Bacterial Production of Poly(β-hydroxyalkanoates) Containing Unsaturated Repeating Units by *Rhodospirillum rubrum*

Herbert W. Ulmer,† Richard A. Gross,‡ Mario Posada,§ Paul Weisbach,§ R. Clinton Fuller,§ and Robert W. Lenz*,‡

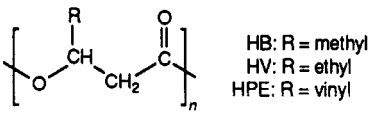
Department of Chemistry, Polymer Science and Engineering Department, and Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, Massachusetts 01003

Received May 25, 1993; Revised Manuscript Received December 17, 1993•

ABSTRACT: *Rhodospirillum rubrum* was grown on 4-pentenoic acid (PEA) and on an equimolar mixture of PEA and pentanoic acid (PA). With both substrates the bacterium produced polymers containing 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 3-hydroxy-4-pentenoate (HPE) repeating units. The poly(β-hydroxyalkanoates) (PHAs) obtained from PEA and from the PEA/PA mixture had number-average molecular weights of 110 000 and 155 000, respectively. The polymers containing the unsaturated unit, HPE, possessed the same crystal structure as PHAs containing a high mole fraction of 3HV units.

Introduction

Poly(β-hydroxyalkanoates) (PHAs) are a class of naturally occurring polyesters, which accumulate as inclusion bodies in many, diverse bacteria, with the general structure shown below:



PHA

The PHA inclusion bodies are generally accumulated by the bacteria during a time of metabolic stress caused by nutrient-limiting conditions in the presence of excess carbon. These PHAs are later utilized as an energy reserve material for cellular growth by the bacteria.[1]

Naturally occurring poly(β-hydroxybutyrate) (PHB) has been found to be readily biodegradable[2] in a wide variety of environments, so it was of interest to determine if PHAs with unusual (nonnatural) pendant groups would also be biodegradable. In recent years, ICI Ltd. has been successful in scaling up the production of P(HB-*co*-HV) copolymers using a mutant strain of *Alcaligenes eutrophus*, which can produce random copolyesters with varying molar amounts of HV units from a culture feed containing glucose and propionic acid. These copolyesters have also been shown to be biodegradable in natural environments.

Along with the widely occurring P(HB-*co*-HV) polyesters, a range of other PHAs can be obtained by feeding various bacteria either alkanes or alkanoic acids to produce monomers that generate units closely related in structure to the substrate. For example, *A. eutrophus* can metabolize butyric acid to 4-hydroxybutyric acid, which is polymerized to form the HB unit in the polyester.[3] Similarly, the production of PHAs with longer *n*-alkyl pendant groups (C_3–C_9) can occur with the bacterium *Pseudomonas oleovorans* when it is grown on either the corresponding *n*-alkanes or, preferably, the *n*-alkanoic acids.[4,5] This bacterium can also produce polyesters with branched alkane pendant groups when fed branched alkanoic acids.[6]

The possibility of producing PHAs containing functional pendant groups by bacteria is especially interesting because it should then be possible to develop a new group of biodegradable polymers by polymer modification reactions of the reactive functional site in those PHAs. The synthesis of graft copolymers, the attachment of bioactive compounds, and the application of controlled cross-linking reactions are just a few of the many possible modification reactions which could be carried out on such PHAs. These functional polymers could become the precursors of a large array of versatile biodegradable polymers, which could be easier to obtain from microorganisms than by chemical synthesis. For example, *P. oleovorans* has been shown to produce PHAs containing vinyl pendant groups when grown on various alkenes and alkenoic acids.[7,8]

