Biosynthesis and Characterization of a New Bacterial Copolyester of 3-Hydroxyalkanoates and 3-Hydroxy-ω-chloroalkanoates

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ABSTRACT: A new bacterial copolyester of 3-hydroxyhexanoate (HH), 3-hydroxyoctanoate (HO), 3-hydroxy-6-chlorohexanoate (HCH), and 3-hydroxy-8-chlorooctanoate (HCO) units was synthesized in *Pseudomonas oleovorans* by using octane and 1-chlorooctane as the carbon sources. The copolyester ($\bar{M}_n$ = 87 000, $\bar{M}_w$ = 201 000) was shown to have a random sequence distribution of four different monomeric units by analysis of the 125-MHz ^{13}C NMR spectrum.

Introduction

A variety of microorganisms accumulate an optically active poly(3-hydroxybutyrate) as an intracellular storage polymer. Recently, poly(3-hydroxyalkanoates) incorporating 3-hydroxyalkanoate units other than 3-hydroxybutyrate have been isolated from several bacterial strains. A copolyester of 3-hydroxybutyrate and 3-hydroxypentanoate has been produced by *Alcaligenes eutrophus* from propionic acid[1,2] or pentanoic acid.[3] The copolyester has been shown to have a statistically random distribution of 3-hydroxybutyrate and 3-hydroxypentanoate units.[4,5] *Pseudomonas oleovorans* is capable of producing unusual copolyesters containing 3-hydroxyalkanoate repeating units with different side chain length (C_1–C_9) from alkanes[6,7] and alkanoic acids.[8,9] Recently, several *Pseudomonas* species[10–12] and *Rhodospillium rubrum*[13] have also been shown to produce poly(3-hydroxyalkanoates) (C_4–C_{10}) from alkanoic acids and alcohols. These bacterial copolyesters have attracted much attention as environmentally degradable thermoplastics for a wide range of agricultural, marine, and medical applications.[1,14]

In this paper we report the production of a new copolyester of 3-hydroxyalkanoate and 3-hydroxy-ω-chloroalkanoate units by *P. oleovorans* from octane and 1-chlorooctane. The composition and sequence distribution of the copolyester have been studied by analysis of the ^1H and ^{13}C NMR spectra.

Experimental Section

Biopolyester Synthesis. Poly(3-hydroxyalkanoates) and poly(3-hydroxyalkanoate-*co*-3-hydroxy-ω-chloroalkanoates) were isolated from *P. oleovorans* (ATCC 29347). The strain was maintained in 10% (w/v) glycerin at −20 °C. The microorganism was grown at 30 °C and pH 7.0 in 1.0 L of mineral medium containing octane (from Tokyo Kasei Co., extra pure grade) and 1-chlorooctane (from Tokyo Kasei Co., guaranteed reagent grade) as the carbon sources under aerobic conditions in a 2.6-L jar fermenter. The mineral medium contained 1.1 g of $(NH_4)_2HPO_4$, 5.8 g of K_2HPO_4, 3.7 g of KH_2PO_4, and 0.12 g $MgSO_4$ per liter of distilled water. In addition, 1 mL of a microelement solution was added to the medium. The microelement solution contained the following (per liter of 1.0 N HCl): 2.78 g of $FeSO_4 \cdot 7H_2O$, 1.98 g of $MnCl_2 \cdot 4H_2O$, 2.81 g of $CoSO_4 \cdot 7H_2O$, 1.67 g of $CaCl_2 \cdot 2H_2O$, 0.17 g of $CuCl_2 \cdot 2H_2O$, and 0.29 g of $ZnSO_4 \cdot 7H_2O$. The cells were cultivated in the medium for 48 h at 30 °C, harvested by centrifugation, and then lyophilized. Polyesters were extracted from the lyophilized cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with methanol.

