Sequence Distribution of β-Hydroxyalkanoate Units with Higher Alkyl Groups in Bacterial Copolyesters

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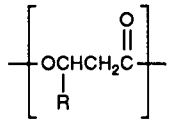
ABSTRACT: Two different samples of copolyesters produced by *Pseudomonas oleovorans* when grown either on nonanoic acid alone or on an equimolar mixture of nonanoic and octanoic acids were evaluated for sequence distribution by controlled methanolysis to their oligomers, separation of the oligomers by HPLC, and analysis of the fractions so obtained by fast atom bombardment mass spectrometry (FAB-MS). The copolyesters contained primarily β-hydroxyheptanoate and β-hydroxynonanoate units, which showed no observable differences in their ^1H and ^{13}C NMR spectra with changes in composition, so those analyses could not be applied. The observed oligomer composition and that calculated on the basis of Bernoullian statistics for sequence distributions in random copolymers of the compositions studied were in good agreement.

Introduction

A wide variety of bacteria produce aliphatic polyesters as an energy reserve storage material. The polymers, which are produced in the form of small granules within the cell, have the general structure



in which R is an *n*-alkyl group varying in size from CH_3 to $C_{12}H_{25}$ and higher, depending on the bacterium. In many cases copolymers are formed, and both ^1H and ^{13}C NMR spectroscopy can be used to determine the sequence distributions of the copolymers in which R is CH_3 and C_2H_5, that is, for copolyesters containing both β-hydroxybutyrate (HB) and β-hydroxyvalerate (HV) units.[1-5]

Some bacteria produce copolyesters containing units with much higher *n*-alkyl groups than methyl or ethyl, and, for these copolymers, NMR analysis cannot be used because, unlike the HB–HV copolymers, there are no detectable differences in the chemical shifts of the units with such longer *n*-alkyl groups. One family of copolyesters in which this limitation applies is that produced by the aerobic bacterium *Pseudomonas oleovorans*, in which R is generally an *n*-pentyl group or higher.[6,7]

In this report we describe how such copolyesters can be analyzed for sequence distribution by fast atom bombardment mass spectrometry (FAB-MS) of the oligomers formed by partial methanolysis of the copolymers. We have previously verified the use of this procedure for the analysis of HB–HV copolymers,[8] but the copolyesters in the present case contained mixtures of units with higher *n*-alkyl pendant groups, which are identified in the following discussion by the following abbreviations: HC, β-hydroxycaproate (R = C_3H_7); HH, β-hydroxyheptanoate (R = C_4H_9); HO, β-hydroxyoctanoate (R = C_5H_{11}); HN, β-hydroxynonanoate (R = C_6H_{13}); HD, β-hydroxydecanoate (R = C_7H_{15}); HU, β-hydroxyundecanoate (R = C_8H_{17}).

Two different samples of poly(β-hydroxyalkanoates) (PHAs) produced by *P. oleovorans* grown on *n*-nonanoic acid were investigated for sequence distributions. The compositions of these samples, as determined by gas chromatography of the methyl esters of the β-hydroxyalkanoic acids obtained after total methanolysis, are given in Table I. Sample 1 was obtained from a culture in which *n*-nonanoic acid was the only carbon source available to the bacteria, while sample 2 was obtained from bacteria grown on an equimolar mixture of *n*-nonanoic acid and *n*-octanoic acid.

Results and Discussion

The procedure used for the partial methanolysis of the two PHA samples studied and for the HPLC fractionation of the oligomers so formed was similar to that previously reported.[8] The methanolysis time was optimized to produce a high proportion of oligomers with molecular masses below the detection limit of the mass spectrometer used of up to ca. 2000 daltons. The repeating-unit compositions of the two samples studied are given in Table I.

Sample 1. The HPLC trace of the partial methanolysis products from sample 1 of Table I is shown in Figure 1. The peaks are well separated, which permitted the collection of fractions corresponding to each peak in Figure 1 up to peak 19. All of the HPLC fractions collected from a single injection of 20 μL of an acetonitrile solution were lyophilized and analyzed by FAB-MS.

