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EXPERIMENTAL ARTICLES

Fatty Acid Composition of *Wautersia eutropha* Lipids under Conditions of Active Polyhydroxyalkanoates Synthesis

G. S. Kalacheva[1] and T. G. Volova

Institute of Biophysics, Siberian Division, Russian Academy of Sciences, Krasnoyarsk, Russia

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Abstract—The fatty acid composition of the lipids of a *Wautersia eutropha* polyhydroxyalkanoate-producing strain was studied by chromato-mass spectrometry. A total of 27 fatty acids were identified; their distribution in the cell fractions was determined. In the cytoplasmic membrane, palmitic, palmitoleic, and *cis*-vaccenic acids were the major components. Long-chain β-hydroxy acids and myristic acids (components of the lipopolysaccharides of the cell envelope) predominated in the fraction of strongly bound lipids. When the polymer was actively synthesized, the content of cyclopropane acids in the easily extracted lipids increased and the content of the corresponding monoenoic acids decreased. The strongly bound lipids had a high content of long-chain β-hydroxy acids (more than 50% of the total fatty acids). These results made it possible to determine the source of polyhydroxyalkanoate (PHA) contamination and to choose the strategy for their purification.

Key words: Wautersia eutropha, polyhydroxyalkanoates, fatty acids.

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The bacterial species *Wautersia eutropha* (formerly assigned to the genera *Hydrogenomonas, Alcaligenes*, and *Ralstonia*) is among the most promising polyhydroxyalkanoate (PHA) producers; it utilizes a number of substrates to synthesize polymers of various chemical structure with a high yield (up to 80–90%) [1–4]. Biocompatible and biodegradable polymers of hydroxyderivatives of fatty acids are a promising material for a range of applications, including pharmaceutical and medical ones. Confirmation of the biocompatibility of new biomaterials and their harmlessness to humans is among the key issues. Cytotoxicity of PHA (mainly polyhydroxybutyrate and copolymers of hydroxybutyrate with hydroxyvalerate) has been reported in some works on animal cell cultures in vitro [5]. Inflammatory and pyrogenic reactions have been detected in the course of in vivo experiments on animals [6]. Analysis of the literature reveals that industrial polymer samples were often used for PHA biomedical studies [7]. The endotoxin concentration in these samples can be as high as 120 U/g [8, 9] due to the presence of residues of microbial cells containing lipopolysaccharide (LPS) and other complexes, which can cause negative cell reactions in vitro and pyrogenic reactions in vivo [10].

The technology of polymer isolation involves the application of solvents. Since they extract both PHA and lipid components, compounds of lipid nature can be the main contaminants in industrial PHA samples. Moreover, the lipid profile is presently widely used in bacterial taxonomy; analysis of the fatty acid composition is a universally accepted method of rapid species identification [11]. Although *W. eutropha* belong to one of the best-studied bacterial species, data on its lipid and fatty acid composition are limited. We know of several publications concerning the lipid and fatty acid composition of the hydrogen-oxidizing *Hydrogenomonas eutropha* H-16 [12–14] and of several *Ralstonia* species [15]; the lipid and fatty acid composition was typical for gram-negative bacteria [16]. We have previously studied the lipid and fatty acid composition of autotrophically grown *Alcaligenes eutrophus* Z-1 [17]; gas–liquid chromatography on prepacked columns was used in the work. In order to predict the source of contamination in the polymer, we specified the fatty acid composition of the biomass of hydrogen bacteria by means of chromato-mass spectrometry.

The goal of the present work was to identify the fatty acids of *Wautersia eutropha* B5786 by means of modern approaches and to determine their distribution among the cell fractions (the cytoplasmic membrane and the outer layers of the cell envelope) under optimal conditions of growth and PHA accumulation.

