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The synthesis of short- and medium-chain-length poly(hydroxyalkanoate) mixtures from glucose- or alkanoic acid-grown *Pseudomonas oleovorans*

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***Pseudomonas oleovorans* NRRL B-778 accumulated mixtures of poly-3-hydroxybutyrate (PHB) and medium-chain-length poly(hydroxyalkanoates) (*mcl*-PHAs) when grown on glucose, octanoic acid or oleic acid, whereas growth on nonanoic acid or undecanoic acid resulted in copolymers of poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate (PHB-*co*-HV). Acetone fractionation verified the presence of PHB/*mcl*-PHA mixtures. The acetone-insoluble (AIS) fractions of the polymers derived from glucose (PHA-glucose), octanoic acid (PHA-octanoic) and oleic acid (PHA-oleic) were exclusively PHB while the acetone-soluble (AS) fractions contained *mcl*-PHA composed of differing ratios of 3-hydroxy-acid monomer units, which ranged in chain length from 6 to 14 carbon atoms. In contrast, both the AIS and AS fractions from the polymers derived from nonanoic acid (PHA-nonanoic) and undecanoic acid (PHA-undecanoic) were composed of comparable ratios of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV). The unfractionated PHA-glucose, PHA-octanoic and PHA-oleic polymers had melting temperatures (T_m) between 177 and 179°C, enthalpies of fusion ($\triangle H_f$) of 20 cal/g and glass transition temperatures (T_g) of 3–4°C. This was due to the large PHB content in the polymer mixtures. On the other hand, the PHA-nonanoic and PHA-undecanoic polymers had thermal properties that supported their copolymer nature. In both cases, the T_m values were 161°C, $\triangle H_f$ values were 7 cal/g and T_g values were −3°C.**
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Keywords: *Pseudomonas oleovorans*; poly(hydroxyalkanoates); PHA mixtures; fatty acids

Introduction

Poly-3-hydroxyalkanoates (PHAs) are poly-β-esters that naturally accumulate within many species of bacteria as carbon and energy reserves when grown under nutrient-limited conditions. Presently, PHA polymers exist in over 100 different structural variations ranging from crystalline, containing short alkyl side chains (e.g., poly-3-hydroxybutyrate, PHB; poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate, PHB-*co*-HV) to amorphous, with side chains containing 3-hydroxy-acid monomer units ranging in length from 6 to 14 carbon atoms (medium-chain-length PHA; *mcl*-PHA) [18].

Many species of *Pseudomonas* belonging to the rRNA homology group I produce PHA from a variety of feedstocks including free fatty acids [4,9,12], triacylglycerols [1,3,6] and sugars [3,8]. When grown on these substrates, the organisms generally produce PHA polymers that contain medium-length alkyl side chains, which make them tacky and unsuitable for structural or fiber applications. In contrast, *Ralstonia eutropha* produces short-chain-length PHA (*scl*-PHA) when grown on appropriate substrates [5,7,10,11]. This contrast in PHA polymer types indicates that the synthase enzymes involved in the polymerization reactions exhibit different substrate specificities that are mutually exclusive, making it difficult to produce natural copolymers consisting of *scl*- and *mcl*-components. It is therefore necessary to provide an organism and/or method by which *scl*-/*mcl*-PHA copolymers can be synthesized in order to provide a material that is strong yet ductile for applications such as fiber formation. To date, only one bacterial isolate (*Pseudomonas* strain GP4BH1) has been found to synthesize *scl*-/*mcl*-PHA polymer blends naturally [19]. A number of *Pseudomonas* strains, however, have been genetically altered to accomplish this task. Specifically, a recombinant strain of *Pseudomonas oleovorans* expressing the *R. eutropha* PHB biosynthesis genes (*phbCAB*) has been constructed and found to accumulate polymer blends of PHB and *mcl*-PHA [19,20]. In another example, recombinant strains of the *Pseudomonas* sp. 61-3 isolate have been shown to accumulate polymer blends of PHB and poly-3-hydroxybutyrate-*co*-3-hydroxyalkanoate (PHB-*co*-HA) [14-16].

In this study, *P. oleovorans* NRRL B-778 was found to produce mixtures of PHB and *mcl*-PHA naturally when grown on glucose or even-carbon-chain fatty acids. When grown on odd-carbon-chain fatty acids, however, the organism produced a PHB-*co*-HV copolymer. The ability of a single bacterial strain to produce both *scl*-PHA and *mcl*-PHA may make this organism a viable candidate for further experimentation into the feasibility of producing *scl*-/*mcl*-PHA copolymers.