Each mass spectrum for peaks 1–13 obtained from the HPLC trace showed the presence of a single oligomer, as evidenced by the molecular ions MH^+ and MNa^+ in the

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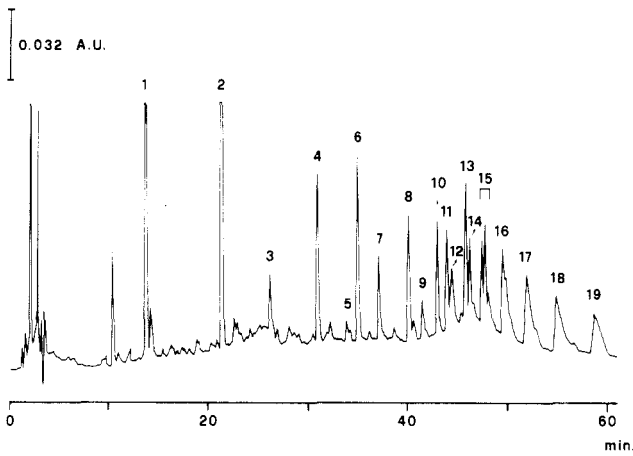


Figure 1. HPLC separation of the methanolysis products from sample 1; structural assignments for the oligomers identified are reported in Table II.

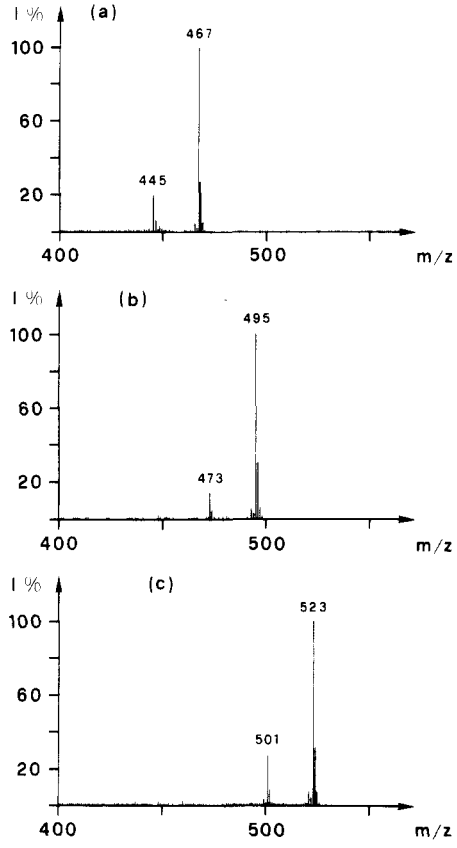


Figure 2. FAB mass spectra corresponding to (a) peak 7, (b) peak 8, and (c) peak 10 in the HPLC trace of the methanolysis products from sample 1.

Table I
Compositions of the PHA Samples Obtained by Gas Chromatography

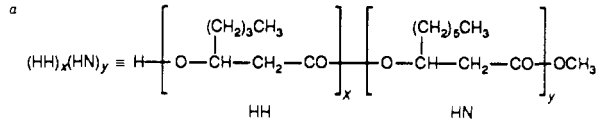
	repeating units present,[a] mol %						
sample	HV	HC	HH	HO	HN	HD	HU
1	2		26		70		2
2	1	5	16	35	40	2	1

[a] See text for abbreviations used.

FAB mass spectra. Representative FAB spectra are shown in Figure 2. The spectrum in Figure 2a, which corresponds to HPLC peak 7 in Figure 1, shows two pseudomolecular ions, which can be assigned to $(HH)_2(HN)H^+$ (m/z 445) and $(HH)_2(HN)Na^+$ (m/z 467). No other peaks, which could have formed from ion fragmentation of this oligomer, were present down to the lowest possible masses, and

Table II
Identification of Oligomer Peaks in HPLC Chromatogram of Figure 1 by FAB-MS

peak	oligomer $(HH)_x(HN)_y$[a]	m/z value from FAB-MS MH^+	MNa^+
1	HH		178[b]
2	HN		206[b]
3	$(HH)_2$	289	311
4	(HH)(HN)	317	339
5	$(HH)_3$	417	439
6	$(HN)_2$	345	367
7	$(HH)_2HN$	445	467
8	$(HH)(HN)_2$	473	495
9	$(HH)_3(HN)$	573	595
10	$(HN)_3$	501	523
11	$(HN)_2$	601	623
12	$(HH)_4(HN)$	701	723
13	$(HH)(HN)_3$	629	651
14	$(HH)_3(HN)_2$	729	751
	$(HH)_5(HN)$	821	851
15	$(HN)_4$	657	679
	$(HH)_2(HN)_3$	757	779
	$(HH)_4(HN)_2$	857	879
	$(HH)_6(HN)$	957	979
16	$(HH)(HN)_4$	785	807
	$(HH)_3(HN)_3$	885	907
	$(HH)_5(HN)_2$		1007
17	$(HN)_5$	813	835
	$(HH)_2(HN)_4$	913	935
	$(HH)_4(HN)_3$	1013	1035
	$(HH)_6(HN)_2$		1135
18	$(HH)(HN)_5$	941	963
	$(HH)_3(HN)_4$	1041	1063
	$(HH)_5(HN)_3$	1141	1163
	$(HH)_7(HN)_2$		1263
19	$(HN)_6$	969	991
	$(HH)_2(HN)_5$	1069	1091
	$(HH)_4(HN)_4$		1191
	$(HH)_6(HN)_3$		1291
	$(HH)_8(HN)_2$		1391