Encouraged by these earlier results, we investigated the possibility of obtaining PHAs with vinyl pendant groups as well as with HB and HV units because the HB/HV copolyesters have shown great promise as biocompatible, degradable materials for medical applications.[9–12] Thus, this report is concerned with the characterization of PHAs produced by *Rhodospirillum rubrum* when grown either on 4-pentenoic acid (PEA) alone or on an equimolar mixture of PEA with pentanoic acid (PA). The bacterial polyesters produced were characterized by gel permeation chromatography, GPC, relative to polystyrene standards, ^1H and ^{13}C Fourier transform nuclear magnetic resonance, FT-NMR, Fourier transform infrared spectroscopy, FT-IR, differential scanning calorimetry, DSC, and wide-angle X-ray diffraction, WAXD, and also oligomers obtained from the polymers by partial methanolysis were analyzed for their compositions by fast atom bombardment mass spectrometry, FAB-MS.

Materials and Methods

Fermentation of *R. rubrum*. *R. rubrum* was obtained from the American Type Culture Collection (ATCC 25903). Stock cultures were grown anaerobically in the light in 25-mL screw-cap test tubes using the 550 R8AH[13] medium which contained malate as the carbon source. The same medium was used to grow *R. rubrum* under PHA-producing conditions except that malate and ammonium sulfate were omitted from the medium. Instead of malate, a variety of other carbon sources were used. Inoculum cultures were prepared by inoculating 250-mL screw-cap bottles containing 200 mL of the PHA-producing media (carbon source, 30 mM malate) with 2 mL of the stock solution and allowing the bacteria to grow for 2 days. Cells were grown from these inocula under anaerobic conditions in the light in 1-L screw-cap bottles. The PHA-producing media containing the

† Department of Chemistry.
‡ Polymer Science and Engineering Department.
§ Department of Biochemistry and Molecular Biology.
• Abstract published in *Advance ACS Abstracts*, February 15, 1994.

0024-9297/94/2227-1675$04.50/0

Table 1. Production and Composition of PHAs Produced by *R. rubrum* Grown on Carbon Sources Containing 4-Pentenoic Acid at 30 °C and Harvested after 5 days

organic substrate[a]	substrate conc (mM)	cell yield (g/L)	PHA content (% cell dry wt)	repeat unit (mol %)[b] HB	HV	HPE
PEA/PA	30/30	0.43	19	14	72	14
PEA	50	0.26	17	11	59	30

[a] PEA is pentenoic acid; PA is pentanoic acid. [b] Determined by integration of ^{1}H NMR spectra.

Table 2. Chemical Shifts of Peaks in the ^{13}C NMR Spectra (75.4 MHz) of the Polymer Containing HPE Units

carbon[a]	repeat units of polyester HB	HV	HPE[b]
1	169.13	169.50	168–169
2	40.73	38.61	39.30
3	67.64	71.87	71.08
4	19.71	26.73	134.70
5		9.33	118.03

[a] The number assignments for the carbons of the repeating units HB, HV, and HPE are shown in Figure 3. [b] A range of shifts is given for carbon 1 because the exact HPE–HPE diad could not be determined as there was no standard HPE homopolymer available.

Table 3. Molecular Weights[a] of PHAs Produced by *R. rubrum* Grown on PEA and PEA/PA Mixtures as Carbon Sources

organic substrate[b]	substrate conc (mM)	M_w	M_n	M_w/M_n
PEA	50	340 000	110 000	3.1
PEA/PA	30/30	480 000	155 000	3.1

[a] Determined from polystyrene standards; M_w = weight-average molecular weight and M_n = number-average molecular weight. [b] See Table 1.

