

In Vivo ¹³C-NMR Spectroscopic Study of Polyhydroxyalkanoic Acid Degradation Kinetics in Bacteria

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Abstract Polyhydroxyalkanoic acid (PHA) inclusion bodies were analyzed *in situ* by ¹³C-nuclear magnetic resonance (¹³C-NMR) spectroscopy. The PHA inclusion bodies studied were composed of poly(3-hydroxybutyrate) or poly(3-hydroxybutyrate-co-4-hydroxybutyrate), which was accumulated in *Hydrogenophaga pseudoflava*, and medium-chain-length PHA (MCL-PHA), which was accumulated in *Pseudomonas fluorescens* BM07 from octanoic acid or 11-phenoxyundecanoic acid (11-POU). The quantification of the ¹³C-NMR signals was conducted against a standard compound, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The chemical shift values for the *in vivo* NMR spectral peaks agreed well with those for the corresponding purified PHA polymers. The intracellular degradation of the PHA inclusions by intracellular PHA depolymerase(s) was monitored by *in vivo* NMR spectroscopy and analyzed in terms of first-order reaction kinetics. The *H. pseudoflava* cells were washed for the degradation experiment, transferred to a degradation medium without a carbon source, but containing 1.0 g/l ammonium sulfate, and cultivated at 35°C for 72 h. The *in vivo* NMR spectra were obtained at 70°C for the short-chain-length PHA cells whereas the spectra for the aliphatic and aromatic MCL-PHA cells were obtained at 50°C and 80°C, respectively. For the *H. pseudoflava* cells, the *in vivo* NMR kinetics analysis of the PHA degradation resulted in a first-order degradation rate constant of 0.075/h ($r^2=0.94$) for the initial 24 h of degradation, which was close to the 0.050/h determined when using a gas chromatographic analysis of chloroform extracts of sulfuric acid/methanol reaction mixtures of dried whole cells. Accordingly, it is suggested that *in vivo* ¹³C-NMR spectroscopy is an important tool for studying intracellular PHA degradation in terms of kinetics.

Key words: PHA degradation kinetics, *in vivo* ¹³C-NMR, bacteria, PHA

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Polyhydroxyalkanoic acids (PHAs) are accumulated in bacterial cells as an intracellular energy source and carbon storage material [12, 15, 18, 19]. Based on early X-ray crystallographic studies on solid poly(3-hydroxybutyrate) [P(3HB)], it was once known that intracellular P(3HB) granules were crystallized [1, 20]. However, several recent investigative studies based on *in vivo* analyses of P(3HB) granules in cells using ¹³C-NMR, X-ray, and differential scanning calorimetry have shown that the intracellular PHA granules are present in an amorphous state [2, 3, 14, 27]. The amorphous nature of these intracellular PHA inclusions may allow the cells to mobilize them easily through intracellular PHA depolymerase(s) [13]. It has also been suggested that ¹³C-NMR spectroscopy is a useful tool to study them *in vivo* [2, 3, 9, 25].

The mechanism of intracellular PHA depolymerization has not yet been well studied despite the recent finding of three P(3HB) depolymerase isozymes in *Wautersia eutropha*, formerly known as *Ralstonia eutropha*, which produces short-chain-length (SCL) PHA [16, 30]. There is another type of PHA, medium-chain-length (MCL) PHA produced by mostly *Pseudomonas* spp., but these are known to have only one intracellular depolymerase [5, 13]. Contrary to extracellular PHA depolymerases, intracellular depolymerases are only known to be active against native PHA granules [13], which can be easily denatured by organic solvents, acids, bases, heat, repeated centrifuging, or other forms of mechanical shock during purification [21]. However, appropriate protocols for isolating PHA granules without damaging the native structure have not been established. Thus, the enzymatic study of intracellular PHA degradation is still in its infant stage.