MATERIALS AND METHODS

The strain of hydrogen-oxidizing bacteria *W. eutropha* (*Ralstonia eutropha, Alcaligenes eutrophus*) B5786 deposited in VKPM [18] was used in the work. The strain is characterized by high PHA production.

[1] Corresponding author; e-mail: kalach@ibp.ru

Bacteria were grown under sterile conditions in batch culture. A 10-l fermenter (working volume 3 l) of the Bio-Flo 110 modular automated computerized fermentation complex (New Brunswick Sci, Inc, United States) was used. The values of pH (7.0) and temperature (30°C) and the rate of oxygen flow were stabilized automatically during the cultivation; the stirring rate was 120 rpm. The cultivation was carried out according to Schlegel's method in a mineral salt medium. Fructose (10 g/l) was used as the source of carbon and energy. The substrate supply into the medium was regulated in the course of the experiment by means of a peristaltic pump. The previously developed two-stage batch mode of bacterial cultivation was used in order to achieve the highest PHA accumulation. During the first stage, bacterial growth was limited by nitrogen (nitrogen supply was 50% of the culture requirements); during the second stage, the culture was grown on nitrogen-free medium [19].

The lipids were extracted from wet biomass according to the Folch procedure with the chloroform–methanol mixture (2 : 1 vol/vol) [20]. In the resulting extract, PHA were separated from the lipids by precipitation with a double volume of hexane. The lipid extract (easily extracted lipids) was dried in a rotary evaporator and treated by methanolysis in order to obtain methyl ethers of fatty acids (MEFA). The fat-free biomass was treated for one hour with 1 N KOH in 95% ethanol on a water bath with a backflow condenser. A double volume of water was then added; the mixture was acidified, and the fatty acids (strongly bound lipids, SBL) were extracted with hexane and converted to MEFA [20]. Thus, the preparations of fatty acids of the phospholipids of the cytoplasmic membrane and of the LPS of the outer layers of the cell envelope were obtained. Methanolysis of fatty acids was carried out for two h in the mixture of methanol and sulfuric acid (50 : 1 vol/vol) at 90°C [21]. The methyl ethers of fatty acids were analyzed on a GCD Plus chromato-mass spectrometer (Hewlett Packard, United States). The chromatographic setup was as follows: carrier gas, helium; flow rate, 1 ml/min; sample inlet temperature, 220°C; temperature increase to 230°C at the rate of 58C/min; capillary column HP-5M; column length, 30 m; column diameter, 0.25 mm; temperature of the transfer line, 250°C; temperature of the ion source, 165°C; electron impact mode at 70 eV; scanning mode of the fragments, from 45 to 450 *m/z* at 0.5 c/s. The fatty acids were identified by comparison of their retention times and mass spectra with the known standards; saturated, monoenoic, di-, tri-, tetra-, and pentaenoic acids with the chain length from 10 to 20, as well as β-hydroxy acids (Serva, Germany and Sigma, United States). For the purpose of identification of unsaturated acids, the ion chromatograms of original MEFA and of hydrated samples were compared. The location of double bonds in monoenoic acids was determined after obtaining the dimethyl disulfide (DMDS) derivatives of the corresponding MEFA [22]. The presence of a propane ring in the FA chain was determined after bromination of the hydrated samples [20, 22]. Long-chain β-hydroxy acids were also identified by analysis of the corresponding trimethyl silile (TMS) derivatives [21].

