Bioassimilation of Atactic Poly[(*R,S*)-3-hydroxybutyrate] Oligomers by Selected Bacterial Strains

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ABSTRACT: Water-soluble a-PHB oligomers (from dimer to dodecamer) were synthesized by anionic oligomerization of (*R,S*)-*β*-butyrolactone and characterized by electrospray ionization tandem mass spectrometry (ESI-MS^n). The oligomers were analogous to the degradation products remaining after enzymatic hydrolysis of synthetic high molecular weight atactic poly[(*R,S*)-3-hydroxybutyrate] by extracellular PHB depolymerases. Selected bacterial strains were used to test bioassimilability of a-PHB oligomers. It was found that not only two PHB-degrading bacteria (*Alcaligenes faecalis* T1 and *Comamonas* sp.) but also a non-PHB-degrading bacterium (*Ralstonia eutropha* H16) could grow on a-PHB oligomers as sole source of carbon and energy. Utilization of a-PHB oligomers by the three bacterial strains (total oligomer consumption and molecular weight distribution changes) was investigated by ESI-MS. Total oligomer consumption (tested after 30 h of bacterial growth) followed the same trend as the observed bacterial growth (*A. faecalis* > *Comamonas* sp. > *R. eutropha*). Mineralization of a-PHB oligomers demonstrates total biodegradability of synthetic high molecular weight atactic poly[(*R,S*)-3-hydroxybutyrate].

Introduction

The heterogeneous enzymatic hydrolysis of natural poly[(*R*)-3-hydroxybutyrate], PHB, is investigated worldwide, and its simple kinetic model has been recently proposed.[1] Among all possible synthetic stereoisomers of PHB, the atactic poly[(*R,S*)-3-hydroxybutyrate], a-PHB, prepared via anionic polymerization of (*R,S*)-*β*-butyrolactone mediated by activated anionic initiators, was recently of our particular interest due to its unusual biodegradation behavior. It was demonstrated by some of us earlier that this amorphous high molecular weight a-PHB (average degree of polymerization DP = 400) does not biodegrade in the pure state but undergoes heterogeneous enzymatic attack (by PHB depolymerase A from *Pseudomonas lemoignei*) in the presence of a second crystalline component.[2−4] Such crystalline polymer was alternatively (i) a natural poly(3-hydroxyalkanoate) (PHA), synthetic poly(*ϵ*-caprolactone) (PCL), or poly(L-lactic acid) (PLLA) in binary blends with a-PHB[2,3] or (ii) a poly(pivalolactone) (PPVL) block in poly[(*R,S*)-3-butyrolactone-*b*-pivalolactone] copolymers.[4] It was shown that heterogeneous enzymatic hydrolysis of a-PHB occurred both when the crystalline component was itself susceptible to enzymatic attack (bacterial PHA) and when—conversely—it was intrinsically nonbiodegradable by the PHB depolymerase employed (PCL, PLLA, PPVL). The water-soluble enzymatic degradation products of atactic PHB in the mentioned binary systems were oligomers containing hydroxyl and carboxylic end groups (up to heptamer),[2,3] higher than those usually found in the case of natural PHB (monomer to trimer).[5,6] This difference was clearly due to stereospecificity of the extracellular PHB depolymerase and to the presence of randomly distributed (*S*) units in synthetic a-PHB. However, the question concerning the fate of the oligomeric degradation products of a-PHB in natural environment (bioassimilability) remained unanswered.

This work aims at answering this question by showing that water-soluble a-PHB oligomers can be assimilated by bacteria isolated from natural environments. For this purpose artificial models of PHB oligomers, analogous to those remaining after enzymatic hydrolysis of high molecular weight atactic PHB, were synthesized by anionic oligomerization of (*R,S*)-*β*-butyrolactone, characterized by electrospray ionization tandem mass spectrometry technique (ESI-MS^n),[7] and subjected to biodegradation by selected bacterial strains. Two PHB-degrading bacteria, namely *Alcaligenes faecalis* T1[8] and *Comamonas* sp.,[9] as well as one non-PHB-degrading bacterium, *Ralstonia eutropha* H16 (formerly *Alcaligenes eutrophus*), were chosen for this study.

