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Application of Recombinant Gene Technology for Production of Polyhydroxyalkanoic Acids: Biosynthesis of Poly(4-hydroxybutyric Acid) Homopolyester*

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Screening of a large number of bacteria revealed several strains, which utilize 1,4-butanediol and/or 4-hydroxybutyric acid (4HB) as a carbon source for growth and for synthesis of polyhydroxyalkanoic acids (PHA) containing 4HB as one constituent among others (mostly 3-hydroxybutyric acid). However, none of the wild-type strains investigated in this study was able to produce a homopolyester consisting solely of 4HB. Only several poly(3-hydroxybutyric acid)-leaky mutants of *Alcaligenes eutrophus* strain JMP222 synthesized poly(4HB) homopolyester, which amounted to approximately 10% (w/w) of the cellular dry matter. If the PHA synthase structural gene of *A. eutrophus* strain H16 was expressed in these mutants, the amount of poly(4HB) was increased to approximately 30% (w/w). The occurrence of poly(4HB) was demonstrated by gas chromatographic as well as ^{1}H and ^{13}C nuclear magnetic resonance spectroscopic analysis.

KEY WORDS: Polyhydroxyalkanoic acids (PHA); 4-hydroxybutyric acid; homopolyester; poly(4-hydroxybutyric acid); biodegradable polyesters.

INTRODUCTION

Polyhydroxyalkanoic acids (PHA) comprise a complex class of naturally occurring polyesters representing the major intracellular storage compound of many bacteria. Approximately 50 hydroxyalkanoic acids have already been detected as constituents of these bacterial polyesters [1–3]. Recently, 4-hydroxybutyric acid (4HB) was also detected, but 4HB was incorporated into PHA only if 4-hydroxybutyrate, 4-butyrolactone, 4-chlorobutyrate, or ω-alkanediols with an even number of carbon atoms such as, e.g., 1,4-butanediol, were provided as a carbon source to *Alcaligenes eutrophus* strain H16, *A. latus*, *Pseudomonas acidovorans*, or *Rhodococcus ruber* or to recombinant strains of the PHA-negative mutant PHB^{-}4 of *A. eutrophus* H16 which expressed the PHA-biosynthesis genes of various anoxygenic photosynthetic sulfur-purple bacteria [4–12]. However, although many constituents have already been detected, most PHA occur as copolyesters. Biosynthesis of only a few homopolyesters other than poly(3-hydroxybutyric acid), poly(3HB), has been reported (Ref. 13 and references cited therein): These are polyesters of 3-hydroxy-5-phenylvaleric acid, 3-hydroxyhexanoic acid, 3-hydroxyheptanoic acid, 3-hydroxyoctanoic acid, or 3-hydroxynonanoic acid. But these homopolyesters either occur in the cells as a blend together with other polyesters, or are produced in only small amounts, or are synthesized by bacteria, which are not well suited for mass cultivation. Recently, the formation of a homopolyester of 3-hydroxyvaleric acid has been reported [13]. Very small amounts of poly(4HB) homopolyester, contributing to only 2% of the cellular dry matter, were synthesized by *A. eutrophus* H16 from 4-hydroxybutyrate if 1.0 or 1.5% (w/v) potassium citrate was added to the medium in addition

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to 4-hydroxybutyrate [14]. In the PHA, which was accumulated from 4-hydroxybutyrate by *P. acidovorans*, 4HB contributed to 99 mol% of the constituents [8].

In this study, we screened a large variety of aerobic Gram-negative bacteria for their ability to utilize 4HB or 1,4-butanediol as carbon sources for growth and for synthesis of PHA, which contained 4-hydroxybutyric acid (4HB) as a constituent at a high molar fraction. In addition, mutants of *A. eutrophus* JMP222, which were defective in genes relevant for biosynthesis and accumulation of PHA, and genetically engineered strains, which harbored additional copies of the PHA-biosynthesis genes from a different strain, were analyzed for their capability to synthesize poly(4HB) homopolyester.

EXPERIMENTAL

Bacterial Strains and Plasmids

Alcaligenes eutrophus H16 (DSM428), *A. eutrophus* JMP222 [15], and the PHA-leaky mutants PHB$^-$102 and PHB$^-$151 as well as the PHA-negative mutant PHB$^-$180 derived from JMP222 were used in this study. In addition, we investigated the bacteria listed in Table I. *Escherichia coli* S17-1 [16] was used as a donor for the plasmids pVK101 :: PP1 (broad-host range vector plasmid harboring the PHA-biosynthesis genes of *A. eutrophus* H16 [17]) and pBL7502 (broad-host range vector plasmid harboring genes of *A. eutrophus* H16 encoding proteins which exhibit homology to components of bacterial sugar:PEP phosphotransferase systems [18]).

