GC/MS Determination of Fatty Acid Picolinyl Esters by Direct Curie-Point Pyrolysis of Whole Bacterial Cells

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A single-step method suitable for cellular fatty acid derivatization to picolinyl esters with the use of a pyrolyzer as a thermochemical micro-reactor was developed for whole bacterial cells. This reduced the preparation time from several hours to less than two minutes. In addition, the minimal bacterial mass required for analysis was reduced from several milligrams to micrograms. The profiling of cellular fatty acids of Gram-positive and Gram-negative bacteria was achieved using three derivatization methods: preparation of methyl esters, β -picolinyl esters by Harvey's method and a new method based on pyrolytic derivatization to β -picolinyl esters. It was shown that there are great similarities between profiles of bacterial fatty acids determined by the pyrolytic derivatization method and traditional preparation methods of picolinyl and methyl esters prior to GC analysis. Results obtained by application of the new technique have immense diagnostic value due to vast similarities between profiles of fatty acids derivatized to either picolinyl and methyl esters. Although the latter are referred to in the literature most often, mass spectra of picolinyl esters contain fragment ions that provide structural information about the chain branching, position of unsaturation, and other substituents. (J Am Soc Mass Spectrom 2003, 14, 58–62) © 2003 American Society for Mass Spectrometry

The composition of cellular fatty acids (CFA) is one of the taxonomic features used for the characterization and identification of bacterial strains at the genus, species, and even subspecies level [1, 2]. Some of the commonly used methods for CFA profiling are based on the analysis of fatty acid derivatives by gas chromatography/mass spectrometry (GC/MS). Most frequently, fatty acids isolated from bacterial cells are derivatized to methyl esters for desirable chromatographic properties. Electron ionization (EI) mass spectra of such derivatives provide some structural information, including molecular weight or the degree of unsaturation. However, since numerous intramolecular rearrangements occur during the fragmentation in the ion source, it is very difficult to establish the exact structure of parent fatty acid molecules by GC/MS or EI MS. In 1982, a new method of fatty acids derivatization to picolinyl esters was developed by Harvey [3]. On electron ionization, the charge is localized on the nitrogen atom of a pyridine ring and causes a hydrogen migration to occur from a given position on the chain, initiating a specific radical-induced cleavage (Scheme

1). This produces a series of ions corresponding to cleavage at each carbon–carbon bond from which structural information on chain branching or position of unsaturation may be deduced.

Traditional methods of CFA derivatization are multi-step and time-consuming. Large excess of the reagents used during extraction or derivatization procedures may cause undesirable contamination of the samples. Moreover, long cultivation times are necessary to obtain milligram amounts of bacterial cells that are required for a single analysis.

In 1988 Dworzanski et al. [4, 5] developed a new technique of pyrolytic derivatization of bacterial fatty acids to methyl esters, using whole bacterial cells in the presence of tetramethylammonium hydroxide as a derivatizing agent. This high-temperature methylation procedure was performed directly on the surface of a ferromagnetic filament and coupled with a GC/MS analysis of in situ generated methyl esters of CFA. It was demonstrated that fatty acid methyl esters formed under such conditions were nearly identical (both qualitatively and quantitatively) to those obtained by classical methods.

In the present work, a single-step method of CFA derivatization to picolinyl esters with the use of a pyrolyzer as a thermochemical micro-reactor was developed for whole bacterial cells. To evaluate the suit-

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Scheme 1

ability of the described method, CFA profiles obtained for selected Gram-negative and Gram-positive bacterial strains were compared to those obtained with the use of Harvey's method and conventional derivatization to methyl esters.

Experimental

Materials and Reagents

Lyophilized Gram-negative (*Escherichia coli* strain B ATCC 11303) and Gram-positive (*Bacillus subtilis* ATCC 6633) bacterial cells and 3-pyridylcarbinol were purchased from Sigma-Aldrich Co. Ltd., (Poznan, Poland). Thionyl sulfide, acetonitrile, hexane, acetone, methanol, diethyl ether, HCl, and NaOH were obtained from Merck Co. Ltd., (Warsaw, Poland). Ferromagnetic wires with a Curie-point temperature (T_c) of 770 °C and 358 °C were obtained from Pye-Unicam Ltd., (Cambridge, UK).

Sample Preparation

Isolation of fatty acids from bacterial cells and preparation of picolinyl esters by Harvey's method. Lyophilized bacterial cells (20–100 mg) were heated with 3 M NaOH (1 ml) for 40 min at 90 °C in a water bath. Two ml of 3.25 M HCl were added and the mixture was heated for 10 min at the same temperature. After cooling, fatty acids were extracted three times with 1 ml of hexane-diethyl ether mixture (1:1, vol/vol). The combined organic layers, separated by centrifugation ($2400 \times g$, 5 min), were evaporated to dryness under a stream of argon. The obtained free fatty acids were derivatized to picolinyl esters according to the method of Harvey [6].

