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Polyester Biosynthesis Characteristics of *Pseudomonas citronellolis* Grown on Various Carbon Sources, Including 3-Methyl-Branched Substrates

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Forty-two different carbon sources were tested for the polyester synthesis of a citronellol-utilizing bacterium, *Pseudomonas citronellolis* (ATCC 13674). These included linear C_2 to C_{10} monocarboxylic acids, C_3 to C_{10} dicarboxylic acids, saccharides, α,ω-diols, hydrocarbons, and 3-methyl-branched substrates such as 3,7-dimethyl-6-octen-1-ol (citronellol), 3-methyl-*n*-valerate, 3-methyl-1-butanol, and 3-methyladipate. Isolated polymers were characterized by gas chromatography, infrared spectroscopy, ^{1}H- or ^{13}C-nuclear magnetic resonance spectroscopy, ^{1}H-^{13}C heteronuclear correlation spectroscopy (^{1}H-^{13}C COSY), ^{1}H-^{1}H homonuclear COSY, and differential scanning calorimetry. Polyesters from nine monocarboxylic acids and two related carbon sources could be metabolically divided into three groups. The first group of C_2 to C_4 carbon sources resulted in copolyesters composed of 61 to 70 mol% 3-hydroxydecanoate, 23 to 33 mol% 3-hydroxyoctanoate, 3.6 to 9.0 mol% 3-hydroxy-5-*cis*-dodecenoate, and 1.8 to 2.6 mol% 3-hydroxy-7-*cis*-tetradecenoate. Carbon sources in group II (C_7 to C_{10}) produced copolyesters composed of 3-hydroxyacid monomer units with the same number of carbon atoms as the substrate (major constituent) and monomer units with either two less or two more carbons. Negligible amounts of 3-hydroxy-5-*cis*-dodecenoate and 3-hydroxy-7-*cis*-tetradecenoate were detected in copolyesters from this group. Copolyesters from group III (C_5 and C_6) had a monomer unit distribution that could be said to be between those of groups I and II. In addition, a novel copolyester, poly(3-hydroxy-7-methyl-6-octenoate-co-3-hydroxy-5-methylhexanoate), was synthesized when grown on citronellol. The ^{1}H-^{13}C heteronuclear COSY spectrum for monomer unit II revealed that both methylene and isopropyl groups, proximately connected in series to a single chiral center, had magnetically diastereotopic natures.

New bacterial polyesters have recently been reported in numerous studies (1, 6, 8, 9, 14, 20, 25, 26, 35, 36). The type of polyester produced depends on the bacterial strains, carbon sources, and culture conditions (1, 6, 8).

Two types of polyesters, short-chain-length polyhydroxyalkanoates (SCL PHAs) and medium-chain-length (MCL) PHAs, are generally synthesized with common carbon sources (34, 35). The SCL monomer units include 3-hydroxypropionate, 3-hydroxybutyrate, 4-hydroxybutyrate, 3-hydroxyvalerate (3HV), 4-hydroxyvalerate (35), and 5-hydroxyvalerate. The MCL monomer units have three to nine more carbon atoms than the SCL monomer units. The MCL monomer units found so far are oxidized at third positions only. It may be due to the long chain of pendant groups in MCL PHAs that functional groups such as halogens (9, 26), olefins (13, 27, 30), branched alkyls (14, 20), cycloalkyls (6), and phenyl (25, 37) can be introduced at or near their terminals.

Alcaligenes eutrophus (8, 31, 32), *Rhodospirillum rubrum* (4), and *Pseudomonas pseudoflava* (2) are known to accumulate copolyesters composed of SCL monomer units only, while *Pseudomonas oleovorans* (3, 16, 22, 23, 27), *Pseudomonas putida* (7, 21, 37), and other fluorescent *Pseudomonas* strains (22) biosynthesize copolyesters principally composed of MCL monomer units. Cellular PHA polymerases may determine the type of monomer unit in PHAs (8, 21, 23).

Careful analysis of the monomer unit composition of PHAs enables us to determine the pathway through which the substrates fed were metabolized to synthesize PHAs (7, 21). For instance, *P. putida* KT2442 cultivated on unrelated substrates, such as glucose, fructose, and glycerol, produced PHAs of similar monomer unit composition (21). In addition to the major unit 3-hydroxydecanoate (3HD), six other monomer units were found to be present in PHA: 3-hydroxyhexanoate (3HC), 3-hydroxyoctanoate (3HO), 3-hydroxydodecanoate, 3-hydroxydodecenoate ($C_{12:1}$), 3-hydroxytetradecanoate, and 3-hydroxytetradecenoate ($C_{14:1}$). From the fact that seven monomer units also occur as sequential intermediates in the fatty acid biosynthetic pathway of bacteria, a possible linkage between de novo fatty acid biosynthesis and PHA synthesis could be suggested (21). However, *P. putida* KT2442 grown on octanoate and decanoate synthesized a PHA composed of 3HO as the major constituent and two other monomer units, 3HC and 3HD, and containing none of the other four closely related monomer units (3-hydroxydodecanoate, $C_{12:1}$, 3-hydroxytetradecanoate, and $C_{14:1}$). This demonstrates that the PHA biosynthetic route is linked to the β-oxidation cycle. The linkage between these two pathways was further confirmed by nuclear magnetic resonance (NMR) analysis of PHAs synthesized by the bacterium grown on the following long-chain fatty acids (7): petroselenic, oleic, and linoleic acids. Such a linkage between them in *P. oleovorans* was also suggested (3, 27).

No detailed study on the characteristics of PHA synthesis involving citronellol-utilizing bacteria has been reported yet. However, *Pseudomonas citronellolis* is known to be able to degrade recalcitrant branched hydrocarbons (10). 3-Methyl-branched substrates are degraded via the citronellol or isovalerate pathway (10, 28). Thus, it was interesting to study the PHA synthesis of the bacterium from branched substrates such as 3,7-dimethyl-6-octen-1-ol (citronellol), 3-methyl-*n*-val-

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erate, 3-methyl-1-butanol (isoamyl alcohol), and 3-methyladipate. In addition to branched substrates, a total of 42 different carbon sources were tested for PHA synthesis in this study. PHA biosynthesis in *P. citronellolis* is discussed in terms of the metabolism of fatty acids and branched substrates at the physiological level.

MATERIALS AND METHODS

Bacterial strain and culture media. The strain, ATCC 13674, of *P. citronellolis* used in this study was purchased from the American Type Culture Collection.

The medium used for PHA accumulation was a mineral salts medium containing a carbon source. The composition of the mineral salts medium was as follows (per liter of distilled water): 0.66 g of $(NH_4)_2SO_4$, 2.3 g of KH_2PO_4, 7.3 g of $Na_2HPO_4 \cdot 12H_2O$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of $NaHCO_3$, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 1 ml of microelement solution. The pH of the medium was adjusted to 7.0. The microelement solution contained 0.58 g of $ZnSO_4 \cdot 7H_2O$, 3.96 g of $MnCl_2 \cdot 4H_2O$, 0.6 g of H_3BO_3, 5.56 g of $FeSO_4 \cdot 7H_2O$, 5.62 g of $CoSO_4 \cdot 7H_2O$, 0.34 g of $CuCl_2 \cdot 2H_2O$, 0.04 g of $NiCl_2 \cdot 6H_2O$, and 0.06 g of $NaMoO_4 \cdot 2H_2O$ per liter of 0.5 N HCl.