Cultivation of Bacteria and Media

All bacteria were cultivated in the basic mineral salts medium (MSM) described by Schlegel *et al.* [19]. Filter-sterilized carbon sources were added as indicated in the text. Solidified media contained 1.5% (w/v) agar.

Fermentations in a 10-L scale were done in glass fermenters (BIOSTAT, Braun Melsungen, Germany), at 30°C. The cultures were aerated with 2 L air/min and agitated at 500 rpm. Ten liters of MSM containing 0.5% (w/v) sodium 4-hydroxybutyrate was inoculated with two 300-ml precultures (medium: 0.8%, w/v, nutrient broth). After 48 h of cultivation 50 g sodium 4-hydroxybutyrate was added, and after approximately 100 h of cultivation the cells were harvested. The pH value of the medium was automatically adjusted to 7.0 by titration with phosphoric acid.

Conjugational Plasmid Transfer

Broad-host range vector plasmids were mobilized from *E. coli* S17-1 to *A. eutrophus* JMP222 or to derivative strains by the spot agar mating technique, and transconjugants were selected on MSM agar plates containing 0.5% sodium succinate and 12.5 μg tetracycline/ml.

Isolation and Gas Chromatographic Analysis of PHA

PHA were isolated from lyophilized cells by extraction with chloroform in a Soxhlet apparatus. The polyester was precipitated from the chloroform solution by the addition of 10 vol of ethanol, and the precipitate was subsequently separated from the solvent by filtration. Remaining solvents were removed by exposure of the polyester to a stream of air.

For quantitative determination of PHA, 3 to 5 mg of lyophilized cell material or isolated polyester was subjected to methanolysis in the presence of 15% (v/v) sulfuric acid, and the hydroxyacyl methylesters were analyzed by gas chromatography exactly as described previously in detail by Brandl *et al.* [20] and Timm *et al.* [21]. 3-Hydroxyfatty acids (C_4 to C_{12}) were obtained from the authors of Ref. 20. These acids as well as the sodium salts of 3-hydroxypropionic acid, 4-hydroxybutyric acid, 4-hydroxyvaleric acid, 4-hydroxyhexanoic acid, 5-hydroxyvaleric acid, and 5-hydroxyhexanoic acid were subjected to methanolysis, too, and the resulting methyl esters were used for calibration.

Assay of PHA Synthase Activity

PHB synthase activity was estimated in crude cellular extracts by a radiometric assay with D-(−)-3-[3-^{14}C]hydroxybutyryl-CoA according to the principle method described by [17], which has been modified recently [22]. Protein concentration was estimated according to the method described by Bradford [23].

Nuclear Magnetic Resonance (NMR) Spectroscopic Analysis

The ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian (Palo Alto, CA) VXR-500S. The 500-MHz ^{1}H-NMR spectra were recorded at 23°C from a $CDCl_3$ solution of the polyesters (15 mg/ml) using 45° pulses, a 4.368-s repetition time, a 7500-Hz spectral width, 64K data points, and 32 accumulations. The 125-MHz ^{13}C-NMR spectra were recorded at 50°C in a $CDCl_3$

Table I. Utilization of 4-Hydroxybutyrate and 1,4-Butanediol as a Carbon Source for Growth[a]