In a previous study, the current authors suggested that, depending on the structural type of the PHA monomer unit, each PHA has its own characteristic first-order degradation rate constant k , [4, 5, 8, 29]. It was also suggested that the

k_i values determined for each PHA may be a measure of the enzymatic specificity of the intracellular depolymerase against the PHA. Thus, without isolating the enzymes, a simple first-order degradation kinetic analysis could reveal the structural heterogeneity of PHA and the relative specific activity of the enzyme. Curley *et al.* [9] already studied the intracellular degradation of MCL-PHA in *Pseudomonas oleovorans* using *in vivo* ^{13}C -NMR spectroscopy, but since their study was on a semiquantitative level, a comparative kinetic analysis was impossible.

Accordingly, this study analyzed the intracellular PHA degradation *in situ* by applying first-order degradation kinetics using *in vivo* ^{13}C -NMR spectroscopy, without isolating the PHA inclusions. For the intracellular degradation of SCL-PHA, P(3HB) and P(3HB-co-4HB) were accumulated in *Hydrogenophaga pseudoflava*. In the case of MCL-PHA, *Pseudomonas fluorescens* BM07 was utilized for the PHA accumulation. The quantification of the *in vivo* ^{13}C -NMR signal intensities for the PHAs in the cells was performed against those for sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the internal standard [9]. The chemical shift values for the *in vivo* NMR experiments were compared with those for the isolated, purified PHAs. The kinetic analysis based on *in vivo* ^{13}C -NMR spectroscopy agreed well with the results from the gas chromatography analysis, implying that *in vivo* ^{13}C -NMR spectroscopy is an important tool for the *in vivo* kinetic analysis of intracellular PHA degradation.

MATERIALS AND METHODS

Bacterial Strain and Culture Media

For the accumulation of MCL-PHA in cells, *P. fluorescens* BM07 (KCTC 10005BP), isolated in the authors' laboratory, was used [17], whereas for SCL-PHA, *H. pseudoflava* ATCC 33668 was employed. A nutrient-rich (NR) medium was used in the seeding, maintenance, and storage of the strain and contained 1% yeast extract, 1.5% nutrient broth, and 0.2% ammonium sulfate. A modified M1 mineral-salts medium was used for both the PHA synthesis and degradation, and its mineral composition was similar to that reported earlier [6]: the M1 medium contained 2.3 g KH_2PO_4 , 7.3 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.06 g $(\text{NH}_4)_2\text{SO}_4$, 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g NaHCO_3 , 0.4 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.03 g $\text{Fe}(\text{NH}_4)_2$ -citrate per 1 l of distilled water, plus 1 ml of a microelement solution (pH 7.2). The culture grown in the NR medium at 30°C or 35°C, 200 rpm for 12 h, was transferred to 500 ml of the mineral-salts medium containing an appropriate amount of a carbon source in a 2-l flask. All the cells were grown to a steady state of growth at 30°C or 35°C, 200 rpm, under aerobic conditions.

Accumulation and Degradation of SCL-PHA in *H. pseudoflava*

P(3HB) Homopolymer. The pre-cultured (nutrient-broth grown) *H. pseudoflava* cells were transferred to 500 ml of the PHA-synthesis mineral medium containing 70 mM glucose and 5 mM ammonium sulfate and cultivated at 35°C for 30 h. The cells were then harvested by centrifugation in a Beckman J2-HS (rotor JA-10, 6,000 rpm for 10 min) and washed with a phosphate buffer. Next, the washed cells were transferred to a degradation medium without a carbon source, but containing 1.0 g/l ammonium sulfate, and cultivated at 35°C for 72 h. Finally, 100 ml of the culture medium was taken at scheduled time intervals to isolate the cells, which were then kept in a deep freezer.

P(3HB-co-4HB) Copolymer. The pre-cultured (nutrient-broth grown) *H. pseudoflava* cells were transferred to 500 ml of the M1 medium containing 15 g/l glucose, 1.68 g/l γ -butyrolactone, and 8 mM ammonium sulfate and cultivated at 35°C for 72 h [7, 29]. A gas chromatographic analysis showed a composition of 70 mol% 3HB and 30 mol% 4HB in the P(3HB-co-4HB). The PHA-accumulated cells were then transferred to a degradation medium and incubated at 35°C for 72 h. Thereafter, the zero and 72 h incubated cells were kept in a deep freezer until further analysis.