Cell growth and polymer isolation. Inocula were grown in 5-ml test tubes containing nutrient-rich (1% yeast extract, 1.5% nutrient broth) media. All growth experiments were performed under aerobic conditions in a temperature-controlled shaker (Korea Instrument Co, Seoul, Republic of Korea) at 30°C and 200 rpm. Cells grown for 22 h were transferred to the PHA synthesis medium with a culture volume of 500 ml in 2-liter Erlenmeyer flasks containing a carbon source. Cells were grown for 3 days or longer, depending on the carbon source used. Cell growth was monitored spectrophotometrically at 660 nm. Cells were then harvested by centrifugation in a Kontron CENTRIKON T-324 centrifuge (6,000 rpm for 10 min), washed with acetone, and dried overnight under vacuum at 50°C.

Nitrogen consumption, biomass increase, and monomer unit composition of the polymer synthesized during cultivation were determined by analysis of the culture medium every 4 or 5 h (3, 31). The remaining NH_4^+ in the medium was measured with Nessler's reagent (11). Polyester contents in cells and the monomer unit composition of polyesters were determined by the method of methyl ester formation (3). The methyl esters of 3-hydroxyacids were analyzed by gas chromatography with a Hewlett-Packard HP5890A gas chromatograph equipped with a Carbowax 20 M column and a flame ionization detector.

Polyesters were extracted from dried cells with hot chloroform in a Pyrex Soxhlet apparatus for 6 h. Concentrated solvent extract was precipitated in rapidly stirred cold methanol. Isolated polyesters were purified by reprecipitating them and were dried overnight under vacuum at 30 to 40°C. Quantitative determination of the monomer units in PHAs was performed by gas chromatography. The peak areas on gas chromatograms were standardized against the molar ratio data from inverse gated decoupling experiments (12, 24) performed with a few purified polyesters. Two minor unsaturated monomer units ($C_{12:1}$ and $C_{14:1}$) in polymers were quantitated by ^{1}H-NMR spectroscopic measurements (21).

Characterization of isolated polymers. Infrared spectra of isolated polyesters were recorded on a Bruker IFS66 FTIR spectrometer.

The ^{1}H- and ^{1}H-noise-decoupled ^{13}C-NMR analysis of the polyester samples was carried out on a Bruker AMX-500 spectrometer in the pulse-Fourier transform mode. Two-dimensional heteronuclear (^{1}H-^{13}C) and homonuclear (^{1}H-^{1}H) correlation spectroscopy (COSY) spectra were recorded at 20°C on a Bruker AMX-500 spectrometer. A $\pi/2$-t_1-$\pi/2$ acquisition pulse sequence was employed in the COSY experiment.

Thermal transitions for polymers were measured by using a DuPont 9900 differential scanning calorimeter equipped with a data station. The solvent-cast and dried samples for the differential scanning calorimetry run were kept at ~20°C for 12 weeks to obtain steady-state structures (15). Experiments were carried out at a heating rate of 10°C/min under a dry nitrogen purge.

RESULTS

PHA accumulation from mono- and dicarboxylic acids. Table 1 shows the PHA synthesis of *P. citronellolis* (ATCC 13674) grown on linear C_2 to C_{10} monocarboxylic acids and C_3 to C_{10} α,ω-dicarboxylic acids. Cells were cultured for 3 days or more, depending on the carbon source used. The bacterium utilized all C_2 to C_{10} monocarboxylic acids to produce polyester. It also utilized γ-butyrolactone despite a rather long culture period. Cultivation on C_2 to C_5 acids resulted in cells with smaller amounts of PHA than did cultivation on C_6 to C_{10} acids at a similar level of carbon source feeding. Likewise, it has previously been reported that *P. oleovorans* produced no or little PHAs from low (C_2 to C_5) monocarboxylic acids (3, 16, 22). The biomasses and PHA contents produced by *P. citronellolis* on these low acids were significantly higher than those produced by *P. oleovorans.*

For the lower C_2 to C_5 carboxylic acids, the major constituent was 3HD, with 3HO as the next major constituent whose content ranged from 21 to 35 mol%. In the case of C_6 to C_9 carboxylic acids, the major repeating unit in the polymer had the same chain length as the *n*-alkanoic acid used for growth. However, it must be also noted that monomer units with two carbon atoms more or less than those of the acid used as the carbon source were also present in fairly small amounts. Meanwhile, the PHA accumulated from decanoate had 3HO as the principal monomer unit, which was shorter than the substrate by one ethylene unit. The PHA samples from heptanoate carried the highest content of a single type of monomer unit (95 mol% 3-hydroxyheptanoic acid). A similar PHA synthesis trend with C_6 to C_{10} monocarboxylic acids was also reported for *P. oleovorans* ATCC 29347 (3, 16, 22), *P. putida* NCIB 9571 (17), and *P. putida* BM01 (37). However, *Pseudomonas aeruginosa* NCIB 9904 (17) grown on decanoate produced a polyester with 3HD as the major constituent.

Linear α,ω-dicarboxylic acids were utilized by *P. citronellolis* for the production of MCL PHAs (Table 1). The monomer unit composition of polyesters from dicarboxylic acids, in which 3HD was the principal constituent, was almost independent of the substrate used. However, these substrates required 1 or 2 days of induction time for cell growth. The polyester contents and monomer unit composition were generally similar to those of C_2 to C_4 monocarboxylic acids, with the exception of the C_3 and C_7 dicarboxylic acids with which no or little polyester was produced.

In addition to saturated monomer units, a significant amount of two unsaturated monomer units, $C_{12:1}$ and $C_{14:1}$, ranging from 5 to 9 mol%, was found to be present in the copolymers synthesized from C_2 to C_4 monocarboxylic acids and C_4 to C_{10} dicarboxylic acids.