Strain	Reference	Carbon source for growth	
		1,4-Butanediol	4-Hydroxybutyrate
Alcaligenes eutrophus A7	[24]	+++	+++
Alcaligenes eutrophus CH34	[25]	+++	+++
Alcaligenes eutrophus H16	DSM 428	−	−
Alcaligenes eutrophus JMP222	[15]	+	+
Alcaligenes eutrophus N9A	DSM 518	−	−
Alcaligenes eutrophus TF93	DSM 531	±	±
Methylobacterium extorquens	DSM 1337	++	+
Methylobacterium extorquens	DSM 1338	++	+
Methylobacterium mesophilicum	DSM 1708	+++	++
Methylobacterium rhodinum	DSM 2163	++	++
Micrococcus radiotolerans	DSM 1819	++	++
Paracoccus denitrificans	DSM 413	− (+SC)	±
Pseudomonas butanovora	DSM 2080	+++	+++
Pseudomonas citronellolis	DSM 50332	−	± (+SC)
Pseudomonas flava	DSM 619	++	++
Pseudomonas fluorescens	DSM 50108	±	++
Pseudomonas glathei	DSM 50014	+	+
Pseudomonas marginalis	DSM 50276	++	+
Pseudomonas mendocina	DSM 50017	++	+++
Pseudomonas oxalaticus	DSM 1105	+	++
Pseudomonas paucimobilis	DSM 1098	± (+SC)	+
Pseudomonas pseudoalcaligenes	DSM 50188	+++	+ (+SC)
Pseudomonas stutzeri	DSM 50227	−	+
Pseudomonas stutzeri	DSM 50027	±	+
Rhodococcus ruber	NCIMB 40126	−	−
27 heavy metal-resistant bacteria	H. G. Schlegel, e.g., [26, 27]	From ± to +++	From − to +++

[a]The bacteria were cultivated on MSM agar plates, which contained 0.5% (w/v) 1,4-butanediol or 0.2% (w/v) 4-hydroxybutyrate as the sole carbon source. +++, excellent growth; ++, good growth; +, poor growth; ±, very poor growth; −, no growth; SC, single colonies occurred after incubation for a prolonged period; DSM, Deutsche Sammlung von Mikrorganismen; NCIMB, National Collection of Industrial and Marine Bacteria.

solution of the polyester (50 mg/ml) using Waltz-16-decoupling, 25° pulses, a 1.193-s repetition time, a 27,500-Hz spectral width, 64K data points, and 1664 accumulations.

Chemicals

4-Hydroxybutyric acid was obtained from Fluka (Buchs, Helvetia); most other chemicals were from Merck (Darmstadt, Germany).

RESULTS AND DISCUSSION

Screening for Wild-Type Bacteria Suitable for Production of PHA Containing 4HB

Approximately 400 aerobic, Gram-negative bacteria from the culture collection of the laboratory, which belong mainly to the genera *Pseudomonas*, *Alcaligenes*, and *Paracoccus* and which were known to be able to synthesize PHA, were incubated on MSM agar plates containing 0.5% (w/v) 1,4-butanediol or 0.2% (w/v) 4-hydroxybutyrate as the sole carbon source. The screening also included 232 new isolates of soil bacteria that exhibited resistance to nickel, cadmium, or cobalt and which were kindly provided by Prof. Dr. H. G. Schlegel. Only 49 strains (including 27 of the heavy metal-resistant bacteria) were able to utilize either compound as a carbon source for growth.

Those strains, which grew on 4-hydroxybutyrate or 1,4-butanediol, were also analyzed for their capability to use these compounds as a carbon source for synthesis of PHA containing 4HB as a constituent. However, most strains synthesized and accumulated copolyesters of 3-hydroxybutyric acid (3HB) and 4HB [(poly(3HB-co-4HB)] from 4-hydroxybutyrate or 1,4-butanediol, and the molar fraction of 4HB in the copolyester only infrequently exceeded 50%. Some pseudomonads synthesized PHA consisting of hydroxyalkanoic

acids of medium chain length with 3-hydroxydecanoic acid as the main constituent from 4-hydroxybutyrate as from, e.g., gluconate (see Ref. 28); however, 4HB was never detected as a constituent in the PHA accumulated by these bacteria. None of the wild types investigated in this study was able to synthesize and accumulate poly(4HB) homopolyester.

If a cell suspension of *Paracoccus denitrificans* was spread on MSM agar plates containing 0.2% 4-hydroxybutyrate as the sole carbon source, spontaneous mutants occurred, which grew much faster than the wild type. After mutagenesis with sodium nitrite, 4-hydroxybutyrate-utilizing mutants could be also isolated from *A. eutrophus* strains H16 and N9A. When these mutants were investigated for their capability to incorporate 4HB into PHA, it turned out that they produced poly(3HB-co-4HB) copolyester from 4-hydroxybutyrate with 4HB at a much lower molar fraction than in the copolyester accumulated by the corresponding wild type. Presumably, the mutations enabled the formation of mutants, which have a higher conversion of 4-hydroxybutyrate to a central intermediate of the metabolism, subsequently resulting in a better utilization of 4-hydroxybutyrate as a carbon source for growth. This assumed alteration of the flux of the metabolites might not only lower the concentration of the intracellular 4HB-CoA pool; since it would also increase the availability of acetyl-CoA from 4-hydroxybutyrate, the probability of synthesis of 3-hydroxybutyryl-CoA and of the incorporation of 3HB into the accumulated PHA would become higher.

