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Biosynthesis of Novel Poly(3-hydroxyalkanoates) Containing Alkoxy Groups by *Pseudomonas oleovorans*

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Abstract Novel poly(3-hydroxyalkanoates), PHAs, having either methoxy or ethoxy groups as the terminal hydrophilic moieties, were biosynthesized by *Pseudomonas oleovorans* grown either solely with 11-alkoxyundecanoic acid or 8-alkoxyoctanoic acid, or grown with a mixture of 6-alkoxyhexanoic acid and nonanoic acid. The PHA synthesized from 11-methoxyundecanoic acid consisted of 88 mol% 3-hydroxy-7-methoxyheptanoate and 12 mol% 3-hydroxy-9-methoxynonanoate. However, the PHA produced from 11-ethoxyundecanoic acid consisted of 56 mol% 3-hydroxy-5-ethoxypentanoate and 44 mol% 3-hydroxy-7-ethoxyheptanoate. The high solubility of the PHAs in methanol and ethanol indicated that the alkoxy groups in the side chains resulted in the formation of PHAs with an enhanced hydrophilicity.

Key words: Alkoxy groups, cometabolism, poly(3-hydroxyalkanoates), PHAs, *Pseudomonas oleovorans*

Bacterial poly(3-hydroxyalkanoates), PHAs, are eco-friendly polyesters that are readily degraded by microorganisms in nature [8]. Medium-chain-length (MCL) PHAs consisting of 3-hydroxyalkanoates longer than 3-hydroxypentanoate are generally produced by several *Pseudomonas* strains, and are either elastomeric or tacky materials with properties that vary according to their composition [7, 14, 20]. Until now, more than 140 MCL-hydroxyalkanoates have been identified as constituents in bacterial PHAs and, of these, approximately 130 constituents have been tailored with either specific substituents or reactive groups [19, 20].

Pseudomonas oleovorans and *P. putida* have been reported to biosynthesize MCL-PHAs containing various functional groups in the side chains, when they were grown with carbon substrates containing the corresponding functional groups or their mixtures with either octanoic acid (OA) or nonanoic acid (NA). Halogens [3, 10], cyano [17], cyclohexyl [6], phenyl [18], phenoxy [11], methylphenoxy [12], and unsaturated groups [4, 5, 7, 21] are examples of the functional groups that have been incorporated into MCL-PHAs. Polymers with enhanced hydrophilicity are of great interest, because they are usually more biocompatible. Some MCL-PHAs with enhanced hydrophilicity have been prepared by incorporating hydrophilic moieties by chemical reaction. Maltosyl [2], hydroxy [16], carboxy [15], and acrylamide [9] groups have been incorporated into MCL-PHAs with carbon-carbon double bonds. However, the microbial production of MCL-PHAs containing hydrophilic groups in substantial quantities has not been reported. The aim of this study was to determine if PHAs with enhanced hydrophilicity can be biosynthesized and accumulated in microorganisms. This paper describes the biosynthesis of novel MCL-PHAs, containing hydrophilic moieties in the terminal region of the side chains, by *P. oleovorans* using various alkoxyalkanoic acids.

The alkoxyalkanoic acids used as the carbon sources were synthesized by a reaction between the corresponding ω-bromoalkanoic acid and sodium alkoxide. The synthesis of 6-ethoxyhexanoic acid is briefly described Toluene (300 ml) and absolute ethanol (36 g, 782 nmole) were added to a 500 ml, three-necked, round-bottomed flask equipped with a cooling condenser. Sodium (18 g, 782 nmole) was added in small portions to this mixture. The mixture was heated gently to ensure a complete reaction between the sodium and the ethanol. When the reaction was completed, 50 g of 6-bromohexanoic acid (256 nmole) was added. After the mixture was refluxed overnight under a nitrogen atmosphere, it was cooled and neutralized with HCl, and then concentrated under reduced pressure. The reaction mixture was dispersed in 150 ml of distilled water and extracted with three portions of 100 ml of ethyl ether. The ethyl ether layer was dried over anhydrous $MgSO_4$ and evaporated. The residue was subjected to vacuum distillation in order to isolate the 6-ethoxyhexanoic acid. The yields were usually in the range of 50%.