PHA accumulation from saccharides, diols, and hydrocarbons. The nature of PHA production from α,ω-linear diols was investigated (Table 2). No cell growth was observed on C-odd (C_5, C_7, and C_9) diols. All C-even diols supported cell growth

TABLE 1. Biosynthesis of PHAs by *P. citronellolis* from low linear mono- and dicarboxylic acids

Carbon source (g/liter)	Culture time (days)[a]	Biomass (g/liter)	PHA content (% by wt)	PHA composition (mol%)[b]								
				3HB	3HV	3HC	3HH	3HO	3HN	3HD	$C_{12:1}$	$C_{14:1}$
Acetate (10)	3	1.69	14.8					33		61	3.6	1.9
Propionate (10)	3	1.22	14.0	7				26		61	3.7	1.8
Butyrate (10)	3	1.39	11.2					27		66	5.0	2.6
Crotonic acid (10)	3	1.32	12.0					30		61	9.0	tr[c]
γ-Butyrolactone (4)	11 (3)	1.28	8.9					23		70	4.3	2.6
Valerate (10)	3	1.14	7.0	4	17			20		53	5.3	0.4
Caproate (10)	3	1.56	20.0	3		75		7		14	1.2	tr[c]
Heptanoate (10)	3	1.53	20.0		3		94		2		0.6	tr[c]
Octanoate (10)	3	1.73	27.1			20		80			0.0	0.0
Octanoate (6)	3	1.10	36.2									
Nonanoate (10)	3	1.12	10.2		3		29		68		0.0	0.0
Decanoate (4)	3	1.32	25.0			11		48		40	0.8	tr[c]
Butanedioate (6)	5 (1)	1.20	10.0					35		56	5.6	3.3
Pentanedioate (6)	5 (1)	1.30	14.4					33		58	9.0	tr[c]
Hexanedioate (6)	5 (1)	1.50	13.5					29		59	9.2	2.5
Heptanedioate (8)	7 (4)	0.74	7.3					37		55	7.9	tr[c]
Octanedioate (8)	5 (2)	1.40	13.2					31		64	1.4	3.3
Nonanedioate (8)	5 (2)	1.09	8.2					28		63	7.3	1.6
Decanedioate (8)	5 (2)	1.60	10.6					35		59	4.4	1.9

[a] Parenthetical values in this column are induction times (in days) for cell growth.
[b] Determined from gas chromatography and ^{1}H-NMR data. 3HB, 3-hydroxybutyric acid; 3HH, 3-hydroxyheptanoic acid; 3HN, 3-hydroxynonanoic acid.
[c] tr, trace amount.

despite the requirement of a rather long induction time (4 to 13 days). However, PHA was produced only on 1,4-butanediol, and the polyester obtained was composed of 29 mol% 3HO, 68 mol% 3HD, 1.3 mol% $C_{12:1}$, and 1.7 mol% $C_{14:1}$. Other C-even diols, such as C_6, C_8, and C_{10}, were utilized only for cell growth without an accumulation of polyester. A similar pattern of PHA synthesis with diols was observed with other bacteria that utilize 3-methyl-branched substrates, such as *P. aeruginosa* ATCC 27853 (5).

The characteristics of PHA synthesis of *P. citronellolis* grown on various saccharides and their derivatives are also shown in Table 2. It has already been reported that several *Pseudomonas* strains produce MCL PHAs when they are grown on glucose. These include *Pseudomonas* sp. strain NCIMB 40135 (18) and *P. putida* KT2442 (21), as well as *P. citronellolis* ATCC 13674 in this study. *P. citronellolis* also utilized gluconate and fructose for PHA production, while no polyester was produced on saccharic acid. Other saccharides, such as D-xylose, D-galactose, L-arabinose, D-mannose, lactose, and D-sorbitol, did not support any cell growth during 3 days of cultivation. *P. aeruginosa* ATCC 27853 showing similar PHA synthesis characteristics to those of *P. citronellolis* also did not grow on these saccharides during an even longer period of 25 days (5). The polyesters obtained on saccharides contained $C_{12:1}$ and $C_{14:1}$ units as well as the major constituent of 3HD and minor ones of 3HO and 3HC. A similar result was reported for *P. putida* KT2442 (21).

PHA accumulation from 3-methyl-branched substrates. Alkyl-branched substrates are generally less susceptible to biodegradation because of the inhibition or prevention of β-oxidation by the branched alkyl side chains (10). Some

TABLE 2. Biosynthesis of PHAs by *P. citronellolis* from saccharides and their derivatives, linear ω-diols, hydrocarbons, and 3-methyl-branched substrates

Carbon source (g/liter)	Culture time (days)[a]	Biomass (g/liter)	PHA content (% by wt)	PHA composition (mol%)[b]						
				I	II	3HC	3HO	3HD	$C_{12:1}$	$C_{14:1}$
Saccharides and their derivatives										
Glucose (10)	3	1.60	26.1			2	25	64	3.3	5.4
Gluconate (10)	3	1.82	27.0				35	56	7.5	1.4
D-Fructose (10)	3	1.24	18.7				14	76	4.9	5.2
ω-Diol, 1,4-butanediol (10)	11 (6)	1.26	13.0				29	68	1.3	1.7
Hydrocarbon, *n*-octane (5)	4 (1)	1.18	17.8			11	80	9	tr[c]	tr[c]
3-methyl-branched substrates										
D,L-Citronellol (5.5)	3	1.37	27.2	65	35					
3-Methyl-*n*-valerate (10)	3	1.33	14.0				31	69	9.6	0.9
Isoamyl alcohol (10)	4	1.00	9.5				31	69	5.0	0.9
3-Methyladipate (6)	5	1.13	15.1				41	59	4.4	1.1

[a] See Table 1, footnote *a*.
[b] Determined from gas chromatography and ^{1}H-NMR data. I, 3-hydroxy-7-methyl-6-octenoate; II, 3-hydroxy-5-methylhexanoate.
[c] See Table 1, footnote *c*.

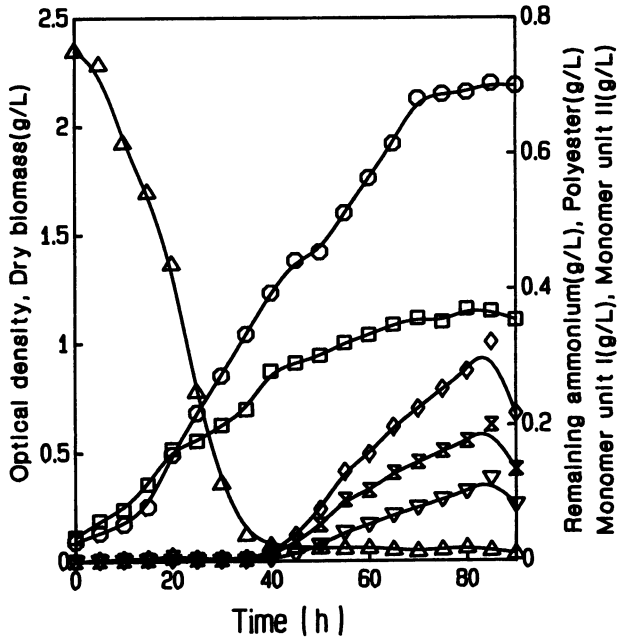

FIG. 1. Accumulation of polyester by *P. citronellolis* grown on citronellol. ○ optical density; □, biomass; △, remaining ammonium concentration; ◇, polyester; ⧖, monomer unit I (3-hydroxy-7-methyl-6-octenoate); ▽, monomer unit II (3-hydroxy-5-methylhexanoate).

pseudomonads, including *P. citronellolis* and *P. aeruginosa*, are known to be capable of degrading 3-methyl-branched substrates such as citronellol and 3-methyl-*n*-valerate. These recalcitrant carbon skeletons are first degraded via the citronellol pathway or the isovalerate pathway to remove branches and then degraded further via the β-oxidation pathway (10, 28).