In addition to the bacteria mentioned above, 12 bacteria, which readily grew on 4-hydroxybutyrate, and 8 bacteria, which readily grew on 1,4-butanediol, were isolated from the soil and investigated for their capability to synthesize PHA containing 4HB as a constituent. It turned out that none of the new isolates was suited to produce PHA with a high content of 4HB. These isolates therefore confirm our conclusion that fast growth on 4-hydroxybutyrate does not parallel efficient incorporation of 4HB into PHA.

Analysis of Mutants and of Genetically Engineered Bacteria

When mutants of *Alcaligenes eutrophus* strain JMP222, which were altered in the accumulation of poly(3HB) from fructose and from gluconate, were investigated for their capability to incorporate 4HB into PHA, an interesting observation was made. PHA-leaky mutants, which were defective in genes presumably regulating the mobilization of poly(3HB) [18], accumulated less PHA than the wild type. From 4-hydroxybutyrate as the sole carbon source, they produced poly(3HB-co-4HB) with an increased molar fraction of 4HB. If 3HB was detected at all, it contributed not more than 10 mol% to the constituents of the accumulated PHA. Some PHA-leaky mutants, such as PHB$^-$102 (Table II) and PHB$^-$151, accumulated small amounts of poly(4HB) homopolyester (Fig. 1), which contributed a maximum of 12% of the cellular dry matter. The biochemical and physiological rationale for this is presently unknown.

If additional copies of the PHA-biosynthesis genes of *A. eutrophus* strain H16 ([17]; for a recent review see Ref. 29) were introduced into the PHA-leaky mutant PHB$^-$102, the content of poly(4HB) homopolyester in the cells synthesized from 4-hydroxybutyrate could be drastically increased (Table II). Recombinant strains of the PHA-leaky mutant PHB$^-$151 and of others harboring pVK101 :: PP1 behaved similar (not shown in Table II). In some recombinant mutants poly(4HB) contributed almost 30% of the cellular dry matter. The recombinant cells expressed approximately five times the poly(3HB) synthase activity measured in the parent strain (Table II). This indicated that the PHA synthase is the bottleneck for poly(4HB) biosynthesis in *A. eutrophus* JMP222 rather than the conversion of 4-hydroxybutyrate to 4-hydroxybutyryl-CoA (Fig. 2).

Cells of recombinant PHA-leaky mutants accumulating poly(4HB) were grown in 10-L fermenters, and the polyester was isolated by chloroform extraction from freeze-dried cells and by subsequent precipitation in the presence of ethanol. Gas chromatographic analysis of the isolated polyesters confirmed the synthesis of poly(4HB) homopolyester.

In PHB$^-$102 harboring plasmid pBL7502, which provides the intact genes from *A. eutrophus* H16 [18] that are presumably defective in these mutants of JMP222, and which restored the phenotype of the wild type regarding the accumulation of poly(3HB) from, e.g., gluconate, the amount and the composition of the accumulated poly(3HB-co-4HB) did not deviate very much from those of the polyester accumulated by the wild type, and also the specific activity of poly(3HB) synthase increased to the level measured in the wild type.

Recombinant strains of the wild type JMP222 harboring plasmid pVK101 :: PP1 did not accumulate more PHA than the parent strain JMP222 from 4-hydroxybutyrate; furthermore, the composition of the PHA was very similar to that of the parent strain. If plasmid pVK101 :: PP1 was transferred to the PHA-negative mutant PHB$^-$180 of *A. eutrophus* JMP222, the

Table II. Incorporation of 4HB into PHA by *A. eutrophus* JMP222 and Derivative Strains[a]

Strain	Characteristics	PHA content (% of CDW)	Composition of PHA (mol%)		Poly(3HB) synthase activity (U/g protein)
			3HB	4HB	
JMP222	Wild type	44.2	31.0	69.0	106.5
JMP222-PHB$^-$102	PHA-leaky mutant	11.3	nd	100.0	12.0
JMP222-PHB$^-$102 (pVK101::PP1)	Recombinant PHA-leaky mutant	27.2	nd	100.0	64.2
JMP222-PHB$^-$102 (pBL7502)	Recombinant PHA-leaky mutant	41.3	25.0	75.0	102.7

[a]Cells were aerobically cultivated for 96 h at 30°C in a mineral salts medium under nitrogen starvation with 4-hydroxybutyric acid (0.5%, w/v) as the sole carbon source. nd, not detectable; CDW, cellular dry weight.

capability to synthesize PHA was restored, but a poly(3HB-co-4HB) copolyester, which contained 4HB at a molar fraction of only approximately 30 to 40 mol%, was accumulated from 4-hydroxybutyrate.

