APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2001, p. 4963–4974 Vol. 67, No. 11
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.67.11.4963–4974.2001

Accumulation of Polyhydroxyalkanoic Acid Containing Large Amounts of Unsaturated Monomers in *Pseudomonas fluorescens* BM07 Utilizing Saccharides and Its Inhibition by 2-Bromooctanoic Acid

HO-JOO LEE,[1] MUN HWAN CHOI,[1] TAE-UN KIM,[2] AND SUNG CHUL YOON[1,3]*

Biomaterials Science Laboratory, Division of Life Science at the College of Natural Sciences[3] and Division of Applied Life Sciences at the Graduate School,[1] Gyeongsang National University, Chinju 660-701, and Department of Clinical Laboratory Science, Catholic University of Pusan, Pusan 609-757,[2] Korea

Received 11 December 2000/Accepted 6 August 2001

A psychrotrophic bacterium, *Pseudomonas fluorescens* BM07, which is able to accumulate polyhydroxyalkanoic acid (PHA) containing large amounts of 3-hydroxy-*cis*-5-dodecenoate unit up to 35 mol% in the cell from unrelated substrates such as fructose, succinate, etc., was isolated from an activated sludge in a municipal wastewater treatment plant. When it was grown on heptanoic acid (C_7) to hexadecanoic acid (C_{16}) as the sole carbon source, the monomer compositional characteristics of the synthesized PHA were similar to those observed in other fluorescent pseudomonads belonging to rRNA homology group I. However, growth on stearic acid (C_{18}) led to no PHA accumulation, but instead free stearic acid was stored in the cell. The existence of the linkage between fatty acid de novo synthesis and PHA synthesis was confirmed by using inhibitors such as acrylic acid and two other compounds, 2-bromooctanoic acid and 4-pentenoic acid, which are known to inhibit β-oxidation enzymes in animal cells. Acrylic acid completely inhibited PHA synthesis at a concentration of 4 mM in 40 mM octanoate-grown cells, but no inhibition of PHA synthesis occurred in 70 mM fructose-grown cells in the presence of 1 to 5 mM acrylic acid. 2-Bromooctanoic acid and 4-pentenoic acid were found to much inhibit PHA synthesis much more strongly in fructose-grown cells than in octanoate-grown cells over concentrations ranging from 1 to 5 mM. However, 2-bromooctanoic acid and 4-pentenoic acid did not inhibit cell growth at all in the fructose media. Especially, with the cells grown on fructose, 2-bromooctanoic acid exhibited a steep rise in the percent PHA synthesis inhibition over a small range of concentrations below 100 μM, a finding indicative of a very specific inhibition, whereas 4-pentenoic acid showed a broad, featureless concentration dependence, suggesting a rather nonspecific inhibition. The apparent inhibition constant K_i (the concentration for 50% inhibition of PHA synthesis) for 2-bromooctanoic acid was determined to be 60 μM, assuming a single-site binding of the inhibitor at a specific inhibition site. Thus, it seems likely that a coenzyme A thioester derivative of 2-bromooctanoic acid specifically inhibits an enzyme linking the two pathways, fatty acid de novo synthesis and PHA synthesis. We suggest that 2-bromooctanoic acid can substitute for the far more expensive (2,000 times) and cell-growth-inhibiting PHA synthesis inhibitor, cerulenin.

Polyhydroxyalkanoic acid (PHA) is accumulated in bacterial cells from many types of carbon sources and it can be used as energy source (3, 25) if the PHA is degraded under certain conditions (10, 39). PHA composition depends on the PHA synthases present, the carbon sources and the route by which the supplied carbon sources are metabolized (25). Medium-chain-length (MCL) PHA-producing bacteria (specifically, the *Pseudomonas* spp. belonging to rRNA group I) can be used for the production of PHA with functional groups, such as phenyl, phenoxy, olefins, halogens, esters, etc. (25), in its side chains. In particular, the incorporation of carbon-carbon double (15, 22, 24, 27, 37) or triple (21) bonds into the side chains of PHA may be important because a large number of applications in pharmacology, agricultural science, etc., are expected after some modification of the olefins by attaching biological active molecules or cross-linking the multiple bonds by γ-irradiation (4), UV light (21), or epoxidation (5, 38). A usual preparation of the modifiable PHA requires a cofeeding of expensive alkenoic acids or alkenes. The fatty acids with more than six carbon atoms are degraded via the β-oxidation pathway. Intermediates from the β-oxidation cycle can be converted to (*R*)-3-hydroxyacyl-coenzyme A (CoA) by a hydratase, epimerase, or reductase activity, and (*R*)-3-hydroxyacyl-CoA is utilized as the substrate of the PHA synthase to polymerize them (25). The introduction of functional groups in the side chains is thus possible by using the fatty acids with the corresponding functional groups as the substrates.

Another type of PHA with unsaturated carbon-carbon double bonds in the side chains is produced by several MCL-PHA-producing bacteria such as *Pseudomonas citronellolis* (9), *P. aeruginosa, P. putida* KT2442 (12, 18, 19, 20), and *Pseudomonas* sp. strain NCIMB 40135 (16), grown on unrelated carbon sources such as fructose, glucose, acetate, butyrate, gluconate, ω-diols, ω-dicarboxylic acids, etc. The unsaturated monomers identified thus far include 3-hydroxy-*cis*-5-dodecenoate ($C_{12:1}$) and 3-hydroxy-*cis*-7-tetradecenoate ($C_{14:1}$), which usually con-

* Corresponding author. Mailing address: Biomaterials Science Laboratory, Division of Life Science, Gyeongsang National University, Gazwa-Dong 900, Chinju 660-701, Korea. Phone: 82-55-751-5942. Fax: 82-55-759-0187. E-mail: scyoon@nongae.gsnu.ac.kr.

stitute minor monomers in the PHA in addition to the major constituents 3-hydroxyoctanoic acid and 3-hydroxydecanoic acid (3HD) (9, 12, 18, 19, 20). However, in most cases reported thus far, the overall contents of the two unsaturated monomers were <10 mol%. These unsaturated monomers are known to derive from the intermediates of the fatty acid de novo synthesis pathway (14, 17, 25). The intermediates are present in a form of acyl carrier protein (ACP). It was suggested that the substrate of MCL-PHA synthase was (*R*)-3-hydroxyacyl-CoA in pseudomonads. Thus, to serve as a substrate for the PHA synthase, (*R*)-3-hydroxyacyl-ACP must be converted to the corresponding CoA derivative. Recently, it was found that 3-hydroxyacyl-ACP:CoA transacylase *phaG* plays the role in linking the two pathways, fatty acid synthesis and PHA synthesis (14, 17, 25, 31).

PHA synthesis-related inhibitors can be used to find the metabolic pathway from which precursors for PHA synthesis (18) are supplied, as well as to channel intermediates of a pathway specific to PHA synthesis (14, 29). Only two inhibitors, acrylic acid and cerulenin, have been applied in literature (14, 18, 29). Acrylic acid has been employed to study the relationship between the β-oxidation cycle and PHA synthesis. It is known to inhibit acyl-CoA synthase and 3-ketothiolase in gram-negative bacteria (18, 29). It was reported that in a wild-type strain such as *P. putida* KT2442, PHA accumulation from fatty acids could be inhibited in the presence of acrylic acid (18). However, in recombinant strains such as *Escherichia coli* (*fadR*) (29) and *P. fragi* (14) harboring a PHA synthase gene, PHA accumulation was enhanced or induced. Cerulenin has been used as an inhibitor for PHA synthesis from saccharides because it inhibits fatty acid synthesis (18, 28). However, cerulenin also inhibits cell growth strongly at the same level of concentration as in PHA synthesis. Thus, it was necessary to find a specific inhibitor to inhibit only PHA synthesis and not cell growth. This could be very useful for an efficient pathway routing for the preparation of a specially designed PHA with the cells in active growth. In this study, a cheaper and more potent inhibitor, 2-bromooctanoic acid, was found to very specifically inhibit PHA synthesis of the bacterium from saccharides without any influence on the cell growth. 4-Pentenoic acid was also found to inhibit PHA synthesis, but it did so less specifically than 2-bromooctanoic acid.

We were interested in screening bacteria to produce PHA with high levels of those unsaturated monomer units from cheaper carbon sources such as saccharides. Recently, we successfully isolated a bacterial strain that is capable of producing MCL-PHA containing the two unsaturated monomers at up to 40 mol% from fructose. This characteristic strain was identified as a psychrotrophic *P. fluorescens*. For the three inhibitors, acrylic acid, 2-bromooctanoic acid, and 4-pentenoic acid, the inhibitory effect on the PHA synthesis of the isolated bacterium was investigated over a wide range of concentrations in order to systematically study the relation between the precursor supplying routes and PHA formation in the bacterium. In addition, several saccharides and monocarboxylic acids including C_2 (acetate) to C_{18} (stearic acid) were tested for their utilization by the bacterium to see what types of PHA are synthesized in the psychrotrophic strain.

MATERIALS AND METHODS

Isolation and characterization of strain BM07. The sample obtained from activated sludge in a municipal wastewater treatment plant in Chinju, Korea, was suspended with sterilized water and inoculated to an M1 mineral salts medium (9) containing 0.05% ammonium sulfate and 1% octanoic acid. After 2 days of incubation at 30°C and 200 rpm for 48 h, the culture was diluted 10^5 fold and 100 μl of the dilution was inoculated onto octanoate M1 mineral agar plates and cultivated for 24 h at 30°C. Several single colonies were picked out according to their opacity, usually caused by PHA synthesis in cells (34), and then purified by a series of spreading steps onto octanoate agar plates. The colony with the highest degree of opacity and the largest size was picked out.