Accumulation of MCL-PHA in *P. fluorescens* BM07

Aliphatic MCL-PHA. The pre-cultured (nutrient-broth grown) *P. fluorescens* BM07 cells were transferred to 500 ml of the M1 medium containing 70 mM fructose and cultivated at 30°C for 48 h [17]. The cells were then harvested by centrifugation and kept in a deep freezer for NMR and GC characterization.

Aromatic MCL-PHA. The pre-cultured (nutrient-broth grown) *P. fluorescens* BM07 cells were transferred to 500 ml of the M1 medium containing 20 mM 11-POU and cultivated at 30°C for 120 h [26]. The cells were harvested by centrifugation, washed with ethanol three or four times to remove any unutilized substrate, and then washed with distilled water. Thereafter, the cells were kept in a deep freezer for NMR and GC characterization.

Gas Chromatographic Analysis

For the GC analysis [11] of the polyesters in the cells, 20 mg of the dried cells were reacted in a mixed solution of 1 ml of chloroform and 1 ml of a methanol/conc. sulfuric acid mixture (87:13 v/v). The reaction mixture was incubated in a closed screw-capped tube at 100°C for 200 min. After cooling the mixture, 1 ml of distilled water was added to separate the organic layer containing the reaction products. The resulting organic layer was then separated, dried over anhydrous Na_2SO_4 , and analyzed using a gas chromatography system (Hewlett Packard 5890A) with an HP-5 (cross-linked 5% PH ME Siloxane) column and FID detector. Each peak was standardized

against standard methyl esters obtained by a methanolysis reaction of purified P(3HB) and P(4HB) homopolymers or a copolyester with a known composition as determined by $^1\text{H-NMR}$ analysis [22].

In Vivo $^{13}\text{C-NMR}$ Analysis

The frozen cells (150 mg) from each medium were resuspended as a slurry in 450 μl of deuterium oxide (D_2O) and transferred to a 10-mm NMR cylindrical-tube insert equipped with a coaxial insert containing 5(v/v)% DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) in D_2O [9]. The use of this double partitioned cylindrical tube was necessary to prevent the bacterial cells from metabolizing the DSS. The parameters used for the NMR (Bruker DRX 500 or 300) spectra acquisition were the following: 5 s pulse repetition, 10 μs pulse width, 30,000 Hz spectral width, 64 K data points, 24,000–28,000 accumulations. Depending on the type of polymer, the data acquisition temperature was changed between 50 and 80°C [9, 25].

Transmission Electron Microscopy

The frozen cells (0.1 g) were resuspended in 1 ml of a 0.1 M sodium phosphate buffer (pH 7.2) and centrifuged. The washed cells were then prefixed with 2.5% glutaraldehyde at 4°C for 4 h, washed with a buffer, and fixed with 1% osmium tetroxide (in a 0.1 M PBS buffer). Thereafter, the fixed cells were imbedded in an agar E-tube, and samples cut to appropriate sizes were then dehydrated by washing with a 60 to 100% ethanol/acetone solution and fixed onto epon resins. Ultrathin sectioning was performed using an LKB-Ultratome with a diamond knife. The sections were then collected on a copper grid coated with a Formvar-carbon film and poststained with lead citrate and uranyl acetate [28]. Electron micrographs were taken with a HITACHI H-600 electron microscope (Tokyo, Japan) under an acceleration voltage of 75 kV.

RESULTS AND DISCUSSION

In Vivo $^{13}\text{C-NMR}$ Analysis of Intracellular P(3HB) Degradation

When the *H. pseudoflava* cells were grown on 70 mM glucose for 30 h, 55 to 65 wt% (in dry cells) of the P(3HB) homopolymer was accumulated in the cells. The intracellular degradation of the P(3HB) was then performed by further incubating the cells in the M1 medium containing 1.0 g/l of ammonium sulfate, free from a carbon source [29]. The remaining P(3HB) was monitored over time by gas chromatographic determination of the 3-hydroxybutyrate methyl ester, as described in Materials and Methods (Fig. 1A). Eighty percent of the intracellular P(3HB) was degraded in 24 h. The degradation rate followed first-order reaction rate kinetics during the initial active degradation