[a] $(HH)_x(HN)_y \equiv$ 

[b] Identified by thermospray MS.

the same is true of the spectra shown in Figure 2b,c. The oligomers corresponding to peaks 1–19 in Figure 1 are identified in Table II. The HPLC fractions corresponding to peaks 1 and 2 in Figure 1 did not yield FAB mass spectra because they represent the HH and HN monomers, both of which were too volatile and evaporated together with the solvent in the lyophilization process. The identification of these peaks was made instead by using a thermospray probe interfaced to the MS and the HPLC to allow the analysis of the HPLC peaks without collection of fractions.

In spectra a and c of Figure 3 are reported the thermospray ionization LC/MS corresponding to HPLC peaks 7 and 10, respectively, of Figure 1. These peaks can be assigned to the $(HH)NH_4^+$ pseudomolecular ion (m/z 178) and to the $(HN)NH_4^+$ pseudomolecular ion (m/z 206) because they are essentially the only high-intensity mass peaks present in the spectra. With the thermospray technique, which uses ammonium acetate as a medium for inducing the ionization of the compounds eluted by the HPLC column (see Experimental Section),[10] the corresponding molecular ions appear in the spectra as clusters with NH_4^+ as shown in Figure 3.

As noted above, HPLC peaks 1–13 in Figure 1 contained only a single oligomer, but FAB analysis of peaks 14–19 revealed that each of those contained several oligomers because the FAB spectra contained several pseudo-

Table III
Experimental[a] and Calculated[b] Relative Amounts of the Methanolysis Products from Sample 1

oligomer $(HH)_x(HN)_y$	obsd from FAB-MS[a]	calcd[b] for HH/HN mole ratio 40/60	35/65	30/70
		Peak 15		
$(HN)_4$	27	20	29	39
$(HH)_2(HN)_3$	51	55	54	51
$(HH)_4(HN)_2$	19	22	15	9.5
$(HH)_6(HN)$	3	3	2	0.5
		Peak 16		
$(HH)(HN)_4$	50	42	53	63
$(HH)_3(HN)_3$	39	45	39	32
$(HH)_5(HN)_2$	11	13	8	5
		Peak 17		
$(HN)_5$	20	12.5	19	28
$(HH)_2(NH)_4$	50	50	54	54
$(HH)_4(HN)_3$	24	31	23	16
$(HH)_6(HN)_2$	6	6.5	4	2
		Peak 18		
$(HH)(HN)_5$	42	30	41	52.5
$(HH)_3(HN)_4$	13	17	11	39
$(HH)_5(HN)_3$	14	20	13	8
$(HH)_7(HN)_2$	2	3	2	0.5
		Peak 19		
$(HN)_6$	18	7	12	19.7
$(HH)_2(HN)_5$	16	13	19	53
$(HH)_4(HN)_4$	28	37	31	23
$(HH)_6(HN)_3$	7	12	7	4
$(HH)_8(HN)_2$	1	2	1	0.3

[a] Relative intensities of MNa^+ ions in the FAB-MS spectrum taken as the average of three samples and analyzed. The spectrum from each sample was averaged over three scans, and deviations in the values of the relative peak intensities were less than 3%. [b] Relative intensities of methanolysis products calculated with eq 1 in the text for three copolymer compositions.

molecular ions. A FAB spectrum representative of the fractions collected is given in Figure 4, which shows that HPLC peak 17 contained four oligomers, all of which are identified in Table II. These FAB spectra of fractions containing mixtures of oligomers are particularly valuable for determining polymer composition and sequence distribution.[8] That is, because no fragmentation of pseudomolecular ions occurred, and if it is assumed that the same response factor to FAB-MS can be used for the oligomers produced in the partial methanolysis product, then the relative abundance of the MNa^+ ions present in the FAB spectrum of each HPLC fraction, which contains more than one oligomer, should yield the polymer composition.[8] The composition can be calculated on the assumption of a random distribution of the HH and HN units in the copolymer.[9]