Experimental Section

Materials. (*R,S*)-*β*-Butyrolactone (from Fluka), bp 47 °C (5 mmHg), was dried as described previously;[10] (*R,S*)-3-hydroxybutyric acid sodium salt (from Sigma) and 18-crown-6 (from Fluka) were used as received. Tetrahydrofuran (THF) purified as described in ref 11 was additionally distilled over a sodium−potassium alloy in an atmosphere of dry argon.

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General Procedure of <bold>**β-Butyrolactone Oligomerization.** The oligomerization of β-butyrolactone initiated by equimolar complex of (*R,S*)-3-hydroxybutyric acid sodium salt with 18-crown-6 was conducted in tetrahydrofuran, as described in refs 2, 7, and 12. The course of the oligomerization was followed by ^{1}H NMR and FTIR spectroscopy. After completion of the reaction, the oligomers were precipitated in hexane and dried in vacuo at room temperature. The crude oligomers were dissolved in dichloromethane, and the acid ion-exchange resin was introduced into the polymer solution. Then, the ion-exchange resin was filtered off, and the oligomers were precipitated in cold pentane and dried in vacuo. The oligomers obtained were characterized by NMR, GPC, and ESI-MS^n techniques.

Bacterial Strains and Culture Conditions. *Ralstonia eutropha* H16 (DSM 428, ATCC 17699), *Alcaligenes faecalis* T1,[8] and *Comamonas* sp.[9] (DSM 6781) were used in this study. The bacteria were grown in a mineral salts solution consisting of (g/L) $Na_2HPO_4 \cdot 12H_2O$ (5.8), KH_2PO_4 (2.3), NH_4Cl (1.0), $MgSO_4 \cdot 7H_2O$ (0.49), $CaCl_2 \cdot 2H_2O$ (0.005), and $FeCl_3 \cdot 6H_2O$ (0.001), pH ~ 7 at 30 °C under shaking. The sample of 0.05 or 0.3% (w/v) sodium (*R,S*)-3-hydroxybutyrate (sterile-filtered solution in water) was added as a carbon source after autoclaving of the growth medium. Alternatively, 0.8 g of a-PHB oligomers was dissolved in ethanol, and the required amount of the solution was added to a glass flask. After evaporation of the solvent, an appropriate amount of mineral salts solution was added (final concentration of the oligomers 0.3%, w/v). The complete mixture (10 mL each) was filter-sterilized (0.2 μm) and added to sterile 100 mL Klett flasks. Washed cells (with carbon source-free growth medium) of a 24 h preculture on 3HB were used as an inoculum at an initial cell density of 15−20 Klett units (Klett-Summerson colorimeter equipped with a 520−580 nm filter). Growth was followed for 30 h at 30 °C. A culture without inoculum served as a control (no bacteria experiment). At the end of growth the bacteria were centrifuged (4500 rpm, 30 min, 4 °C), and the cell-free culture fluids were analyzed by the ESI-MS technique.

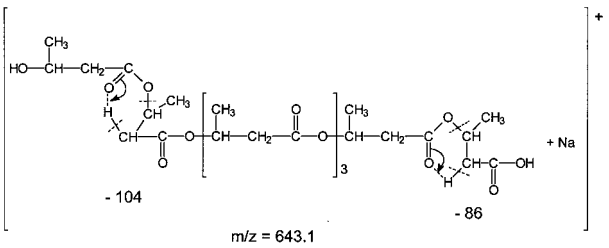
Analytical Procedures. NMR spectra were recorded using a Varian VCR-300 multinuclear spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were run in D_2O using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard.

GPC experiments were conducted in THF solution at 35 °C, at a flow rate of 1 mL/min using a Spectra-Physics 8800 solvent delivery system with a PLgel 3 μm MIXED-E ultrahigh efficiency column and a Shodex SE 61 refractive index detector. Polystyrene standards with low polydispersity were used to generate a calibration curve.