Table 4. Thermal Transitions of PHAs Produced by *R. rubrum* Grown on Carbon Sources Containing PEA[a]

organic substrate[a]	repeat unit (mol %) HB	HV	HPE	T_m range (°C)	T_m max (°C)	T_g[b] (°C)	ΔH_m (cal/g)
50 mM PEA	11	59	30	40–115	93	−11	14.0
30 mM PEA/ 30 mM PA	14	72	14	35–115	96	−13	11.5

[a] See Table 1. [b] T_g was measured at the inflection point in the DSC thermogram of the second heating cycle after quenching the sample from the melt state; a very small inflection was also observed at −18 °C (see Figure 6).

desired carbon source were inoculated with 10 mL of the inoculum and allowed to stand for 5 days with daily stirring. For large-scale fermentations, cells were cultivated anaerobically in the light in a temperature-controlled 12-L fermentor (New Brunswick; 30 °C, 100 rpm) with with desired carbon source. Each culture was inoculated with 200 mL of the inoculum and harvested after 5 days. After the prescribed growth periods, the cells were harvested by centrifugation (Sorvall RC2-B; 5 °C, 10 000*g*), and the whole cell pellets were lyophilized to yield the dry cells. Dry cell weights were determined gravimetrically.

The PHA produced was extracted from the lyophilized cells in a Soxhlet extractor with chloroform, the solutions were filtered through a cottom plug, and the polymer was precipitated in a 10-fold volume excess of rapidly stirred methanol. The polymer was centrifuged (Soryall RC2-B; 5 °C, 10 000*g*) and dried in vacuo (1 mmHg) for 16 h at room temperature. Further purification was done by repeating the same solution and precipitation procedure. The total amount of PHA was determined gravimetrically and calculated as the percentage of cellular dry weight.

Polymer Characterization

Solution ^{1}H NMR spectra and ^{13}C NMR spectra were

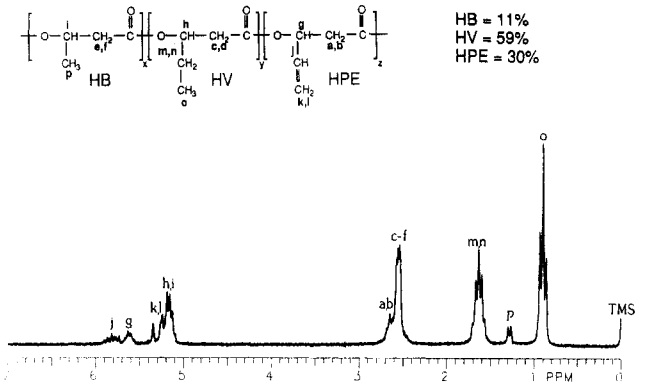


Figure 1. ^{1}H NMR spectrum (200 MHz) of the PHA containing 30 mol % HPE units; recorded at 17 °C in $CDCl_3$.

obtained in chloroform-*d* solutions at 17 °C with a Varian-XL 200 NMR spectrometer at 200 MHz and a Varian-XL 300 NMR spectrometer at 70.4 MHz, respectively. The ^{1}H NMR spectra were referenced to TMS (0.00 ppm) and the ^{13}C NMR spectra to chloroform-*d* (77.0 ppm). FT-IR spectra were obtained on cast PHA films. The PHA films were solution cast from chloroform. The spectra were recorded on a Cygnus 100 Mattson FT-IR with a 2 cm^{-1} resolution at 32 scans.

Molecular weights were determined by GPC with a Waters Model 6000A solvent delivery system, a Mode 401 refractive index detector, and a Model 730 data module with two Ultrastyragel linear columns in series. Chloroform was used as the eluent at a flow rate of 1.0 mL/min. Sample concentrations of 10–15 mg/mL and injection volumes of 100 μL were used. A calibration curve was generated with seven polystyrene standards of low polydispersities, which were purchased from Polysciences.

The glass transition (T_g) and melting (T_m) temperatures and the heat of fusion (ΔH_m) were measured on the PHA samples using a DuPont DSC 2000. The weight of each sample was typically 5 mg. PHA samples were heated at a rate of 20 °C/min from −80 to +200 °C, quickly cooled, and scanned a second time using the same heating rate and temperature range as the first scan. The values of T_m and ΔH_m were determined from the thermograms of the first DSC scan, while T_g was determined from the thermograms of the second DSC scan. Indium was the standard used to calibrate the DSC.