The isolated strain was characterized by using the API 20NE and ATB ID-32-GN Identification System and identified as *P. fluorescens* (% identification [%ID] = 99.5). The strain was named *P. fluorescens* BM07 and deposited in Korean Collection for Type Cultures (strain no. KCTC 10005BP).

16S rRNA gene sequencing. The strain was also identified by using 16S rRNA gene sequence homology. Genomic DNA was obtained from bacteria grown overnight at 30°C in 5 ml of Luria-Bertani medium. The Marmur procedure (26) was employed for the isolation of the genomic DNA.

The PCR mixture consisted of 1.0 μl of the genomic DNA template solution; 10× PCR buffer; 50 ng each of two primers (forward, 5′-TATGGATCCTTCT ACGGAGAGTTTGATCC-3′; reverse, 5′-TATGGATCCCACCTTCCGCTAC GGCTACC-3′) (11); 0.25 mM concentrations each of dATP, dCTP, dGTP, and dTTP; and 2.5 U of *Taq* polymerase in a final volume of 50 μl. Thermal cycling was undertaken by initially denaturing the DNA at 94°C for 5 min, followed by 50 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final step at 72°C for 5 min. The PCR products were cloned into pGEM-T Easy vector (Promega, Madison, Wis.).

A portion of the reaction mixture was used to visualize PCR products on 1% (wt/vol) agarose gels. PCR products in the remainder of the reaction mixture were purified using a QIAquick PCR purification kit (Qiagen, Ltd., Crawley, West Sussex, United Kingdom) and sequenced by using an ABI PRISM BigDye terminator cycle ready reaction kit according to the manufacturer's instructions (PE Applied Biosystems, Warrington, United Kingdom). Sequence data obtained were compared with known 16S ribosomal DNA (rDNA) sequences of *P. fluorescens* strains (accession no. AF094726, AJ278814, AJ278813, and D84013) by using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) (1).

Culture media and PHA accumulation in *P. fluorescens* BM07. Nutrient-rich (NR) medium was used in the seeding, maintenance, and storage of the isolated strain and contained 1% yeast extract, 1.5% nutrient broth, and 1% ammonium sulfate. A modified M1 mineral salts medium of the same composition as that reported earlier (9) was used as the PHA synthesis medium. The culture (5 ml) grown in NR medium at 30°C at 180 rpm for 12 h was transferred to 500 ml of M1 mineral salts medium containing an appropriate amount of a carbon source and 1.0 g of ammonium sulfate/liter in a 2-liter flask and cultivated long enough to grow maximally. The cells were then harvested, washed with methanol, and dried under a vacuum at room temperature. In the cultivation with insoluble substrates such as C_{14}, C_{16}, and C_{18} carboxylic acid as a carbon source, a two-phase slurry cultivation technique reported earlier (35) was used for an efficient cultivation of the strain.

The cell growth was monitored by turbidity measurements at 660 nm with a Spectronic 20 spectrophotometer. The concentrations of ammonium ion remaining in the media were measured by using the Nessler reagent method. The concentration of fructose remaining in the medium was determined by using the 3,5-dinitrosalicylic acid method. The concentration of organic acid remaining in the medium was measured by reacting the chloroform extract of NaCl-saturated medium with sulfuric acid-methanol mixture, followed by gas chromatographic (GC) determination of the resultant methyl esters (8, 34). In time course experiments, PHA formation was similarly followed by determining the content of PHA in cells by using the sulfuric acid-methanol reaction mixture of the dried cells followed by GC determination of the resulting 3-hydroxymethyl esters. Gas chromatograms were obtained on a Hewlett-Packard 5890A gas chromatograph equipped with an HP-1 column and a flame ionization detector.

Polyester isolation and characterization. Polyesters were extracted from an appropriate amount of cells, which had been dried overnight at 50°C under a vacuum, with hot chloroform in a Pyrex Soxhlet apparatus for 6 h. After concentration, the solvent extract was precipitated in rapidly stirred cold methanol. The isolated polymers were dried overnight under a vacuum at ambient temperature and then weighed. Quantitative determination of the monomer units in the polymers was performed by GC as described above. The standardization of each GC peak was made against the PHA of known structure characterized by quantitative nuclear magnetic resonance (NMR) analyses (34, 35).

The ^{1}H- and ^{1}H-noise-decoupled ^{13}C-NMR analyses of the polyester samples were carried out on a Bruker-DRX 500 MHz spectrometer in the pulse-Fourier transform mode. Thermal transitions of the polyesters were measured under a nitrogen purge by using a TA differential scanning calorimeter (DuPont 2100, DSC V4.0B) equipped with a data station. The heating rate was 20°C/min. The scanning range was between −100 and 200°C.

Transmission electron microscopy. The washed cells were doubly fixed with 2% glutaraldehyde and 1% osmium tetroxide. Ultrathin sectioning was performed by using an LKB-Ultratome with a diamond knife. These sections were then collected on a copper grid coated with a Formvar-carbon film and were poststained with lead citrate and uranyl acetate (34). Electron micrographs were obtained with a Hitachi H-600 electron microscope (Tokyo, Japan) under an acceleration voltage of 75 kV.

Inhibition of PHA synthesis and cell growth. Among the several compounds tested, the three compounds, acrylic acid, 2-bromooctanoic acid, and 4-pentenoic acid were found to be potent inhibitors for PHA synthesis in *P. fluorescens* BM07. After cultivation of the cells in NR medium at 30°C for 24 h, the cells (2.79 g/liter in dry biomass) were transferred to a modified M1 PHA synthesis medium containing 70 mM fructose or 40 mM octanoate and cultivated at 30°C for 48 h in the presence of an appropriate amount of each inhibitor. The medium contained 1.0 g of ammonium sulfate per liter. The NR medium-grown cells did not contain PHA. Almost an equal amount of NR medium-grown cells (2.79 g/liter in dry cell weight) was added to each culture medium containing a different level of inhibitor. The content of PHA and its monomer composition were measured by GC analysis of the inhibitor-treated cells. Minimum triplicate experiments were carried out and statistically averaged. The standard deviations for each determination were within 5%. Five batches of noninhibitor cells were grown on 70 mM fructose and 40 mM octanoate, respectively, since the control experiments for both carbon sources and their biomasses were measured and averaged for each carbon source. The average values were determined to be 5.281 and 3.665 g/liter for fructose- and octanoate-grown cells, respectively, and were used as the control biomass in the calculation of the percent inhibition. For the three inhibitors, no systematic and significant change in the monomer ratio of the PHA recovered was observed compared to the PHA synthesized in the absence of inhibitor. Irrespective of the level of each inhibitor, a maximum ± 3% (2-bromooctanoic acid) to ± 7% (acrylic acid and 4-pentenoic acid) of nonsystematic GC signal variation was observed for each GC peak. So, the calculation of the percent inhibition was based on the change of the signal intensities of two strong GC peaks, $C_{12:1}$ peak for fructose-grown cells and 3-hydroxyoctanoate (3-HO) peak for octanoate-grown cells.

The percent inhibition of PHA accumulation is defined as $[(\text{PHA wt\%})_{\text{control}} - (\text{PHA wt\%})_{\text{inhibitor}}]/(\text{PHA wt\%})_{\text{control}}$, where $(\text{PHA wt\%})_{\text{control}}$ is the weight percent (wt%) of PHA in the control dry biomass and $(\text{PHA wt\%})_{\text{inhibitor}}$ is the weight percent of PHA accumulated in the presence of inhibitor. Similarly, the apparent percent inhibition of cell growth is defined as $[(\text{g of PHA-free dry cell mass/liter})_{\text{control}} - (\text{g of PHA-free dry cell mass/liter})_{\text{inhibitor}}]/(\text{g of PHA-free dry cell mass/liter})_{\text{control}}$, where $(\text{g of PHA-free dry cell mass})_{\text{control}}$ is the grams of PHA-free dry cell mass per liter in the absence of inhibitor, calculated by subtracting the weight of PHA from the weight of total averaged control biomass, and $(\text{g of PHA-free dry cell mass/liter})_{\text{inhibitor}}$ is the grams of PHA-free dry cell mass per liter in the presence of inhibitor. The determined control value for the grams of PHA-free dry cell mass/liter in the absence of inhibitor was 4.273 and 3.098 g/liter for fructose- and octanoate-grown cells, respectively. The limiting values corresponding to the theoretically allowed maximum percent inhibition of cell growth were calculated to be 35 and 10% for fructose- and octanoate-grown cells, respectively, because the $(\text{g of PHA-free dry cell mass/liter})_{\text{inhibitor}}$ values cannot be theoretically less than 2.79 g/liter, which was the amount of NR-grown cells equally added to each culture medium containing a certain level of inhibitor. Thus, the calculated apparent percent inhibition (virtually not normalized) of cell growth exceeding the limiting value was considered 100% inhibition.

Assay of the inhibitors remaining in media. 2-Bromooctanoic acid that remained in a culture medium was extracted with chloroform, and the chloroform extract was reacted in a sulfuric acid-methanol mixture. The methyl ester in the organic layer was analyzed by using GC. The concentration of acrylic acid and 4-pentenoic acid that remained in media was analyzed in a rather different way. Five milliliters of the culture medium was saturated with magnesium sulfate, and 0.5 ml of 2 M HCl was added to the saturated solution in order to increase the efficiency of transfer of the remaining inhibitor into an organic phase. After the mixture was vortex mixed, the free acid was extracted by adding 2 ml of chloroform. The chloroform extract was methanolized in a similar manner, and the resulting methyl ester was analyzed by using a Hewlett-Packard 5890A gas chromatograph equipped with a DB-WAXETR column (30 m by 0.53 mm by 0.25 μm; J&W Scientific, Folsom, Calif.). A typical GC run condition is as follows: initial temperature, 60°C for methylacrylate and 50°C for methyl-4-pentenoate; initial time, 2 min; heating rate, 10°C/min; and final temperature, 120°C. The methyl ester of acrylic acid and 4-pentenoic acid was eluted at retention times of 3.2 and 2.8 min, respectively. The same treatment procedure was applied to the samples for the standardization curves of the two inhibitors.