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Materials and methods

Materials

All simple salts, glucose, octanoic, nonanoic, undecanoic and oleic acids were obtained from Sigma (St. Louis, MO). All solvents were

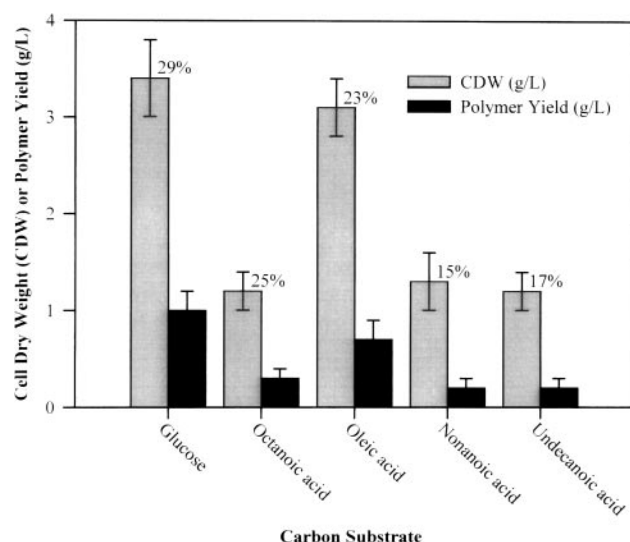


Figure 1 Growth and PHA polymer yield of *P. oleovorans* NRRL B-778 grown on glucose or various free fatty acids (n=4). The numerical percentages above each graph represent the cellular productivity of the bacterium on each substrate. They are calculated by: (PHA yield/cell dry weight)×100.

HPLC grade and purchased from Burdick and Jackson (Muskegon, MI). The silylation reagent (N,O-bis(trimethylsilyl)-trifluoroacetimide, BSTFA) was purchased from Aldrich Chemical (Milwaukee, WI).

Strain information and polymer synthesis

P. oleovorans NRRL B-778 was obtained from the ARS Culture Collection, NCAUR, ARS, US Department of Agriculture (Peoria IL) and used as the producer strain in all experiments.

Shake flask production: Nonanoic acid and undecanoic acid were tested as feedstocks in shake flask experiments conducted in 500-ml volumes in Medium E* (pH 7.0). Medium E* contained the following, in g/l: $(NH_4)_2HPO_4$, 1.1; K_2HPO_4, 5.8; KH_2PO_4, 3.7; $MgSO_4{\cdot}7H_2O$, 0.25; and 1 ml of microelement solution. The microelement solution consisted of the following, in g/l of 1 N HCl: $FeSO_4{\cdot}7H_2O$, 2.78; $MnCl_2{\cdot}4H_2O$, 1.98; $CoSO_4{\cdot}7H_2O$, 2.81; $CuCl_2{\cdot}2H_2O$, 0.17; $ZnSO_4{\cdot}7H_2O$, 0.29; $CaCl_2{\cdot}2H_2O$, 1.67. The inoculum was prepared by aseptically scraping an individual colony of *P. oleovorans* NRRL B-778 from a nutrient agar plate, inoculating it into 50 ml of Luria broth and incubating it at 30°C with agitation at 250 rpm. At 24-h incubation, 2.0 ml of the culture was aseptically transferred to a 1-l Erlenmeyer flask containing 250 ml of Luria broth and incubated at 30°C, at 250 rpm for 24 h. After 24 h, cells from the 250-ml Luria broth cultures were aseptically harvested by centrifugation (8000×g, 20 min, 4°C), washed once with sterile deionized water, resuspended in 25 ml of sterile Medium E* and transferred into 1-l Erlenmeyer flasks containing 500 ml of sterile Medium E*. Filter-sterilized nonanoic acid or undecanoic acid was added directly to the shake flasks at a concentration of 15 mM. The inoculum was 3.2±0.3 g of cells (wet cell weight)/flask. Each shake flask was incubated at 30°C, at 250 rpm for 8 days. At the appropriate time, the cells were pelleted by centrifugation as described above, washed twice with deionized water and lyophilized (~24 h) to constant weight.