X-ray diffraction measurements were made under reduced pressure using a Statton camera and a Siemens K710H generator operating at 40 kV and 30 mA. The X-ray beam was pinhole collimated. Nickel-filtered Cu Kα radiation (λ = 0.1542 nm) was used. The PHA films were solution-cast from chloroform. The distance between the polymer sample and the film was 74.7 mm for moderate angle ($10° < 2\theta < 30°$), and the samples were exposed for 6 h.

Results and Discussion

R. rubrum generally grows well on the lower alkanoic acids (C_4–C_6), and our previous studies have shown that *R. rubrum* is a more versatile microorganism than *A. eutrophus* for the production of PHAs other than the usual P(HB-*co*-HV) copolymers. Earlier results, for example, have shown the incorporation of *n*-propyl pendant groups of up to 15 mol % into the biopolyester is possible by *R. rubrum*,[14] and the PHAs obtained in the present study further demonstrate the adaptability of *R. rubrum* for producing unusual polyesters.

In the present study, it was found that *R. rubrum* was able to incorporate up to 30 mol % of units with the vinyl pendant group, HPE, in the PHA product obtained when

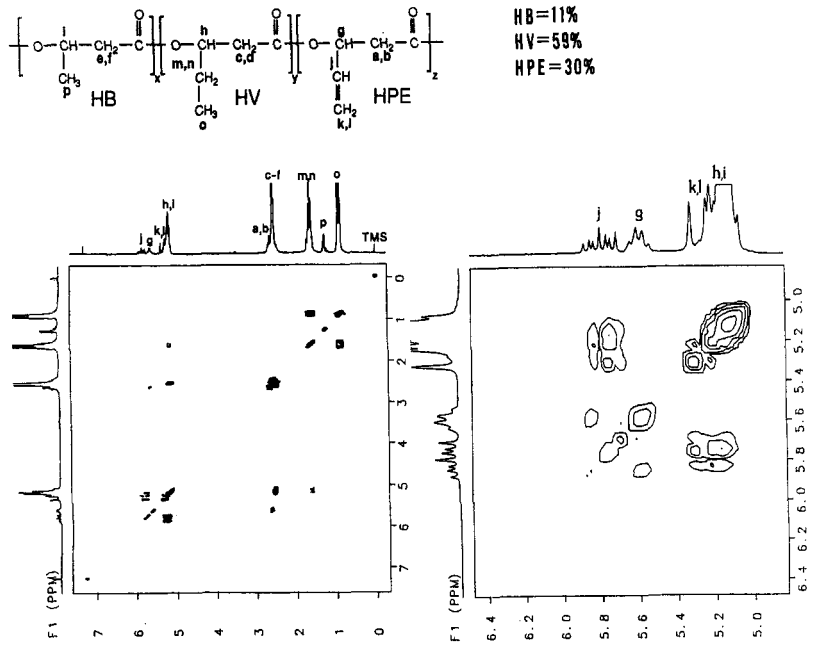


Figure 2. ^{1}H two-dimensional homonuclear correlated (COSY) spectrum (200 MHz) of the PHA containing 30 mol % HPE units; recorded at 17 °C in $CDCl_3$.

it was grown solely on 4-pentenoic acid (PEA) as the carbon source. The remainder of the composition was HB and HV units. That is, the polyester product obtained had three different repeating units, as shown in the generalized equation below.

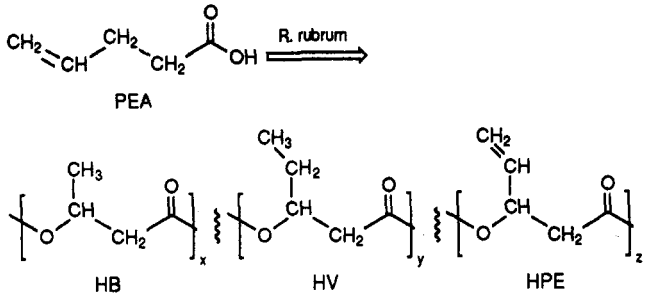
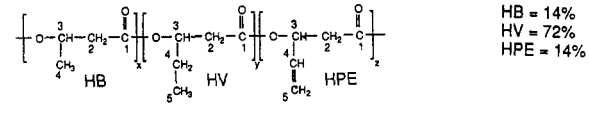


