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ОРИГИНАЛЬНЫЕ СТАТЬИ

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PROFILING OF BACTERIAL CELLULAR FATTY ACIDS BY PYROLYTIC DERIVATIZATION TO 3-PYRIDYLCARBINOL ESTERS

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Pyrolytic derivatization to 3-pyridylcarbinol esters coupled with gas chromatography–mass spectrometry (**GC–MS**) was applied to determine cellular fatty acid (**CFA**) profiles of *Escherichia coli*, *Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *Clostridium perfringens*. The structures of bacterial CFAs were inferred from the interpretation of mass spectra with electron ionization of the corresponding 3-pyridylcarbinol esters. In the method used, the entire bacterial cells are pyrolyzed in the presence of a derivatizing reagent, and CFA isolation prior to the GC–MS analysis is not required. The method is argued to be a cheap, fast and accurate alternative to classical esterification to 3-pyridylcarbinol esters, while still providing more detailed results than methods based on esterification to methyl esters. Significat reduction in the required minimum bacterial mass allows for fast identification of the microorganisms, in particular slow growing bacteria species.

Keywords: pyrolysis–GC–MS, 3-pyridylcarbinol fatty acid esters, fast microorganism identification, bacterial taxonomy, cellular fatty acid identification.

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Fatty acids are of great importance for bacterial cells as they define the structure and characteristics of the cell membranes [1–3]. Their composition is dynamic and can be influenced by environmental factors such as temperature, osmolarity, salinity and pH [4, 5] or the presence of substances affecting microbial growth and metabolism [6]. The adaptation of the membranes' chemical content is necessary to maintain the cell homeostasis. The cell achieves this by embedding lipids with an alternative fatty acid structure, which regulates biophysical properties of the cell membranes [2, 4]. Moreover, cellular fatty acid profiles vary across genera and species and can be used in phenotypic microorganism identification [7, 8]. Gathered over many years, the results of bacterial lipid analyses show a significant diagnostic value of the CFA profiling in classification and fast identification of microorganisms [9–12].

To determine the bacterial CFA profile the fatty acids must be released and converted to volatile derivatives. The most common derivatisation technique is esterification to methyl esters [11, 12]. Fatty acid methyl esters have excellent chromatographic properties and information such as molecular mass or degree of unsaturation can be inferred from their mass spectra. Unfortunately, fatty acid methyl esters fragmentation in the ion source follows complicated pathways and subsequent regroupings, so that the precise identification of the molecule structure might prove to be impossible. In 1982 Harvey designed a synthesis method of 3-pyridylcarbinol (**3-PC**) fatty acid esters, which follow simpler fragmentation paths when ionized with electron stream (**EI**). On EI ionization, a radical-induced cleavage at each of the carbon–carbon bonds leads to the formation of the series of ions stabilized by the charge that is localized on the nitrogen atom of the pyridine ring. Structural features of fatty acid molecule, in particular position of unsaturation or chain branching, can be easily deduced from the resulting fragmentation pattern [13–16].

In order to determine the CFA profile with a gas chromatography technique the investigated sample has to be processed chemically, so as to release the fatty acids from cellular structures and to derivatize them to compounds volatile enough for chromatographic analysis. The techniques, which are becoming popular nowadays, introduce derivatization directly in the injector or the pyrolyzer, before injecting the sample into the chromatographic column. Chavari et al. [17] developed *in situ* pyrolysis and silylation for analysis of lipid materials used in paint layers, while for CFA pro-

filing Dworzanski et al. [18, 19] developed a 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. That technique was further developed by Kurkiewicz et al. [20], who introduced a method of CFA pyrolytic derivatization to 3-PC esters.

The goal of presented work was to obtain the CFA profiles of a few common bacteria species using the aforementioned pyrolytic derivatization to 3-pyridinylcarbinol esters. Entire bacterial cells were used and the structure of detected fatty acids was determined. The following bacterial species were chosen for the research: Gram-negative *Escherichia coli*, *Aerobacter aerogenes*, *Pseudomonas fluorescens*, and Gram-positive *Bacillus subtilis* and *Clostridium perfringens*.

EXPERIMENTAL

Materials and reagents. Lyophilized bacterial cells of *E. coli* strain B ATCC 11303, *A. aerogenes* Type III (*Enterobacter aerogenes*), *P. fluorescens* Type II ATCC 13430, *B. subtilis* ATCC 6633 and *C. perfringens* ATCC 13124, and 3-pyridylcarbinol were purchased from Sigma-Aldrich (Poznań, Poland). Acetone and sodium hydroxide were obtained from Merck (Warsaw, Poland). Ferromagnetic wires with a Curie-point temperature (T_c) of 770°C were obtained from Pye-Unicam (Cambridge, UK).

Preparation of 3-pyridylcarbinol esters of bacterial CFA. Pyrolytic wires with T_c of 770°C were coated with lyophilized bacterial cells (20–400 μg). After coating the wires, 1 μL of a derivatization mixture (aqueous solution containing 10% NaOH and 5% 3-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 grad/min, then to 265°C at a rate of 1 grad/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°C and 100°C, respectively. An electron energy of 30 eV was an optimized value for ionization of 3-PC 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. 3-PC esters were separated on a fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm 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. A Hewlett-Packard ChemStation G1034C version C.02.00 software was used for the data collection and the mass spectra processing.