RESULTS AND DISCUSSION

The composition of *W. eutropha* B5786 fatty acids under the conditions of optimal growth and active PHA accumulation determined by mass spectral analysis are presented in the table. In the fatty acid spectrum of easily extracted and strongly bound lipids, 27 fatty acids were identified with chain lengths from12 to 18 carbon atoms. Palmitic acid ($C_{16:0}$) was the main unsaturated acid (37–42% of the total fatty acids). The content of myristic acid ($C_{14:0}$) varied from 0.7 to 25% depending on the fraction; the content of stearic acid, from 0.4 to 1.8%. Other saturated acids (laurinic, pentadecanic, and heptadecanic) were the minor components; their ratio did not exceed 0.1%. Branched fatty acids with the *iso-* and *anteiso-* position of the methyl groups were also detected, although they were present in trace amounts. Palmitoleic acid ($C_{16:1}$) and *cis*-vaccenic acid ($C_{18:1}$) were the major monoenoic acids; their ratio was 28 and 22%, respectively. Their molecular masses, 268 and 296, respectively, were determined from the spectra of the original MEFA samples. Moreover, their isomers were also revealed; their ratio in the overall FA spectrum was ca. 1.5%. Other monoenoic acids ($C_{14:1}$, $C_{15:1}$, and $C_{17:1}$) were the minor components; their ratio varied from traces to 1.5 %. The position of double bonds in the aliphatic chain was determined from the mass spectra of the DMDS derivatives of monoenoic acids. For example, the molecular mass of DMDS isomers of the hexadecenoic acid was 362, and the *m/z* values of the diagnostic fragments into which the molecule decomposes after binding of dimethyl sulfide residues to the carbon atoms forming a double bond were 217 and 145 for $C_{16:1}\omega7$ (Fig. 1a), and 245 and 117 for $C_{16:1}\omega5$. For the DMDS isomers of octadecenoic acid, the molecular mass was 390; the *m/z* values of the diagnostic fragments were 245 and 145 for $C_{18:1}\omega7$ (*cis*-vaccenic) (Fig. 1b) and 217 and 173 for $C_{18:1}\omega9$ (oleic). The positions of double bonds in the molecules of minor moenoic acids ($C_{14:1}\omega5$, $C_{15:1}\omega6$, $C_{17:1}\omega9$, $C_{17:1}\omega6$, and $C_{17:1}\omega8$) were determined in the same way (Table). In the case of active PHA synthesis, high amounts of C_{17}-cyclopropanic acid (more than 20% of the total fatty acids) and somewhat lower amounts of C_{19}-cyclopropanic acid (approximately 2%) were revealed. Their identification was confirmed after the treatment of hydrated MEFA samples with bromine. This resulted in the disappearance of the peaks of the corresponding fatty acids from the chromatogram (Fig. 2).

Mass spectrometry clearly indicated the presence of long-chain β-hydroxy acids among the fatty acids of the strongly bound lipids. For the methyl ethers of such acids, *m/z* = 103 is the diagnostic fragment (Table).

Fatty acid composition of *Wautersia eutropha* B5786 lipids under conditions of optimal growth and active PHA synthesis