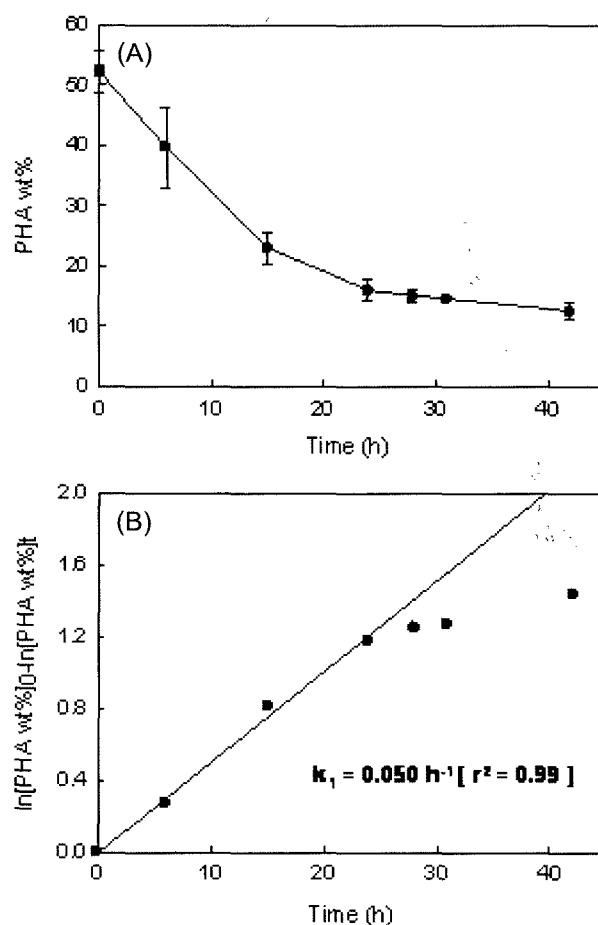


Fig. 1. P(3HB) degradation in *H. pseudoflava* in the presence of ammonium sulfate (1.0 g/l). (A) Time-course profile of remaining PHA content in cells; (B) First-order kinetics analysis of intracellular P(3HB) degradation.

period, throughout which degradation limiting factors are considered to be minimal [29]. A linear regression analysis gave the first-order rate constant k_1 of 0.050 h^{-1} ($r^2=0.99$ for the initial 24 h) (Fig. 1B).

Without isolating the intracellular P(3HB) granules in *H. pseudoflava*, the intracellular degradation was followed *in vivo* by comparing the C-13 signals associated with P(3HB) over the degradation time (0, 6, 15, 24 h) using a 75 MHz $^{13}\text{C-NMR}$ spectrometer (Fig. 2). To increase the chain mobility of the P(3HB), and thus increase the signal sensitivity, the temperature of the NMR spinning region was increased to 70°C. In addition, the high temperature was expected to allow a more sensitive collection of the NMR signals, while preventing any further PHA degradation in the cells by residual activity of intracellular PHA depolymerase(s). Four 3HB-carbon associated signals were observed at the chemical shifts of 168.51, 66.99, 40.01, and 18.82 ppm, which agreed well with literature values [10, 23]. Meanwhile, the standard compound DSS exhibited four absorption peaks at 56.87, 21.49, 17.67, and 0.00 ppm [9].