Electrospray tandem mass spectrometric analysis (ESI-MS^n) was performed with a Finnigan LCQ ion trap mass spectrometer. In qualitative analysis 50 μL of each sample was dissolved in 300 μL of 50/50 (v/v) water/methanol solution, and such solutions were introduced to the ESI source through continuous infusion by means of the syringe pump with the rate of 3 μL/min. In quantitative analysis the samples were injected through LCQ injector with a 5 μL loop and measured in single-ion display (SID) mode. The mobile phase was methanol at an isocratic flow rate of 40 μL/min, supplied by Spectra SERIES P100 solvent delivery system. The ESI source was operated at 4.25 kV, and the capillary heater was set to 200 °C. For ESI-MS^n experiments mass-selected molecular adduct ions were isolated monoisotopically in the ion trap and were collisionally activated with 32% ejection rf amplitude at standard He pressure. The experiments were performed in the positive-ion mode.

Results

Synthesis and Characterization of Water-Soluble a-PHB Oligomers. Polymerization of β-butyrolactone mediated by activated anionic initiators is an effective PHB synthetic method, yielding at room temperature "living" polymers with desired microstructure and well-defined chemical structure of the end groups.[7,12,13] In the present study the "living" water-soluble a-PHB of a calculated M_n = 600 was synthesized from racemic β-butyrolactone in the presence of sodium (*R,S*)-3-hydroxybutyrate/18-crown-6 complex and after protonation was characterized by GPC, NMR, and ESI-MS^n spectrometry. The obtained oligomers possessed M_n-(GPC) = 580 and M_w/M_n(GPC) = 1.16, as revealed by GPC experiments. The NMR analysis indicated that the PHB obtained was atactic[13] and contained hydroxybutyrate end groups.[12] The ESI-MS analysis indicated the presence of the sodium adduct ions of the a-PHB dimer (*m/z* = 213), trimer (*m/z* = 299), tetramer (*m/z* = 385), pentamer (*m/z* = 471), hexamer (*m/z* = 557), heptamer (*m/z* = 643), octamer (*m/z* = 729), nonamer (*m/z* = 815), decamer (*m/z* = 901), undecamer (*m/z* = 987), and dodecamer (*m/z* = 1073) of the following structure:



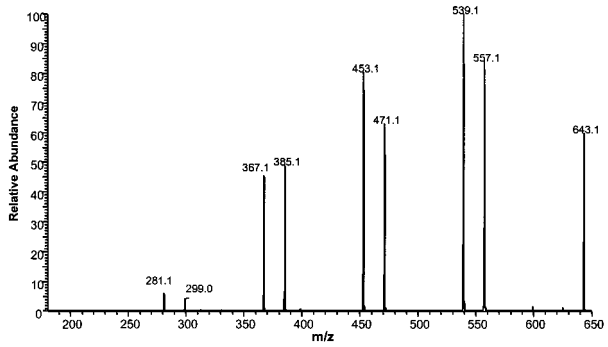
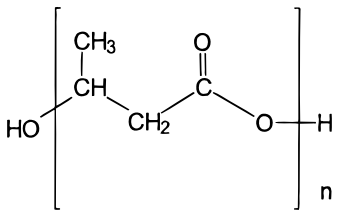


Figure 1. MS^2 fragmentation spectrum of heptamer a_7, parent ion [M + Na] at *m/z* 643.1.



a-PHB; n = 2 - 12

The intensities of the peaks were maximal for the heptamer and decreased to higher and lower oligomers in a Gauss-like distribution with a peak-to-peak mass increment of 86 g mol^{-1}, which is equal to the molecular weight of the PHB repeating unit. The fragmentation experiments of the sodium adduct ions of a-PHB were performed using the ESI-MS^n technique (Figure 1).