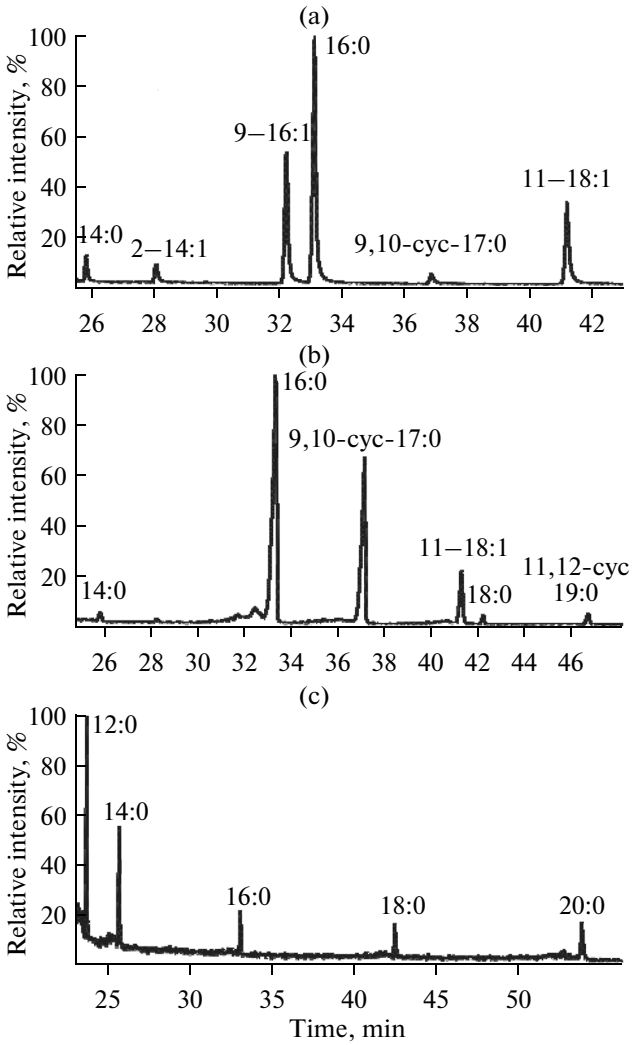

Fig. 1. Reconstructed chromatograms (m/z 92–93) of CFA 3-pyrdinylcarbonyl esters obtained through the pyrolytic derivatization method from whole bacterial cells of *A. aerogenes* (a), *P. fluorescens* (b), and *C. perfringens* (c). For peak designation: see Table footnote.

RESULTS AND DISCUSSION

Figure 1 shows specimen chromatograms of CFA 3-pyrdinylcarbonyl esters obtained through the pyrolytic derivatization method from entire bacterial cells of *A. aerogenes* (a), *P. fluorescens* (b), and *C. perfringens* (c). The results are shown as chromatograms reconstructed from the sum of the m/z 92 and m/z 93 ions. The mechanism by which these ions are formed has

CFA profiles obtained by pyrolytic derivatization method from whole bacterial cells (percent of the total CFA peak area, mean ± standard deviation, $n = 3$)

Fatty acid*	*E. coli*	*A. aerogenes*	*P. fluorescences*	*B. subtilis*	*C. perfringens*
$C_{12:0}$	2.3 ± 0.1	–	–	–	37.7 ± 1.9
i-$C_{14:0}$	–	–	–	2.9 ± 0.2	–
$C_{14:0}$	5.3 ± 0.2	5.6 ± 0.5	2.3 ± 0.2	–	24.2 ± 1.0
i-$C_{15:0}$	–	–	–	12.2 ± 0.5	–
2-$C_{14:1}$	4.1 ± 0.2	9.1 ± 0.7	–	–	–
ai-$C_{15:0}$	–	–	–	44.9 ± 2.5	–
$C_{15:0}$	3.7 ± 0.3	–	–	–	–
i-$C_{16:0}$	–	–	–	8.0 ± 0.3	–
9-$C_{16:1}$	–	23.1 ± 1.4	–	–	–
$C_{16:0}$	40.8 ± 3.3	43.1 ± 1.3	59.3 ± 1.8	6.1 ± 0.3	9.8 ± 0.5
i-$C_{17:0}$	–	–	–	8.1 ± 0.4	–
ai-$C_{17:0}$	–	–	–	17.9 ± 1.1	–
9,10-cyc$C_{17:0}$	23.3 ± 2.1	2.1 ± 0.1	26.8 ± 1.5	–	–
11-$C_{18:1}$	5.3 ± 0.4	17.1 ± 1.0	7.7 ± 0.3	–	–
$C_{18:0}$	–	–	2.2 ± 0.1	–	12.1 ± 0.7
11,12-cyc$C_{19:0}$	15.3 ± 0.8	–	1.7 ± 0.1	–	–
$C_{20:0}$	–	–	–	–	16.2 ± 1.0

* The 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; *i*, *iso*; *ai*, *anteiso* acids.

been proposed by Harvey [16] and is typical for all 3-PC esters. The *m/z* 92 and 93 ions were found to be formed during the cleavage of the bond linking oxygen from the ester bond and the alcoholic carbon. Reconstructed chromatograms were used to enhance the signal to noise ratio and to eliminate peaks indicating impurities. The Table shows CFA profiles of the investigated bacteria. The fatty acids found in the bacterial lipids represent various structural types: the samples were identified to contain unbranched fatty acids both saturated and unsaturated, branched fatty acids and fatty acids with cyclopropane ring as well as hydroxy-acids.