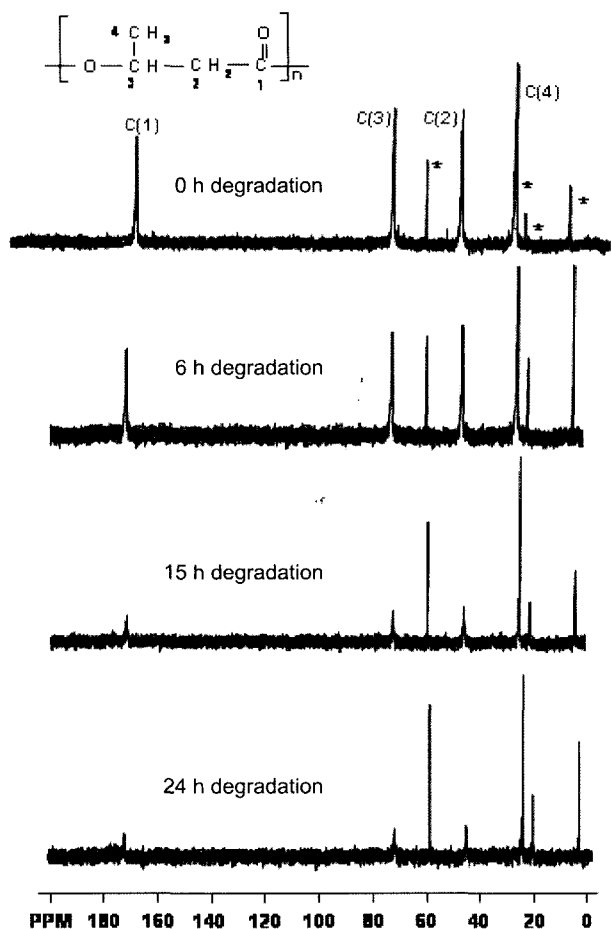


Fig. 2. Degradation-time dependent 75 MHz ^{13}C -NMR spectra of P(3HB) degradation in *H. pseudoflava* cells grown on 70 mM glucose at 35°C.

The signals for the DSS standard are indicated with the symbol *. The NMR data collection experiments were carried out at 70°C.

The NMR signal ratio {peak height of carbonyl C(1) for P(3HB)/peak height of 56.87 ppm for DSS} was used as an index for the degree of intracellular P(3HB) degradation. Here, the same Lorentzian shape was assumed for the two well-separated peaks. For each indicated time, the intensity of the signals associated with DSS remained relatively constant over the degradation time owing to the addition of an equal amount of DSS [5(v/v)%] to each frozen cell sample (150 mg) suspended in 450 μl D_2O at the indicated time. Thus, the value of the ratio of the peak height of C(1) carbon in the P(3HB) to that of the resonance at 56.87 for DSS was substituted for the concentration term in the first-order rate equation. The decreasing pattern of the PHA content determined by GC (open circle in Fig. 3A) proceeded in parallel with the decrease of the NMR C(1) signals (filled square in Fig. 3A). For the two methods, the logarithmic plots for the data in Fig. 3A are also presented in Fig. 3B to determine the first-order rate constant from the slopes. The k_1 value for

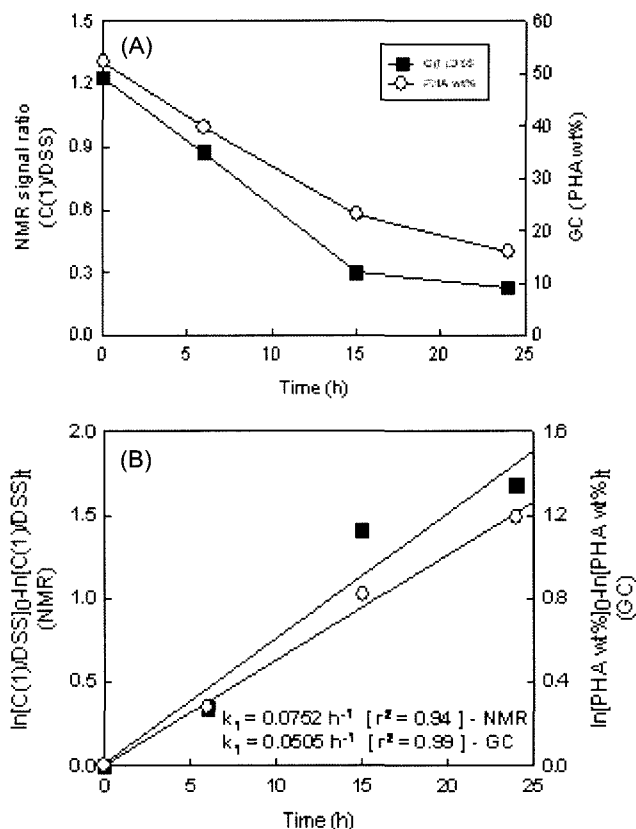


Fig. 3. Comparison of intracellular degradation at 35°C of P(3HB) in *H. pseudoflava* cells between two different methods, GC (○) and *in vivo* ^{13}C -NMR spectroscopy (■). (A) Time course profile of remaining PHA content in cells; (B) First-order kinetics analysis of intracellular P(3HB) degradation.