Ten-liter fermentation: Glucose, octanoic acid and oleic acid were tested as substrates for the production of *scl-/mcl*-PHA mixtures at the 10-l scale. The fermenter was a BioFlo 3000 (New Brunswick Scientific, Edison, NJ) equipped with a 12-l fermentation tank. Medium E* (composition described previously) was used as the production medium. The inoculum was prepared as described above, except that at 24-h incubation time, 2.0 ml of the Luria broth culture was aseptically transferred to twelve 1-l Erlenmeyer flasks each containing 250 ml of Luria broth and incubated at 30°C, at 250 rpm for 24 h. At 24 h, the 250-ml Luria broth cultures were aseptically harvested as

Table 1 Compositions of the PHA polymers and their acetone fractions[a] produced by *P. oleovorans* NRRL B-778 from glucose, octanoic acid, oleic acid, nonanoic acid or undecanoic acid

PHA isolate	β-Hydroxymethyl esters (mol%)[b,c]								
	3HB	3HV	3HHx	3HO	3HD	3HDd	3HDde	3HT	3HTe
PHA-glucose	99	–	–	Tr[d]	Tr	–	Tr	–	–
AIS	100	–	–	–	–	–	–	–	–
AS	–	–	3	13	42	5	37	Tr	–
PHA-octanoic	99	–	–	Tr	Tr	Tr	–	–	–
AIS	100	–	–	–	–	–	–	–	–
AS	–	–	3	38	42	11	–	–	6
PHA-oleic	93	–	Tr	2	2	1	–	–	2
AIS	100	–	–	–	–	–	–	–	–
AS	–	–	1	34	30	12	–	–	23
PHA-nonanoic	85	15	–	–	–	–	–	–	–
AIS	87	13	–	–	–	–	–	–	–
AS	87	13	–	–	–	–	–	–	–
PHA-undecanoic	84	16	–	–	–	–	–	–	–
AIS	87	13	–	–	–	–	–	–	–
AS	85	15	–	–	–	–	–	–	–

[a]Acetone fractions correspond to an AIS fraction and an AS fraction.
[b]β-Hydroxymethyl ester column headings correspond to 3HB, 3HV, 3-hydroxyhexanoic acid (3HHx), 3HO, 3HD, 3HDd, 3HDde, 3-hydroxytetradecanoic acid (3HT) and 3HTe.
[c]Each numerical value is an average of three separate measurements (n=3).
[d]Tr (trace)=less than 1 mol%.

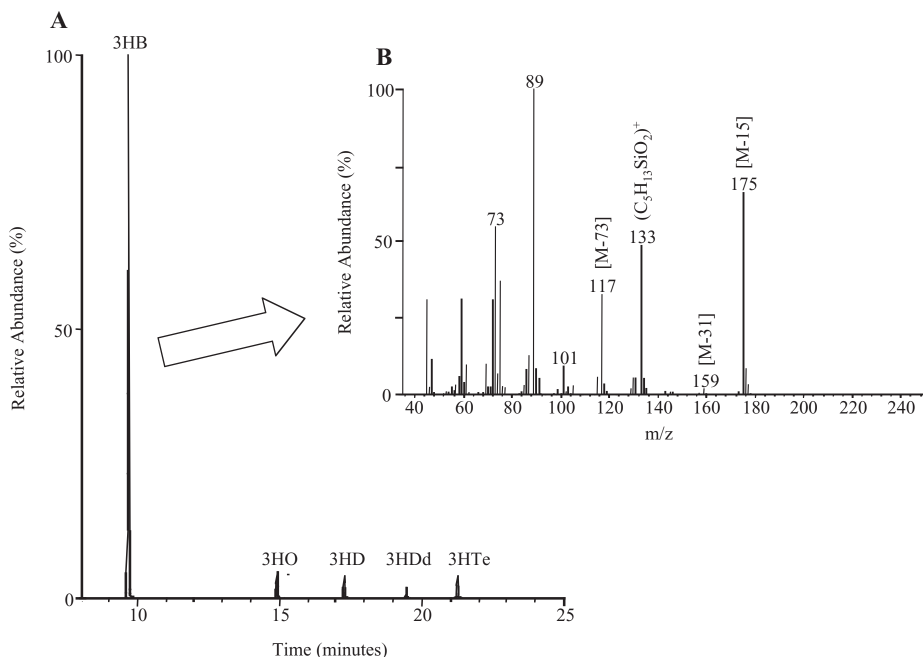


Figure 2 The gas chromatogram of the silylated 3-hydroxymethyl esters derived from PHA-oleic (A) and the mass spectrum of silylated 3-hydroxybutyric acid methyl ester (B). Abbreviations are as follows: 3-hydroxybutyric acid (3HB), 3-hydroxyoctanoic acid (3HO), 3-hydroxydecanoic acid (3HD), 3-hydroxydodecanoic acid (3HDd) and 3-hydroxytetradecenoic acid (3HTe). For the MS of the silylated medium-chain 3-hydroxymethyl esters, see Ref. [13].