Palmitic acid (C16:0) was found to be the most wide-spread CFA as it was present in the profiles of all investigated bacteria species; for Gram-negative *E. coli*, *A. aeorogenes* and *P. fluorescens* it was also the CFA with the highest percentage of the total CFA content. Furthermore, these bacteria species were shown to contain cyclopropanoic acids and other unbranched and unsaturated acids. The samples from *E. coli* and *A. aerogenes* were shown to contain 3-PC 2-C14:1 ester. This is formed during the chemical processing of the sample as a product of dehydration of 3-hydroxymyristic acid [20].

The CFA inventory of *C. perfringens* is much less complicated. The species contains saturated acids only, with varied alkyl chain lengths (C12–C20). Interestingly, the CFA profile of *B. subtilis* consists mostly of saturated, branched fatty acids of both *iso*- and *anteiso*-type. These types of branching are the most popular among bacterial CFAs. Fig. 2 shows the mass spectra of 3-PC *iso*- and *anteiso*-heptadecanoic acid (*i*-C17:0 and *ai*-C17:0, respectively) esters from *B. subtilis*, along with the most important fragmentation routes. The CFA with methyl branching can be easily recognized in the chromatogram because of the 28 mass units gap, which marks the exact place of branching and represents elimination of the secondary alkyl radical along with the methyl group. It is characteristic for the mass spectra of this type of CFA to have higher intensities of the ions neighbouring the 28 mass units gap [13, 16].

The CFA structure of all five bacterial species was determined by interpretation of the mass spectra obtained by pyrolytic derivatization of entire bacterial cells to 3-PC esters. Resulting CFA profiles are nearly identical to the profiles obtained by conventional methods using Harvey esterification to 3-PC and esterification to methyl esters. The method used here allows reduction of the minimum required bacterial mass to only about 20 μg; this is a significant decrease when compared to the few milligrams needed for conventional methods, and thus allows the process of growing the bacterial culture to be shortened or even omitted. This can prove to be essential in the case of slow growing bacteria

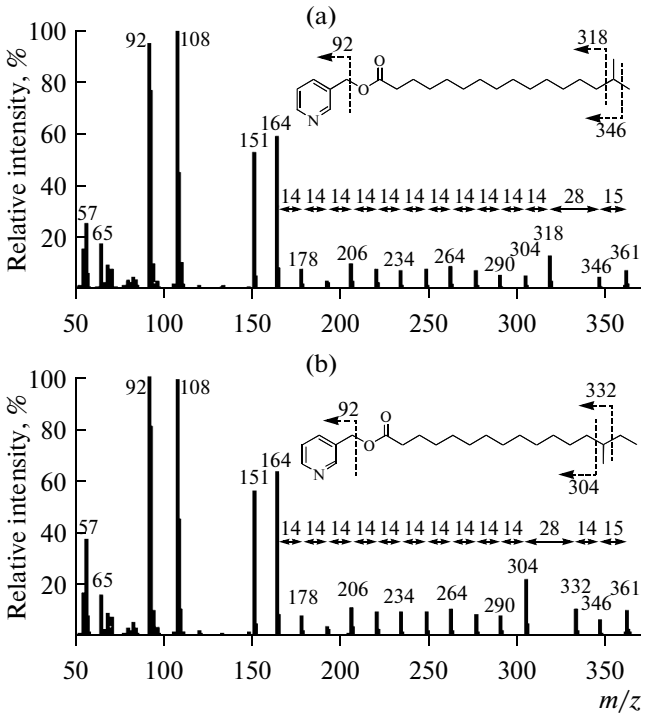

Fig. 2. The EI mass spectra (30 eV) of 3-pyridylcarbinol esters of *iso*- (a) and *anteiso*- (b) heptadecanoic acid. The esters were obtained by the pyrolytic derivatization method from whole *B. subtilis* cells.

species, as it allows quick diagnosis. The time of the analysis preparation is also shortened to just a few minutes, and the amount of reagents is significantly reduced.

* * *

The method of pyrolytic derivatization allows determination of the CFA profiles of *E. coli*, *A. aerogenes*, *P. fluorescens*, *B. subtilis* and *C. perfringens*. Using this technique, the total bacterial mass as well as the amount of reagents used can be diminished, reducing the total cost of analysis. Moreover, interpretation of the mass spectra of 3-PC CFA esters provides information on the structure of CFAs present in researched bacterial species.

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