the NMR method was determined to be 0.075/h ($r^2=0.94$) for the initial 24 h and close to 0.050/h as determined by GC, which were slightly lower than the literature value of 0.12/h [29]. However, the discrepancy was probably due to different culture conditions, such as the concentration of glucose and medium composition. Nonetheless, the similarity of the two values between the two different methods for the same batch cells clearly demonstrated that the intracellular PHA degradation followed first-order kinetics. The first-order dependence with respect to the concentration of the polymer substrate may suggest a relatively constant number density of the depolymerase on the granular surface throughout the degradation [29]. Thus, *in vivo* NMR measurements may be used as a tool to “quantitatively and simply” analyze intracellular PHA degradation without resorting to a multistep process, including an acid methanolysis reaction.

***In Vivo* ^{13}C -NMR Analysis of P(3HB-co-4HB) Copolymer Degradation**

When *H. pseudoflava* was grown in the M1 medium containing 15 g/l glucose and 1.68 g/l γ -butyrolactone,

Table 1. Gas chromatographic analysis of intracellular degradation of P(3HB-co-4HB) in *H. pseudoflava*.

Degradation time (h)	Dry cell wt (g/l)	Total PHA wt%	Mol%		wt%	
			3HB	4HB	3HB	4HB
0	3.3	44.0	70.0	30.0	31.7	12.4
26	2.5	28.8	59.5	40.5	18.1	10.7
48	2.3	23.8	56.2	43.8	14.3	9.5
60	2.2	24.0	55.5	44.5	14.3	9.7
72	2.1	20.5	53.6 (16.4%↓)	46.4 (16.4%↓)	13 (18.7%↓)	7.5 (4.9%↓)

the resulting polymer was composed of a 70 mol% 3HB-unit and 30 mol% 4HB-unit. The GC analysis for the time-course profile of the copolymer degradation is shown in Table 1. After 72 h of intracellular degradation, the monomer composition was changed from 70 mol% 3HB and 30 mol% 4HB to 54 mol% 3HB and 46 mol% 4HB. This preferential degradation of the 3HB-unit demonstrates the higher specificity of the intracellular *H. pseudoflava* PHA depolymerase(s) to the 3HB-unit rather than to the 4HB-unit, as well as the blend-type nature of the synthesized polyester already noted in a previous report [29].

Figure 4 shows the 125 MHz ^{13}C -NMR spectra before and after degradation of the 3HB/4HB polymer when the data collection was made at zero and after 72 h. The NMR spinning temperature was 70°C. The two carbonyl carbons were absorbed at around 169–173 ppm, whereas the other six peaks were absorbed at around 15–70 ppm, as expected [10]. To analyze the NMR microstructure changes [10, 29]