described above, combined and aseptically transferred into the fermenter vessel containing 10 l of Medium E*. Glucose was added from a filter-sterilized 20% (wt/vol) solution to a final concentration of 0.5% (wt/vol). Filter-sterilized octanoic acid or oleic acid was added to the fermenter to a final concentration of 15 mM. The inoculum was 38.6±3.9 g of cells (wet cell weight)/fermentation. The fermenter was run at 30°C with an impeller speed of 400 rpm and aeration at 2 SLPM (standard liters per minute) for 2 days (glucose), 3 days (oleic acid) or 8 days (octanoic acid). The pH was not controlled. At the appropriate time, the bacterial cells were pelleted by centrifugation as described above, washed twice in deionized water and lyophilized (~24 h) to constant weight.

Polymer isolation

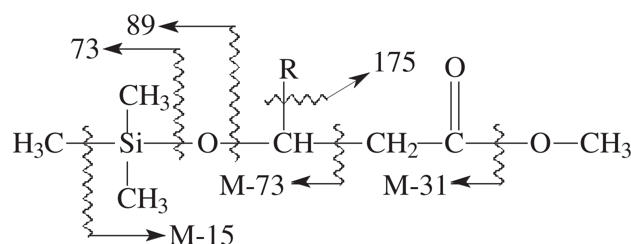
All PHA polymers were isolated by chloroform extraction at 30°C overnight at 250 rpm. Cellular debris was removed by filtration through Whatman no. 1 filter paper and the chloroform fraction rotary-evaporated to dryness to give the crude polymer. Crude polymer was dissolved in a small volume of chloroform and reprecipitated by dropwise addition to cold methanol. The polymer was recovered, placed into a tared vial and dried *in vacuo* for 24 h.

Acetone fractionation

To determine the presence of *scl-/mcl*-PHA mixtures, each isolated polymer was subjected to acetone solubility tests. Each of the polymers was placed into a clean vial with excess acetone and allowed to stir overnight at room temperature using a stir bar. The fractions were then separated by filtration through Whatman no. 1 filter paper and the acetone-insoluble (AIS) fraction was placed into a dry, tared vial. The acetone-soluble (AS) fraction was pipetted into a clean, tared vial and the acetone was removed under a nitrogen stream. Both the AIS and AS samples were dried *in vacuo* overnight.

Instrumental procedures

Each PHA polymer (parental polymer, AIS fraction and AS fraction) was characterized with respect to repeat unit composition, molar mass and thermal properties. Repeat unit compositions were determined by gas chromatography/mass spectrometry (GC/MS) of the silylated 3-hydroxymethyl esters obtained by acid hydrolysis of the polymers according to Brandl *et al* [4]. Samples were silylated by reacting 10 μl of each sample with 250 μl of BSTFA and 200 μl of pyridine. The mixtures were heated at 70°C for 30 min and allowed to cool to room temperature. Finally, 150 μl of hexane was added to each sample and the samples were analyzed by GC/MS as described elsewhere [2]. Electron impact fragmentation of silylated 3-hydroxymethyl esters occurred as shown below:



Percent composition was obtained by selecting the mass 175 ion (indicative of silylated 3-hydroxymethyl esters) and identifying the molecular ion−15 amu (M-15; $-CH_3$), molecular ion−31 amu (M-31; $-OCH_3$) and molecular ion−73 amu (M-73; $-CH_2COOCH_3$) ions.