In addition to citronellol and 3-methyl-*n*-valerate, isoamyl alcohol and 3-methyladipate were used to test the accumulation of polyesters by *P. citronellolis* (Table 2). The citronellol used as a carbon source was a DL-racemic mixture. Circular dichroic measurement showed that the bacterium utilized both of the optical isomers (data not shown). Figure 1 shows the growth kinetics of the bacterium grown on citronellol. Only a slight pH change was observed during cultivation, changing from the initial 7.0 to 6.3 finally. A complete depletion of NH_4^+ (initial concentration, 0.75 g/liter) occurred after 40 h of cultivation when cells started to accumulate polyesters. Maximal polyester accumulation was observed after 80 h of cultivation. However, further cultivation decreased PHA content in cells probably because of intracellular degradation by depolymerase (8). The increase in biomass was biphasic. The initial increase was due to exponential cell growth, and the further increase was due to subsequent PHA accumulation. The increase in optical density after 40 h of cultivation is presumed to be caused by the increase in polyester granule content in cells (31).

The polyester from citronellol-grown cells consisted of two monomer units as described below. The monomer unit composition of the polyester was slightly affected by changes in culture times. For instance, a gradual decrease of monomer unit I in the polyester was observed during growth on citronellol (Table 3); the content level of monomer unit I changed from 73.8 mol% after 40 h of cultivation to 62.0 mol% after 90 h of cultivation. However, the monomer unit composition of the polyester formed in 3-methyl-*n*-valerate-grown cells remained constant (~25 mol% 3HO and ~75 mol% 3HD) throughout the cultivation experiment (Table 3). Two other examples of such invariability in monomer unit composition with culture times have been reported, poly(3HC-co-3HO) synthesized by *P. oleovorans* grown on *n*-octane (27) and poly(3HO-co-3HD) synthesized by *P. putida* BM01 grown on octanoate (37). It was found that the monomer unit composition rarely changed with changes in the cultivation parameters, such as C/N ratio, temperature, pH, phosphate concentration, and buffer capacity (33). We analyzed 22 PHA samples obtained from optimization experiments of PHA production by *P. putida* BM01 grown on octanoate. Results showed that the 3HO contents varied between 74.1 and 85.4 mol%, with an average of 80.5 mol% ± 2.4 mol%. The content levels of the other two monomer units, 3HC and 3HD, varied between 14.2 to 21.9 and 0.0 to 7.5 mol%, respectively.

TABLE 3. Compositional variation of monomer units in polyesters during cultivation of *P. citronellolis* on 3-methyl-branched substrates

Substrate	Time (h)	Monomer unit (mol%)		Monomer unit (mol%)[a]	
		I[b]	II[c]	3HO	3HD
Citronellol	20	77.3	22.7		
	25	71.7	28.3		
	30	67.7	32.3		
	35	68.0	32.0		
	40	73.8	26.2		
	45	72.0	28.0		
	50	69.3	30.7		
	55	68.0	32.0		
	60	ND[d]	ND[d]		
	65	65.7	34.3		
	70	64.9	35.1		
	75	64.1	35.9		
	80	63.3	36.7		
	85	62.2	37.8		
	90	62.0	38.0		
3-Methyl-*n*-valerate	24			19.8	80.2
	28			21.1	78.9
	32			23.4	76.6
	36			22.3	77.7
	40			22.9	77.1
	44			25.1	74.9
	48			24.2	75.8
	52			24.0	76.0
	56			26.2	73.8
	60			27.3	72.7
	64			ND[d]	ND[d]
	68			25.5	74.5
	72			23.7	76.3
	92			24.8	75.2
	96			26.0	74.0

[a] Determined from gas chromatography data.
[b] I, 3-hydroxy-7-methyl-6-octenoate.
[c] II, 3-hydroxy-5-methylhexanoate.
[d] ND, not determined.

P. citronellolis also utilized two other methyl-substituted substrates, isoamyl alcohol and 3-methyladipate, for the production of polyester. The polyesters produced consisted of 3HO, 3HD, $C_{12:1}$, and $C_{14:1}$ monomer units. The 3HD monomer unit is the major constituent of polyesters from branched substrates other than citronellol.

Structural characterization of a novel copolyester with methyl-substituted olefinic and methyl-branched groups. Figure 2 shows the 125-MHz ^{13}C-NMR spectrum of the polymer synthesized by *P. citronellolis* grown on citronellol. The chem-

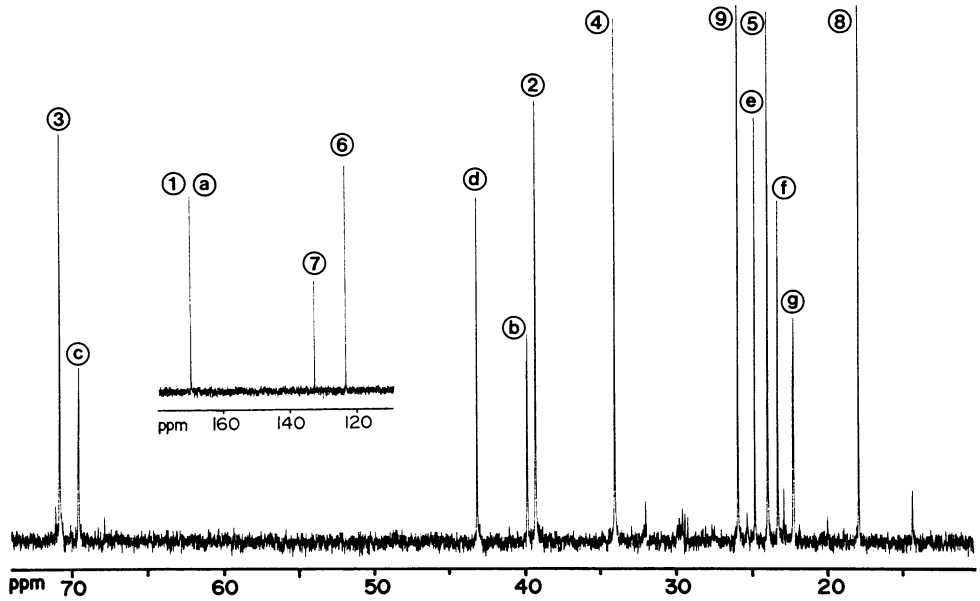

FIG. 2. ^{13}C-NMR spectra (125 MHz) of polyester synthesized by *P. citronellolis* grown on citronellol.