Table 1 gives the cell yields, polymer yields, and polymer compositions for the PHAs produced by *R. rubrum* when grown on either PEA or on an equimolar PEA/PA mixture as carbon sources for 5 days at 30 °C. The mole percent of the repeating units in the product was determined by the integrated of the corresponding ^{1}H NMR spectrum. The production of one or more polyesters containing HB, HV, and HPE units by *R. rubrum* when grown on 50 mM PEA represents a highly complex metabolic synthesis in the polymer production, and polymer formation took place during culture conditions which were not nutrient limiting. Possibly, the presence of PEA as the carbon source may have induced the stress upon the bacterium which caused polymer production within the cells. In that regard, PEA has been shown to be an inhibitor for fatty acid oxidation in rat mitochondria.[15] It is possible that PEA had a similar effect in *R. rubrum*, and this effect provided the stress which induced polymer production by the bacterium. This type of induced stress could also account for the relatively low cell yields obtained with this substrate.

The ^{1}H NMR spectrum of the polymeric products containing HB, HV, HPE units produced by *R. rubrum* when grown on 50 mM PEA is shown in Figure 1. The PHA product obtained was found to contain 11 mol % HB units, 59 mol % HV units, and 30 mol % HPE units by integrating the ^{1}H NMR spectrum. The peak assign-



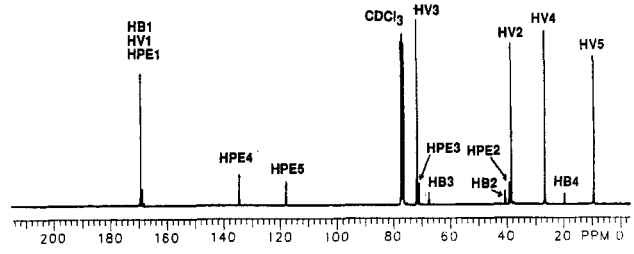


Figure 3. ^{13}C NMR spectrum (75.4 MHz) of the PHA containing 14 mol % HPE units; recorded at 17 °C in $CDCl_3$.

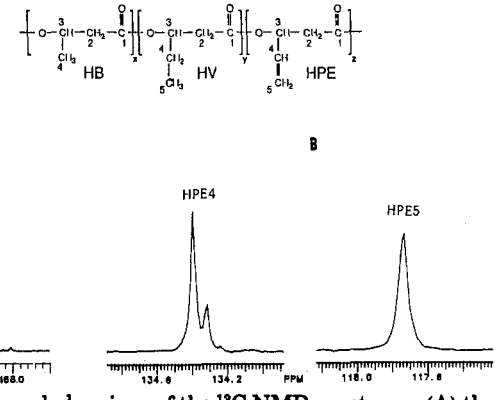
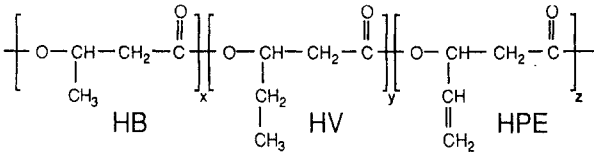


Figure 4. Expanded regions of the ^{13}C NMR spectrum: (A) the carbonyl carbon peaks for the HB, HV, and HPE units; (B) the vinylic carbon peaks of HPE.

ments of the ^{1}H NMR spectrum were made using the results from the two-dimensional homonuclear (^{1}H) correlated spectra (COSY) of the same sample shown in Figure 2. In the 2D ^{1}H-COSY NMR spectra, the HPE methine proton showed two spin groups a,b–g and g–j, which indicated the sequence a,b–g–j. The HPE j vinyl proton showed two spin groups g–j and j–k,l, representing the sequence g–j–k,l. The terminal vinyl protons, k,l, showed only one spin group representing the sequence j–k,l. Thus,