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P. oleovorans ATCC 29347 was used in this study as the polymer-producing organism and was cultivated on a mineral salt medium [14] containing the alkanoic acid(s) to a total concentration of 10 mM. Batch fermentations were conducted using a 5-l jar fermentor, as previously described [5]. The PHAs were isolated from the lyophilized cells with hot chloroform using a Soxhlet apparatus [1]. The extracted crude PHA was purified by repeated precipitation by cold methanol/water (50:50) solution, which was stirred gently. The compositions of the PHAs were determined by nuclear magnetic resonance (NMR) spectroscopy and gas chromatography/mass spectrometry (GC/MS) analyses as described previously [12].

The fermentation results for the PHA synthesis by *P. oleovorans* grown with various alkoxyalkanoic acids or with mixtures containing NA are shown in Table 1. 6-Methoxyhexanoic acid (6-MHx) and 6-ethoxyhexanoic acid (6-EHx) did not support cell growth for 5 days when used as the sole carbon sources. However, *P. oleovorans* utilized 8-methoxyoctanoic acid (8-MO), 8-ethoxyoctanoic acid (8-EO), 11-methoxyundecanoic acid (11-MUD), and 11-ethoxyundecanoic acid (11-EUD) as sole carbon sources for growth and PHA production. The PHA contents in the cells grown with 8-MO, 8-EO, and 11-MUD were less than 5% of dry cell weight. Of the alkoxyalkanoic acids examined, 11-EUD supported the highest biomass yield (0.5 g/l) and PHA content (8 wt%). As shown in Table 1, 6-MHx and 6-EHx produced PHAs bearing the respective methoxy and ethoxy groups, when they were co-fed with NA. The PHA contents were usually over 10% of dry cell weight. These values were approximately three times higher than those obtained when *P. oleovorans* was grown with the same concentration of 8-MO, 8-EO, or 11-MUD. Several reports have shown that *P. oleovorans* and *P. putida* can produce MCL-PHAs containing the corresponding functional groups from carbon substrates with certain functional groups only in the presence of a good polymer-producing substrate, such as OA and NA [5, 10, 17]. Accordingly, the bioconversion of 6-alkoxyhexanoic acids into PHAs and the significant increase in the total cell and PHA yields caused by NA addition is possibly an example of cometabolism. Meanwhile, Kim *et al.* [5] reported that *P. putida* did not metabolize any alkoxyhexanoic acids, when used solely for growth and PHA biosynthesis. In addition, *P. putida* did not produce any PHAs containing the corresponding functional groups from the alkoxyalkanoic acids examined in this study, even when NA was provided as a cosubstrate. These differences between *P. oleovrans* and *P. putida* might be closely linked with either the carbon uptake system in the cell membrane or a specific PHA synthase, which can catalyze the polymerization of (*R*)-3-hydroxyalkoxyalkanoyl-CoA molecules.

The ion fragmentation pattern and molecular mass of the methyl esters of each repeating unit with a methoxy or ethoxy group in the synthesized PHAs, as determined by GC/MS, are listed in Table 2. The ion fragments with *m/z* values of 71 and 103 in the electron impact (EI) mass spectra were in accordance with the characteristic ones, which could be produced from methyl 3-hydroxyalkanoates [21]. In particular, data in Table 2 revealed that methyl 3-methoxyalkanoates could be cleaved to produce an ion fragment with a m/z value of 32, corresponding to the mass of methanol. For example, electron ionization of methyl 3-hydroxymethoxyhexanoate (*m/z*: 176) and methyl 3-hydroxymethoxyheptanoate (*m/z*: 190) resulted in the formation of ion fragments with *m/z* values of 143 and 157, respectively, indicating the absence of methoxy groups in the original molecules. Likewise, in the case of methyl 3-hydroxyethoxyhexanoate (*m/z*: 190), electron ionization of this molecule produced an ion fragment with a *m/z* value of 143, together with an ion fragment with a *m/z* value of 46 that corresponded to the mass of the ethanol. Chemical ionization (CI) GC/MS spectrometry clearly showed that each monomer unit in the PHAs produced from methoxy- and ethoxyalkanoic acids contained the corresponding alkoxy groups. The monomer structures of

Table 1. Fermentation results for the synthesis of PHAs containing alkoxy groups by *P. oleovorans* grown with various alkoxyalkanoic acids or their mixtures with nonanoic acid.