RESULTS

Characterization of the isolated strain. Table 1 shows the morphological, biochemical, and nutritional characteristics for the isolated strain. The strain has three to five polar flagella, and it exhibited motility. The strain is able to grow at 4°C. Activities of cytchrome oxidase and arginine dihydrolase were present. Denitrification was observed. Esculin was not hydrolyzed, but gelatin was hydrolyzed. Glucose, L-arabinose, and D-mannose were utilized for growth. Phenylacetate was not utilized. Thus, from the test result obtained by using the API identification program (ID-32-GN strip test) in accordance with standard methods (23), the isolate was identified as a strain of *P. fluorescens* (%ID = 99.5) and named *P. fluorescens* BM07. The strain was also identified by 16S rRNA gene sequence homology (98%) as *P. fluorescens*. An almost complete 16S rDNA sequence (1,469 nucleotides) was determined and compared with known 16S rDNA sequences.

Growth of the strain on a nutrient agar at 30°C led to the formation of pulvinate colonies. Even though the agar plate was stored in a 4°C refrigerator, vigorous cell growth was observed. The colony became translucent, probably due to the slime formed around the colony, with the colony shape changed to a rather diffuse convex form after storage at 4°C. The growth characteristics at 4°C indicate that *P. fluorescens* BM07 is a psychrotrophic strain.

PHA synthesis from unrelated carbon sources. Among the seven saccharides tested, fructose was the best carbon source for both cell growth and PHA production. The growth curve for the cells grown at 30°C on 70 mM fructose in a mineral salts medium containing 1.0 g of ammonium sulfate/liter (molar C/N ratio of ~10) is shown in Fig. 1. Cell growth and PHA accumulation occurred in a growth-associated fashion without any time lag. The dry cell weight was 4 g/liter in a 500-ml batch culture. The yield of PHA synthesis was 25% (wt/wt) dry cells.

The PHA isolated from the cells grown with fructose at 25°C was analyzed by using a 500-MHz ^{1}H-NMR spectrometer (Fig. 2). Detailed chemical-shift assignments for all of the carbons and protons were published elsewhere (12, 18). The four absorption peaks, designated g, h, j, and k, appeared at 5 to 6 ppm in the ^{1}H-NMR spectrum (Fig. 2A) and are ascribable to the protons attached to the two ethylene carbons (-CH═CH-) associated with the unsaturated monomers, $C_{12:1}$ and $C_{14:1}$, present in the PHA. A 125-MHz ^{13}C-NMR spectrum for the PHA revealed two pairs of resonances at 120 to 140 ppm ascribable to the two ethylene carbons in $C_{12:1}$ and $C_{14:1}$ (Fig. 2B). The expanded spectrum for the carbonyl band region showed well-resolved doublet peaks at 169.32 and 169.20 ppm, respectively (Fig. 2B, inset). The stronger absorption at 169.32 ppm was assigned to the carbonyls of the saturated 3-hydroxyl monomers, whereas the weaker one at 169.20 ppm was ascribable to those of $C_{12:1}$ and $C_{14:1}$. The methine carbon (oxygen-bound -CH- group in the backbone) also showed doublet absorptions at 70.81 and 70.43 ppm, respectively (Fig. 2B, inset). One more doublet occurred at 39.49 and 38.83 ppm, associated with the methylene carbon (-CH_2-) in the backbone chain

TABLE 1. Characterization of the isolated MCL-PHA-producing bacterium

Test	Result
Miscellaneous	
Cell shape	Rod
Gram staining	−
Motility	+
Flagellum number	3–5
Growth on nutrient agar	+
Growth on MacConkey agar	+
Growth at 4°C	+
Biochemical test	
Cytochrome oxidase	+
Arginine dihydrolase	+
Urease	−
Nitrate reduction	+
Indole test	−
Glucose acidification	−
Hydrolysis of:	
Esculin	−
Gelatin	+
p-Nitrophenyl-β-D-galactopyranoside	−
Utilization of:	
Glucose	+
L-Arabinose	+
D-Mannose	+
L-Rhamnose	−
Maltose	−
D-Ribose	+
D-Saccharose	+
D-Melibiose	−
Fucose	−
Alanine	+
Salicine	−
Histidine	+
L-Serine	+
L-Proline	+
Phenylacetate	−
Mannitol	+
D-Sorbitol	+
Glycogen	−
Utilization of:	
Propionate	+
Valerate	+
Caprate	+
Adipate	−
Malate	+
Acetate	+
Malonate	−
D-Lactate	+
Citrate	+
Itaconate	−
Suberate	−
Gluconate	+
5-Ketogluconate	−
2-Ketogluconate	+
3-Hydroxybutyrate	+
3-Hydroxybenzoate	−
4-Hydroxybenzoate	+
N-Acetylglucosamine	−

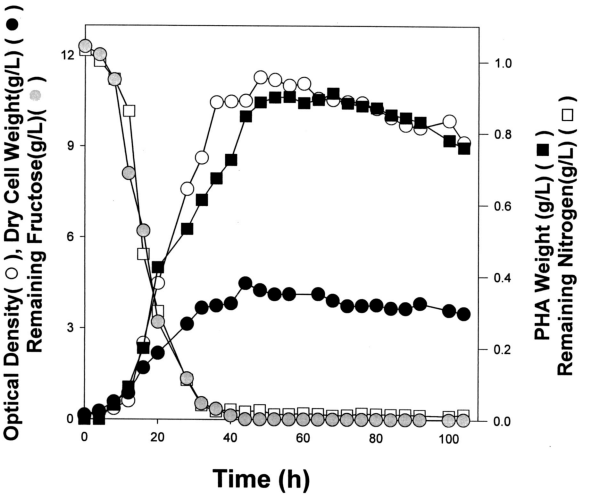

FIG. 1. Time course profiles for growth-associated PHA synthesis in *P. fluorescens* BM07 grown in PHA synthesis mineral salts medium containing 70 mM fructose and 1.0 g of ammonium sulfate/liter at 30°C. PHA formation was monitored by determining the content of PHA in cells by using a sulfuric acid-methanol reaction mixture and GC determination of the resulting 3-hydroxy-methylesters.

(visible in the expanded spectrum [not shown]). For all of the three doublet peaks, the downfield peaks are associated with the saturated monomer units and the upfield ones due to the unsaturated monomer units. Thus, from the peak area ratios of the doublets, the relative amounts of total unsaturated monomers in PHA can be calculated. The percentages of unsaturated monomer units calculated for each doublet to the carbonyl, methine, and backbone methylene were 38.1, 38.8, and 36.0, respectively. The GC-determined mol% of unsaturated monomers in the same PHA sample was 36.8, showing almost the same value as that determined by the ^{13}C-NMR peak area ratio method described above.

GC analysis showed that the PHA synthesized at 30°C from fructose or succinate was principally composed of 36 mol% 3HD and 30 mol% $C_{12:1}$. The third major monomer was 3-hydroxydodecanoic acid (3HDD), which is a reduced form of $C_{12:1}$. The longer monomers $C_{16:1}$ and 3-hydroxyhexadecanoic acid (3HHD) were also detected as minor monomers in the PHA from succinate-grown cells.

PHA synthesis from monocarboxylic acids. The lower aliphatic acids (acetic, propionic, butyric, valeric, and capric acids) supported only cell growth and not PHA accumulation. The dry cell mass was ca. 2 g/liter for the five carbon sources. For the longer carbon sources, heptanoic acid (C_7) to hexadecanoic acid (C_{16}), the monomer composition of PHA depends on the length of substrate molecules (Table 2), similar to what is observed in other pseudomonads belonging to rRNA homology group I (23). For the three carbon sources, C_7, C_8, and C_9 acids, the monomer unit that has the same number of carbon atoms as the carbon source used was a major constituent in the PHA produced. Decanoic acid (C_{10})-grown cells produced the PHA whose major monomer was 3HO, shorter than the substrate by one ethylene unit. For the carboxylic acids longer than C_{10}, 3HO and 3HD almost equally constituted major monomers. In the PHA from the cells grown on C_{10} to C_{16}, the longer monomer 3HDD was incorporated into the polymer at a significant level (17 to 23 mol%). In the cells grown with C_{14} and C_{16}, the relatively long 3-hydroxyacid monomers, 3-hydroxytetradecanoic acid and 3HHD, were also polymerized without being degraded via β-oxidation. The incorporation