The MS^2 spectrum of sodium adduct parent ion of heptamer (*m/z* = 643.1; Figure 1), presented as an example, shows fragmentation at both ends of the polymer molecule. Fragment ions at *m/z* = 557.1, 471.1, 385.1, and 299.0 were formed by the fragmentation involving expulsion of crotonic acid (86 Da). On the other hand, the fragment ion at *m/z* = 539.1 (Figure 1)

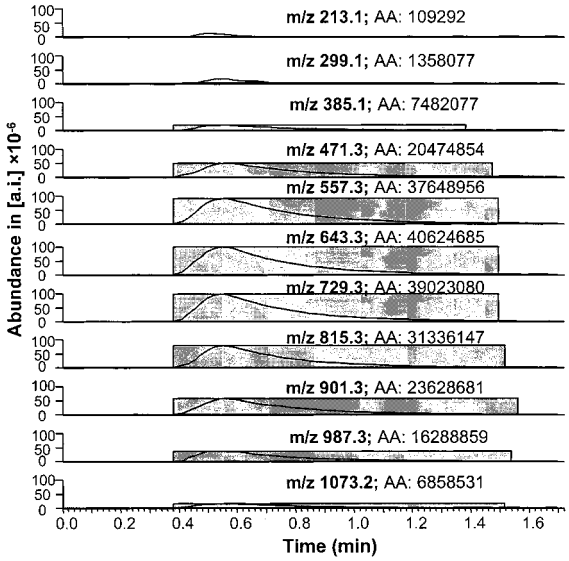


Figure 2. SID traces of individual a-PHB oligomers from dimer (m/z = 213.1) to dodecamer (m/z = 1073.2). AA is the peak area in arbitrary units.

indicates the loss of hydroxybutyric acid (104 Da).[7] It was therefore unequivocally demonstrated, with the aid of ESI-MS^n "fingerprint", that the oligomers prepared contained a_2 up to a_{12} oligomers which bear hydroxyl and carboxylic end groups.

To estimate the concentration of individual a-PHB oligomers in mineral salts medium without the bacterial strains, the 5 μL sample of a-PHB oligomers in mineral salts solution (0.3%, w/v) was analyzed by the ESI-MS technique in the selected ion display mode obtaining the SID traces shown in Figure 2. The ions selected were those of the a-PHB dimer (a_2; m/z = 213), trimer (a_3; m/z = 299), tetramer (a_4; m/z = 385), pentamer (a_5; m/z = 471), hexamer (a_6; m/z = 557), heptamer (a_7; m/z = 643), octamer (a_8; m/z = 729), nonamer (a_9; m/z = 815), decamer (a_{10}; m/z = 901), undecamer (a_{11}; m/z = 987), and the dodecamer (a_{12}; m/z = 1073).

On the basis of the integration of the signals recorded for the selected ions (AA, Figure 2) and the known total concentration of a-PHB oligomers (0.3% w/v), the concentration of the individual a-PHB oligomers (c_i) was estimated and presented in Figure 3 (no bacteria sample). Because the number of molecules of the individual a-PHB oligomer (n_i) is related to c_i by the formula: $n_iM_i = c_iV$ (where M_i represents the molecular weight of individual oligomer in the volume V), the number-average molecular weight of oligomers M_n(ESI) $= \sum n_iM_i/\sum n_i = \sum c_i/\sum c_iM_i^{-1} = 636$ and weight-average molecular weight M_w(ESI) $= \sum n_iM_i^2/\sum n_iM_i = \sum M_ic_i/\sum c_i = 683$ were estimated together with the polydispersity index, calculated as M_w/M_n(ESI) = 1.07. The estimated values are comparable with those determined by GPC experiment, and the difference between M_n(GPC) and M_n(ESI) is lower than the molecular weight of one PHB repeating unit (86 g mol^{-1}).

Table 1. Growth Yields of Different Bacteria on (*R,S*)-3-Hydroxybutyrate and on Synthetically Prepared Oligomers of Atactic 3-Hydroxybutyrate

	OD (Klett units)			
bacterial strain	no carbon source	0.05% w/v 3-HB	0.3% w/v 3-HB	0.3% w/v a-PHB
R.eutropha	18	168	438	192
Comamonas sp.	7	140	370	192
A. faecalis	25	167	396	365

The ESI-MS analysis of a-PHB oligomers without the bacterial strains was essentially the same before and after 30 h of incubation in sterile mineral salts medium and revealed a mixture of a_2 to a_{12} oligomers with a maximum for the heptamer. This result confirmed that a-PHB oligomers are chemically stable under the conditions applied, and no chemical hydrolysis takes place at 30 °C during the experiments conducted.