Fatty acid*	MEFA		DMDS and TMS derivatives of monoenoic and β-OH MEFA		Fatty acids (% of total)			
					Complete medium		Nitrogen-free medium	
	Mol. ion	Basic ion	Mol. ion	*m/z* of the diagnostic fragments	EEL	SBL	EEL	SBL
$C_{12:0}$	212	74			0.12	0.81	0.01	0.24
$C_{14:0}$	242	74			1.89	25.61	0.71	10.81
$C_{14:1}\omega5$	240		334	217, 117	0.06	–	Tr.	0.20
aiso-$C_{15:0}$	256	74			0.15	0.12	1.82	0.87
i-$C_{15:0}$	256	74			0.24	0.40	0.39	2.13
$C_{15:0}$	256	74			0.11	0.27	0.06	0.05
$C_{15:1}\omega6$	254		348	217, 131	Tr.	–	Tr.	–
i-$C_{16:0}$	270	74			0.16	0.19	0.74	1.90
$C_{16:0}$	270				42.06	8.33	37.37	5.21
$C_{16:1}\omega7$	268		362	217, 145	27.77	4.77	10.49	6.52
$C_{16:1}\omega5$	268		362	245, 117	0.98	1.77	0.57	–
i-$C_{17:0}$	284	74			Tr.	0.11	0.24	0.66
$C_{17:0}$	284	74			0.09	0.10	0.09	–
$C_{17:1}\omega9$	282		376	203, 173	1.47	–	Tr.	–
$C_{17:1}\omega6$	282		376	245, 131	Tr.	–	Tr.	–
$C_{17:1}\omega8$	282		376	217, 159	Tr.	–	Tr.	–
c-$C^{**}_{17:0}$	282				0.88	–	21.62	0.26
β-OH $C_{12:0}$		103	302	73, 89, 175, 287	–	0.21	–	0.05
2-OH $C_{14:0}$	258		330	73, 89, 103, 271, 315	–	5.41	–	14.74
$C_{18:0}$	298	74			1.17	1.79	0.73	0.45
$C_{18:1}\omega7$	296		390	245, 145	21.99	1.80	21.75	0.53
$C_{18:1}\omega9$	296		390	217, 173	0.57	0.55	–	0.98
2-OH $C_{16:0}$	286		358	73, 89, 103, 299, 343	–	0.44	–	0.30
β-OH $C_{14:0}$		103	330	73, 89, 175, 315	–	46.75	–	51.64
β-OH $C_{16:0}$		103	358	73, 89, 175, 343	–	0.39	–	1.34
c-$C^{**}_{19:0}$	310				–	–	2.83	–
β-OH $C_{18:0}$		103	386	73, 89, 175, 371		Tr.	–	0.70

Notes: * The first number indicates the number of carbon atoms; the second, the number of double bonds; and the third, the position of the double bond from the methyl end.

** Cyclopropanic acid. Tr, traces. MEFA, methyl ethers of fatty acids; DMDS, dimethyl disulfide derivatives of MEFA; TMS, trimethyl silile derivatives of fatty acids; EEL, easily extractable lipids; SBL, strongly bound lipids.

However, the molecular mass of these acids was not determined; the chain length was therefore established by comparison of the retention times of the β-OH acids of the sample with the standards, β-OH-C_{14}, β-OH-C_{12}, and β-OH-C_{16} (Sigma, United States) and by chromatography of the TMS derivatives. The mass spectra of TMC-hydroxy acids practically coincided with those known from the literature; their major characteristics are presented in the table. The predominant acid was β-OH-C_{14} (more than 46% of the total acids of strongly bound lipids). The minor components included β-OH-C_{12}, β-OH-C_{16}, and β-OH-C_{18}; their ratio did not exceed 1.3%. Among hydroxy acids, 2-OH-C_{14} acid (molecular mass 258, content from 5 to 14%) and the minor 2-OH-C_{16} (content did not exceed 0.44%) were revealed.

The fatty-acid composition of the cytoplasmic membrane (easily extracted lipids) and of the outer layer of the cell envelope (strongly bound lipids) differed significantly. Extracted lipids were acylated mostly by palmitic acid and monoenoic and cyclopropanic acid (over 95%). Myristic acid constituted ca. 2% and stearic acid ca. 1%. It has been previously estab-