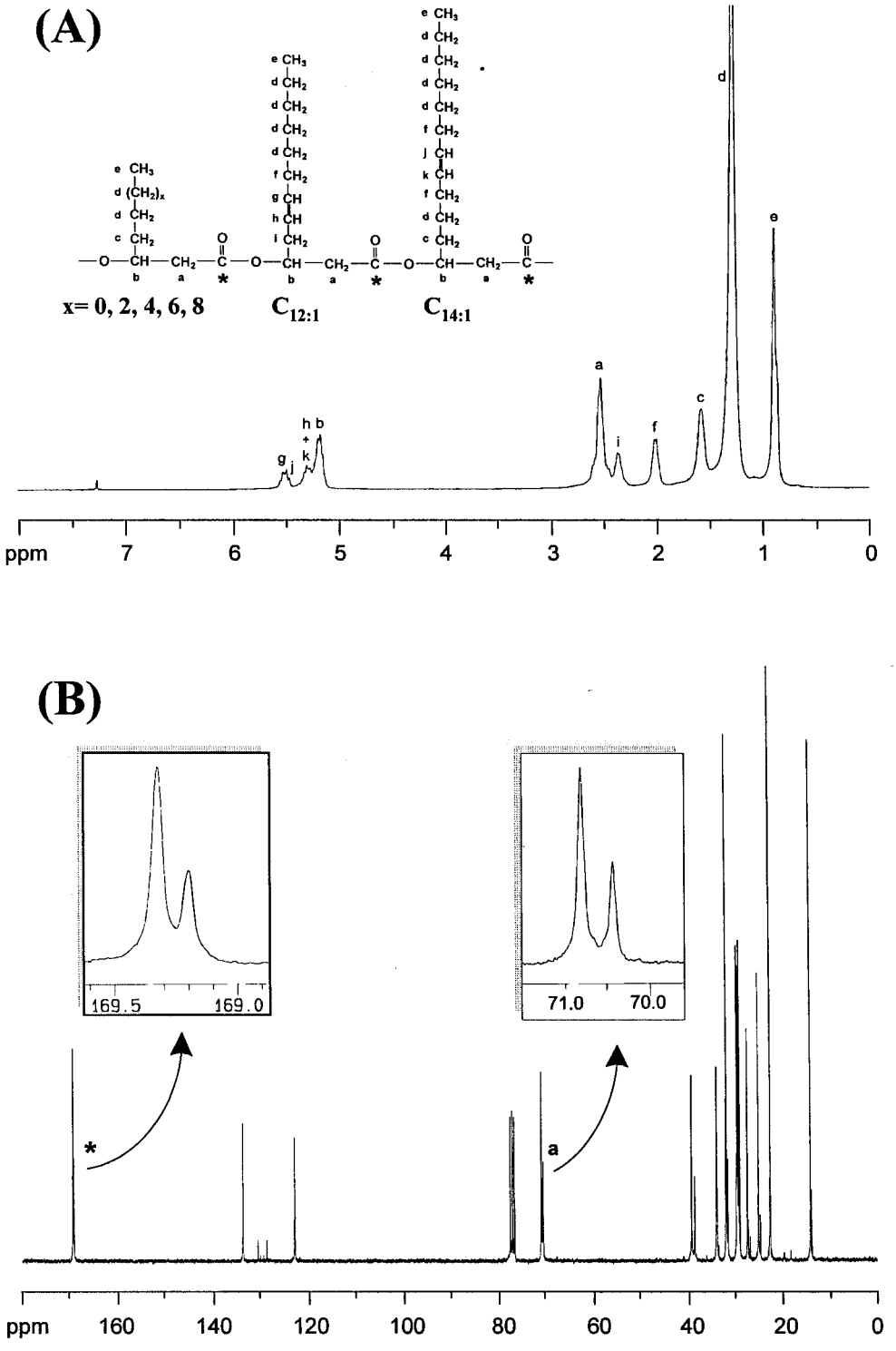

FIG. 2. 500 MHz ^{1}H-NMR spectrum (A) and 125 MHz ^{13}C-NMR spectrum (B) of PHA synthesized by *P. fluorescens* BM07 cells grown on 70 mM fructose at 25°C. For the inserted doublet peaks (B), the downfield peaks are associated with saturated monomer units and the upfield ones are due to unsaturated monomer units ($C_{12:1}$, $C_{14:1}$, etc.). Thus, from the peak area ratios of the doublets, the relative amount of total unsaturated monomers in PHA can be calculated. The calculated percentage of unsaturated monomer units was 38%, a result that agreed well with the percentage determined by GC (37%). See the text for details.

TABLE 2. Biosynthesis of PHA in *P. fluorescens* BM07 from various carbon sources at 30°C[a]

Carbon source	Concn (mM)	Culture time (h)	Dry cell wt (g/liter)	Polyester content (wt%)	Monomer composition (mol%)[b]													Thermal transitions		
					HB	HV	HC	HH	HO	HN	HD	HDD	$C_{12:1}$	HTD	$C_{14:1}$	HHD	$C_{16:1}$	T_g (°C)	T_m (°C)	ΔH (J/g)
Fructose	70	44	4.00	25.2					5.5		37.5	18.2	30.8	3.5	4.5			−52	0	3.7
Galactose	70	48	3.50	24.1					5.8		35.3	19.2	32.6	2.6	4.5					
Mannose	70	48	2.02	9.4					5.2		36.8	19.5	30.9	3.3	4.3					
Glucose	70	48	1.53	8.2					5.3		35.5	19.3	32.5	2.8	4.6					
Arabinose	70	48	2.97	10.4					5.8		35.5	19.3	32.1	3.1	4.2					
Xylose	70	72	1.16	ND[c]																
Sucrose	40	48	3.35	16.1					5.3		37.3	19.6	32.0	2.1	3.7					
Succinate	70	44	2.13	21.2	0.1		1.5		8.4		35.7	16.9	29.8	2.5	4.2	0.6	0.3			
Heptanoate	40	96	2.25	25.1		6.0		90.0		4.0										
Octanoate	40	44	2.47	23.3	0.4		11.8		84.4		1.8	0.4	0.4	0.4	0.1	0.2	0.2	−35	50	19.7
Nonanoate	30	72	2.62	34.3		4.0		30.0		66.0										
Decanoate	25	48	2.29	24.0			8.0		40.0		32.0	20.0								
Dodecanoate	23	50	2.88	38.1			6.1		33.8		32.5	22.8	3.8	0.6	0.4					
Tetradecanoate	20	50	2.70	35.3					38.4		35.6	17.0	2.0	7.0				−41	46	9.3
Hexadecanoate	13	50	2.48	27.0			4.7		32.6		33.7	19.7	2.4	0.8		5.0	1.2	−38	46	14.7
Octadecanoate	10	82	2.42	0																

[a] For the carbon sources such as fructose, glucose, succinate, octanoate, decanoate, and higher carboxylic acids, a minimum of two determinations were made. For the other carbon sources, one determination was made. The data for dry cell weight and PHA content were reproducible within a maximum of ±10%, and those for monomer composition were reproducible within ±5%. The thermal transition data were within ±5% to 10%.

[b] Calculated from the GC data. HB, 3-hydroxybutyric acid; HV, 3-hydroxypentanoic acid; HC, 3-hydroxyhexanoic acid; HH, 3-hydroxyheptanoic acid; HO, 3-hydroxyoctanoic acid, HN, 3-hydroxynonanoic acid; HD, 3-hydroxydecanoic acid; HDD, 3-hydroxydodecanoic acid; $C_{12:1}$, 3-hydroxydodecenoic acid; HTD, 3-hydroxytetradecanoic acid; $C_{14:1}$, 3-hydroxytetradecenoic acid; HHD, 3-hydroxyhexadecanoic acid; $C_{16:1}$, 3-hydroxyhexadecenoic acid.

[c] ND, not detected by GC analysis.

levels were 7 and 5 mol% for 3-hydroxytetradecanoic acid and 3HHD, respectively. It seems highly probable that *P. fluorescens* BM07 could incorporate the monomer with a protected reactive endgroup attached to its terminal into the polymer chain without being modified; the resulting PHA could be used for the production of functional PHA.

Stearic acid (C_{18}) was also tested for PHA production (Table 2). The chloroform extract of the stearic acid-grown cells was reacted in a sulfuric acid-methanol mixture. The resultant methyl esters were analyzed by using GC. Only one major peak appeared at the retention time when the methyl ester of stearic acid itself was detected. No other peaks ascribable to the expected monomers of PHA were observed. Differential scanning calorimetric (DSC) analysis revealed that the dried solid powder recovered from the chloroform extract melted at 69°C (the melting point of pure stearic acid), confirming the presence of stearic acid transported into the cells. Stearic acid is insoluble in water. Therefore, as described in Materials and Methods, the bacterium was cultivated in an emulsive solid stearic acid-liquid slurry medium. It seemed that stearic acid adhered to the cell surface, contributing to the extractable component. However, an ethanol washing of the cells resulted in the same result as for the unwashed cells. It was concluded that stearic acid supported only cell growth, and the transported stearic acid was stored in cells without being chemically modified.

The amount of stearic acid stored in cells was 5% (wt/wt) in dry cells. It was previously reported that crystallizable bacterial inclusions respond to a heating in a DSC cell, exhibiting a melt transition (36). To investigate a possible existence of free stearic acid inclusions, DSC analysis was performed against the dried stearic acid-grown cells. However, no melt transition was observed for the cells, which means that stearic acid may exist in a noncrystallizable form or may be localized near the membrane. For further characterization about the localization of stearic acid in the cell, electron microscopic photographs were taken for the cells grown on palmitic acid (control) and stearic acid (Fig. 3). The palmitate-grown cells clearly demonstrated the presence of large PHA inclusion granules, but the 1-ethylene-unit-longer stearic acid-grown cells revealed tiny electron-transparent inclusions. The electron-dense spots in the two photographs may represent polyphosphate granules (2). However, considering the amount of stearic acid in cells, it is likely that the tiny inclusions represent stearic acid granules. The absence of any melt transition for the dried stearic acid-grown cells suggests that the related granules are impure, probably mixed with other lipid-like molecules. At any rate, from the physiological point of view, it is interesting that *P. fluorescens* BM07 utilizes an economical energy storage method without resorting to the PHA synthesis metabolism.