Growth of the Bacteria. Two PHB-degrading bacteria, namely *Alcaligenes faecalis* T1 and *Comamonas* sp., as well as one non-PHB-degrading bacterium, *Ralstonia eutropha* H16, were grown in mineral salts medium with carbon sources as indicated. All bacteria grew well on 0.05% (*R,S*)-3-hydroxybutyrate (3HB) and on 0.3% 3HB. As expected, the final cell yield was significantly lower with 0.05% 3HB (ca. 150 Klett units) compared to 0.3% 3HB (ca. 400 Klett units) and confirmed that the carbon source was the growth-limiting nutrient (Table 1).

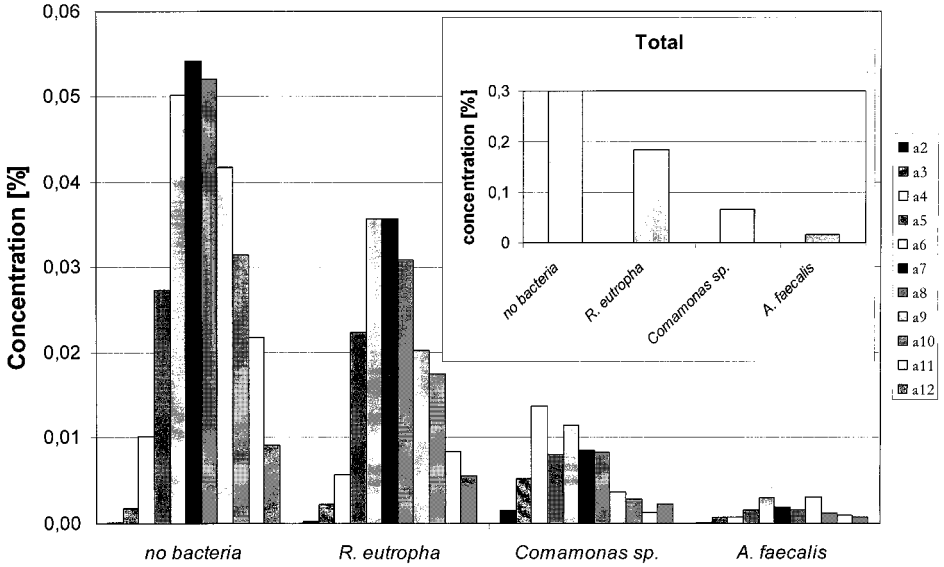


Figure 3. Concentration of individual a-PHB oligomers (a_2−a_{12}) and total a-PHB concentration (insert), after 30 h in mineral salts solution without and with bacterial strains.

When a-PHB oligomers were the only carbon source (0.3% w/v), the best growth was observed with *A. faecalis*. Hardly any difference in the growth curve could be detected compared to growth on 0.3% 3HB (doubling time [td] = 2.0 ± 0.3 h) (Figure 4A) and indicated that a-PHB oligomers have been utilized almost completely by *A. faecalis*. The other PHB-degrading bacterium, *Comamonas* sp., was also able to utilize a-PHB oligomers, and growth was similar to that with 3HB as a carbon source (td = 2.3 ± 0.3 h) (Figure 4B). However, growth flattened already at about 110 Klett units. This indicated that only a part of the a-PHB oligomers had been used for growth. The *R. eutropha* was also able to utilize a-PHB oligomers for growth. This is astonishing because this bacterium does not produce any extracellular PHB depolymerase activity (unpublished results). In contrast to *A. faecalis* and *Comamonas* sp., growth of *R. eutropha* on a-PHB oligomers was significantly slower (td = 6.0 ± 0.6 h) compared to growth on 3HB (td = 2.6 ± 0.3 h; Figure 4C). This indicated that the mechanism of a-PHB oligomers utilization is different and/or less efficient compared to the cases of *A. faecalis* and *Comamonas* sp.

After 30 h of bacterial growth the concentrations of individual a-PHB oligomers as well as the total concentration of a-PHB in the medium were estimated from ESI-MS (as described above for the no-bacteria sample) and are presented in Figure 3.