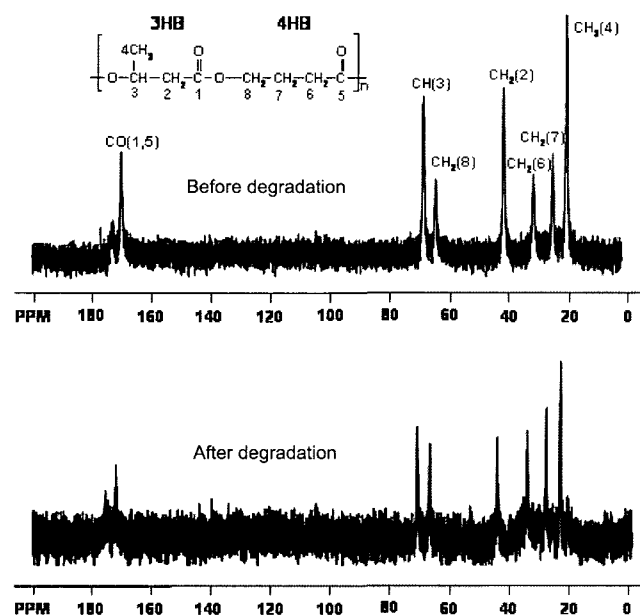


Fig. 4. 125 MHz ^{13}C -NMR spectra of 3HB/4HB (initial mole ratio, 70:30) copolymer in *H. pseudoflava* cells before and after degradation.

The degradation continued for 72 h at 35°C. The NMR experiment was carried out at 70°C.

of the polymer before and after degradation, each absorption peak was expanded, yet no characteristically resolved peaks were seen, as the signal-to-noise ratios were too low in this *in vivo* experiment. However, a comparison between the zero and 72 h NMR spectra showed that the level of the 3HB-units, as determined from the signal intensities associated with the 3HB-units (C2, C3, and C4), preferentially decreased with time compared with that of the 4HB-units. This semiquantitative interpretation is reasonable because of the same NMR run conditions for the two samples. These *in vivo* NMR data also support the conclusion that the intracellular *H. pseudoflava* PHA depolymerase(s) had a higher specificity to the 3HB-unit than to the 4HB-unit, the same conclusion as in the above GC analysis (Table 1).

In Vivo ^{13}C -NMR Analysis of MCL-PHA in *P. fluorescens* BM07

The PHA synthesized in *P. fluorescens* BM07 in the M1 medium containing 70 mM fructose was composed of various lengths of 3-hydroxy acids, including large amounts of unsaturated monomer units: 3-hydroxyoctanoic acid, 3-hydroxydecanoic acid, 3-hydroxy-*cis*-5-dodecenoate (a major monomer-unit), 3-hydroxydodecanoic acid, 3-hydroxy-*cis*-7-tetradecenoate (less than 5 mol%), and 3-hydroxytetradecanoic acid [17]. A similar signal pattern was observed for the polymer in the cells as for the solvent-extracted polymer, yet the *in vivo* spectrum revealed several additional signals presumably related with other unidentified metabolites (Fig. 5). The *in vivo* 125 MHz ^{13}C -NMR experiment was carried out at 50°C because of the melting point around 50–60°C [17], thereby expecting a high chain mobility. All the chemical shift values associated with the PHA agreed well with those for the chloroform-extracted polymer. For example, the two absorptions designated g and h around 120–140 ppm were attributed to the two ethylenic carbons C-5 and C-6 of $\text{C}_{12:1}$. The inserted TEM photograph in Fig. 5 shows the aliphatic MCL-PHA granules in the cells, based on which the NMR data were collected with a 20 k scan number. As such, a glance at the TEM picture and the data in Fig. 2 show that 10% (v/v) or more of the PHA granules in the cells was adequate for characterization by *in vivo* ^{13}C -NMR spectroscopy.

Another MCL-PHA composed of the aromatic monomer units 3-hydroxy-5-phenoxyvalerate, 3-hydroxy-7-

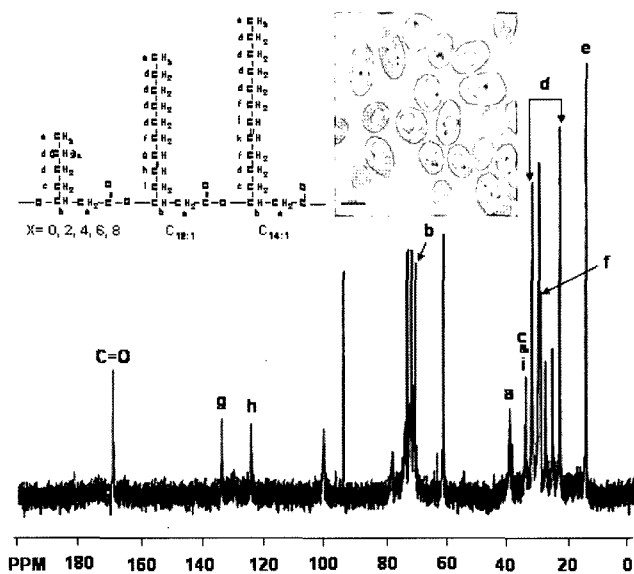


Fig. 5. 125 MHz *in vivo* ^{13}C -NMR spectrum of PHA in *P. fluorescens* BM07 cells grown on 70 mM fructose at 30°C. The NMR data collection was made at 50°C. The transmission electron micrograph of the cells indicates that the cell volume contains ~40 v/v% PHA.