The statistical probability $P_{x,y}$ of finding a given $(HH)_x(HN)_y$ sequence, assuming Bernoullian (random) statistics for each oligomer, is given by

$$P_{x,y} = \binom{x+y}{y} P_{HH}{}^x P_{HN}{}^y \quad (1)$$

where P_{HH} and P_{HN} are the molar fractions of HH and HN units in the copolymer. The binomial coefficient in this equation is the number of possible sequence arrangements of the $(HH)_x(HN)_y$ oligomers. By starting with an arbitrary set of P_{HH} and P_{HN} values, it is possible to calculate the statistical abundances, $P_{x,y}$, for a series of oligomers actually present in a chromatographic fashion. The best match between the experimentally observed oligomer distributions and the calculated statistical abundances gives the copolymer composition.

The results of these calculations for sample 1 are reported in Table III. The experimental data used here are the relative intensities of the MNa^+ ions appearing in the FAB spectra of HPLC peaks 15–19 in Figure 1. The agreement between the observed and calculated values was a function of P_{HH}/P_{HN} ratio, and as seen in Table III the best fit was for a ratio 35/65, which contrasts to a 27/73 ratio obtained from GC compositions in Table I. However, considering that in order to simplify the calculations the presence of two minor components in the polymer, the HV and HU units, were neglected, this level of agreement can be regarded as satisfactory, and the results confirm the assumption that the polymer possessed a random distribution of repeating units.

In an alternate set of calculations the relative intensities of the three HPLC peaks containing the dimers, peaks 3, 4, and 6 in Figure 1, were used, and eq 1 was again applied to estimate the sequence distribution by restricting the analysis to the dimeric species. The observed HPLC peak intensities and those calculated according to eq 1 are reported in Table IV. The best agreement between the calculated values and the experimental ones was found for an HV/HH/HN/HU composition of 2/31/65/2, which is very close to the H/N ratio of 35/65 calculated from the FAB-MS data in Table III.

Sample 2. The HPLC trace of the partial methanolysis products from sample 2 is shown in Figure 5. The number of HPLC peaks for this sample was higher than for the methanolysis products of sample 1 in Figure 1 because of the higher number of component units present in this polymer, as seen in Table I. All of the HPLC fractions collected were lyophilized and analyzed by FAB-MS except for peaks 1–3 in Figure 5, which were analyzed by the thermospray probe as discussed above. In Figure 6a–c are given the thermospray spectra obtained. As discussed above, the pseudomolecular ions in thermospray mass spectra appear as clusters with NH_4^+, and the HPLC peaks 1–3 in Figure 5 can be readily identified as arising from the HH, HO, and HN units.

FAB mass spectra corresponding to peaks 14–16 in the HPLC trace in Figure 5 showed the presence of a single oligomer in each peak. The three representative mass spectra in Figure 6 all show two pseudomolecular ions, MH^+ and MNa^+, with negligible traces of ion fragmentation.

The FAB analysis of HPLC peaks 17–30 in Figure 5 revealed that each peak contained several oligomers because all of these FAB spectra contained several pseudomolecular ions. For example, in Figure 7 is given the spectrum of the fraction corresponding to peak 28 in the HPLC trace in Figure 5. Figure 7 shows that this peak contained several oligomers, which are identified as reported in Table V. Because sample 2 contained so many different units, in order to apply the statistical approach expressed by eq 1 to estimate sequence distributions, the analysis had to be restricted to the dimeric species present. For this purpose, the relative intensities were measured for the six HPLC peaks containing the dimers in Figure 5. The experimentally observed intensities were compared with those calculated according to eq 1, and the results are reported in Table VI. The best agreement of the calculated values with the experimental ones was found for an HV/HC/HH/HO/HN/HD/HU composition of 2/6/16/37/33/4/2, which is close to the repeating-unit ratio obtained by GC analysis in Table I. This result confirms the assumption that sample 2 also possessed a random distribution of units even though it was grown on a mixture of two different alkanoic acids.