Analytical Procedures. The ^1H and ^{13}C NMR spectra of the polyesters were recorded on a JEOL GX-500 spectrometer. The 500-MHz ^1H NMR spectra were recorded at 27 °C in a $CDCl_3$ solution of polyester (3 mg/mL) with a 3.5-μs pulse width (45° pulse angle), 5-s pulse repetition, 5000-Hz spectral width, 32K data points, and 100 accumulations. The 125-MHz ^{13}C NMR spectra were recorded at 27 °C in a $CDCl_3$ solution of polyester (25 mg/mL) with a 10-μs pulse width (45° pulse angle), 5-s pulse repetition, 25 000-Hz spectral width, 64K data points, and 15 000 accumulations. Tetramethylsilane (Me_4Si) was used as an internal chemical shift standard.

The melting temperatures of the polyester samples were recorded on a Shimadzu DSC-50. The 3-mg samples were encapsulated in aluminum pans and heated at 10 °C/min up to 200 °C.

Molecular weight data were obtained at 40 °C by using a Shimadzu 6A GPC system. Chloroform was used as eluant at a flow rate of 0.5 mL/min, and a sample concentration of 1.0 mg/mL was used. Polystyrene standards with a low polydispersity were used to make a calibration curve.

Results and Discussion

The copolyester (sample 1) of 3-hydroxyoctanoate (HO), 3-hydroxy-8-chlorooctanoate (HCO), 3-hydroxyhexanoate (HH), and 3-hydroxy-6-chlorohexanoate (HCH) units was produced in *P. oleovorans* by using octane and 1-chlorooctane as the carbon sources. As reported in a previous paper,[7] the copolyester (sample 2) of HO and HH units was produced by *P. oleovorans* from octane. In this paper, the carbon species in the four monomeric units are denoted by numbers 1–28.

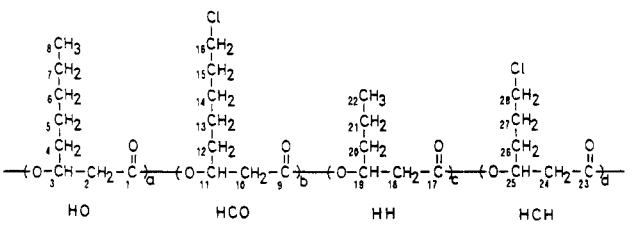

Table I gives the fermentation conditions and compositions of two copolyester samples. The compositions of the samples were determined from ^1H and ^{13}C NMR spectra, as described later. Table II lists the properties and elemental analysis data of two copolyester samples. The number-average molecular weights ($\bar{M}_n$) were ca. 90 000, and the polydispersities ($\bar{M}_w/\bar{M}_n$) were in the range 2.0–2.3. The melting temperature (T_m) of sample 2 was 59.1 °C. In contrast, sample 1 was an amorphous polymer, and the enthalpy of fusion (ΔH_m) was not observed. The elemental analysis data showed that sample 1 contained 16.7 wt % of chlorine.

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Table I
Production of Copolyesters of 3-Hydroxyoctanoate (HO), 3-Hydroxy-8-chlorooctanoate (HCO), 3-Hydroxyhexanoate (HH), and 3-Hydroxy-6-chlorohexanoate (HCH) by *Pseudomonas oleovorans* at 30 °C for 48 h

sample	carbon source, g/L		cell dry wt, g	polyester content,[a] wt %	copolyester compn,[b] mol %			
	octane	1-chlorooctane			HO	HCO	HH	HCH
1	33	40	2.6	5	29	59	2	10
2	36	0	1.4	19	91	0	9	0

[a] Polyester content in dry cells. [b] Determined by ^1H and ^{13}C NMR spectra.

Table II
Analytical Data of Copolyester Samples

sample	mol wt[a]		T_m,[b] °C	ΔH_m,[b] J/g	elem anal.,[c] wt %		
	$\bar{M}_n \times 10^{-3}$	$\bar{M}_w/\bar{M}_n$			C	H	Cl
1	87	2.3		0	57.1 (57.3)	7.9 (7.9)	16.7 (15.1)
2	90	2.0	59.1	13.6	66.9 (67.3)	9.8 (9.8)	0.2 (0.0)

[a] Determined by GPC. [b] Melting temperature (T_m) and enthalpy of fusion (ΔH_m) were measured at 10 °C/min. [c] The values in parentheses are calculated for NMR compositional data in Table I. A small amount of Cl found in sample 2 may arise from certain contaminants such as chloroform.