HB = 11%
HV = 59%
HPE = 30%

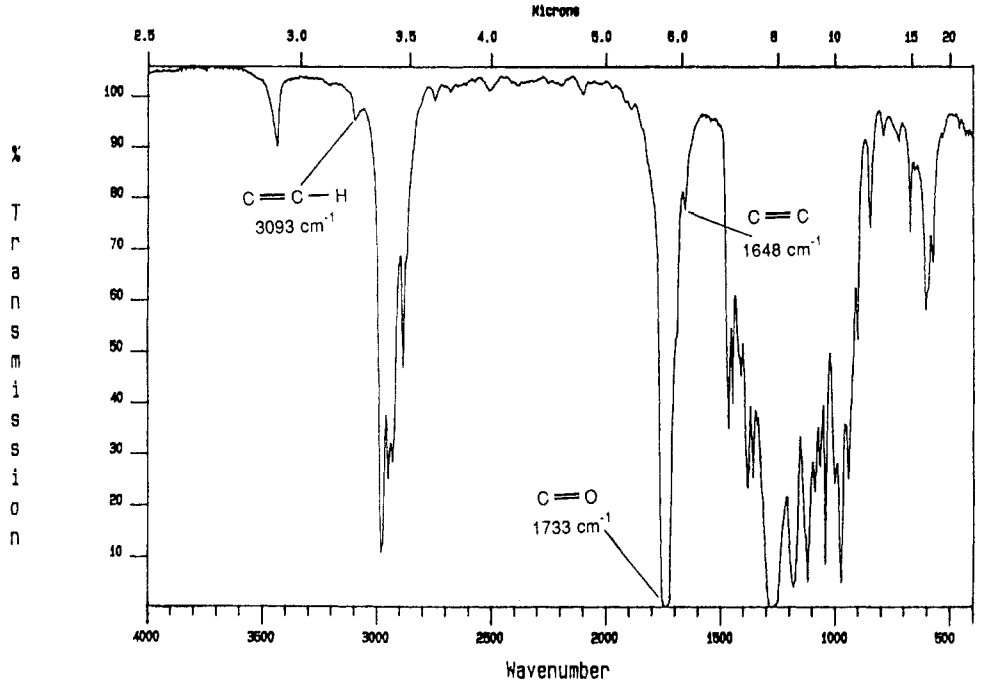


Figure 5. FTIR spectrum of the PHA containing 30 mol % HPE units.

Table 5. Interplanar *d*-Spacing (Å) for PHAs Biosynthesized by *R. rubrum*

repeat unit (mol %)			diffraction maxima (reflections)[a] (Å)								
HB	HV	HPE	d_1	d_2	d_3	d_4	d_5	d_6	d_7	d_8	d_9
100	0	0	6.62 (M)	5.26 (M)	4.49 (W)	3.29 (W)					
33	67	0	6.85 (S)	5.03 (M)	4.73 (W)	4.42 (S)	3.38 (M)	3.13 (W)	3.00 (W)		
14	72	14	6.91 (S)	5.02 (S)	4.77 (M)	4.42 (S)	3.41 (S)	3.15 (W)	3.02 (W)	2.86 (W)	2.74 (W)
11	59	30	6.91 (S)	5.02 (M)	4.43 (M)	3.43 (M)	3.14 (W)	3.02 (W)			

[a] Intensities of reflections are given as: S, strong; M, medium; W, weak.

the overall sequence of proton coupling was a,b–g–j–k,l which correlated very well with the HPE structure proposed.