The ESI-MS analysis of samples after bacterial growth showed that the total concentration of a-PHB had decreased in all cases (Figure 3). Nearly 95% decrease of the initial total concentration of a-PHB oligomers was observed for the sample treated with *A. faecalis*, which is in agreement with the observed good growth of *A. faecalis* on a-PHB oligomers. The M_n(ESI) = 589 and M_w/M_n(ESI) = 1.12 of the remaining 5% of a-PHB oligomers (estimated as described above for the no-bacteria sample) indicate partial redistribution of the individual oligomers. The ESI-MS analysis of a-PHB oligomers after growth of *Comamonas* sp. and *R. eutropha* showed 80% and 38% decrease of the total oligomer content, respectively (Figure 3). A significant redistribution of the concentrations of remaining individual a-PHB oligomers observed for the *Comamonas* sp. sample with the maximum shifted to tetramer (c_4 = 0.014%; Figure 3) and also the average number molecular weight decrease (M_n(ESI) = 475) and increase of polydispersity (M_w/M_n(ESI) = 1.16) of the remaining 20% a-PHB oligomers indicated that extracellular hydrolytic enzymes had cleaved the a-PHB oligomers to lower oligomers. On the contrary, nearly no redistribution of the remaining a-PHB oligomers after treatment with *R. eutropha* was observed (Figure 3; M_n(ESI) = 610 and M_w/M_n(ESI) = 1.07), which is in agreement with the absence of any extracellular PHB depolymerases in *R. eutropha*.

Discussion

In the present study we have demonstrated that the ESI-MS^n technique is a powerful tool in the qualitative and quantitative analysis of the biodegradation of individual molecules, in the case of a mixture of a-PHB oligomers. We demonstrated that a-PHB oligomers, prepared by polymerization of racemic β-butyrolactone in the presence of sodium (*R,S*)-3-hydroxybutyrate/18-crown-6 complexes, consisted of 2−12 3HB repeating units with a maximum for the heptamer. The water-diluted oligomers were stable in the presence of medium components at 30 °C for at least 2 days. We have shown that the a-PHB oligomers supported growth of PHB-

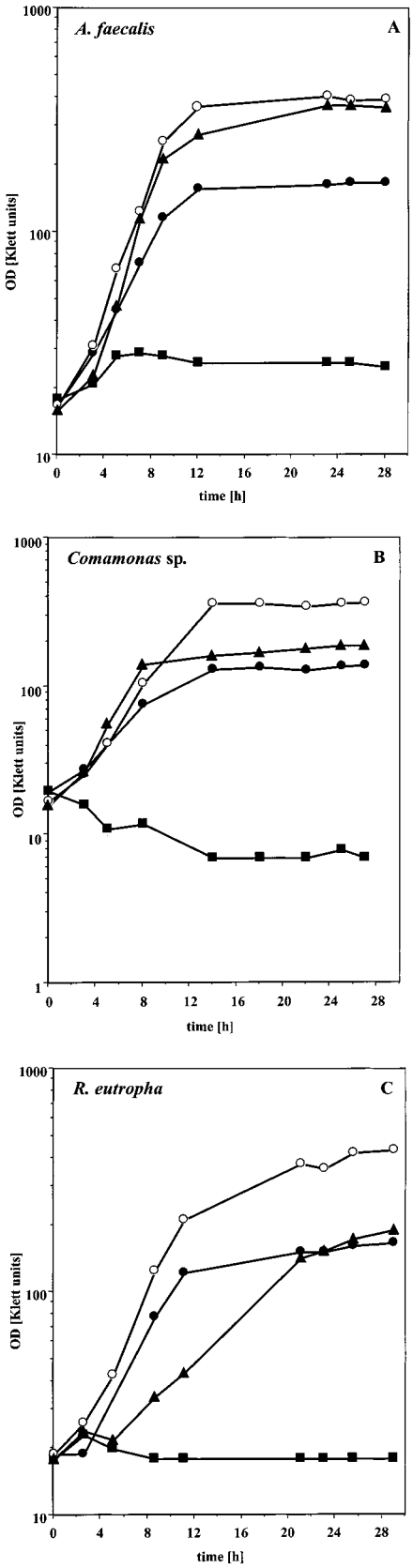


Figure 4. Growth of selected bacterial strains (A, *A. faecalis*; B, *Comamonas* sp.; C, *R. eutropha*) in the presence of (■) no carbon source, (●) 0.05% sodium (*R,S*)-3-hydroxybutyrate, (○) 0.3% sodium (*R,S*)-3-hydroxybutyrate, and (▲) 0.3% a-PHB oligomers.