We do not know why the bacterium is unable to synthesize PHA from stearic acid. For the growth of the cells on stearic acid, first of all, stearic acid should be degraded via the β-oxidation pathway, resulting in metabolites with fewer carbon atoms (C_{16}, C_{14}, etc.). These shortened metabolites could be precursors of PHA. However, it was found that no PHA was formed at all throughout the growth when the cells were grown on 3.4 g of stearic acid/liter for 95 h (data not shown). The growth on stearic acid required at least 36 h of induction period and reached a plateau steady-state region (2.5 g of dry biomass/liter) after 80 h. The accumulation of free stearic acid in cells continued steadily until 64 h, finally reaching 4.5% (wt/wt) of dry cells equivalent to 3.2% of the initial stearic acid. From these data, it can only be speculated now that the stearyl-CoA or one of its C_{18} derivatives acts as an inhibitor for one of the enzymes related to PHA synthesis. Further detailed molecular level study on this substrate-carbon-length-dependent PHA synthesis is under way.

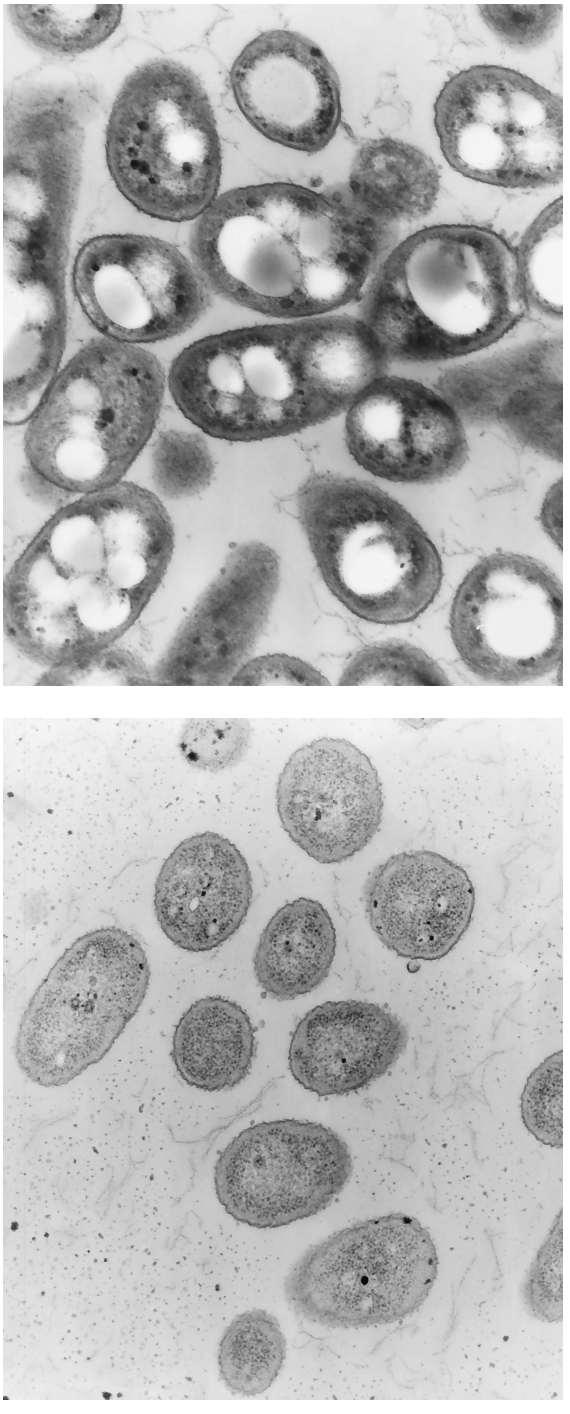

FIG. 3. Electron microscopic photographs of PHA inclusion granules in *P. fluorescens* BM07 cells cultivated with C_{16} (palmitic acid) (top panel) for 50 h and C_{18} (stearic acid) (bottom panel) for 82 h at 30°C. The palmitate-grown cells contained 27% (wt/wt) of dry cells of PHA. The palmitate-grown cells clearly showed the presence of large PHA inclusion granules. However, the stearic acid-grown cells revealed tiny electron-transparent inclusions. The tiny inclusions are considered to probably represent the granules containing free stearic acid (5% [wt/wt] dry cells).

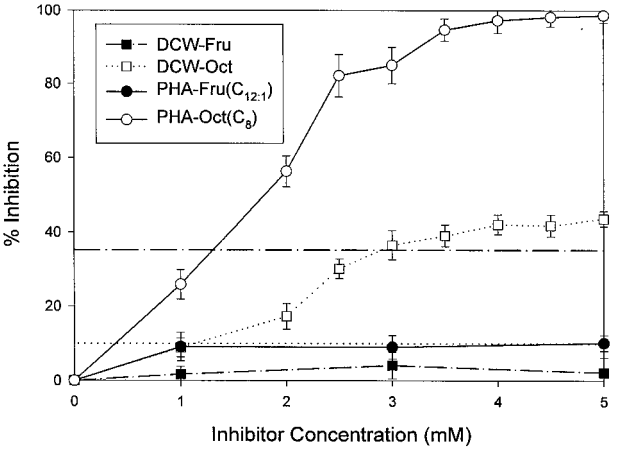

FIG. 4. Inhibition of PHA synthesis (○) and cell growth (□) by acrylic acid when *P. fluorescens* BM07 was grown on 40 mM octanoic acid (open symbols) or 70 mM fructose (solid symbols) at 30°C. NR medium-grown cells were transferred to PHA synthesis medium containing the indicated level of acrylic acid and were grown for 48 h. Cell growth was determined by measuring the dry cell weight (DCW). The calculation of the percent inhibition of PHA synthesis was based on the change of the signal intensities of two strong GC peaks, a $C_{12:1}$ peak for fructose-grown cells and a 3-HO peak for octanoate-grown cells. Minimum triplicate experiments were carried out and statistically averaged. The percent inhibitions for PHA accumulation and cell growth are defined in Materials and Methods. The limiting value corresponding to the theoretically allowed maximum percent inhibition of cell growth was calculated to be 35% for fructose-grown (— · — · — · —) and 10% for octanoate-grown (· · · · · · · ·) cells. Thus, the calculated apparent percent inhibition (virtually not normalized) of cell growth exceeding the limiting value was considered to be 100% inhibition of cell growth.

Thermal properties of PHA. Table 2 also shows thermal transition data for PHA synthesized in *P. fluorescens* BM07. The precipitated and dried polymer samples were fully crystallized at an ambient temperature. The PHA prepared from fructose had a lower glass transition temperature (T_g) than that from octanoic acid by 17°C. It did not exhibit a melt transition ca. 50°C, whereas the PHA from carboxylic acid-grown cells did. The absence of the melt may be caused by the *cis*-type olefinic bonds in the side chains (e.g., $C_{12:1}$ and $C_{14:1}$ monomers) perturbing side chain packing for crystallization (27). These planar olefin bonds, however, may contribute to a lowering of T_g, resulting from an increased side chain mobility. A small endothermic peak observed at 0°C for the first-run sample of the PHA from fructose-grown cells appeared again during the reheating process after the first run scanned up to 150°C. Thus, the melting peak at 0°C may be ascribed to a structural characteristic of the PHA and not to an artifact. The polyesters prepared from C_{14} and C_{16} substrates have a higher percentage of longer monomers, 3HD and 3HDD (total, 50 mol%), than that from octanoic acid. However, the melting point of the longer side chain PHA is not significantly different from that of the 3-HO-dominant PHA, which means that the uniformity of side chain length may not be a principal factor in determining the crystallinity of MCL PHA.

Inhibition of PHA synthesis and cell growth by inhibitors. The dependence of inhibitor concentration on PHA synthesis and cell growth was investigated for all three inhibitors: acrylic

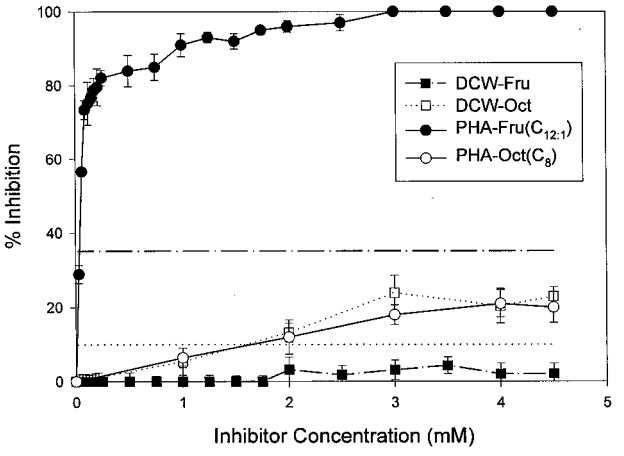

FIG. 5. Inhibition of PHA synthesis (○) and cell growth (□) by 2-bromooctanoic acid when *P. fluorescens* BM07 was grown on 40 mM octanoic (open symbols) acid or 70 mM fructose (solid symbols). The limiting value corresponding to the theoretically allowed maximum percent inhibition of cell growth was calculated to be 35% for fructose-grown (— · — · — · —) and 10% for octanoate-grown (· · · · · · ·) cells. Thus, the calculated apparent percent inhibition (virtually not normalized) of cell growth exceeding the limiting value was considered to be 100% inhibition of cell growth. See the legend in Fig. 4 for the other details.

acid, 2-bromooctanoic acid, and 4-pentenoic acid (Fig. 4, 5, and 6). The addition of an inhibitor to a cell growth medium could affect the growth and PHA accumulation kinetics of *P. fluorescens* BM07. Therefore, in order to compare the inhibitory effects of the three inhibitors in terms of strength and specificity, it is essential to determine the sampling time for each culture exhibiting a steady-state maximum cell growth