The 75.4-MHz ^{13}C NMR spectrum of a solution of the PHA containing 14 mol % HPE units is shown in Figure 3. The ^{13}C peak assignments were made by analyzing the distortionless enhancement by the polarization transfer (DEPT) ^{13}C NMR spectrum and the proton-coupled spectrum of the same polymer. The chemical shift assignments for the ^{13}C NMR spectrum of the HB, HV, and HPE units are given in Table 2. The expanded ^{13}C NMR spectrum of the carbonyl region and vinyl carbon region in Figure 4 suggested that the unit sequences of the HPE containing copolymer were most likely random because the diad sequencing of the HB–HV units was similar to that found by Marchessault and co-workers for statistically random copolymers of P(HB-*co*-HV).[16] However, the exact diad sequencing of HPE units could not be determined by NMR because no standard HPE homopolymer was available, so the samples were also analyzed by FAB-MS analysis of the oligomers obtained from the copolymers by partial methanolysis and HPLC separation. The analysis, which was carried out at the University of Catania, Italy, showed that the compositions of the oligomers followed Bernoullian statistics, indicating that both types of copolyesters were random in sequence distribution.[17]

The FTIR spectrum of the polymer produced by *R. rubrum* grown on 50 mM PEA is shown in Figure 5. The 30 mol % HPE-containing polymer produced by *R. rubrum* shows the =CH stretch at 3093 cm^{-1} and the C=C stretch at 1648 cm^{-1}.

The number (M_n) and weight average (M_w) molecular weights of the PHAs produced by *R. rubrum* when grown on PEA as the sole carbon source were determined by GPC. The 30 mol % HPE copolymer produced had an M_n of 110 000 (see Table 3), which was considerably lower than the M_n of the copolymer containing 14 mol % HPE units that was obtained by growth of the bacterium on a 1:1 molar mixture of PEA and PA, although the polydispersities (M_w/M_n) for both polymers were the same, 3.1. In general, P(HB-*co*-HV) copolymers produced by *R. rubrum* generally have higher molecular weights with M_n values of approximately 200 000, compared to those of the HPE-containing polymers. Thus, there is a trend to decreasing molecular weight of the PHA with increasing HPE content in the copolymer. All of the observed GPC chromatograms were unimodal in molecular weight distribution, but relatively large polydispersities were observed for both of the PHAs.

The DSC thermograms for the polymers produced by *R. ubrum* containing 14 and 30 mol % HPE units are shown in Figure 6. The thermal transitions and heats of fusion (ΔH_m) of the HPE-containing polyesters are given

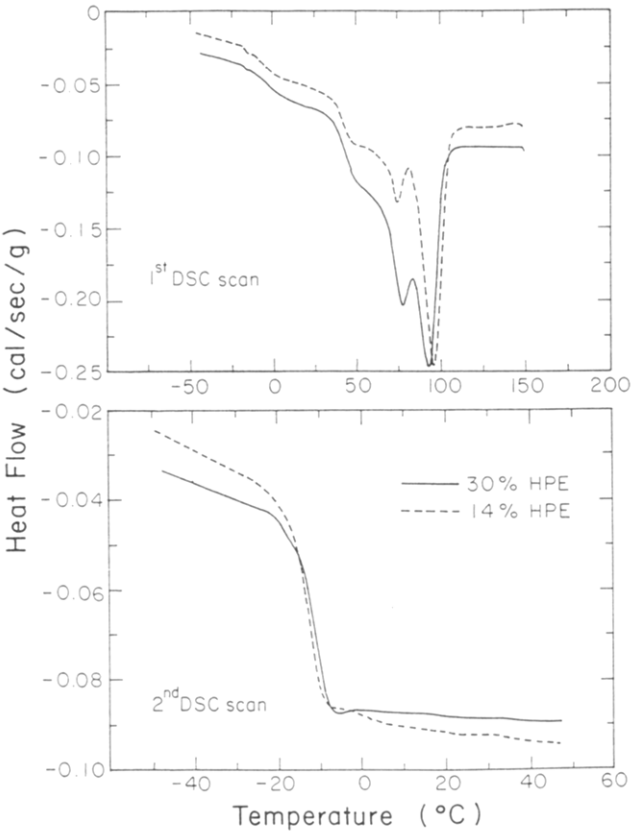


Figure 6. DSC thermograms for the first heating cycle and second heating cycle after quenching of the PHAs containing 14 and 30 mol % HPE units.

