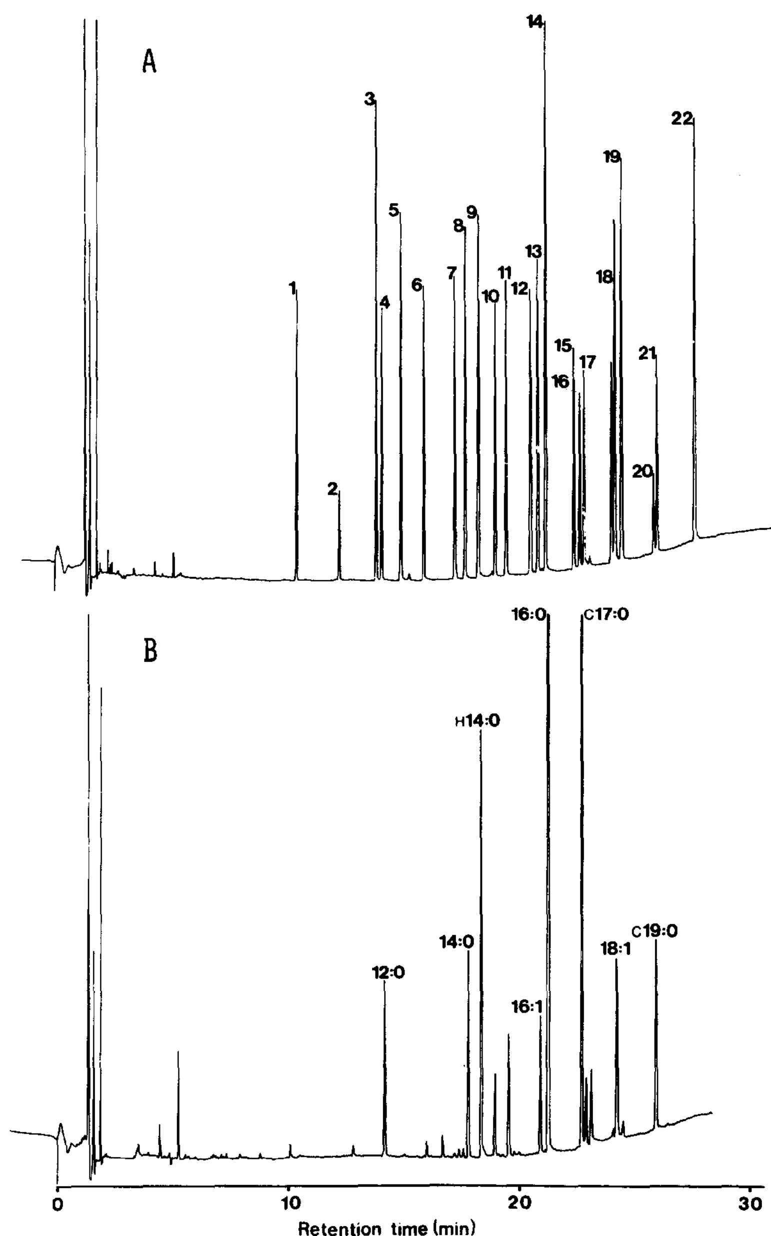

Fig. 1. (A) Chromatogram of the bacterial fatty acid reference mixture. About 20 μg of each ester was derivatized. Peaks: 1 = PFB 2-O-PFPO-decanoate; 2 = PFB undecanoate; 3 = PFB 2-O-PFPO-dodecanoate; 4 = PFB dodecanoate; 5 = PFB 3-O-PFPO-dodecanoate; 6 = PFB tridecanoate; 7 = PFB 2-O-PFPO-tetradecanoate; 8 = PFB tetradecanoate; 9 = PFB 3-O-PFPO-tetradecanoate; 10 = PFB 12-methyltetradecanoate; 11 = PFB pentadecanoate; 12 = PFB 2-O-PFPO-hexadecanoate; 13 = PFB palmitoleate; 14 = PFB palmitate; 15 = PFB 14-methylhexadecanoate; 16 = PFB *dl-cis*-9,10-methylenehexadecanoate; 17 = PFB heptadecanoate; 18 = PFB oleate; 19 = PFB stearate; 20 = PFB *dl-cis*-9,10-methyleneoctadecanoate; 21 = PFB nonadecanoate; 22 = PFB eicosanoate. (B) Chromatogram of the cellular fatty acids of *E. coli*. About 0.4 mg of lyophilized cells were hydrolysed and derivatized. Peaks: 12:0 = PFB dodecanoate; 14:0 = PFB tetradecanoate; H14:0 = PFB 3-O-PFPO-tetradecanoate; 16:1 = PFB palmitoleate; 16:0 = PFB palmitate; C17:0 = PFB *dl-cis*-9,10-methylenehexadecanoate; 18:1 = PFB oleate; C19:0 = PFB *dl-cis*-9,10-methyleneoctadecanoate.

yields. No degradation of the acid-labile cyclopropane-substituted acids occurred unless the TCE and PFB esters (with either PFB-Br or PFB-OH) were prepared at high reaction temperatures [8], and no dehydration of the hydroxy acids (leading to formation of unsaturated PFB esters) was observed. The disposable silica columns were very efficient in removing excess reagents with no noticeable loss of the ester derivatives.

The ECD response to myristic acid PFB ester was ca. 1.9 times higher than that to the TCE ester. The response to HFBO-derivatized PFB hydroxy acids was somewhat higher than to the corresponding PFPO derivatives, as previously described [8]; however, the HFBO-PFB derivatives of 2-hydroxydodecanoic and dodecanoic acid, and of 3-hydroxymyristic and 12-methylmyristic acid, were not separated completely. The PFB and PFPO-PFB esters were therefore selected as the preferred derivatives.

Fig. 1B shows a chromatogram of PFB- and PFPO-PFB-derivatized fatty acids of the *E. coli* cells. The sample was found to contain the same unsaturated, saturated, cyclopropane-substituted and hydroxylated acids as reported previously [12].

The PFB- and PFPO-PFB-derivatized fatty acids are strong electrophores and can be analysed with ECD at femtomolar levels. PFB esters have also proved very suitable for use in negative-ion chemical-ionization mass spectrometry, providing excellent sensitivity and selectivity [5,8]. The method reported here is useful for analysing bacterial fatty acids at high sensitivity, provided that contaminating fatty acids, universally present in the environment and thus capable of affecting the analytical results [2,3,6], can be reduced.

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