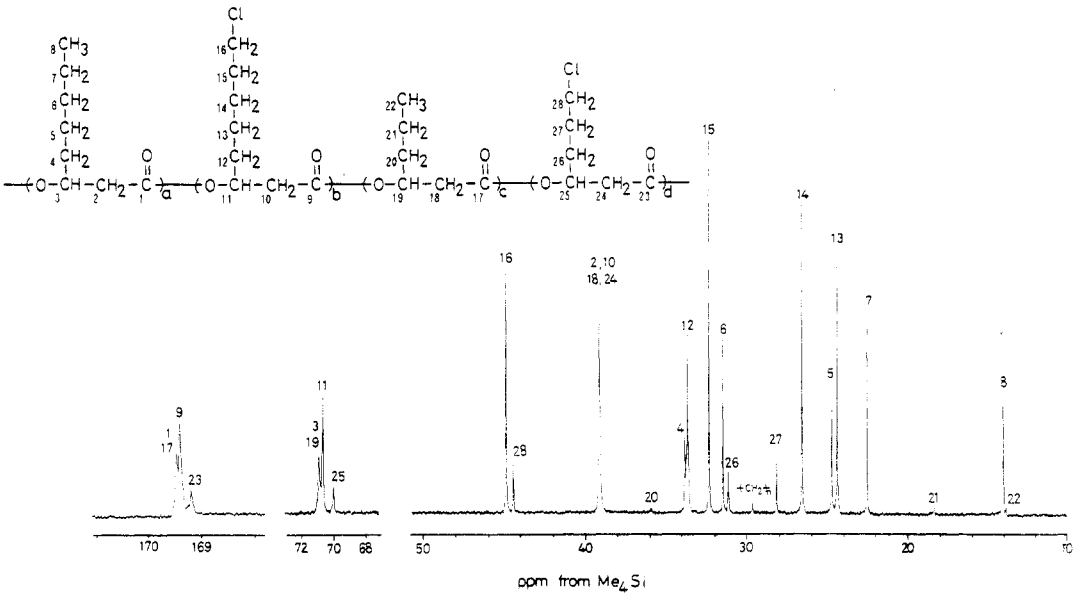

Figure 1. 125-MHz ^{13}C NMR spectrum of sample 1 in chloroform.

Table III
Chemical Shifts (ppm) of ^{13}C Resonances in Copolyester Sample 1 of 3-Hydroxyoctanoate (HO), 3-Hydroxy-8-chlorooctanoate (HCO), 3-Hydroxyhexanoate (HH), and 3-Hydroxy-6-chlorohexanoate (HCH)

HO		HCO		HH		HCH	
carbon	ppm	carbon	ppm	carbon	ppm	carbon	ppm
1	169.46	9	169.40	17	169.46	23	169.19
2	39.12	10	39.12	18	39.12	24	39.12
3	70.90	11	70.68	19	70.80	25	70.03
4	33.77 33.86	12	33.69 33.76	20	35.90	26	31.16 31.21
5	24.73	13	24.39	21	18.30	27	28.18
6	31.51	14	26.58	22	13.78	28	44.46
7	22.51	15	32.38				
8	14.00	16	44.90				

Figure 1 shows the 125-MHz ^{13}C NMR spectrum of sample 1, together with the chemical shift assignment for each carbon resonance. The chemical shift assignment was made on the basis of the empirical relations proposed by Wehrli and Wirthlin.[15] Table III gives the chemical shifts of the ^{13}C resonances of sample 1.

Figure 2 shows the 500-MHz ^1H NMR spectrum of sample 1. The chemical shift assignment for each proton resonance was made by analysis of the two-dimensional ^{13}C–^1H COSY NMR spectrum of sample 1. The mole fractions of 3-hydroxyalkanoate units (HH and HO) and 3-hydroxy-ω-chloroalkanoate units (HCH and HCO) were determined from the intensity ratio of methyl proton resonances 8 and 22 to methylene proton resonances 16 and 28. The mole fraction of HH or HO units was determined from the relative peak area of methyl carbon resonance 8 to methyl carbon resonance 22 in the ^{13}C NMR spectrum (see Figure 1), and the mole fraction of HCH or HCO units was determined from the relative peak areas of methylene carbon resonance 16 to methylene carbon resonance 28. The mole fractions of HO, HCO, HH, and HCH units in sample 1 are given in Table I.