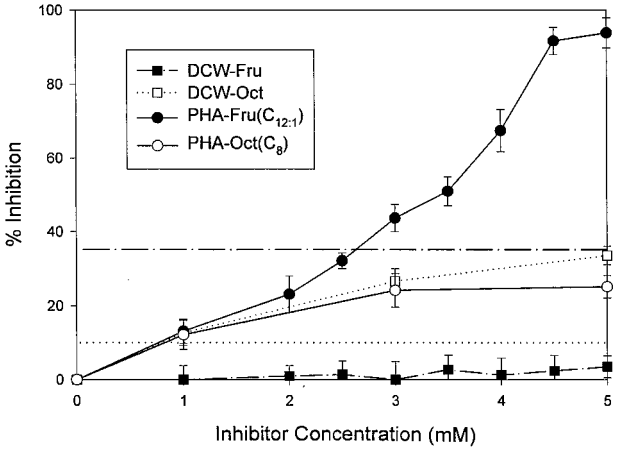

FIG. 6. Inhibition of PHA synthesis (○) and cell growth (□) by 4-pentenoic acid when *P. fluorescens* BM07 was grown on 40 mM octanoic acid (open symbols) or 70 mM fructose (solid symbols). The limiting value corresponding to the theoretically allowed maximum percent inhibition of cell growth was calculated to be 35% for fructose-grown (— · — · — · —) and 10% for octanoate-grown (· · · · · · ·) cells. Thus, the calculated apparent percent inhibition (virtually not normalized) of cell growth exceeding the limiting value was considered to be 100% inhibition of cell growth. See the legend in Fig. 4 for the other details.

and PHA synthesis in the presence of inhibitor. As shown in Fig. 1, the increasing patterns of the time course profiles of optical density (OD), cell yield, and PHA content are in parallel with one another. Furthermore, it was reported that an increase in PHA content linearly and significantly increased the OD of a nitrogen-free culture medium in which *R. eutropha* H16 was grown (33). Thus, a steady-state plateau OD value must be attributed to both maximal cell growth and PHA accumulation. Thus, we investigated the time courses for the growth of NR medium-grown cells on fructose in the presence of 5 mM acrylic acid, 1 mM 2-bromooctanoic acid, or 3 mM 4-pentenoic acid and on octanoate in the presence of 3 mM acrylic acid, 3 mM 2-bromooctanoic acid, or 3 mM 4-pentenoic acid (data not shown). For fructose-grown cells, none of the three inhibitors shifted the time to reach the plateau OD compared to the control growth experiment without inhibitor (the four OD profiles almost exactly overlapped one another). For octanoate-grown cells, the OD profiles for the three inhibitor-treated cells were shifted by 10 h compared to the inhibitor-free control experiment, due to the almost equally increased induction periods. However, the time leading to the plateau OD was in a similar range 45 to 50 h. Thus, both the maximal cell growth and PHA synthesis were considered to occur within 42 to 48 h of cultivation for all experiments. Therefore, the sampling was made after 48 h of cultivation for all inhibition experiments.

Acrylic acid completely inhibited PHA accumulation at 3.5 mM or higher concentrations in cell suspensions containing 40 mM octanoic acid as the sole substrate (Fig. 4). The inhibiting level of acrylic acid is similar to that reported for *P. putida* KT2442 (18). The calculated apparent percent inhibition of cell growth exceeded the limiting value of 10% at ca. 1.5 mM acrylic acid, where the limiting value for octanoate-grown cells is a theoretically allowed maximum value calculated from $[(3.098\text{ g of PHA-free dry cell mass/liter})_{control} - (\text{g of PHA-free dry cell mass/liter})_{inhibitor}]/(3.098\text{ g of PHA-free dry cell mass/liter})_{control}$ as defined in Materials and Methods. The value of $(\text{grams of PHA-free dry cell mass/liter})_{inhibitor}$ cannot be theoretically less than 2.79 g/liter because it was the amount of NR medium-grown cells equally added to each culture medium containing a certain level of inhibitor. Thus, in the case of octanoate-grown cells, the limiting value was calculated to be $[(3.098 - 2.79)/3.098] \times 100 = 10\%$. The calculated percent inhibition higher than the limiting value 10% therefore means that cell growth was completely inhibited at concentrations of acrylic acid exhibiting a percent inhibition of >10% (e.g., 1.5 mM or higher). The gradual, significant increase in the apparent percent inhibition over the limiting value at the concentrations of acrylic acid higher than 2.5 mM was considered mostly due to a remarkable cell lysis causing a large cell loss during recovery. At any rate, complete inhibition of PHA synthesis and cell growth occurred at 3.5 and 1.5 mM acrylic acid, respectively, when the cells were grown on octanoic acid. This indicates that, at acrylic acid concentrations between 1.5 and 3.5 mM, PHA synthesis was still occurring, whereas cell growth stopped. However, for the cells grown with 70 mM fructose, 10% or less inhibition of PHA synthesis and negligible cell growth inhibition compared to the limiting value of 35% (calculated similarly for octanoate grown cells described above) were observed irrespective of the acrylic acid concentration.

TABLE 3. Catabolism of three inhibitors, acrylic acid, 2-bromooctanoic acid, and 4-pentenoic acid, after 48 h of second-step cultivation at 30°C

Inhibitor	Carbon source[a]	Initial concn (mM ± SD) of inhibitor in medium	Concn (mM ± SD) of inhibitor remaining in medium after 48 h	% Inhibitor catabolized after 48 h
Acrylic acid	Fructose	1.93	ND[b]	100
		3.95	ND	100
	Octanoate	2.07	ND	100
		3.67	ND	100
		5.22	ND	100
2-Bromooctanoic acid	Fructose	0.237 ± 0.009	0.168 ± 0.015	29
		1.311 ± 0.021	1.214 ± 0.056	8
		2.497 ± 0.022	2.409 ± 0.031	4
	Octanoate	2.521 ± 0.040	2.471 ± 0.038	2
		5.368 ± 0.274	5.328 ± 0.285	1
4-Pentenoic acid	Fructose	3.42	ND	100
		4.96	ND	100
	Octanoate	3.62	ND	100
		4.99	ND	100

[a] The fed concentration was 70 mM for all experiments with fructose, and the fed concentration was 40 mM for all experiments with octanoate. All media initially contained 1 g of ammonium sulfate/liter.
[b] ND, not detected.

This indicates that acrylic acid had little effect on the catabolism of fructose and the consequent cell growth and PHA accumulation. Thus, the PHA synthesis in the bacterium from fructose does not occur via the β-oxidation pathway, whereas the PHA synthesis from octanoic acid strictly occurs via the β-oxidation pathway (18, 25).

Figure 5 shows that 2-bromooctanoic acid strongly inhibited PHA-synthesis in *P. fluorescens* BM07 cells incubated on 70 mM fructose. It exhibited a steep rise in the percent inhibition of PHA synthesis over a small range of concentration below 100 μM. The inhibition profile takes the shape of a hyperbolic curve with a high curvature. The K_i value was calculated to be 60 μM, assuming a single-site binding of the inhibitor at a specific inhibition site. The complete inhibition occurred at 2 mM or higher. However, 2-bromooctanoic acid negligibly inhibited cell growth over the concentration range studied. This means that 2-bromooctanoic acid specifically inhibits PHA synthesis from fructose, but the supplying route of acetyl-CoA for cell growth is never inhibited. For the cells grown on 40 mM octanoic acid, an increase in the concentration of 2-bromooctanoic acid also increased the percent inhibition of PHA synthesis but led to only a maximum 20% inhibition at 4 mM as shown in Fig. 5. However, for octanoate-grown cells, the apparent percent inhibition of cell growth was calculated to exceed the limiting value of 10% (the theoretically maximum allowed inhibition) at 1.5 mM 2-bromooctanoate. This means that, when the cells are grown on octanoate in the presence of ≥1.5 mM 2-bromooctanoic acid, cell growth was completely inhibited, whereas PHA synthesis was still occurring. All these results suggest that 2-bromooctanoate acts as a weak inhibitor for the β-oxidation enzyme(s), whereas it acts as a strong inhibitor for the enzyme(s) involved in the pathway leading to PHA synthesis from fructose.

4-Pentenoic acid almost completely inhibited PHA accumulation in cell suspensions containing fructose as the substrate at the concentration of 4.5 mM or higher (Fig. 6). At lower concentrations, it showed a severely depressed concentration dependence of inhibition compared to 2-bromomooctanoic acid. However, similar to findings observed with 2-bromooctanoic acid, 4-pentenoic acid did not substantially inhibit cell growth over the concentration range of 1 to 5 mM. In the case of cell suspensions containing octanoic acid, 4-pentenoic acid exhibited only a maximum of 20% inhibition of PHA synthesis, a result similar to that for 2-bromooctanoic acid. When the level of 4-pentenoic acid was increased to 5 mM, the apparent percent inhibition of cell growth was calculated to be 30%, far exceeding the threshold value 10% described above. This high apparent percent inhibition of cell growth over the limiting value can be explained in the same way as described above for acrylic acid and 2-bromooctanoic acid. At any rate, it is thought that, when grown on octanoic acid, 4-pentenoate almost completely inhibited cell growth over the concentration range tested (1 to 5 mM). Thus, both 2-bromooctanoic acid and 4-pentenoic acid have significantly different characteristics in the inhibition of PHA synthesis in *P. fluorescens* BM07 grown on fructose, a finding indicative of totally different inhibition mechanisms.