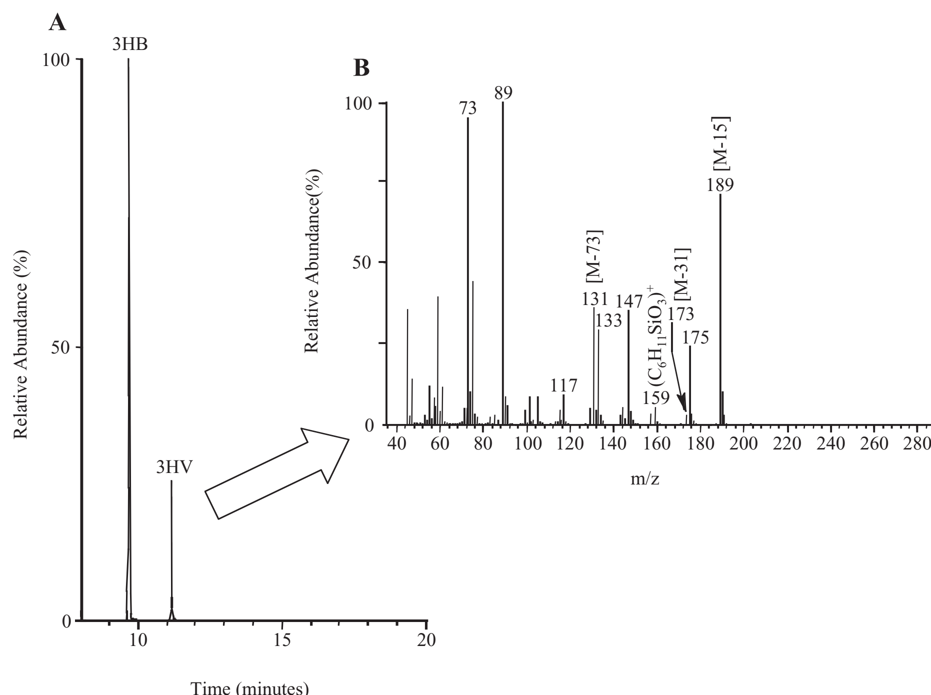


Figure 3 The gas chromatogram of the silylated 3-hydroxymethyl esters derived from PHA-nonanoic (A) and the mass spectrum of silylated 3-hydroxyvaleric acid methyl ester (B). Abbreviations are: 3-hydroxybutyric acid (3HB) and 3-hydroxyvaleric acid (3HV).

Molar mass averages were determined by gel permeation chromatography (GPC) as described elsewhere [6]. Styragel HR1, HR3, HR4 and HR6 columns (Waters, Milford, MA) were connected in series and polystyrene standards (Polyscience, Warrington, PA) with narrow polydispersities were used to generate a calibration curve. Chloroform was used as the eluent at a flow rate of 1 ml/min. The sample concentrations were 0.1% (wt/vol) for the parental polymers and the AIS fractions due to viscosity concerns and 0.3% for the AS fractions. The injection volume in all cases was 200 μl.

Thermal properties were measured for each PHA sample using a Pyris 1 differential scanning calorimeter (Perkin-Elmer, Norwalk, CT) at a heating rate of 10°C/min under a dry nitrogen purge. The instrument was calibrated using both indium (T_m=156°C) and cyclohexane (transition temperatures at −87 and 6°C). The T_g was taken as the midpoint temperature and the T_m as the peak of the melting endotherm.

Results and discussion

Many pseudomonads belonging to the rRNA homology group I have the ability to produce PHA polymers from a number of different substrates. The mechanism of synthesis from free fatty acids involves β-oxidation to produce (*R*)-3-hydroxyacyl CoA intermediates, which constitute the committed step in the polymer-

Table 2 Molar mass and thermal properties of the PHA polymers and their acetone fractions[a] produced by *P. oleovorans* NRRL B-778 from glucose, octanoic acid, oleic acid, nonanoic acid or undecanoic acid[b]

PHA isolate	M_n ($\times10^3$)	M_w ($\times10^3$)	M_w/M_n	T_m (°C)	ΔH_f (cal/g)	T_g (°C)
PHA-glucose	1628	3386	2.08	179	20	3
AIS	1651	3252	1.97	178	19	4
AS	92	341	3.71	39	1	−53
PHA-octanoic	1683	2962	1.76	177	20	3
AIS	1677	2801	1.67	177	21	3
AS	52	144	2.77	40	1	−49
PHA-oleic	1514	2680	1.77	178	20	4
AIS	1617	2377	1.47	178	18	4
AS	43	124	2.88	37	1	−51
PHA-nonanoic	1353	2354	1.74	161	7	−3
AIS	1410	2214	1.57	164	8	−3
AS	1327	2136	1.61	162	6	−3
PHA-undecanoic	1537	2997	1.95	161	7	−3
AIS	1512	2812	1.86	163	7	−3
AS	1574	2817	1.79	163	5	−4

[a]Acetone fractions correspond to an AIS fraction and an AS fraction.
[b]Each numerical value is an average of three separate measurements (n=3).