The sequence distribution of the four monomeric units in sample 1 was determined by analysis of side-chain me-

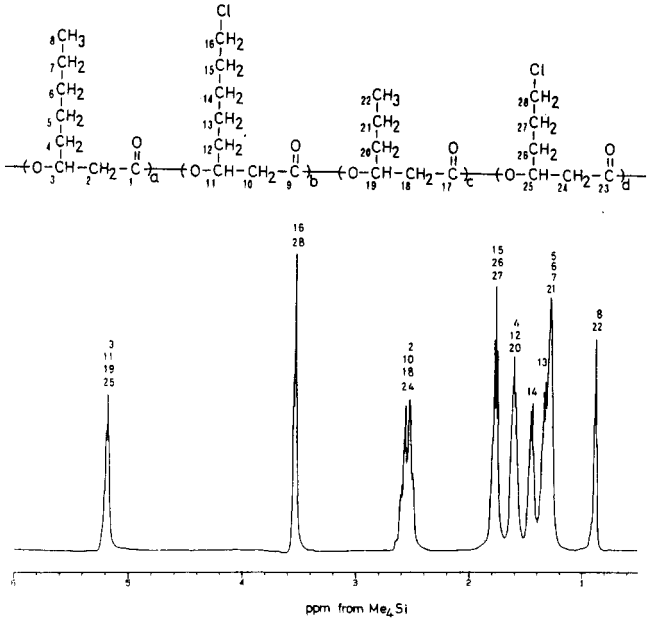

Figure 2. 500-MHz ^{1}H NMR spectrum of sample 1 in chloroform.

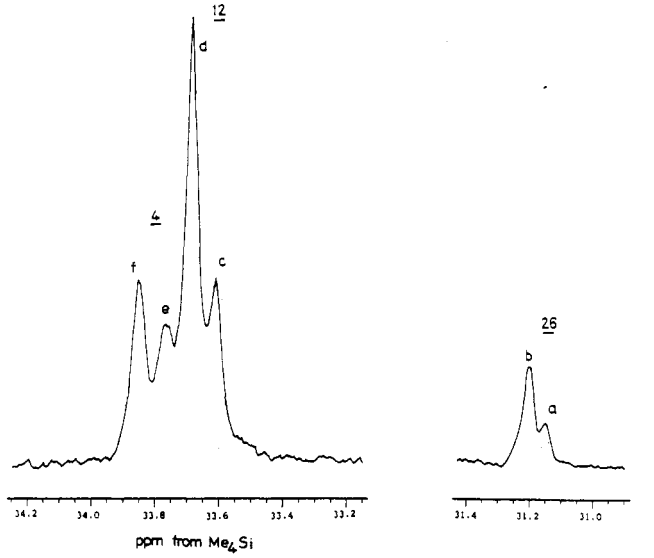

Figure 3. Expanded 125-MHz ^{13}C NMR spectrum of side-chain methylene carbon resonances 4, 12, and 26.

Table IV
Diad Sequence Distributions of the Four Monomeric Units HO, HCO, HH, and HCH in Sample 1

carbon	peak[a]	chem shift, ppm	sequence	rel intens obsd[b]	rel intens calcd
26	a	31.16	HO·*HCH + HH·*HCH	0.03	0.03
	b	31.21	HCO·*HCH + HCH·*HCH	0.07	0.07
12	c	33.61	HO·*HCO + HH·*HCO	0.16	0.18
	d	33.69	HCO·*HCO + HCH·*HCO	0.42	0.41
4	e	33.77	HO·*HO + HH·*HO	0.12	0.09
	f	33.86	HCO·*HO + HCH·*HO	0.18	0.20
20		35.90	HO·*HH + HH·*HH + HCO·*HH + HCH·*HH	0.02	0.02

[a] Peaks in Figure 3. [b] Determined from relative peak areas.