Carbon substrate (mM)	Culture time (h)	Dry cell weight (g l^{-1})	PHA yield (mg l^{-1})	PHA content (%wt)
6-MHx (10)	120	-	ND[a]	ND
6-EHx (10)	120	-	ND	ND
8-MO (10)	14	0.3	8	3
8-EO (10)	14	0.4	15	4
8-MO (5)+NA (5)	13	0.8	104	13
11-MUD (10)	10	0.4	15	4
11-EUD (10)	14	0.5	40	8
6-MHx (5)+NA (5)	15	0.8	120	15
6-MHx (7)+NA (3)	12	0.6	67	11
6-EHx (5)+NA (5)	15	0.8	92	12
6-EHx (7)+NA (3)	13	0.6	80	13

[a]Not determined.

Table 2. Ion fragmentation pattern and molecular mass of methyl esters of each constituent.

Methyl ester of constituent	*m/z* in EI-MS	Molecular mass (CI-MS)
Methyl ester of 3-hydroxy-4-methoxybutyrate	130, 117, 103, 99, 85, 75, 71, 61, 59	148
Methyl ester of 3-hydroxy-6-methoxyhexanoate	143, 128, 116, 103, 87, 85, 71, 58	176
Methyl ester of 3-hydroxy-7-methoxyheptanoate	157, 140, 125, 117, 103, 85, 71, 56	190
Methyl ester of 3-hydroxy-8-methoxyoctanoate	205, 187, 155, 123, 103, 95, 71, 57	204
Methyl ester of 3-hydroxy-9-methoxynonanoate	168, 145, 136, 113, 103, 95, 84, 71	218
Methyl ester of 3-hydroxy-4-ethoxybutyrate	144, 131, 117, 103, 89, 85, 71, 61	162
Methyl ester of 3-hydroxy-5-ethoxypentanoate	158, 130, 114, 103, 85, 71, 59	176
Methyl ester of 3-hydroxy-6-ethoxyhexanoate	159, 143, 128, 117, 103, 85, 71, 59	190
Methyl ester of 3-hydroxy-7-ethoxyheptanoate	157, 142, 125, 112, 103, 85, 71, 59	204

the PHAs synthesized in this study were also identified by ^{1}H and ^{13}C NMR spectroscopies (data not shown).

The compositions of the PHAs bearing alkoxy groups, as determined by ^{1}H-NMR spectroscopy, are listed in Table 3. The total fraction of repeating units bearing the methoxy groups in the PHAs biosynthesized from an equimolar mixture of 6-MHx or 8-MO with NA was less than 40 mol%, while that of the repeating units containing ethoxy groups in the polyester from an equimolar mixture of 6-EHx and NA was over 60 mol%. This suggests that 3-hydroxy-4-ethoxybutyryl-CoA and 3-hydroxy-6-ethoxyhexanoyl-CoA are more appropriate substrates of *P. oleovorans* PHA synthase than other intermediate molecules derived from the methoxyalkanoates or NA. It has been reported that *P. oleovorans* grown with undecanoic or undecenoic acid produces a copolyester consisting of three corresponding repeating units with carbon atoms, 7, 9, and 11 [7, 13]. The PHA synthesized from 10-undecynoic acid also included repeating units of C_9 and C_{11} bearing carbon-carbon triple bonds, although the 3-hydroxy-6-heptynoate unit was not detected [4]. However, the PHA biosynthesized from 11-MUD contained the two monomers, 88 mol% 3-hydroxy-7-methoxyheptanoate and 12 mol% 3-hydroxy-9-methoxynonanoate (Fig. 1a). No 3-hydroxy-11-methoxyundecanoate was detected in the PHA. Similarly, it was reported that the PHAs synthesized from 11-cyanoundecanoic acid (11-CUD) [17] and 11-bromoundecanoic acid (11-BUD) [10] contained only two repeating units of C_7 and C_9 bearing the corresponding functional groups. Therefore, it is believed that the absence of the 3-hydroxyundecanoate units bearing the corresponding substituents in the PHAs produced from 11-MUD, 11-CUD, and 11-BUD, might be due to the presence of substituted groups in the terminals of the carbon substrates, which can be recognized as additional atoms. Meanwhile, the PHA biosynthesized from 11-EUD by *P. oleovorans* consisted of 56 mol% 3-hydroxy-5-ethoxypentanoate and 44 mol% 3-hydroxy-7-ethoxyheptanoate (Fig. 1b). These results are comparable to those obtained in the PHA synthesized from 11-MUD. This is because 3-hydroxy-9-ethoxynonanoate which can be produced by the deacetylation of 3-hydroxy-11-ethoxyundecanoate was not detected in the PHA. Considering that the 3-hydroxy-9-methoxynonanoate unit was included in the PHA synthesized from 11-MUD, the absence of a 3-hydroxy-9-ethoxynonanoate unit in the PHA indicates that the PHA synthase from *P. oleovorans* was not specific to the 3-hydroxy-9-ethoxynonanoyl-CoAs having 12 atoms. These results are in good agreement with the fact that the PHA synthase from *P. oleovorans* preferentially incorporates 3-hydroxyoctanoate and 3-hydroxynonanoate units with 8 or 9 atoms into the growing polymer chains, but exhibits weak activity for the longer chain 3-hydroxyalkanoates [13].