Preparation of fatty acid methyl esters. Methyl esters of bacterial CFA were obtained according to the procedure described by Miller and Berger [7]. Briefly, fatty acids released by an alkaline hydrolysis of bacterial cells in the presence of methanol were derivatized to methyl esters in an HCl-containing methanolic medium. The derivatives were then extracted with a hexane-diethyl ether mixture.

Preparation of picolinyl esters by pyrolytic derivatization of whole bacterial cells. Pyrolytic wires with T_c of 770 °C were coated with lyophilized bacterial cells (20–400 μ g). For quantitative measurements, aliquots (5 μ l) of

the suspension of bacterial cells in acetone (0.07 mg/ μ l) were placed on the tip of pyrolysis wires. Immediately after coating the wires, 1 μ l of a derivatization mixture (aqueous solution containing 10% NaOH and 5% pyridylcarbinol) was added and the solvents were evaporated under a stream of nitrogen. The wires were inserted into the Curie-point pyrolyzer and pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) analyses were carried out.

The Conditions of Py-GC/MS Analysis

The pyrolysis cell was kept at 220 °C, while the total pyrolysis time was 4 s. The GC oven temperature was programmed from 40 °C (isothermal for 2 min) to 235 °C at a rate of 20 °C/min, then to 265 °C at a rate of 1 °C/min. The final temperature was held for 5 min. Helium was used as the carrier gas at a flow rate of 1.8 ml/min. and the GC column outlet was connected directly to the ion source of a mass spectrometer. The GC/MS interface was kept at 250 °C, while the ion source and the quadrupole analyzer were at 200 and 100 °C, respectively. An electron energy of 30 eV was an optimized value for ionization of picolinyl esters of fatty acids.

Equipment

The analyses were performed with the use of a Hewlett-Packard (Palo Alto, CA) 5890 series II gas chromatograph interfaced to a Hewlett-Packard 5989A mass spectrometer. Both methyl and picolinyl esters were separated on a fused-silica capillary column (30 m $\times$ 0.25 mm i.d., 0.25 μ m film thickness) coated with a chemically bonded HP5-MS phase (95% polydimethylsiloxane, 5% diphenylsiloxane). A Pye-Unicam (Cambridge, UK) Curie Point Pyrolyser type 795050 was coupled directly to a GC column. For the analysis of methyl esters as well as picolinyl esters obtained by Harvey's method, a split/splitless-type injector was applied. A Hewlett-Packard ChemStation G1034C version C.02.00 software was used for the data collection and the mass spectra processing.

Results and Discussion

Gram-negative *E. coli* and Gram-positive *B. subtilis* strains were chosen for CFA profiling because of a large diversity of fatty acid structural types synthesized by these organisms. Typical fatty acids of *E. coli* are: Saturated and unsaturated straight-chain fatty acids, acids containing cyclopropane rings, and hydroxy acids. The CFA profile of *B. subtilis* is dominated by the saturated, branched-chain fatty acids of the iso- and anteiso-type.

Representative chromatograms of β -picolinyl esters of bacterial CFA are presented in Figure 1 (for *E. coli*) and Figure 2 (for *B. subtilis*). Figures 1a and 2a show the total ion current chromatograms (TIC, m/z 45–500) of

Figure 1. CFA profiles of *E. coli* determined as picolinyl esters: (a) Total ion current chromatogram (TIC, m/z 45–500) of the products obtained by Harvey's method, (b) TIC (m/z 45–500) of the products from the pyrolytic derivatization of whole bacterial cells, (c) reconstructed ion chromatogram (m/z 92–93) from the TIC shown in panel (b). Peak designation: Number before the colon refers to the number of carbon atoms of the fatty acid and the number after the colon is the number of double bonds; cyc, cyclopropane acid.

the picolinyl esters prepared by Harvey's method, while Figures 1b and 2b display the TIC (m/z 45–500) profiles of the products obtained by the pyrolytic derivatization of fatty acids from whole bacterial cells. To improve the signal-to-noise ratio for the analytes and to eliminate peaks corresponding to some impurities, the reconstructed ion chromatograms (RIC) for ions m/z 92–93 were chosen because these ions arise from the cleavage of β -picolinyl ester bonds and as a result are characteristic for all CFA picolinyl esters. Profiles for these ions are presented in Figures 1c and 2c as RIC from the total ion chromatograms shown in Figures 1b and 2b.

Individual fatty acids were identified using retention time characteristics and standard rules for interpretation of CFA β -picolinyl esters mass spectra [3, 6]. To enhance the relative abundance of the diagnostic ions, the EI mass spectra were recorded at 30 eV. The use of an ionization energy much lower than 70 eV was postulated earlier by Harvey [3] to obtain higher quality picolinyl ester spectra.