thylene carbon resonances 4, 12, and 26. Carbon resonances other than 4, 12, and 26 were not resolved in the ^{13}C NMR spectra at 125 MHz. Figure 3 shows the expanded ^{13}C NMR spectrum of side-chain methylene carbons 4 (HO), 12 (HCO), and 26 (HCH). These methylene carbon resonances are clearly resolved into two peaks, arising from different diad sequences of connecting 3-hydroxyalkanoate (HH or HO) and 3-hydroxy-ω-chloroalkanoate (HCH or HCO) units. The ^{13}C chemical shift assignment of peaks a–f in the methylene carbon resonances are given in Table IV, together with the relative area of each peak. Peak e at 33.77 ppm was assignable to methylene carbon 4 in HO·*HO and HH·*HO sequences, since its chemical shift was consistent with that of methylene carbon 4 in copolyester sample 2 of HH and HO units. Therefore, peak f at 33.86 ppm was assigned to methylene carbon 4 in HCO·*HO and HCH·*HO sequences. Peaks a–d were assigned as given in Table IV, on the assumption that methylene carbon resonances 12 and 26 of the HCO and HCH units are shifted toward a high magnetic field by bonding with the 3-hydroxyalkanoate units of HH and HO.

The diad sequence distribution data for four monomeric units were compared with the Bernoullian statistics applicable to a statistically random copolymerization. In the Bernoullian model, the mole fraction F_{ij} of diad sequence ij can be expressed with the mole fractions F_i and F_j of i and j units as $F_{ij} = F_iF_j$. Table IV gives the diad fractions calculated from the mole fractions of HO, HCO, HH, and HCH units in Table I. The calculated diad fractions are in good agreement with the observed values. Thus, it may be concluded that the sequence distribution of four monomeric units in sample 1 is statistically random.

In conclusion, a new random copolyester of 3-hydroxyalkanoate and 3-hydroxy-ω-chloroalkanoate units is produced in *P. oleovorans* from octane and 1-chlorooctane.

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References and Notes

(1) Holmes, P. A. *Phys. Technol.* **1985**, *16*, 32.
(2) Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1987**, *20*, 2988.
(3) Doi, Y.; Tamaki, A.; Kunioka, M.; Soga, K. *Appl. Microbiol. Biotechnol.* **1988**, *28*, 330.
(4) Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1986**, *19*, 2860.
(5) Bluhm, T. L.; Hamer, G. H.; Marchessault, R. H.; Fyfe, C. A.; Veregin, R. P. *Macromolecules* **1986**, *19*, 2871.
(6) De Smet, M.; Eggink, G.; Witholt, B.; Kingma, J.; Wynberg, H. *J. Bacteriol.* **1983**, *154*, 870.
(7) Lageveen, R. G.; Huisman, G. W.; Preusting, H.; Ketelaar, P.; Eggink, G.; Witholt, B. *Appl. Environ. Microbiol.* **1988**, *54*, 2924.
(8) Brandl, H.; Gross, R. A.; Lenz, R. W.; Fuller, R. C. *Appl. Environ. Microbiol.* **1988**, *54*, 1977.
(9) Gross, R. A.; DeMello, C.; Lenz, R. W.; Brandl, H.; Fuller, R. C. *Macromolecules* **1989**, *22*, 1106.
(10) Haywood, G. W.; Anderson, A. J.; Chu, L.; Dawes, E. A. *Biochem. Soc. Trans.* **1988**, *16*, 1046.
(11) Ramsay, B. A.; Ramsay, J. A.; Copper, D. G. *Appl. Environ. Microbiol.* **1989**, *55*, 584.
(12) Haywood, G. W.; Anderson, A. J.; Dawes, E. A. *Biotechnol. Lett.* **1989**, *11*, 471.
(13) Brandl, H.; Knee, E. J., Jr.; Fuller, R. C.; Gross, R. A.; Lenz, R. W. *Int. J. Biol. Macromol.* **1989**, *11*, 49.
(14) Byrom, D. *Trends Biotechnol.* **1987**, *5*, 246.
(15) Wehrli, F. W.; Wirthlin, T. *Interpretation of Carbon-13 NMR Spectra*; Heyden: London, 1976.