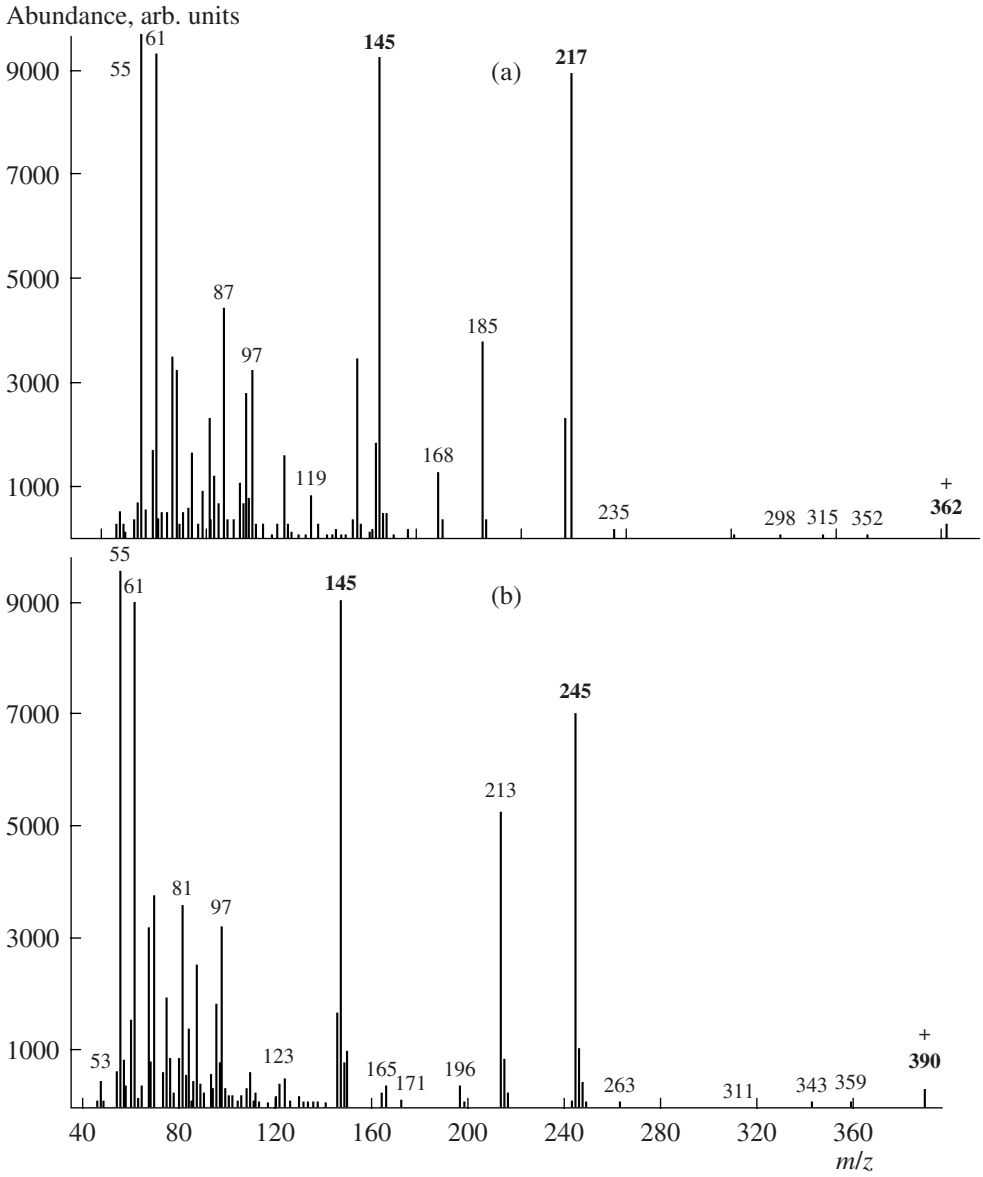

Fig. 1. Mass spectra of the DMDS derivatives of methyl ethers of palmitoleic (a) and *cis*-vaccenic (b) acids.

lished that strongly bound lipids are represented by the components of the cell envelope lipopolysaccharides of gram-negative bacteria; the spectrum of fatty acids which acylate lipid A differs from the composition of intracellular fatty acids [23]. The fatty acid composition of strongly bound lipids of strain B5786 was characterized by a high content of long-chain hydroxy acids (their ratio was 50%) and increased content of myristic acid (over 20% of the total fatty acids of strongly bound lipids). Cyclopropanic acids were not revealed in lipopolysaccharides.