phenoxyheptanoate, and 3-hydroxy-9-phenoxy-nonanoate, which was accumulated (43.8 wt%) in *P. fluorescens* BM07 from 20 mM 11-POU [26], was analyzed by 75 MHz ^{13}C -NMR *in vivo* (Fig. 6). The NMR data collection at 50°C was not successful, probably because of the higher chain rigidity of the aromatic PHA developed by the presence of side-chain phenyl rings. In fact, the glass transition temperature of this aromatic PHA was previously reported

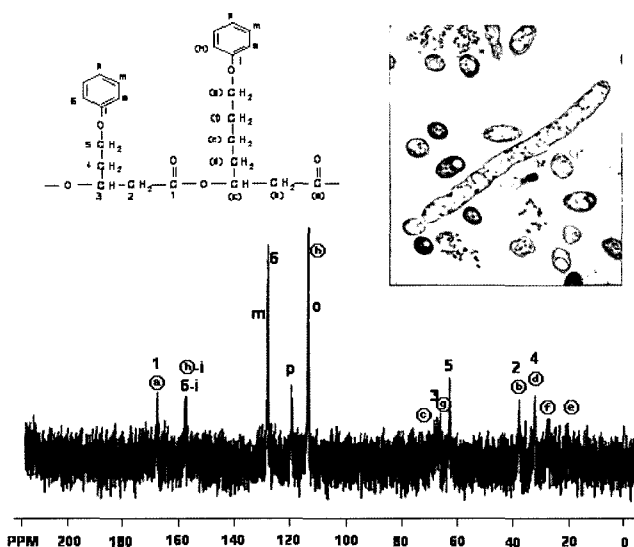


Fig. 6. 75 MHz *in vivo* ^{13}C -NMR spectrum of PHA in *P. fluorescens* BM07 cells grown on 20 mM 11-POU at 30°C. The NMR data collection was made at 80°C. The transmission electron micrograph for the cells shows a significantly elongated cell morphology.

to be 14°C, significantly higher than -40 to -50°C for aliphatic MCL-PHA [16, 26]. Thus, the data collection temperature was increased to 80°C for the aromatic MCL-PHA cells to obtain the spectrum successfully (Fig. 6). All the absorption peaks were observed at the corresponding chemical shifts, as in the isolated polymer system, even though the signal-to-noise ratio was rather low compared with that for the spectrum of the isolated polymer [26]. The inserted TEM micrograph shows that, when the BM07 strain was cultivated on 20 mM 11-POU, some cells showed a highly elongated morphology. According to some reports [24], the unusual elongation of cells has already been observed, especially for recombinant cells harboring no phasin gene. Thus, the cultivation of the bacterium on 11-POU may result in a different level of phasin(s) to that when using ordinary carbon sources.

From our studies, we could conclude four things as shown below.

1. *In vivo* ^{13}C -NMR spectroscopy measurements for cells containing 10–50 wt% PHA exhibited well-resolved, strong absorption signals at the same chemical shifts as observed for chloroform-extracted PHA polymers.
2. A kinetic analysis of first-order intracellular PHA degradation traced by GC agreed well with the results of *in vivo* NMR spectroscopy.
3. Depending on the type of PHA synthesized in cells, a different data collection temperature should be employed to obtain strong signals owing to different chain rigidities in the cells.
4. *In vivo* ^{13}C -NMR spectroscopy can be used for a structural characterization and intracellular degradation kinetics analysis of various types of PHA without isolating the PHA from the cells.

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