in Table 4. The melting temperature (T_m) and ΔH_m were determined from the first DSC scan, and the glass transition (T_g) was determined from the second scan after rapidly cooling the sample pair with liquid nitrogen. A very broad melting transition with multiple endotherms was observed for both of the HPE-containing polymers. The occurrence of multiple endotherms in polyesters is fairly common and is generally indicative of the presence of crystalline regions of varying size and perfection. After quenching the two samples in liquid nitrogen, the second DSC scan of the polymer containing 30 mol % HPE showed a major T_g at −11 °C and possibly a much smaller T_g at −18 °C. The thermogram of the polymer containing 14 mol % HPE units showed only one T_g at −13 °C. The thermal stability of the two HPE-containing copolymers was also evaluated by heating the copolymers to 150 °C for 15 min, but no indication of either thermal degradation or structural changes was observed.

Table 5 gives the interplanar *d*-spacings obtained by WAXD on the two different PHA samples produced by *R. rubrum* grown on PEA. Values for the PHB and PHV homopolymers and for a copolymer containing only HB and HV units are also given for comparison. The X-ray diffractograms of films of the PHAs containing HPE units exhibited the same *d*-spacings as the diffractogram fo the PHA containing 67 mol % HV units. Because a P(HB-*co*-HV) copolymer of this composition is known to crystallize in a PHV lattice, it can be concluded that the PHAs containing HPE units had the same crystal structure as PHV. The X-ray diffractogram for the PHA film containing 30 mol % HPE units is shown in Figure 7.

Conclusion

The production of P(HB-*co*-HV) copolyesters containing repeating units with vinyl pendant groups by *R. rubrum*

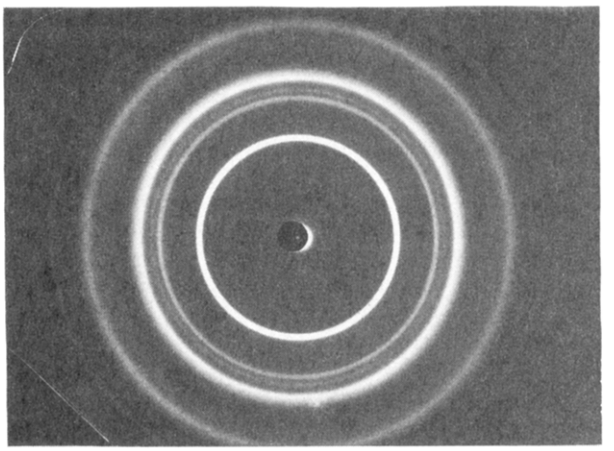


Figure 7. X-ray diffractogram of the polymer containing 30 mol % HPE units.

demonstrates that new, functionalized biopolymers can be produced by bacteria, and these PHAs may possess many of the desired properties of P(HB-*co*-HV) copolymers. Also, the production by bacteria of various PHAs containing units from monomers that were biosynthesized as a result of their growth on unnatural carbon sources shows the need to study a much wider range of microorganisms for the production of new and useful PHAs. However, the longer growth times and lower cell and polymer yields obtained with *R. rubrum*, when compared to other bacteria, may make this bacterium a poor candidate for the production of large amounts of PHAs in a practical manner. On the other hand, the ability of this bacterium to produce unusual, functional PHAs may make it an excellent candidate for genetic engineering.

Acknowledgment. We thank Edward Knee, Jr., for his technical assistance. We gratefully acknowledge the financial support from the Office of Naval Research, Molecular Biology Program, under Grant No. N00014-86K-0369, and from the Rohm and Haas Co. for this study.

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