Under the conditions of active PHA synthesis by *W. eutropha*, the fatty acid composition of both easily extractable and strongly bound lipids changed significantly (Table). In the fatty acid spectrum of easily extractable lipids, the content of monoenoic fatty acids (mostly of $C_{16:1}\omega7$) decreased, while the ratio of C_{17}-cyclopropanic acid increased (from 0.88 to 21.6%); C_{19}-cyclopropanic acid was detected. Synthesis of cyclopropanic acids under unfavorable growth conditions is a usual reaction of a number of bacteria, with monoenoic acids predominant in the fatty acid composition [24]. In the strongly bound lipids of *W. eutropha* grown under nitrogen limitation, the ratio of myristic acid decreased, and the ratios of hydroxy acids (2-OH-$C_{14:0}$ and of all β-OH acids, except for β-OH-C12:0) increased.

In contrast to our previous results for strain Z-1 [17], we did not reveal differences in the qualitative composition of the fatty acids of easily extractable lipids of strain B5786. The ratio of the major acids changed somewhat: the content of $C_{16:0}$ increased (ca. 40%)

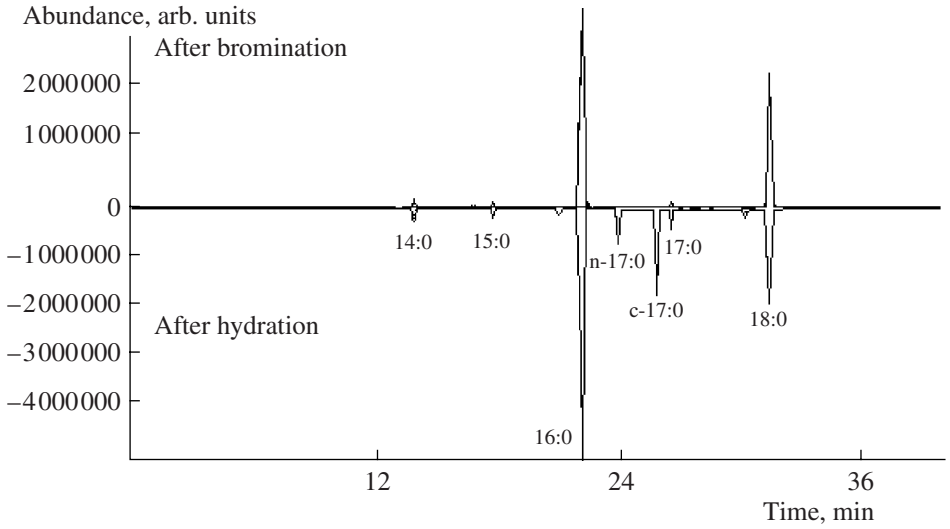

Fig. 2. Ion chromatogram of methyl ethers of fatty acids from *Wautersia eutropha* B5786 lipids after hydration and after bromination.

compared to Z-1 (31%), while the content of monoenoic acids decreased ($C_{16:1}\omega7$ – 28 and 32%; $C_{18:1}\omega7$ – 22 and 28.5/% for strains B5786 and Z-1, respectively). The fatty acid composition of strongly bound lipids was significantly different from the previously obtained values. A broader spectrum of β-OH acids was revealed; 2-OH were identified. Previously, we had detected only one hydroxy acid, which was identified as β-OH-$C_{14:0}$. The results obtained for strain B5786 conform well to the data presented in the work [15]. The fatty acid composition of the lipopolysaccharides of two *Ralstonia* strains, NCIMB 40529 and NCIMB 11842, is characterized by the high content of myristic acid (15–18%), 2-OH-$C_{14:0}$ (14–16%), and β-OH-$C_{14:0}$ (59%). However, the authors did not report the presence of other hydroxy acids. This may be due either to strain differences or to the fact that the fatty acid composition of phenol-extracted pure lipopolysaccharides was determined.

Thus, all the fatty acids of the PHA-producing *W. eutropha* strain are presently revealed. Their distribution in the major lipid fraction is established. These data provide the information required to determine the source of the polymer contamination and to develop the relevant strategy for its purification.

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