Table 3 Acetone fractionation of the PHA polymers produced by *P. oleovorans* NRRL B-778 from glucose, octanoic acid, oleic acid, nonanoic acid or undecanoic acid[a]

PHA isolate	AS[b] (% recovery)	AISb (% recovery)
PHA-glucose	1.2±0.2	98.8±0.2
PHA-octanoic	0.8±0.2	99.2±0.2
PHA-oleic	6.9±0.5	93.1±0.5
PHA-nonanoic	49.6±3.7	50.4±3.9
PHA-undecanoic	46.9±4.4	53.1±4.2

[a]Each numerical value is an average of three separate measurements ±standard deviation.
[b]AS and AIS refer to the acetone-soluble and acetone-insoluble fractions, respectively.

ization reaction. These polymers generally contain 3-hydroxyalkanoic acid repeat units with alkyl side chains that vary between 3 and 11 carbon atoms. In this study, three different strains of *P. oleovorans* (NRRL B-778, NRRL B-14682 and NRRL B-14683) were screened by fermentation for their ability to produce PHA polymers from glucose and free fatty acids. Each strain synthesized a different PHA polymer from oleic acid. Specifically, strain NRRL B-14682 produced PHB while strain NRRL B-14683 produced *mcl*-PHA; however, neither of these two strains produced any PHA polymer from glucose. In fact, strain NRRL B-14682 did not grow on glucose while strain NRRL B-14683 grew but did not produce PHA. *P. oleovorans* strain NRRL B-778 was unique in that it naturally synthesized a mixture of PHB and *mcl*-PHA from glucose (PHA-glucose), octanoic acid (PHA-octanoic) and oleic acid (PHA-oleic). Bacterial growth on these substrates resulted in cell dry weights (CDW) of 3.4±0.4, 1.2±0.2 and 3.1±0.3 g/l, respectively, and average (n=3) cellular productivities ranging from 23% to 29% of the CDW (Figure 1). Glucose was the preferred substrate for cell growth and PHA production. Cell growth reached a maximum at 2 days of cultivation time while 3 days were required to reach maximum cell growth on oleic acid. Octanoic acid was not as readily utilized as glucose or oleic acid. Growth on octanoic acid increased the lag time in the culture and the maximum cell yield was not achieved until 8 days post-inoculation. These results suggested that the organism has the ability to synthesize the (R)-3-hydroxyacyl CoA intermediates both through fatty acid biosynthesis (from glucose) and through β-oxidation from fatty acids.

Each round of β-oxidation releases one molecule of acetyl CoA, which is the metabolic precursor of PHB in a number of other organisms including *R. eutropha* and *Alcaligenes latus*. These two organisms rely on a cascade of three enzymatic reactions each catalyzed by a distinct enzyme (β-ketoacyl-CoA thiolase, PhbA; acetoacetyl-CoA reductase, PhbB; and PHB polymerase, PhbC) to produce the mature PHB polymer from acetyl CoA. That *P. oleovorans* NRRL B-778 produces PHA with a large concentration of *scl*-monomers indicates that this organism contains genes for biosynthesis that are analogous to the *phbCAB* operon of *R. eutropha* and probably also uses acetyl CoA as the metabolic precursor for PHB biosynthesis. This theory is supported by the fact that PHA-glucose was composed of 99% 3-hydroxybutyric acid (3HB; Table 1), most likely due to the formation of large quantities of acetyl CoA from pyruvate upon oxidative decarboxylation by the pyruvate dehydrogenase complex of enzymes.

Growth on nonanoic acid and undecanoic acid followed the same pattern as that seen with octanoic acid: long lag times with minimal growth and cellular productivity (15–17% of the CDW). The metabolism of nonanoic acid and undecanoic acid, however, also resulted in incorporation of 3-hydroxyvaleric acid (3HV) into the *scl*-PHA. In fact, the PHA polymers derived from nonanoic acid (PHA-nonanoic) and from undecanoic acid (PHA-undecanoic) were both composed of comparable ratios of 3HB (84–85 mol%) and 3HV (15–16 mol%). These results are analogous to those seen in *R. eutropha* grown on propionic acid. This was not unexpected due to the formation and availability of propionyl CoA