Spectroscopic Analysis of Poly(4HB)

To confirm the GC data, which provided evidence that only 4HB was present in the polyesters isolated from cells of the PHB-leaky mutants of *A. eutrophus* JMP222 and from its derivative strains, which were obtained from 4-hydroxybutyric acid in 10-L fermentations that were carried out as described under Experimental, ^{1}H- and ^{13}C-NMR spectra were recorded (Figs. 3 and 4). The ^{13}C as well as the ^{1}H signals of poly(4HB) isolated from the recombinant strain of *A. eutrophus* JMP222-PHB$^-$102 were identical to those obtained for 4HB from the hydrogen and carbon atoms of 4HB in poly(3HB-co-

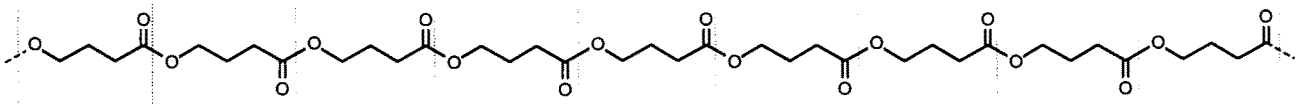


Fig. 1. Structural formula of poly(4HB).

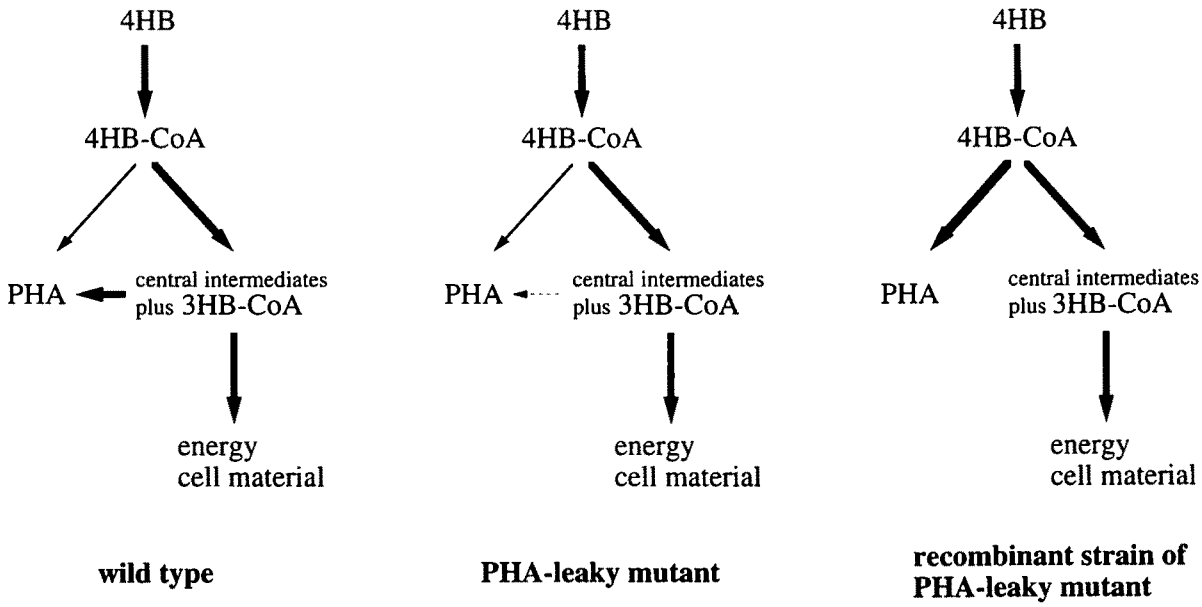


Fig. 2. Physiological basis of PHA biosynthesis from 4-hydroxybutyric acid in the wild type of *A. eutrophus* JMP222 and in derivative strains.