The PHAs containing alkoxy groups prepared from either 11-MUD or 11-EUD were highly soluble in methanol and ethanol. In addition, the PHAs containing more than 60 mol% 3-hydroxyalkoxyalkanoates were soluble even in a

Table 3. The compositions of the PHAs produced from various carbon substrate mixtures.

Carbon substrate (mM)	Relative amount of repeating units[a] in PHAs (mol%)										
	3HMB	3HMHx	3HMHp	3HMO	3HMN	3HEB	3HEP	3HEHx	3HEHp	3HHp	3HN
6-MHx (5)+NA (5)	14	17								18	51
6-EHx (5)+NA (5)						46		18		10	26
8-MO(5)+NA (5)	12	13		14						15	46
11-MUD (10)			88		12						
11-EUD (10)							56		44		

[a]3HMB, 3-hydroxy-4-methoxybutyrate; 3HMHx, 3-hydroxy-6-methoxyhexanoate; 3HMHp, 3-hydroxy-7-methoxyheptanoate; 3HMO, 3-hydroxy-8-methoxyoctanoate; 3HMN, 3-hydroxy-9-methoxynonanoate; 3HEB, 3-hydroxy-4-ethoxybutyrate; 3HEP, 3-hydroxy-5-ethoxypentanoate; 3HEHx, 3-hydroxy-6-ethoxyhexanoate; 3HEHp, 3-hydroxy-7-ethoxyheptanoate; 3HHp, 3-hydroxyheptanoate; 3HN, 3-hydroxynonanoate.

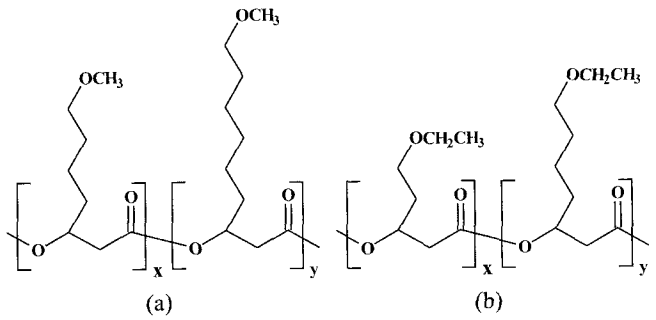

Fig. 1. A PHA produced from 11-methoxyundecanoic acid (a) and a PHA produced from 11-ethoxyundecanoic acid (b).

methanol/water (70:30) solution. This shows that the inherent hydrophobic nature of the PHAs was significantly altered by the introduction of alkoxy groups into the side chains, which resulted in the formation of polymers with an enhanced hydrophilicity. Considering that hydrophilicity is one of the most important factors affecting the use of biomedical polymers in *in vivo* applications, polymers bearing the alkoxy moiety as their hydrophilic groups may be useful for various applications, including medical devices. Further characterization of the PHAs, including their mechanical properties and hydrophilicity, is currently underway.

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