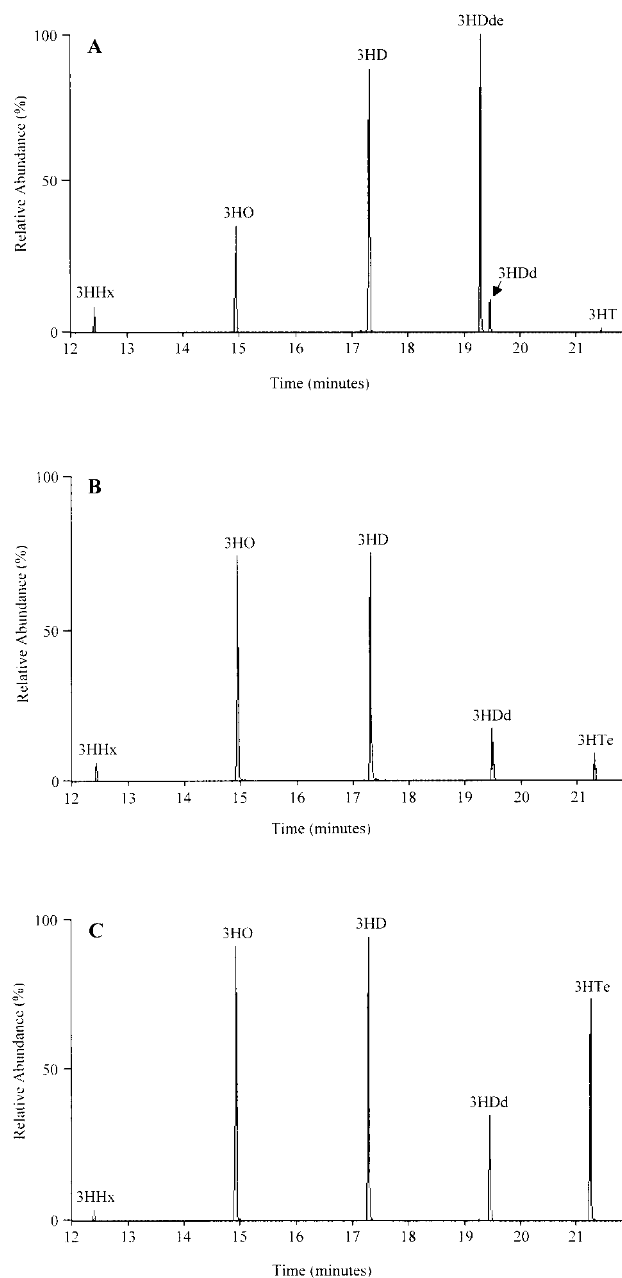


Figure 4 Gas chromatograms of the silylated 3-hydroxymethyl esters derived from the AS fractions of PHA-glucose (A), PHA-octanoic (B) and PHA-oleic (C). Abbreviations are: 3-hydroxyhexanoic acid (3HHx), 3-hydroxyoctanoic acid (3HO), 3-hydroxydecanoic acid (3HD), 3-hydroxydodecanoic acid (3HDd), 3-hydroxydodecenoic acid (3HDde), 3-hydroxytetradecanoic acid (3HT) and 3-hydroxytetradecenoic acid (3HTe). For the mass spectra of each silylated medium-chain 3-hydroxymethyl ester, see Ref. [13].

as a final product of the efficient β-oxidation of odd-chained fatty acids.

Interestingly, *mcl*-monomers also comprised a small percentage of the PHA polymers. The best example of this was in PHA-oleic. Figure 2 shows the GC/MS data pertaining to PHA-oleic. In this instance, the majority of the monomer repeat units (93 mol%) of PHA-oleic were 3HB while 7 mol% of the monomers had chain lengths of eight carbon atoms or greater. The presence of *mcl*-monomers suggested that the *phaC1* and *phaC2* genes (*mcl*-PHA biosynthetic genes) were not only present but also active. This was independently confirmed by a polymerase chain reaction (PCR) protocol developed previously (data not shown) [17]. The GC/MS data pertaining to PHA-nonanoic are seen in Figure 3. The use of nonanoic acid and undecanoic acid as substrates for PHA production resulted in the inclusion of 3HV into the polymers. In each case, it was initially assumed that copolymers were formed, which consisted of PHB-*co*-HA from glucose, octanoic acid or oleic acid and PHB-*co*-HV from nonanoic acid or undecanoic acid. However, this was not the case. Calorimetric analysis of each polymer showed definitive melting temperatures (T_m) from PHA-glucose, PHA-octanoic and PHA-oleic between 177 and −179°C and glass transition temperatures (T_g) at 3–4°C, each corresponding to PHB (Table 2). However, these PHA polymers also contained *mcl*-monomers (indicated by GC/MS), which, if copolymeric, should have decreased the T_m and T_g of the polymers. This suggested that the original polymers were not copolymeric but were mixtures of PHB and *mcl*-PHA. To verify the presence of polymer blends, acetone fractionation was used to separate the PHB (AIS fraction) from any *mcl*-PHA (AS fraction). The AIS fractions from PHA-glucose, PHA-octanoic and PHA-oleic were all recovered at >93% while the AS fractions ranged from 0.8% to 6.9% recovery (Table 3). GC/MS showed that the AIS fraction from each polymer was indeed PHB (Table 1) and that the AS fractions were composed of *mcl*-PHA with monomer repeat units ranging from 6 to 14 carbon atoms (Figure 4).