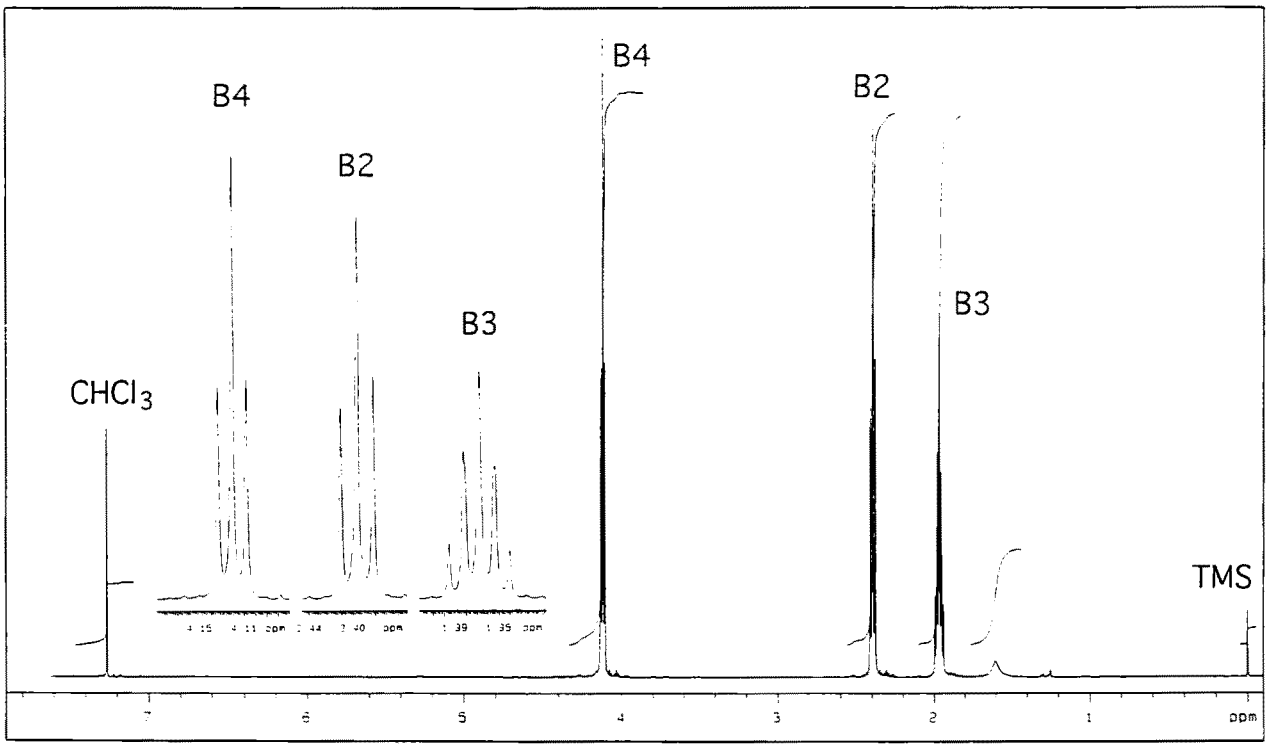


Fig. 3. ^{1}H-Nuclear magnetic resonance spectrum (500 MHz) of poly(4HB). The polyester was isolated from *A. eutrophus* JMP222-PHB-151 (pVK100::PP1) cultivated with 4-hydroxybutyric acid as the sole carbon source, and the spectrum was recorded from the isolated polyester in $CDCl_3$ as described under Experimental. Parts of the spectrum were expanded and are shown as insets. The assignments of signals refer to the carbon atom numbers shown in the structural formula for poly(4HB) provided in Fig. 5.