Metabolism of three inhibitors in PHA synthesis medium. Analysis of the metabolic fates of the three inhibitors must be helpful for understanding the above inhibition profiles in terms of their specificities. Thus, the catabolism of the three inhibitors in *P. fluorescens* BM07 was investigated (Table 3). NR medium-grown cells were cultured in PHA synthesis medium with an appropriate level of an inhibitor. The medium contained 70 mM fructose or 40 mM octanoate as a carbon source and 1 g of ammonium sulfate/liter. The inhibitor remaining in medium was analyzed by GC. When added at millimolar concentrations, both acrylic acid and 4-pentenoic acid completely disappeared after 48 h of cultivation. On the contrary, 2-bromooctanoic acid was catabolized very little in both fructose and octanoic acid medium. In addition, the disappearance of 2-bromooctanoic acid is a function of the initial fed concentration, a result strongly suggestive of highly specific inhibition by one of its metabolites. However, none of the three inhibitors were

utilized by *P. fluorescens* BM07 when they were fed as a sole carbon source at millimolar levels (1 to 5 mM). Acrylic acid is known to be metabolized to CO_2 and acetyl-CoA via a secondary pathway of propionic acid catabolism in mouse tissues (6). Thus, 3-hydroxypropionic acid could be a probable inhibitor. However, 3-hydroxypropionic acid did not affect PHA accumulation from both octanoic acid and fructose at the level of 5 mM. Its ineffectiveness may be due to no transportation of the molecule into the cell because *P. fluorescens* BM07 did not grow on 40 mM 3-hydroxypropionic acid as a sole carbon source.

DISCUSSION

High production of unsaturated 3-hydroxy acids from saccharides. As far as we know, this is the first report of a bacterial strain capable of producing such a high amount of the unsaturated monomers 3-hydroxy-*cis*-5-dodecenoate ($C_{12:1}$) and 3-hydroxy-*cis*-7-tetradecenoate ($C_{14:1}$) from unrelated carbon sources such as saccharides. Hence, it is suggested that this strain can be used as a producer of the two unsaturated fatty acids, which presumably are useful for pharmaceutical applications, from cheap saccharides. We do not know at present why the bacterium produces such high levels of these unsaturated 3-hydroxy acids from saccharides. However, in light of the good growth at 5°C, the high level of production of the unsaturated 3-hydroxy acids may be related to a means for survival in cold environments. This idea was supported by our preliminary experimental finding that a lowering of the cultivation temperature led to an increase in the level of unsaturated derivatives in PHA, as well as in membrane lipids (data not shown).

Acrylic acid and 4-pentenoic acid are multiple-site PHA synthesis inhibitors. The three inhibitors exhibited different inhibitor concentration dependencies on PHA synthesis. This may imply that their curve shapes strongly reflect their catabolism behavior. In particular, the inhibition curves for acrylic acid and 4-pentenoic acid indicate a probable existence of multiple-site inhibition by one inhibitory species or more than one different species with different K_i values. As shown in Table 3, acrylic acid and 4-pentenoic acid were completely catabolized after 48 h of cultivation. Thus, it is probable that two or more metabolites contribute to their inhibition. Acrylic acid is known to be metabolized to CO_2 and acetyl-CoA via a catabolic pathway of propionic acid in mouse tissues (6). However, since a probable intermediate 3-hydroxypropionic acid was found to be unable to inhibit PHA synthesis, we propose one other intermediate species may inhibit two different enzymes. Thus, the sigmoidal shape of the percent PHA synthesis inhibition curve for acrylic acid (Fig. 4) suggests that acrylic acid, probably in the form of CoA, has at least two target enzymes for inhibiting both PHA accumulation and cell growth. Acrylic acid is known to inhibit the 3-ketoacyl-CoA thiolase, which catalyzes the final step of β-oxidation, i.e., the release of acetyl-CoA from 3-ketoacyl-CoA (18). It is also known to inhibit acyl-CoA synthase (18). Such sigmoidal inhibition may indicate a dual inhibition for two different enzymes with different K_i values by acrylic acid. However, in recombinant strains such as *E. coli* (*fadR*), acrylic acid was not significantly catabolized (29). Compared to the case of acrylic acid, 4-pentenoic acid showed no such characteristic concentration dependence on PHA synthesis from fructose but rather demonstrated a featureless dependence. 4-Pentenoic acid is known to be metabolized to 2,4-pentadienyl-CoA, 3-keto-4-pentenoyl-CoA, etc. 3-Keto-4-pentenoyl-CoA acts both as a reversible and as an irreversible inhibitor of the 3-ketoacyl-CoA thiolase in rat heart mitochondria (32). 3-Keto-4-pentenoyl-CoA is known as a rather unspecific inhibitor because it inhibits other enzymes such as carnitine acetyltransferase and acetoacetyl-CoA thiolase. (7, 40). The monotonously increasing and broad featureless inhibition curve may thus indicate the presence of at least two or more enzymes interacting with the intermediates derived from 4-pentenoic acid.

4-Pentenoic acid and 2-bromooctanoic acid inhibit the enzyme(s) linking the two pathways, fatty acid synthesis and PHA synthesis. The addition of 4 to 5 mM acrylic acid to fructose medium caused little effect on PHA synthesis and cell growth, but its addition to octanoic acid medium completely inhibited both PHA synthesis and cell growth. This means that most CoA monomers necessary for PHA synthesis in fructose-grown cells may be directly supplied via the route linking fatty acid synthesis and PHA synthesis. It is recently known that the enzyme (*R*)-3-hydroxylacyl-ACP-CoA transferase converting (*R*)-3-hydroxylacyl-ACP to (*R*)-3-hydroxylacyl-CoA plays the role in linking the two pathways (14, 17, 31). 4-Pentenoic acid is known to be a β-oxidation inhibitor in animal cells, as described previously. However, 4-pentenoic acid inhibited PHA synthesis strongly in fructose-grown *P. fluorescens* BM07 cells but only weakly in octanoate-grown cells. This indicates that one of the derived intermediates may act as a strong inhibitor for the enzyme involved in the linkage between fatty acid synthesis and PHA synthesis.

Compared to 4-pentenoic acid, 2-bromooctanoic acid more strongly and more specifically inhibited PHA synthesis in fructose-grown cells at micromolar concentrations in the hundreds. However, like 4-pentenoic acid, 2-bromooctanoic acid had little effect on cell growth. This means that the carbon flow for cell growth in fructose medium is not blocked by 2-bromooctanoic acid. This very specific PHA synthesis inhibition strongly suggests that an inhibitor molecule derived from 2-bromooctanoic acid specifically targets the enzyme bridging the two pathways, PHA synthesis and fatty acid synthesis, essential for cell growth. The relatively small effect of 2-bromooctanoic acid on PHA synthesis from octanoic acid implies that the enzyme(s) in the β-oxidation pathway is affected very weakly. Little inhibition of cell growth in fructose medium by 2-bromooctanoic acid implies that the enzymes in the fatty acid de novo synthesis pathway are not target enzymes. 2-Bromooctanoic acid is converted to 2-bromooctanoyl-CoA and 2-bromo-3-ketooctanoyl-CoA in rat liver mitochondria (30). However, 2-bromo-3-ketooctanoyl-CoA is the specific inhibitor for the β-oxidation enzyme 3-ketothiolase I (acyl-CoA–acetyl-CoA *C*-acyltransferase). The fed concentration-dependent and insignificant decrease in the concentration of 2-bromooctanoate during cultivation (Table 3) suggests that one of the two CoA intermediates acts as an inhibitor against the bridging enzyme in *P. fluorescens* BM07 without further catabolism of the 3-keto-CoA. Especially, in fructose-grown cells, an increase in the level of 2-bromooctanoic acid resulted in a drastic decrease in the percentage of the inhibitor catab-

olized. Since the number of cells (approximated as the PHA-free dry cell mass) grown with fructose was relatively constant in spite of the increase in the inhibitor level as seen in Fig. 5, the fed concentration-dependent metabolism of 2-bromooctanoic acid may reflect a constant number of the inhibitor molecules converted in the cell. This may additionally account for the high specificity of the probable species against the target enzyme.

Both ACP and CoA have a common prosthetic group, phosphopantetheine, to which acyl moieties are bound (13). An acyl-ACP-CoA transferase may have a conformationally flexible binding motif into which acyl-ACP and acyl-CoA could be fitted in a stepwise manner. The inhibiting CoA-type molecule derived from 2-bromooctanoic-acid (e.g., 2-bromo-3-keto-octanoyl-CoA) could be fitted into the binding cleft. The target enzyme of the inhibiting species could thus be (*R*)-3-hydroxylacyl-ACP-CoA transferase. Further genetic and enzymatic studies are under way to elucidate this in more detail. In any case, it can be concluded that a derivative from 2-bromooctanoic acid specifically inhibits a key enzyme involved in the supplying route of PHA monomer precursors generated via the fatty acid synthesis pathway.

2-Bromooctanoic acid is more potent and specific than cerulenin. Cerulenin inhibits the growth of *P. putida* KT2442 at 1.34 mM (18). It binds to the active sites of keto-acyl-ACP synthases I and II in the fatty acid synthesis pathway. In glucose-grown cells cerulenin completely inhibited PHA synthesis. Cerulenin also inhibited PHA synthesis by up to 69% in the cells grown on 10 mM octanoate. However, both 4-pentenoic acid and 2-bromooctanoic acid inhibited PHA synthesis by only ~20% in *P. fluorescens* BM07 grown on octanoic acid. Thus, 2-bromooctanoic acid may inhibit PHA synthesis more specifically than cerulenin. It is therefore suggested that 2-bromooctanoic acid can substitute for the more expensive (2,000 times) and less-specific (cell growth-inhibiting) inhibitor, cerulenin, in the inhibitor study of PHA synthesis.

ACKNOWLEDGMENTS

S.C.Y. acknowledges the financial support of the Korea Research Foundation (KRF) made in the program years 1997 and 2000 (KRF-2000-0157DS0038). H.-J.L. was supported by a graduate scholarship through the BK21 program to KRF. M.H.C. acknowledges a postdoctoral fellowship provided through the BK21 program to KRF.