CFA profiles obtained for a given bacterial strain were qualitatively nearly identical, regardless of the

Figure 2. CFA profiles of *B. subtilis* determined as picolinyl esters: (a) Total ion current chromatogram (TIC, m/z 45–500) of the products obtained by Harvey's method, (b) TIC (m/z 45–500) of the products from the pyrolytic derivatization of whole bacterial cells, (c) reconstructed ion chromatogram (m/z 92–93) from the TIC shown in panel (b). Peak designation: i, iso; ai, anteiso acids; for remaining designations, see Figure 1.

method used for the preparation of picolinyl derivatives. Additional peaks at 25.3 and 27.5 min present in the pyrolytically generated chromatograms of *E. coli* (Figure 1b and c) were difficult to identify. Their mass fragmentation patterns do not correspond to fatty acid picolinyl esters and were not found in the commercially available mass spectra libraries.

The profiles of bacterial CFA obtained by various derivatization methods are compared in Figure 3 (for *E. coli*) and Figure 4 (for *B. subtilis*). All the analyses were run in triplicate and the quantities are mean percentages ($\pm$ standard deviation) of total peak areas calculated from the appropriate TIC profiles (m/z 45–500 for picolinyl esters and m/z 45–450 for methyl esters). Although each of the approaches yields reproducible and comparable CFA profiles, some differences in the yield of individual fatty acids can be observed between derivatization methods. In particular, only derivatization to methyl esters allows direct determination of 3-hydroxymyristic acid (3-OH- $C_{14:0}$) because the experimental conditions caused decomposition of the picolinyl ester of this acid.

This can be substantiated as follows: the analysis of the mass spectrum shown in Figure 5 indicates that the

Figure 3. A comparison of CFA profiles of *E. coli* strain B (ATCC 11303) determined by three derivatization methods. The yield of individual fatty acids are expressed as mean ($n = 3$) percent of total peak area calculated from the TIC. For the CFA designations, see Figure 1. An asterisk denotes that the starred peaks are actually observed as 14:1 but are believed to form from thermal degradation of picolinyl esters of 3-OH-14:0 (see text).

peak with a retention time of 28.2 min (Figure 1) represents the picolinyl ester of a monounsaturated fatty acid with unusual retention time characteristics. The mass spectrum of this compound contains an abundant molecular ion at m/z 317 and this mass is two mass units lower than in the mass spectrum of the corresponding saturated acid. The fragment ion at m/z 302 clearly results from the elimination of a methyl group and is followed by a series of ions due to successive elimination of alkyl radicals differing by 14 mass units (until m/z 190). The decreased abundance of the m/z 164 ion (characteristic in mass spectra of picolinyl esters of C2–C3 saturated fatty acids) combined with the presence of m/z 177 and 190 (that are typical for picolinyl esters of fatty acids with a double bond localized at the second carbon atom) allow the identification of this compound as the β -picolinyl ester of 2-tetradecenoic acid (2-C_{14:1}) [8]. We postulate that this compound arises from the decomposi-

Figure 5. The EI mass spectrum of the β -picolinyl ester of 2-tetradecenoic acid (2-C_{14:1}).

tion of a 3-OH-C_{14:0} acid and as result can be regarded as a marker for this characteristic component of bacterial lipopolysaccharides, typical for Gram-negative microorganisms.

In addition, the retention time of this analyte is very characteristic. Unlike most esters (both picolinyl and methyl) of unsaturated fatty acids that elute from nonpolar phases before the saturated counterpart, the β -picolinyl ester of 2-C_{14:1} acid has a retention time much longer than that of myristic acid (C_{14:0}) (Figure 1).

The CFA profile of *E. coli* determined by the pyrolytic method indicates a lower percentage of fatty acids with shorter hydrocarbon chains, in particular C_{12:0} and C_{14:0} acids. This can be explained by the fact that these compounds elute on the slope of the pyridylcarbinol, which can decrease the ionization efficiency of the acids. However, it is also possible that the higher volatility of the lower molecular weight acids could influence the derivatization process.

To evaluate the yields of the methods, the amount of palmitic acid (C_{16:0}) in *E. coli* cells determined by the pyrolytic derivatization method was compared to that determined by the Harvey's method by spiking the cells with palmitic acid as a standard. In this series of experiments, both CFA and palmitic acid esters prepared by the Harvey's method were placed on the surface of pyrolytic wires with T_c of 358 °C and inserted into the pyrolyzer. The esters were thermally desorbed from the wires in the pyrolytic cell and analyzed using conditions matching those for Py-GC/MS experiments. Mean amounts of palmitic acid ($n = 3$) determined by Harvey's and the pyrolytic derivatization methods were 46.2 ± 1.4 and 39.1 ± 2.0 μ M, respectively, calculated per 1 g of bacterial mass. Hence, the yield of the pyrolytic method with respect to Harvey's method was around 85%.

In conclusion, this work demonstrates that pyrolytic derivatization of CFA to picolinyl esters coupled on-line with GC/MS analysis enables rapid generation of reproducible CFA profiles, which are comparable to those obtained by conventional methods. A substantially diminished amount of the biological material is an additional advantage of the described technique.

Figure 4. A comparison of CFA profiles of *B. subtilis* (ATCC 6633) determined by three derivatization methods. The yield of individual fatty acids are expressed as mean ($n = 3$) percent of total peak area calculated from the TIC. For the CFA designations, see Figure 2.

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