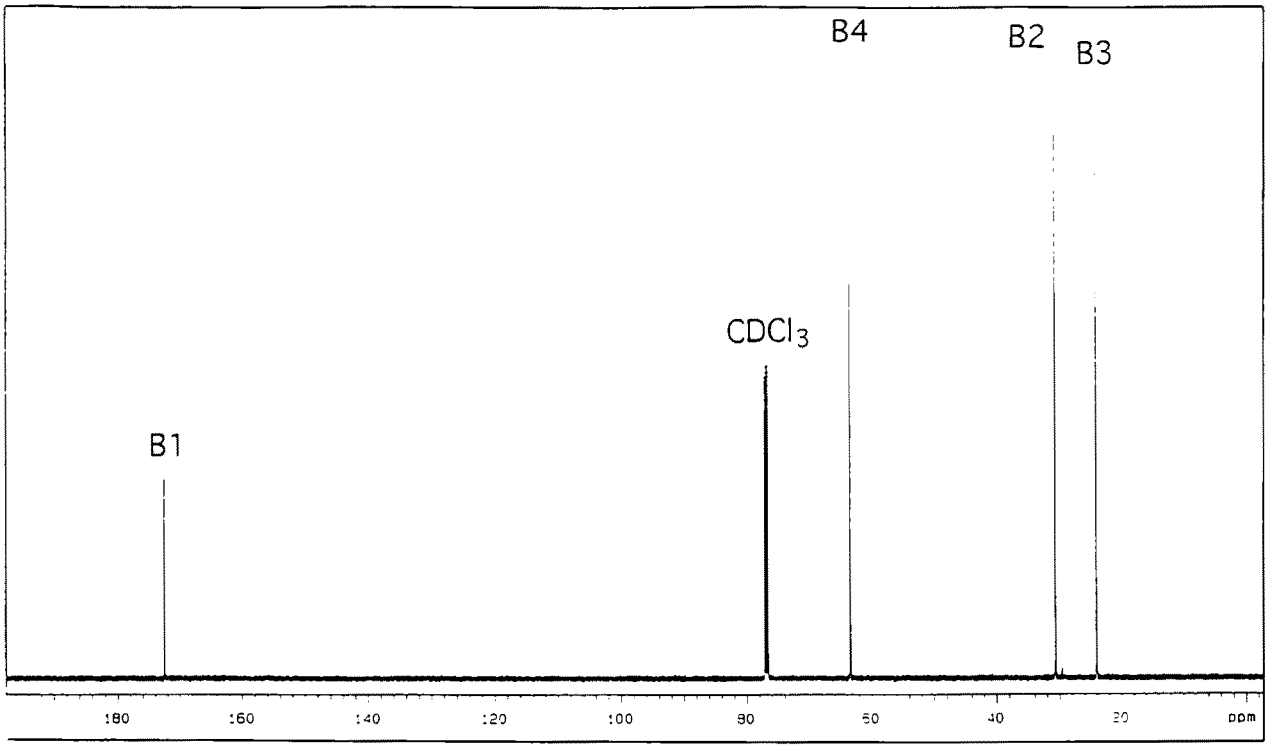
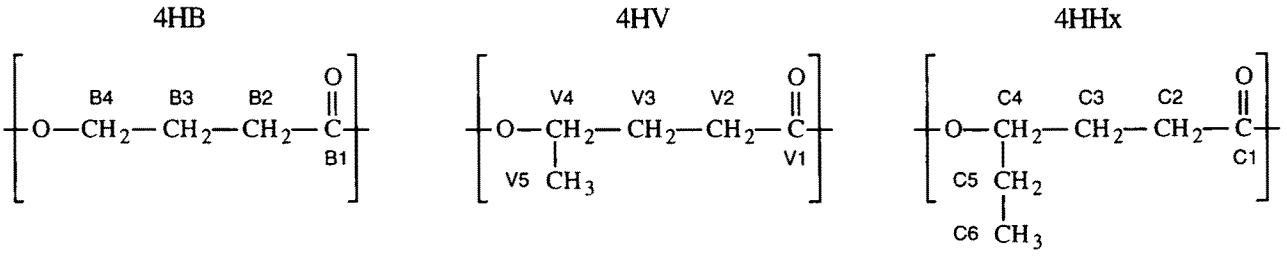


Fig. 4. ^{13}C-Nuclear magnetic resonance spectrum (125 MHz) of poly(4HB). The polyester was isolated from *A. eutrophus* JMP222-PHB-151 (pVK100::PP1) cultivated with 4-hydroxybutyric acid as the sole carbon source, and the spectrum was recorded from the isolated polyester in $CDCl_3$ as described under Experimental. The assignments of signals refer to the carbon atom numbers shown in the structural formula for poly(4HB) provided in Fig. 5.