ical shifts of carbon atoms in the polymer are listed in Table 4. Absorption bands at 169.34, 169.14, 70.59, and 69.36 ppm indicate that the isolated polymer is a polyester which consists of at least two kinds of hydroxy acid monomer units (I and II) (16, 30). The absorptions at 169.34 and 169.14 ppm are ascribable to the carbonyl carbons in the backbone chain, and the last two absorptions (70.59 and 69.36 ppm) are ascribable to the methine carbons singly bonded to ester oxygen. The gas chromatogram of the methyl ester obtained from methanolysis of the polyester displayed only two major peaks (data not shown). The two absorptions in the downfield region (123.04 and 132.43 ppm) indicate the presence of two olefinic carbons (24, 30). The presence of the olefinic group (C=C—H) is also confirmed by the infrared spectrum which shows an olefinic C—H stretching absorption at 3,010 cm^{-1} (27) (Fig. 3). For detailed assignments of both the carbon and proton spectra, we obtained the 500-MHz two-dimensional ^{1}H-^{13}C heteronuclear COSY spectrum of the polymer from citronellol (Fig. 4 and 5). As shown in Fig. 4, no cross-peak between carbonyl carbon and any protons appeared, as expected. C-7 also does not show a cross-peak because of its quaternary nature. The proton signal, which absorbs at 5.06 ppm, correlates with the C-6 signal, indicating that it is an olefinic proton. The previous assignment of the methine carbons C-3 and the carbon designated c (Fig. 4) is consistent with the chemical shifts assigned to the protons on them. The two cross-peaks pertaining to C-3 and the carbon designated c (Fig. 4) confirm the presence of two kinds of monomer units in the polymer.

Figure 5 is an expanded plot of the upfield COSY spectral portion of Fig. 4. The carbon signals C-2 and the carbon designated b (Fig. 5) correlate with the proton signals absorb-

TABLE 4. Chemical shifts of carbon atoms in the 125-MHz ^{13}C-NMR spectrum of polyester synthesized by *P. citronellolis* grown on citronellol as the sole carbon source

Carbon atom[a]	Absorption (ppm) of 3-hydroxyalkanoate repeating unit	
	I	II
1 (a)	169.34	169.14
2 (b)	39.04	39.62
3 (c)	70.59	69.36
4 (d)	33.81	42.93
5 (e)	23.72	24.57
6 (f)	123.04	23.04
7 (g)	132.43	21.99
8	17.68	
9	25.68	

[a] Numerals are for repeating unit I, 3-hydroxy-7-methyl-6-octenoate, and letters are for repeating unit II, 3-hydroxy-5-methylhexanoate.

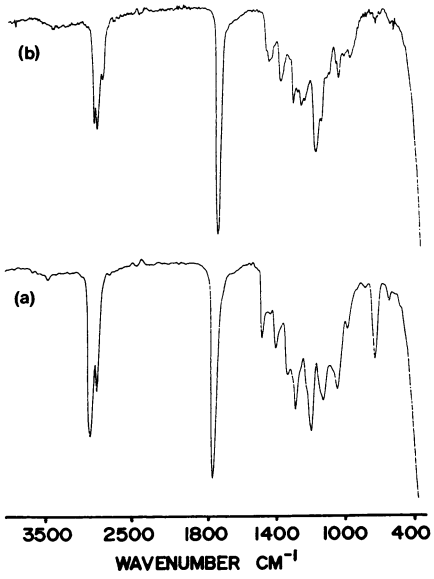

FIG. 3. Infrared spectra of polyesters synthesized by *P. citronellolis* from acetate (a) and citronellol (b).

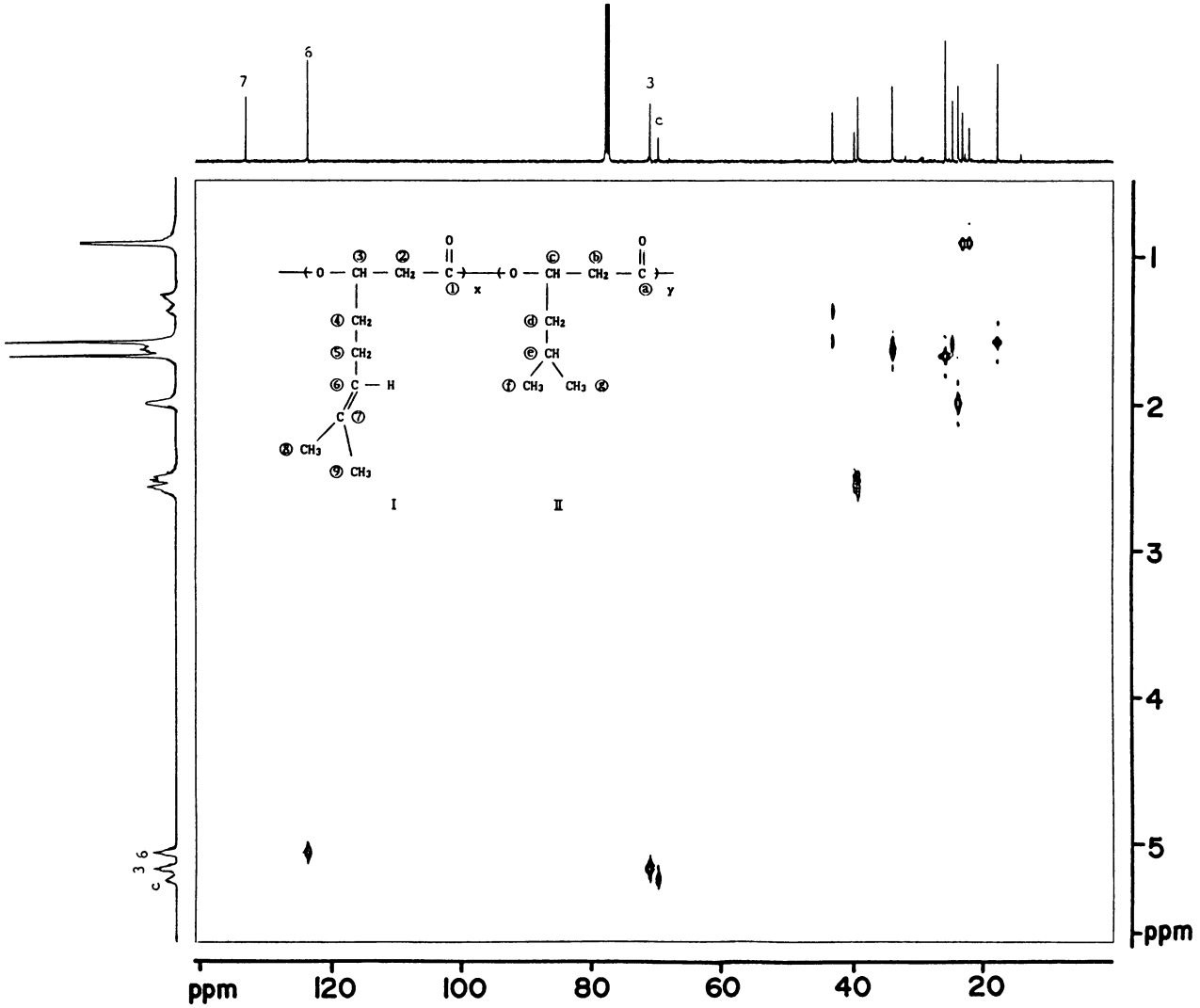

FIG. 4. Two-dimensional ^{1}H-^{13}C heteronuclear COSY spectrum of polyester synthesized by *P. citronellolis* grown on citronellol.