Table IV
Experimental[a] and Calculated[b] Amounts of the Dimers Obtained from the Methanolysis of Sample 1

HPLC peak	dimer	HPLC area[a]	calcd[b] for HV/HH/HN/HU mole ratio 2/26/70/2	2/31/65/2	0/40/60/0
3	$(HH)_2$	14	10	13	16
4	(HH)(HN)	41	38	42	48
6	$(HN)_2$	45	52	45	36

[a] Relative HPLC areas for the dimer species obtained by methanolysis as shown in Figure 1 and Table II; deviations in the values of the areas were less than 2%. [b] Relative abundances of dimer species as calculated by eq 1 in the text for three copolymer compositions.

Table V
Identification of Oligomer Peaks in the HPLC Chromatogram of Sample 2 in Figure 5 by FAB-MS

peak	oligomers	m/z values from FAB-MS MH+	MNa+	peak	oligomers	m/z values from FAB-MS MH+	MNa+
1	HH		178[a]	22	C_{33} tetramers	615	637
2	HO		192[a]		C_{37} pentamers	701	723
3	HN		206[a]		C_{41} hexamers		809
4	(HV)(HO), (HC)(HH)		297	23	C_{34} tetramers	629	651
5	(HV)(HN), (HC)(HO), $(HH)_2$	289	311		C_{38} pentamers	715	737
6	C_{18} trimers[b]	375	397		C_{39} pentamers	729	751
7	(HV)(HD), (HC)(HN), (HH)(HO)	303	325		C_{42} hexamers		823
8	C_{19} trimers	389	411		C_{43} hexamers		837
9	(HV)(HV), (HC)(HD), (HH)(HN), $(HO)_2$	317	339	24	C_{35} tetramers	643	665
10	C_{20} trimers	403	425		C_{40} pentamers	743	765
11	(HC)(HU), (HH)(HD), (HO)(HN)	331	353		C_{44} hexamers		851
12	C_{21} trimers	417	439	25	C_{36} tetramers		679
13	(HH)(HU), (HO)(HD), (HN)(HN)	345	367		C_{40} pentamers		765
14	C_{22} trimers	431	453		C_{41} pentamers	757	779
15	C_{26} tetramers	517	539		C_{45} hexamers		865
16	C_{23} trimers	445	467		C_{46} hexamers		879
17	C_{24} trimers	459	481		C_{50} heptamers		965
	C_{28} tetramers		567	26	C_{42} pentamers	771	793
	C_{32} pentamers		653		C_{47} hexamers	871	893
18	C_{25} trimers		495		C_{51} heptamers		979
	C_{29} tetramers		581	27	C_{43} pentamers		807
	C_{33} pentamers		667		C_{48} hexamers		907
19	C_{26} trimers		509		C_{52} heptamers		993
	C_{30} tetramers		595		C_{53} heptamers		1007
	C_{34} pentamers		681		C_{57} octamers		1093
	C_{38} hexamers		767	28	C_{44} pentamers		821
20	C_{27} trimers		523		C_{49} hexamers		921
	C_{31} tetramers		609		C_{54} heptamers		1021
	C_{35} pentamers		695		C_{58} octamers		1107
	C_{39} hexamers		781	29	C_{45} pentamers		835
21	C_{39} hexamers	601	623		C_{50} hexamers		935
	C_{36} pentamers	687	709		C_{55} heptamers		1035
	C_{40} hexamers		795		C_{59} octamers		1121
	C_{60} octamers		1135	30	C_{51} hexamers		949
	C_{63} nonamers		1207		C_{56} heptamers		1049
					C_{61} octamers		1149
					C_{65} nonamers		1235

[a] Identified by thermospray MS. [b] All possible trimers containing 18 carbon atoms that could be obtained from the monomers are indicated as C_{18} trimers, including $(HC)_3$, (HV)(HC)(HH), and $(HV)_2(HO)$; the identity of the remaining C_n oligomers could be deduced in the same way.

Table VI
Experimental[a] and Calculated[b] Relative Amounts of the Dimeric Species Obtained from the Methanolysis of Sample 2

HPLC peak	HPLC area[a]	calcd[b] for HV/HC/HH/HO/HN/HD/HU mole ratio 1/5/16/35/40/2/1	2/6/16/37/33/4/2	1/5/16/25/50/2/1
4	5	2	4	2
5	11	7	9	6
7	14	15	17	14
9	29	26	27	23
11	26	30	28	27
13	14	19	15	27

[a] Relative HPLC area of the dimeric species produced by methanolysis as shown in Figure 5 and Table V; deviations in values of the areas were less than 2%. [b] Relative abundances of dimeric species as calculated by eq 1 in the text for three copolymer compositions.