degrading bacteria but also of a non-PHB-degrading bacterium (*A. faecalis* > *Comamonas* sp. > *R. eutropha*).

A. faecalis. The *A. faecalis* assimilated almost all oligomers available (95%) and showed a very good growth which was undistinguishable from growth on the same concentration of 3HB (Table 1, Figures 3 and 4A). *A. faecalis* is known to have a highly active extracellular PHB depolymerase.[8,14] Since more than 95% of the a-PHB oligomers have been used by *A. faecalis* and because the *A. faecalis* PHB depolymerase is not able to hydrolyze S−S or R−S linkages,[15] this bacterium must have an additional activity that is responsible for utilization of the S-3HB-containing oligomers.

***Comamonas* sp.** showed significant growth on the oligomers. However, the final cell density was lower as compared to that of *A. faecalis*. This means that the bacteria apparently used only a portion of the carbon source. The total concentration of a-PHB oligomers decreased by 80% upon bacterial growth (Figure 4B). This indicated that most of the oligomers were used by the bacteria and is in agreement with the observed growth of *Comamonas* sp. on the a-PHB oligomers (Table 1, Figure 4B). The distribution of the remaining 20% of a-PHB oligomers had changed significantly compared to the control with the maximum shifted from heptamer to tetramer (Figure 3). This indicated that *Comamonas* sp. secreted hydrolytic enzyme(s), presumably its extracellular PHB depolymerase,[9] which hydrolyzed a large portion of the oligomers to low molecular products. We assume that *Comamonas* sp. has the ability to cleave at least some linkages in the oligomers and to utilize most of the degradation products.

***R. eutropha*.** Growth of *R. eutropha* on a-PHB oligomers was significantly slower (td = 6 h) compared to growth on the same concentration of 3HB (td = 2.6 h; Figure 4C), and the growth yield was also significantly reduced. Analysis of the remaining a-PHB oligomers showed that only a portion (38%) of the oligomers have been consumed, which agrees with the observed reduced growth. In contrast to *A. faecalis* and *Comamonas* sp., the distribution of the remaining oligomers was nearly the same as for the control culture (no bacteria, Figure 3). This is indicative of the absence of extracellular hydrolysis of a-PHB oligomers and is in agreement with the known inability of *R. eutropha* to produce extracellular PHB depolymerase. We assume that *R. eutropha* is able to take up atactic 3HB oligomers and to hydrolyze the oligomers by intracellularly located hydrolytic enzymes. Some of us have shown previously that *R. eutropha* has a cytoplasma-localized highly active esterase activity (measured as the hydrolysis of *p*-nitrophenylbutyrate).[16] This activity might be responsible for intracellular utilization of a-PHB oligomers.

Conclusions

The results of the present study revealed for the first time that water-soluble oligomers of a-PHB are assimilated by selected bacterial strains and that the biodegradation behavior of single a-PHB oligomer in solution of mineral salts can be determined by the ESI-MS^n technique. To our surprise we found utilization of a-PHB oligomers not only by typical PHB-degrading bacteria (i.e., *A. faecalis* and *Comamonas* sp.) but also by a typical non-PHB-degrading bacterium (*R. eutropha*). Since the a-PHB oligomers used in this work were synthesized to mimic the oligomeric products remaining after enzymatic hydrolysis of high molecular weight atactic poly[(*R,S*)-3-hydroxybutyrate] by extracellular PHB depolymerases, the present results demonstrate complete mineralization, i.e., total biodegradability, of this synthetic polymer.

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