ing at ~2.5 ppm owing to the backbone methylene protons of 3-hydroxyalkanoate polymers. This again proves that the polymer from citronellol is a copolymer. The two strong proton signals at 1.58 and 1.67 ppm of equal intensity (peaks 8 and 9 [Fig. 5]) may be due to the geminal two methyl groups attached to the olefinic group. The carbon (C-9) or proton signals associated with the methyl group *cis* bonded relative to the olefinic proton absorbs in the region further downfield compared with those *trans* bonded (24). From a comparison of signal intensity ratios between protons, it could be concluded that proton signals 3, 5, 6, 8, and 9 (Fig. 5) belong to monomer unit I. In addition, the difference in carbon signal intensity between the carbon designated b (Fig. 5) and C-2 reveals that peak 2 is associated with monomer unit I. Analysis of the carbon signal intensity ratio of the two proposed monomer units also indicates that carbon signals designated b, c, d, e, f, and g (Fig. 5) must be from monomer unit II.

More accurate assignments of the carbon and proton signals and determinations of the sequential order of carbon atoms may be made through an analysis of the ^{1}H-^{1}H homonuclear COSY spectrum of the copolymer (Fig. 6). The homonuclear COSY spectrum reveals cross-peaks indicative of the correlation between coupled proton signals belonging to neighboring protons of the structure. In the ^{1}H-^{1}H COSY spectrum (Fig. 6), the presence of cross-peaks associated with methylene group 4 shows that it belongs to monomer unit I. In the region of proton resonances for monomer unit I, six cross-peaks, designated 2-3, 3-4, 4-5, 5-6, 6-8, and 6-9, were detected, indicating that the carbon sequence in monomer I is C-2–C-3–C-4–C-5–C-6. For monomer unit II, six cross-peaks, designated b-c, c-d_A, c-d_B, d_A-e, e-f, and e-g (Fig. 6), were detected while the cross-peak designated d_B-e was hardly detected because of its presence near the diagonal region as a result of nearly the same chemical shifts of the corresponding proton signals (Fig. 6). Thus, the carbon sequence for monomer unit II can be determined as carbons b-c-d-e-(f,g). The two monomer units may be named 3-hydroxy-7-methyl-6-octenoate (monomer I) and 3-hydroxy-5-methylhexanoate (monomer II), respectively.

The molar fractions of the two monomer units from the copolyester sample were determined from the intensity ratio of each proton resonance pertaining to monomer units I and II.

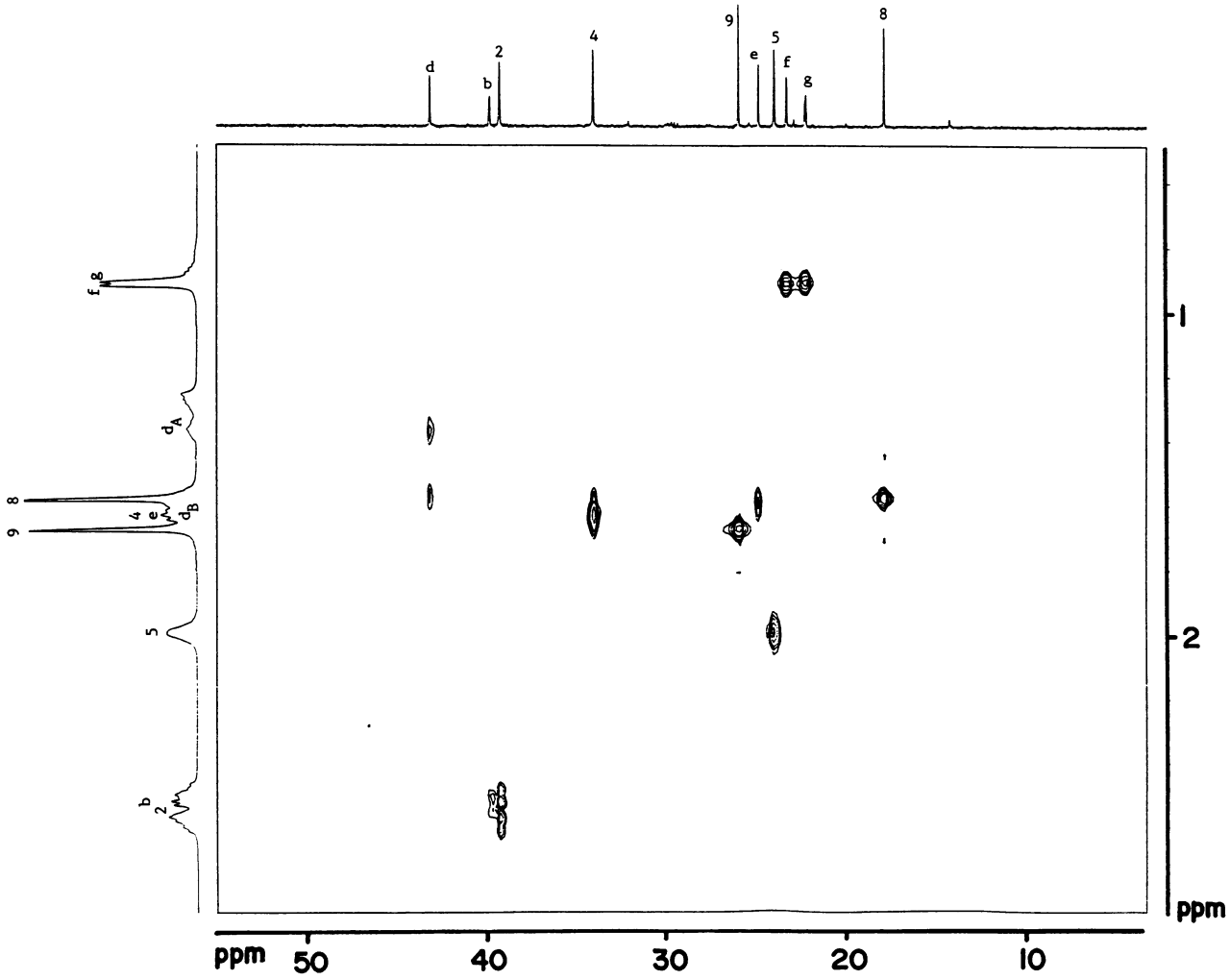

FIG. 5. Expanded upfield ^1H-^{13}C heteronuclear COSY spectrum of polyester synthesized by *P. citronellolis* grown on citronellol.

The polyester contained 65 mol% monomer I and 35 mol% monomer II. This was confirmed by gas chromatography.