Chemical shifts in ^1H nuclear magnetic resonance analysis (ppm)

4HB[a]		4HB[b]		4HV[d]		4HHx[e]	
CH_2 (B2)	2.40	CH_2 (B2)	2.40	CH_2 (V2)	2.32	CH_2 (C2)	2.26
CH_2 (B3)	1.97	CH_2 (B3)	1.97	CH_2 (V3)	1.87	CH_2 (C3)	1.84
CH_2 (B4)	4.12	CH_2 (B4)	4.12	CH (V4)	4.92	CH (C4)	4.80
				CH_3 (V5)	1.22	CH_2 (C5)	1.55
						CH_3 (C6)	0.85

Chemical shifts in ^{13}C nuclear magnetic resonance analysis (ppm)

4HB[a]		4HB[c]		4HV[d]		4HHx[e]	
C=0 (B1)	172.54	C=0 (B1)	171.85 - 172.67	C=0 (V1)	171.85 - 172.02	C=0 (C1)	171.91 - 172.61
CH_2 (B2)	30.72	CH_2 (B2)	30.59 - 30.88	CH_2 (V2)	30.43 - 30.56	CH_2 (C2)	30.46 - 30.50
CH_2 (B3)	24.13	CH_2 (B3)	23.95 - 24.00	CH_2 (V3)	30.88 - 30.94	CH_2 (C3)	28.61 - 28.86
CH_2 (B4)	63.52	CH_2 (B4)	63.54 - 63.64	CH (V4)	70.36 - 70.40	CH (C4)	74.66 - 74.95
				CH_3 (V5)	19.65 - 19.85	CH_2 (C5)	26.80 - 26.95
						CH_3 (C6)	9.38 - 9.42

Fig. 5. Chemical shifts of ^1H- or ^{13}C-nuclear magnetic resonances occurring with polyester containing 4-hydroxyalkanoic acids obtained from NMR spectra. The assignments refer to those shown in the structural formulas. Superscripts: (a) this study; (b) data taken from Ref. 4; (c) data taken from Ref. 6; (d) data taken from Ref. 30; (e) data taken from Ref. 31. 4HB, 4-hydroxybutyric acid; 4HV, 4-hydroxyvaleric acid; 4HHx, 4-hydroxyhexanoic acid.

4HB) copolyester or in poly(3HB-co-3HV-co-4HB) terpolyester, which were obtained from *A. eutrophus* strain H16 in earlier studies (Fig. 5) [4, 6]. The chemical shifts of ^1H and ^{13}C NMR signals of 4HB, 4-hydroxyvaleric acid, and 4-hydroxyhexanoic acid are summarized and compared in Fig. 5. In the spectra of the biosynthetic poly(4HB) homopolyester, there was no sign of a 21.56-ppm peak which would represent the CH_2 methylene group at carbon 2 of 3HB, and there was also no other unexpected repeat unit NMR signal, thus confirming the conclusions from the gas chromatographic analysis that no constituents other than 4HB occurred in the isolated polyester.

Some Properties of Biosynthetic Poly(4HB) Homopolyester

Poly(4HB) was extracted from lyophilized cells of *A. eutrophus* with chloroform at a much lower rate than other PHA such as, e.g., poly(3HB) from cells of the same strain. Whereas poly(3HB) was completely extracted within usually 1 day, the same amount of poly(4HB) was extracted from the same amount of cells of *A. eutrophus* in the same Soxhlet apparatus and under otherwise identical conditions within 3 to 4 days only. The homopolyester could be readily precipitated from chloroform in the presence of an excess of ethanol, and in contrast to poly(3HB) or poly(3HB-co-3HV), it precipitated as a highly fibrous material which easily and almost quantitatively ended up on a glass rod if the latter was used for stirring during precipitation.

Dry poly(4HB) is a white material, and it exhibited a much higher elasticity than, e.g., poly(3HB), as revealed by manual examination. The molecular masses of poly(4HB) samples as determined by gel-permeation chromatography were relatively high. For three samples from independent fermentations the values were M_w = 1.14×10^6 (M_w/M_n = 2.79), M_w = 0.99×10^6 (M_w/

M_n = 3.25), and $M_w = 0.79 \times 10^6$ (M_w/M_n = 3.82). Only one sample exhibited a lower value, $M_w = 0.20 \times 10^6$ (M_w/M_n = 2.52); the reason for this remained unclear.

CONCLUSIONS

Genetically engineered derivatives of mutants of *A. eutrophus* JMP222, which were almost impaired for synthesis of poly(3HB) and which contained multiple copies of a heterologous PHA synthase structural gene, produced a poly(4HB) homopolyester if 4-hydroxybutyric acid was used as the carbon source. The biosynthetic poly(4HB) exhibited some interesting properties, and its molecular weight was relatively high. Since this homopolyester was accumulated in large amounts in the cells, it is now available to evaluate whether it is suitable for some technical applications. In addition, studies on the biodegradability of the biopolymer and on the access of PHA depolymerases and of other hydrolytic enzymes for this polyester are now possible.

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