Experimental Section

Polyester Production. The biosynthesis of the two samples was carried out by growing *P. oleovorans* under aerobic conditions as 12-L batch cultures using 10 MM carbon source concentrations. The E* medium used for growing *P. oleovorans* contained the following components (per liter): $(NH_4)_2HPO_4$, 1.1 g; K_2HPO_4, 5.8 g; KH_2PO_4, 3.7 g. Ten milliliters of a 100 mM $MgSO_4$ solution and 1 mL of a microelement solution were added. The microelement solution contained the following components (per liter of

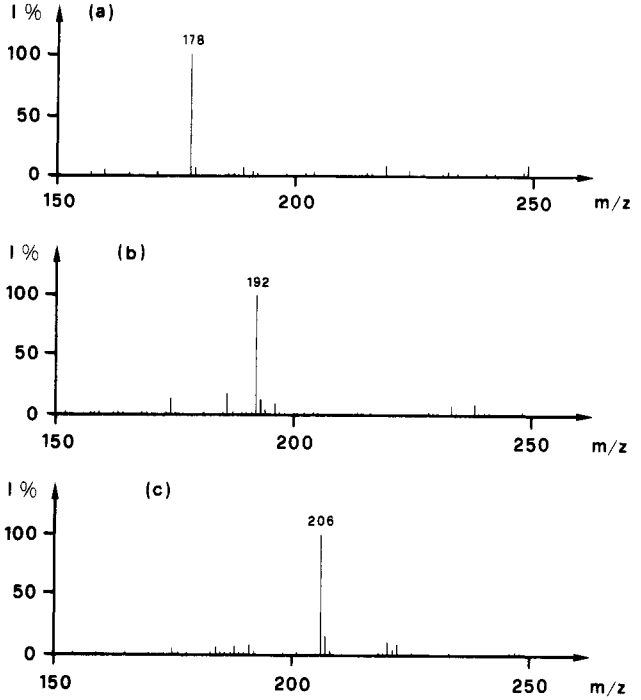


Figure 3. Thermospray LC/MS corresponding to (a) peak 1 and (c) peak 2 in the HPLC trace of the methanolysis product from sample 1; in the HPLC trace of the methanolysis products from PHA sample 2, the thermospray LC/MS correspond to (a) peak 1, (b) peak 2, and (c) peak 3.

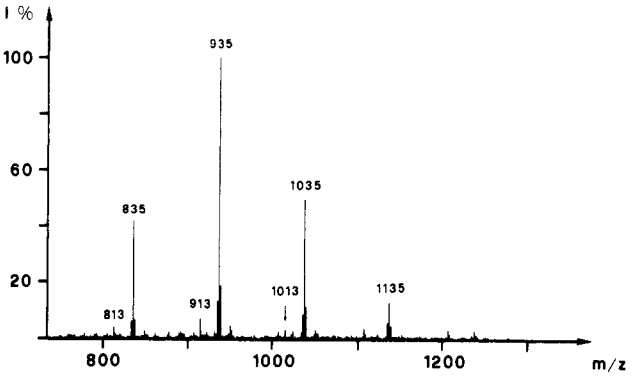


Figure 4. FAB mass spectra corresponding to peak 17 in the HPLC trace of sample 1.

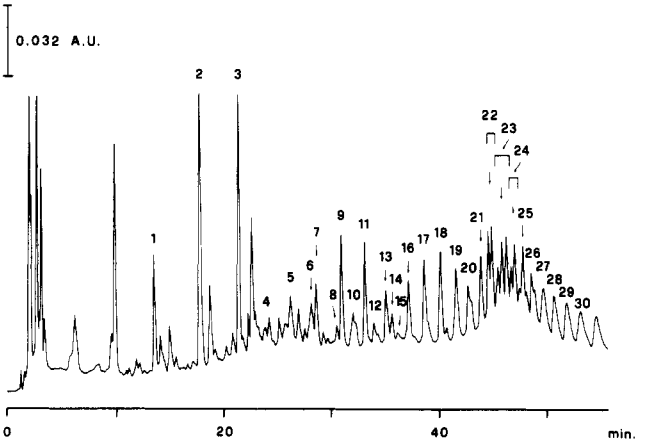


Figure 5. HPLC separation of the methanolysis products from sample 2; structural assignments for the oligomers identified are reported in Table V.