DISCUSSION

Unsaturated monomer units semiquantitatively correlate with saturated monomer units. As shown in Tables 1 and 2, all polyesters having 3HD as the major monomer unit and 3HO as the next major monomer unit contained significant levels of two unsaturated monomer units, $C_{12:1}$ and $C_{14:1}$. PHA composition analysis also reveals that chain elongation of the original carbon skeleton should precede PHA synthesis when grown on lower carbon (C_2 to C_4) sources. Therefore, a certain correlation between the saturated (3HD and 3HO) and unsaturated ($C_{12:1}$ and $C_{14:1}$) monomer units may exist. Thus, the presence of these unsaturated monomer units indicates that the precursors of 3HO and 3HD synthesized from C_2 to C_4 monoacids, all diacids, and unrelated substrates may be principally derived via the fatty acid synthesis pathway. It is not known whether the acyl moieties of *R*-3-hydroxyalkanoates-acyl carrier protein can be directly incorporated into the polymer or whether a transfer to coenzyme A (CoA) prior to polymerization is required (21).

Copolyesters from the longer C_6 to C_{10} acids consisted primarily of 3-hydroxyalkanoate with the same carbon chain length as the substrate. Furthermore, they contained negligible amounts of unsaturated monomer units. The monomer unit precursors in the form of CoA must have been derived mostly via the β-oxidation pathway (4, 21).

From such differences in monomer unit composition between PHAs from all monocarboxylic acids, linear acids can be categorized into three groups. The C_2 to C_4 acids in group I may be metabolized via a similar pathway mentioned above to synthesize PHAs since the polyesters from these acids contain similar levels of 3HO, 3HD, and the two unsaturated monomer units. The C_7 to C_{10} acids, which metabolize principally via the β-oxidation pathway, are in group II. PHAs from the intermediate C_5 and C_6 acids in group III exhibited a rather complicated monomer unit distribution. For example, the polyester synthesized from valerate contained monomer units of 3HO, 3HD, $C_{12:1}$, and $C_{14:1}$ presumably derived from the intermediates of fatty acid biosynthesis pathway and a monomer unit of 3HV presumably derived from the intermediate of β-oxidation. Cultivation on caproate produced a copolyester composed of a β-oxidation derivative as the major monomer unit

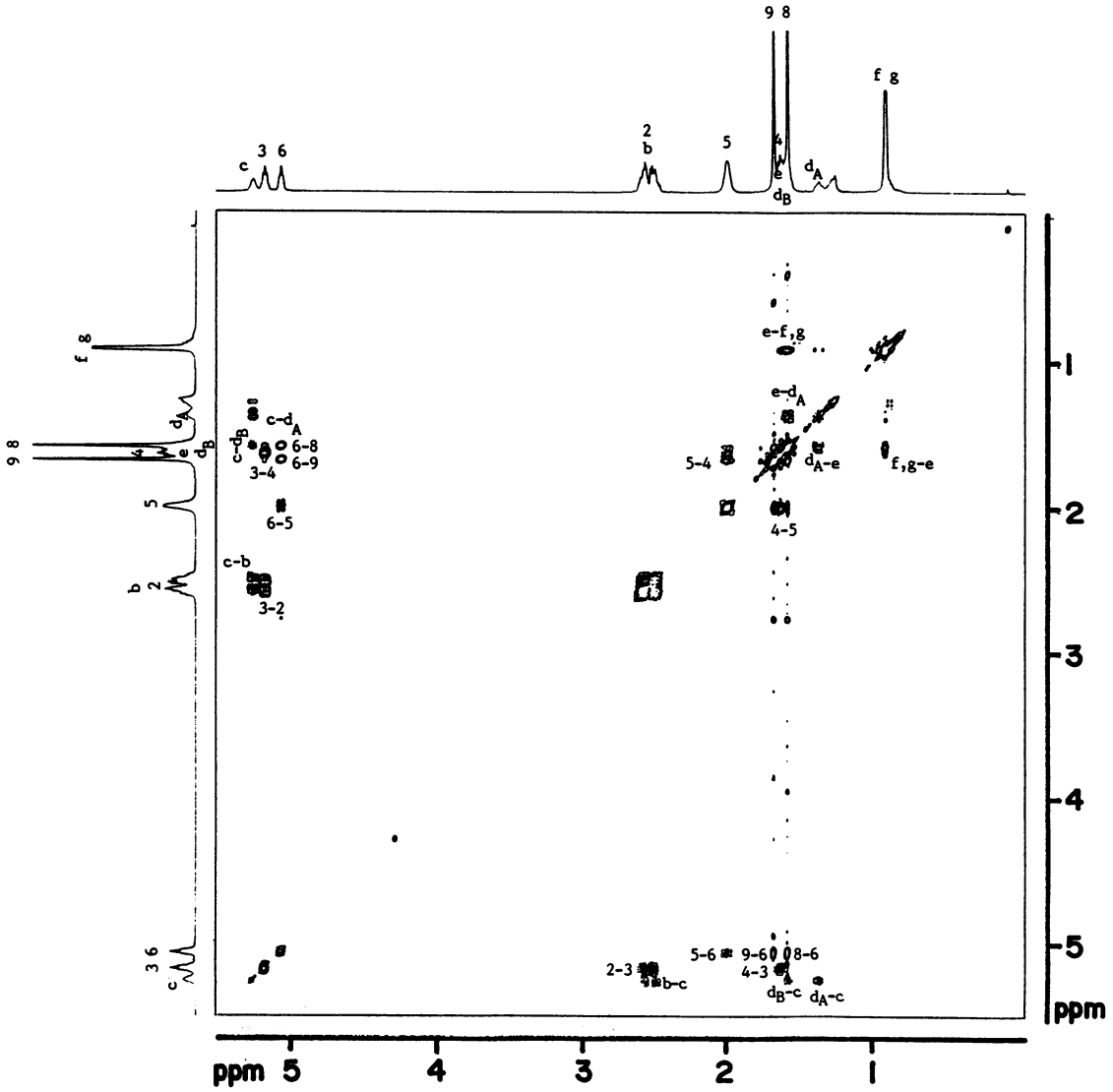

FIG. 6. Two-dimensional ^1H-^1H homonuclear COSY spectrum of polyester synthesized by *P. citronellolis* grown on citronellol.

and fatty acid synthesis derivatives as minor components. Thus, the linear mono- and dicarboxylic acids are metabolized via different pathways for the synthesis of polyesters according to chain length. It should be mentioned that from the analysis of monomer unit composition, 1,4-butanediol may be classified in group I.

Metabolism of 3-methyl-branched substrates for PHA synthesis. It is interesting that, contrary to the case of *n*-valeric acid, 3HV units were not present in PHAs from cells grown on 3-methyl-*n*-valerate. It has been proposed that growth on 3-methyl-*n*-valerate produces acetyl-CoA and 3-ketovaleric acid via the isovalerate pathway (28). The 3-ketovaleryl group may be metabolized either via the β-oxidation pathway or via the fatty acid synthesis pathway. However, the absence of 3HV units may favor PHA synthesis from intermediates via the fatty acid synthesis pathway.