We thank the reviewers for helpful suggestions.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Alvarez, H. M., O. H. Pucci, and A. Steinbüchel.** 1997. Lipid storage compounds in marine bacteria. Appl. Microbiol. Biotechnol. **47:**132–139.
3. **Anderson, A. J., and E. A. Dawes.** 1990. Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. **54:**450–472.
4. **Ashby, R. D., T. A. Foglia, C. K. Liu, and J. W. Hampson.** 1998. Improved film properties of radiation-treated medium-chain-length poly(hydroxyalkanoates). Biotech. Lett. **20:**1047–1052.
5. **Ashby, R. D., T. A. Foglia, D. K. Y. Solaiman, C. K. Liu, A. Nuñez, and G. Eggink.** 2000. Viscoelastic properties of linseed oil-based medium chain length poly(hydroxyalkanoate) films: effects of epoxidation and curing. Int. J. Biol. Macromol. **27:**355–361.
6. **Black, K. A., L. Finch, and C. B. Frederick.** 1993. Metabolism of acrylic acid to carbon dioxide in mouse tissues. Fundamental Appl. Toxicol. **21:**97–104.
7. **Boulanger, Y., H. Wong, J. Noel, J. Senecal, A. Fleser, A. Gougoux, and P. Vinay.** 1993. Heterogeneous metabolism and toxicity of 4-pentenoate along the dog nephron. Ren. Physiol. Biochem. **16:**182–202.
8. **Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller.** 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. **54:**1977–1982.
9. **Choi, M. H., and S. C. Yoon.** 1994. Polyester biosynthesis characteristics of *Pseudomonas citronellolis* grown on various carbon sources, including 3-methyl-branched substrates. Appl. Environ. Microbiol. **60:**3245–3254.
10. **Choi, M. H., S. C. Yoon, and R. W. Lenz.** 1999. Production of poly(3-hydroxybutyric acid-co-4-hydroxybutyric acid) and poly(4-hydroxybutyric acid) without subsequent degradation by *Hydrogenophaga pseudoflava*. Appl. Environ. Microbiol. **65:**1570–1577.
11. **Collins, M. D., S. Cockcroft, and S. Wallbanks.** 1994. Phylogenetic analysis of a new LL-diaminopimelic acid-containing coryneform bacterium from herbage *Nocardioides plantarum* sp. nov. Int. J. Syst. Bacteriol. **44:**523–526.
12. **de Waard, P., H. van der Wal, G. N. M. Huijberts, and G. Eggink.** 1993. Heteronuclear NMR analysis of unsaturated fatty acids in poly(3-hydroxyalkanoates). J. Biol. Chem. **268:**315–319.
13. **Elovson, J., and D. Vegelos.** 1968. Acyl carrier protein. X. Acyl carrier protein synthetase. J. Biol. Chem. **243:**3603–3611.
14. **Fiedler, S., A. Steinbüchel, and B. H. A. Rehm.** 2000. PhaG-mediated synthesis of poly(3-hydroxyalkanoates) consisting of medium-chain-length constituents from nonrelated carbon sources in recombinant *Pseudomonas fragi*. Appl. Environ. Microbiol. **66:**2117–2124.
15. **Fritzsche, K., R. W. Lenz, and R. C. Fuller.** 1990. Production of unsaturated polyesters by *Pseudomonas oleovorans*. Int. J. Biol. Macromol. **12:**85–91.
16. **Haywood, G. W., A. J. Anderson, D. F. Ewing, and E. A. Dawes.** 1990. Accumulation of a polyhydroxyalkanoate containing primarily 3-hydroxydecanoate from simple carbohydrate substrates by *Pseudomonas* sp. strain NCIMB 40135. Appl. Environ. Microbiol. **56:**3354–3359.
17. **Hoffmann, N., A. Steinbüchel, and B. H. A. Rehm.** 2000. Homologous functional expression of cryptic *phaG* from *Pseudomonas oleovorans* establishes the transacylase-mediated polyhydroxyalkanoate biosynthetic pathway. Appl. Microbiol. Biotechnol. **54:**665–670.
18. **Huijberts, G. N., T. C. de Rijk, P. de Waard, and G. Eggink.** 1994. ^{13}C nuclear magnetic resonance studies of *Pseudomonas putida* fatty acid metabolic routes involved in poly(3-hydroxyalkanoate) synthesis. J. Bacteriol. **176:**1661–1666.
19. **Huijberts, G. N. M., G. Eggink, P. de Waard, G. W. Huisman, and B. Witholt.** 1992. *Pseudomonas putida* KT2442 cultivated on glucose accumulates poly(3-hydroxyalkanoates) consisting of saturated and unsaturated monomers. Appl. Environ. Microbiol. **58:**536–544.
20. **Huisman, G. W., E. Wonink, R. Meima, B. Kazemier, P. Terpstra, and B. Witholt.** 1991. Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*. J. Biol. Chem. **266:**2191–2198.
21. **Kim, D. Y., Y. B. Kim, and Y. H. Rhee.** 1998. Bacterial poly(3-hydroxyalkanoates) bearing carbon-carbon triple bonds. Macromolecules **31:**4760–4763.
22. **Kim, Y. B., R. W. Lenz, and R. C. Fuller.** 1995. Poly-3-hydroxyalkanoates containing unsaturated repeating units produced by *Pseudomonas oleovorans*. J. Polymer Sci. Polymer Chem. **33:**1367–1374.
23. **Krieg, N. R., and J. G. Holt.** 1984. Gram-negative aerobic rods and cocci, p. 140. *In* R. G. E. Murray and D. J. Brenner (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore, Md.
24. **Lageveen, R. G., G. W. Huisman, H. Preusting, P. Ketelaar, G. Eggink, and B. Witholt.** 1988. Formation of polyesters by *Pseudomonas oleovorans* : Effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates) and poly-(*R*)-3-hydroxyalkenoates. Appl. Environ. Microbiol. **54:**2924–2932.
25. **Madison, L. L., and G. W. Huisman.** 1999. Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiol. Mol. Biol. Rev. **63:**21–53.
26. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3:**208–218.
27. **Preusting, H., A. Nijenhuis, and B. Witholt.** 1990. Physical characteristics of poly(3-hydroxyalkanoates) and poly(3-hydroxyalkenoates) produced by *Pseudomonas oleovorans* grown on aliphatic hydrocarbons. Macromolecules **23:**4220–4224.
28. **Price, A. C., K. H. Choi, R. J. Heath, Z. Li, S. W. White, and C. O. Rock.** 2001. Inhibition of β-ketoacyl-[acyl carrier protein] synthases by thiolactomycin and cerulenin: structure and mechanism. J. Biol. Chem. **276:**6551–6559.
29. **Qi, Q., A. Steinbüchel, and B. H. A. Rehm.** 1998. Metabolic routing towards polyhydroxyalkanoic acid synthesis in recombinant *Escherichia coli* (*fadR*): inhibition of fatty acid β-oxidation by acrylic acid. FEMS Microbiol. Lett. **167:**89–94.
30. **Raaka, B. M., and J. M. Lowenstein.** 1979. Inhibition of fatty acid oxidation by 2-bromooctanoate. J. Biol. Chem. **254:**6755–6762.
31. **Rehm, B. H. A., N. Kröger, and A. Steinbüchel.** 1998. A new metabolic link between fatty acid synthesis and polyhydroxyalkanoic acid synthesis. J. Biol. Chem. **273:**24044–24051.
32. **Schulz, H.** 1983. Metabolism of 4-pentenoic acid and inhibition of thiolase by metabolites of 4-pentenoic acid. Biochemistry **22:**1827–1832.
33. **Song, J. J., Y. C. Shin, and S. C. Yoon.** 1993. P(3HB) accumulation in *Alcaligenes eutrophus* H16 (ATCC 17699) under nutrient-rich condition and

its induced production from saccharides and their derivatives. J. Microbiol. Biotechnol. **3:**115–122.
34. **Song, J. J., and S. C. Yoon.** 1994. Isolation of *Pseudomonas putida* BM01 accumulating high amount of MCL-PHA. J. Microbiol. Biotechnol. **4:**126–133.
35. **Song, J. J., and S. C. Yoon.** 1996. Biosynthesis of novel aromatic copolyesters from insoluble 11-phenoxyundecanoic acid by *Pseudomona putida* BM01. Appl. Environ. Microbiol. **62:**536–544.
36. **Song, J. J., S. C. Yoon, S. M. Yu, and R. W. Lenz.** 1998. Differential scanning calorimetric study of poly(3-hydroxyoctanoate) inclusions in bacterial cells. Int. J. Biol. Macromol. **23:**165–173.
37. **Ulmer, H. W., R. A. Gross, M. Posada, P. Weisbach, R. C. Fuller, and R. W. Lenz.** 1994. Bacterial production of poly(β-hydroxyalkanoates) containing unsaturated repeating units by *Rhodospirillum rubrum*. Macromolecules **27:** 1675–1679.
38. **van der Walle, G. A. M., G. J. H. Buisman, R. A. Weusthuis, and G. Eggink.** 1999. Development of environmentally friendly coatings and paints using medium-chain-length poly(3-hydroxyalkanoate) as the polymer binder. Int. J. Biol. Macromol. **25:**123–128.
39. **Yoon, S. C., and M. H. Choi.** 1999. Local sequence dependence of polyhydroxyalkanoic acid degradation in *Hydrogenophaga pseudoflava*. J. Biol. Chem. **274:**37800–37808.
40. **Zhong, J., J. C. Fong, and H. Schulz.** 1985. Inhibition of carnitine acetyltransferase by metabolites of 4-pentenoic acid. Arch. Biochem. Biophys. **240:**524–529.