1 N HCl): $FeSO_4 \cdot 7H_2O$, 2.78 g; $CaCl_2 \cdot 2H_2O$, 1.67 g; $CuCl_2 \cdot 2H_2O$, 0.17 g; $ZnSO_4 \cdot 7H_2O$, 0.29 g. To 12 L of E* medium 120 mmol of the carbon sources was added, so 19.6 g of 98% nonanoic acid

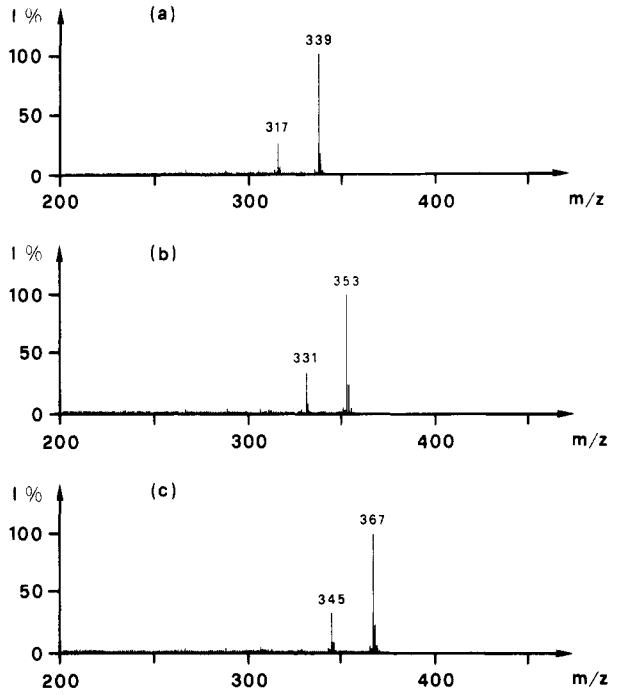


Figure 6. FAB mass spectra corresponding to (a) peak 9, (b) peak 11, and (c) peak 13 in the HPLC trace of the methanolysis products from sample 2.

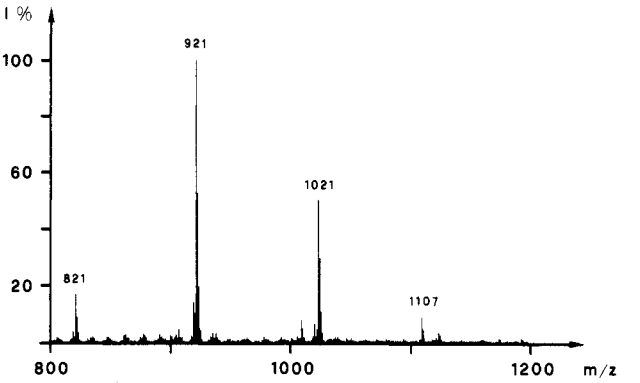


Figure 7. FAB mass spectrum corresponding to peak 28 in the HPLC trace of sample 2.

was added to produce sample 1, while 9.8 g of 98% nonanoic acid and 8.8 g of 98% octanoic acid were added to produce sample 2. The pH was adjusted to 7.0, and the medium was autoclaved. Inoculums were prepared by growing *P. oleovorans* on the same medium containing only nonanoic acid as 250-mL batch cultures. The optical density of the inoculum was between 1.2 and 1.4 AU (at 660 nm) after 14-h growth. To ensure reproducibility, if the optical density of an inoculum deviated from that value, it was discarded. The amount (milliliters) of the inoculum added to the 12-L medium was determined by dividing 150 by the optical density of the inoculum. A temperature-controlled fermenter (New Brunswick) was used (30 °C, 1000 rpm, 2 L of air/min). The change of the optical density at 660 nm was monitored, and cells were harvested when the optical density reached the maximum plateau by centrifugation (Sorvall RC2-B; 4 °C, 12000*g*). The pellets were resuspended and washed once with 10 mM Tris-HCl buffer (pH 7.5) and water, and the harvested cells were lyophilized.

The polymers were isolated from lyophilized cells by extraction with chloroform in a Soxhlet extractor. The extraction was carried out for 6 h, after which the solvent was evaporated and the weight of crude extract was measured. The crude polymer product was dissolved in chloroform at a ratio of the volume of chloroform (milliliters) to weight of the crude extract (grams) of 5:1. The solution was passed through a glass funnel containing a cotton plug into rapidly stirred methanol. The ratio of methanol to chloroform was 10:1 (v/v). After 30 min of stirring, the liquid

layer was decanted, and the precipitated polymer was dried in vacuo (1 mmHg) at a temperature above 35 °C for 16 h. The product was weighed and the precipitation was repeated twice more to obtain the final product.