Isoamyl alcohol and 3-methyladipate are presumed to be degraded via the isovalerate pathway and ω-oxidation pathway, respectively, considering their closely related structure to isovalerate. The presence of significant amounts of $C_{12:1}$ and $C_{14:1}$ monomer units in these polyesters implies that the monomer units may be derived via the isovalerate pathway followed by the fatty acid biosynthesis pathway. This implication can be reinforced further by a comparison of major monomer units in PHAs from group I substrates and those from decanoate. Growth on group I substrates led to the production of PHAs with the 3HD unit as the primary monomer unit and 3HO in a smaller amount along with significant amounts of the two unsaturated monomer units, while decanoate-grown cells produced mostly 3HO and then 3HD, with negligible amounts of the two unsaturated monomer units. The dissimilar distribution of 3HO and 3HD between PHAs from different substrates may suggest the existence of different routes for supplying the precursors used for polymerization in cells.

Monomer unit I of the polyester from citronellol has nine carbon atoms. This confirms that citronellol is degraded via the citronellol pathway to produce a substrate with one less carbon than the original molecule. The polyester does not have a linear 3-hydroxy acid monomer unit. The additional monomer

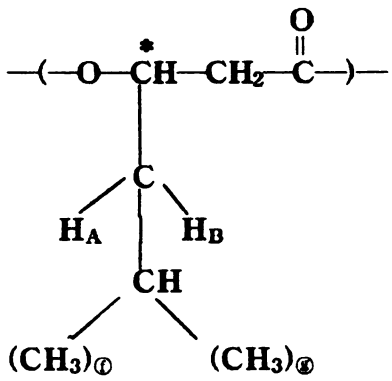

FIG. 7. Example to demonstrate magnetic inequivalence revealed by methylene and isopropyl groups proximately connected in series within the same molecule. The asterisk indicates the chiral center.

unit II, 3-hydroxy-5-methylhexanoate, is thought to be derived from the precursor of monomer unit I whose double bond was reduced and two carbons were cut away in the β-oxidation pathway. Thus, polyester synthesis occurs via minimum steps of modification of the substrate without resorting to any further rearrangement of the carbon skeletons.

The branched monomer unit was also found in *P. oleovorans* cells grown on the corresponding branched acid (14, 20). *P. oleovorans* produced 3-hydroxy-7-methyloctanoate as the major monomer unit and 3-hydroxy-5-methylhexanoate as the minor monomer unit (10 mol%) when the bacterium was grown on 7-methyloctanoate (14, 20). They also found a small amount of 4-methyl-substituted monomer unit in the copolymers obtained from 6-methyloctanoate. A polyester with an even bulkier group at the fifth position, such as poly(3-hydroxy-5-phenylvalerate), could be produced in MCL-PHA-producing bacteria, including *P. oleovorans* (25), *P. citronellolis* (5), and *P. putida* (37). 3-Hydroxyacyl-CoA precursors with bulky groups substituted at, at least, two carbon atoms away from the third position are likely to be polymerized by polymerases in these bacteria.

Two diastereotopic groups connected in series to a chiral center. The isobutyl pendant group attached to the chiral center in monomer unit II shows interesting NMR absorption splittings. As shown in Fig. 5, the two methylenic protons on the carbon atom designated d exhibit two well-separated ($\Delta\nu$ = 134 Hz) absorptions. The spin system of two methylene protons may be defined as an ABCX system. Each of the two spectral peaks was too broad to resolve. However, it is generally known that if a methylene group is attached to a chiral group, the geminal hydrogen atoms are diastereotopic (12). Thus, they have different magnetic shieldings and consequently give separate signals. The magnetic inequivalence can also be applied to the isopropyl group in the isobutyl group. If we consider the ^{1}H one-dimensional spectrum only, the doublet at 0.90 ppm could be ascribed to a first-order spin coupling of two methyl groups to the methine proton designated e (Fig. 5). However, the heteronuclear COSY data show that the geminal methyl groups are also diastereotopic, giving separate carbon signals and cross-peaks. As shown in Fig. 7, this is an interesting example to demonstrate the magnetic inequivalence revealed by both the methylene and isopropyl groups proximately connected in series within the same molecule.

Thermal characteristics of kinetically stabilized polyesters. The crystallization rate at 20°C, far above the glass transition temperature, of MCL PHAs is so low that it takes at least 3 to 7 weeks to get stable crystalline structures (15). The polyester sample from octanoate, which showed a melting endotherm in the first run, exhibited a negligible level of endotherm upon another immediate heating after cooling, as was reported for polyesters synthesized by *P. oleovorans* (data not shown). Other crystallizable samples obtained in this study generally showed similar hysteresis behavior. Annealing of the polyesters from octanoate at 20°C significantly reduced the endothermic shoulder probably ascribable to undefined metastable structures. A 12-week annealing at 20°C may be long enough to obtain thermal properties, demonstrating the structural differences among polyesters with steady-state structures.

The quenched sample of poly(3-hydroxybutyrate) with a methyl group as a side branch has a glass transition temperature (T_g) between 2.1 and 10°C (8, 29). The increase of one methylene unit in the R group (e.g., 3HV) decreased the T_g value to −10°C (8, 19). The significant decreases in the T_g values of MCL PHAs may be explained in terms of the fast movement of *n*-pentyl, *n*-hexyl, or *n*-heptyl side chains acting as a diluent (29).

Two polyesters from hexanoate and heptanoate showed no discernible melting transition. This indicates no crystalline region in these samples, results similar to those with the corresponding polymers from *P. oleovorans* (16). The melting temperatures (T_ms) of crystallizable polyesters were in the range of 40 to 59°C. The T_gs of MCL PHAs ranged between −24 and −45°C. Generally, an increase in 3HV content and/or monomer unit complexity lowered the T_m and fusion enthalpy values of PHA. From X-ray crystallography (16), differential scanning calorimetry (15, 16), and solid-state ^{13}C-NMR (29) studies, it has been suggested that an MCL PHA with an *n*-alkyl pendant group equal to or greater than *n*-pentyl may crystallize in a manner similar to that described for other comb-type polymers with lengthy (C_{15} to C_{18}) side chains, such as poly(*r*-*n*-octadecyl L-glutamate).

The differential scanning calorimetry scan of polyester from citronellol showed a glass transition at −20°C and no melting transition. Its T_g is higher than those of polyesters with 55 and 50 mol% of unsaturation at the terminals of R groups synthesized by *P. oleovorans* from 1-decene and 1-octene, showing T_gs of −43 and −37°C, respectively (30). The polymers produced with citronellol had the highest T_gs among the polymers analyzed. The single glass transition exhibited by the polyester poly(3-hydroxy-7-methyl-6-octenoate-co-3-hydroxy-5-methylhexanoate) from citronellol may indicate that it is a copolyester and not a blend of each homopolymer. This was further confirmed by gas chromatographic analysis of the methanolized products of two fractions which were fractionated by precipitation in *n*-propanol as the supernatant and precipitate. Gas chromatography analysis of the two fractions showed similar molar monomer unit ratios of 65:35 and 70:30, respectively.

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