In most instances, 3-hydroxyoctanoic acid (3HO) and 3-hydroxydecanoic acid (3HD) predominate in *mcl*-PHA polymers. This was the case for the AS fractions of PHA-octanoic and PHA-oleic; this was not the case, however, for PHA-glucose. The AS fraction of PHA-glucose was composed of 37 mol% of 3-hydroxydodecenoic acid (3HDde). The formation of *mcl*-PHA from glucose is associated with *de novo* fatty acid biosynthesis. Unlike the β-oxidation of unsaturated fatty acids where olefinic groups can be conserved, a double bond must be generated from glucose in order to be present in the final PHA polymer. This reaction involves the formation of 3-*cis*-enoyl-ACP. In order to synthesize 3HDde, (*R*)-3-hydroxydecanoyl-ACP undergoes 3-hydroxydecanoyl-ACP dehydrase-catalyzed dehydration, resulting in the isomerization of 2-*trans*-decenoyl-ACP to 3-*cis*-decenoyl-ACP. An additional round in the biosynthetic cycle would then result in (*R*)-3-hydroxy-5-dodecenoyl-ACP, which can then be enzymatically converted to (*R*)-3-hydroxy-5-dodecenoyl-CoA by the gene product of the *phaG* gene and channeled into the PHA polymer. The lack of other unsaturated monomers in PHA-glucose shows that this dehydration step is preferential for (*R*)-3-hydroxydecanoyl-ACP in this bacterial system.

The AS fraction of PHA-oleic was composed predominantly of 3HO (34 mol%), 3HD (30 mol%) and 3-hydroxytetradecenoic acid (3HTe; 23 mol%). The latter monomer unit can be explained by the conservation of double bonds during β-oxidation. However, the AS fraction from PHA-octanoic had a large 3HD fraction as well as a 3-hydroxydodecanoic acid (3HDd) fraction and a 3HTe fraction, suggesting that this *mcl*-PHA was synthesized through a combination of β-oxidation and fatty acid biosynthesis. This may explain the extended fermentation times required to induce bacterial growth and PHA production with octanoic acid as the substrate.

As mentioned earlier, the use of odd-carbon-chain fatty acids (nonanoic acid or undecanoic acid) as substrates resulted in extended fermentation times, but unlike octanoic acid, these substrates resulted in copolymers of PHB-*co*-HV. It is known that organisms such as *R. eutropha* and *A. latus* produce PHB-*co*-HV when grown on substrates like propionic acid. Although *R. eutropha* and *A. latus* do not produce *mcl*-PHA, the fact that they do produce PHB-*co*-HV copolymers under the appropriate conditions suggests that *P. oleovorans* NRRL B-778 has *scl*-PHA polymerization enzymes whose mode of action is similar to those of *R. eutropha* and/or *A. latus*.

The molar masses were reflective of the polymer compositions. In all cases, the molar masses (M_n) of the parental polymers were approximately 1.5×10^6 g/mol, appreciably larger than other *Pseudomonas*-derived *mcl*-PHA (Table 2). However, when the parental polymers were fractionated by acetone, the AS (*mcl*-PHA) fractions had molar masses (M_n) that were less than 100,000 g/mol, in line with *mcl*-PHAs produced by other bacterial species [1,6]. In contrast, the *scl*-PHA fractions (AIS fractions) had molar masses comparable to the parental polymers, which reflected the small *mcl*-PHA concentrations (AS fraction) in the parental polymers.

In conclusion, we have identified a wild-type bacterial strain that has the ability to produce *scl*-/*mcl*-PHA mixtures from glucose or even-carbon-chain fatty acid substrates while producing copolymers of PHB-*co*-HV from odd-carbon-chain fatty acid substrates under the same growth conditions under which other pseudomonads synthesize *mcl*-PHA. These results suggested that the genes necessary for the utilization of both coenzyme A-thioesters of 3-hydroxyalkanoic acids with four to five carbon atoms and the coenzyme A-thioesters of 3-hydroxyalkanoic acids with six or more carbon atoms are present and functional in this bacterial strain. Because of the mutual exclusivity of the *scl*-PHA and *mcl*-PHA synthase enzymes, bacteria do not normally synthesize *scl*-/*mcl*-PHA copolymers. Nevertheless, the ability of a single bacterial strain to produce both *scl*- and *mcl*-3-hydroxy-acid monomers from the same substrate indicates that *P. oleovorans* NRRL B-778 may be a good candidate for continued research into the production of *scl*-/*mcl*-PHA copolymers for applications such as fibers where strength and flexibility are required.

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