GC Assay. The sample compositions were determined by gas chromatography analysis of the acid-catalyzed methanolysis products of the polymers. Into a screw cap test tube 3–5 mg of the polymer was weighed, and 1 mL of 3% (v/v) sulfuric acid solution in methanol and 1 mL of chloroform were added. The mixture was allowed to stand in an oil bath at 100 °C for 4 h, after which the mixture was cooled to room temperature, 1 mL of distilled water was added, and the mixture was shaken vigorously. The organic layer was dried over anhydrous $MgSO_4$. About 2.5 μL was injected into a Perkin-Elmer 8500 GC equipped with a Durabond-Carbowax-M15 megabore capillary column (15 m × 0.54 mm; J&W Scientific) and a flame ionization detector. Identification and quantification of the β-hydroxyalkanoic acid methyl esters formed by methanolysis were carried out by the use of calibration curves obtained from standard materials.[6]

Molecular Weight Measurement. Molecular weights were determined by gel permeation chromatography (GPC) using polystyrene standards. The instruments used include a Waters Model 6000A solvent delivery system, Model 401 refractive index detector, and a PC as the data processor. One Ultrastyragel linear column and one 300-Å Ultrastyragel column were used. Chloroform was used as eluent at a flow rate of 1 mL/min. The sample concentratino was 15 mg/mL, and 60 μL was injected. The number-average molecular weight was approximately 70 000, and the ratio of weight- to number-average molecular weights was about 1.7 for both samples.

Partial Methanolysis. A 1 N solution of HCl in dry methanol was prepared by bubbling gaseous HCl through redistilled anhydrous methanol. The amount of HCl dissolved was determined gravimetrically. The concentration was then adjusted by adding an appropriate volume of methanol. About 0.1 g of each sample was dissolved in 20 mL of $CHCl_3$, and 3 mL of a freshly prepared 1 N solution of HCl in methanol was added. The mixture was allowed to react at room temperature for 48 h, after which the solvent was evaporated. The residue was taken up with 2 mL of acetonitrile and transferred for HPLC analysis.

HPLC Fractionation. The fractionation of the methanolysis products was performed by HPLC, using a Varian VISTA 5500 HPLC system equipped with a Rheodyne injector with a 50-μL loop, a Varian 2050 UV detector, and a Microbondapak column (C18, Waters) of 30 cm × 4 mm. Twenty microliters of the acetronitrile solution was injected, using an elution gradient starting with a 20/80 acetonitrile/water composition and ending with 100% acetonitrile in 40 min, with 1 mL/min flow and UV detection at 205 nm.

FAB Mass Spectra. A double-focusing Kratos MS 50S equipped with the standard FAB source and a DS 90 data system was used to obtain mass spectra. The FAB gun (Ion Tech) was operated with a 7–8-keV xenon beam. The instrument was scanned from m/z 2000 to m/z 60, with a scan rate of 10 s/decade. The accelerating voltage was 6–8 kV. Cesium and rubidium iodides (50/50 w/w) were used for computer calibration. The resolution was approximately 2500.

Lyophilized samples to be analyzed were dissolved in acetonitrile. About 2 μL of the sample solution was placed on the copper target end of the direct insertion probe and mixed with 3-nitrobenzyl alcohol doped with NaCl. Peak intensity values shown in the mass spectra represent the average of three separate mass spectra.

Thermospray LC/MS. Thermospray LC/MS was carried out with the MS 50S instrument interfaced with a Kratos LC/MS thermospray apparatus[10] using the same HPLC set described above. The interface was operated at a vaporizer temperature of 220 °C and at the ion source temperature of 230 °C. Poly(ethylene glycol) (MW = 600) was used for computer calibration. Twenty microliters of an acetonitrile solution of the mixture of the methanolysis products was injected, using an elution gradient starting with a 20/80 ratio of acetonitrile/0.1 M CH_3COONH_4 in water and ending with 100% acetonitrile in 40 min, with a flow rate of 1 mL/min. Another mode of operation consisted in flushing the thermospray interface with a 0.1 M solution of CH_3COONH_4 in water and injecting an HPLC fraction. This operation mode was therefore equivalent to the FAB-MS analysis of the lyophilized HPLC fractions, as described above except for the ionization mode used.

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