Appl Microbiol Biotechnol (1994) 40:710–716

Applied and Microbiology Biotechnology

Identification of 4-hydroxyhexanoic acid as a new constituent of biosynthetic polyhydroxyalkanoic acids from bacteria

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Received: 5 August 1993/Accepted: 1 September 1993

Abstract. Various aerobic Gram-negative bacteria were analysed for utilizing 4-hydroxyhexanoic acid (4HHx) as a carbon source for growth and for synthesis of polyhydroxyalkanoic acids (PHA). Although many wild types grew on 4HHx, only recombinant strains of the PHA-negative mutants *Pseudomonas putida* GPp104 and *Alcaligenes eutrophus* PHB$^-$4, which harboured plasmid pHP1014::E156 with the PHA-biosynthesis genes of *Thiocapsa pfennigii,* incorporated 4HHx up to a molar fraction of 47 or 1.4%, respectively, into PHA if the cells were cultivated in the presence of 4HHx as sole carbon source and under nitrogen starvation. A terpolyester consisting of 3-hydroxybutyric acid (3HB), 3-hydroxyhexanoic acid (3HHx) and 4HHx was synthesized, as revealed by gas chromatographic analysis of the accumulated polyester and as confirmed by nuclear magnetic resonance spectroscopic analysis of the isolated polyester. 4HHx was also detected in PHA accumulated by *Rhodococcus ruber* if 4HHx was used as a carbon source. However, it occurred at a molar fraction of maximally 1.3 mol% only beside 3HB, 3-hydroxyvaleric acid and 3HHx.

Introduction

Many bacteria accumulate polyhydroxyalkanoic acids (PHA) as carbon and energy storage compounds or synthesize these polyesters as a sink for reducing equivalents (Anderson and Dawes 1990; Steinbüchel 1991a). More than 40 different constituents of these biodegradable thermoplastics and elastomers have been detected. This includes straigth-chain saturated 3-hydroxyalkanoic acids with three to twelve carbon atoms, unsaturated 3-hydroxyalkanoic acids with five to fourteen carbon atoms and one or two double bonds as well as 3-hydroxyalkanoic acids with branched, halogenated or aromatic side chains (for review: see Steinbüchel 1991 a, b). PHA synthases, which represent the key enzyme of PHA biosynthesis, have been investigated in detail at a molecular level (Steinbüchel et al. 1992 and references cited therein). Mainly physiological studies indicated the occurrence of two types of PHA synthases, which are distinguished with respect to their substrate specificity (Steinbüchel et al. 1992). The majority of PHA-accumulating bacteria possess a PHA_{SCL} synthase, which incorporates preferably 3-hydroxyalkanoic acids of short chain length with three to five carbon atoms. Some bacteria that possess a PHA_{SCL} synthase can, in addition to 3-hydroxyalkanoic acid, also incorporate 4-hydroxybutyric acid (4HB) (Kunioka et al. 1988), 4-hydroxyvaleric acid (4HV) (Valentin et al. 1992) and 5-hydroxyvaleric acid (Doi et al. 1987) into PHA. Molecular analysis revealed two different classes of this type, which are distinguished by their structure (Steinbüchel et al. 1992). Only pseudomonads belonging to the rRNA homology group I possess PHA_{MCL} synthases, which preferably incorporate 3-hydroxyalkanoic acids of medium chain length with six or more carbon atoms. The latter do not or only at a marginal level incorporate 3-hydroxybutyric acid (3HB) into PHA; some of them can obviously also incorporate 3-hydroxyvaleric acid (3HV) (Gross et al. 1989; Preusting et al. 1990).

Recently, evidence for a PHA synthase was obtained, the substrate specificity of which deviated from those mentioned above. When a PHA-negative mutant of *Pseudomonas putida,* which harboured a plasmid with the PHA-biosynthesis genes of *Thiocapsa pfennigii,* was cultivated on octanoic acid, a polyester was accumulated that consisted of almost equimolar amounts of 3HB and 3-hydroxyhexanoic (3HHx) plus small amounts of 3-hydroxyoctanoic acid (3HO) (Liebergesell et al. 1993). Under different conditions this PHA synthase also conferred the capability to synthesize PHA consisting of 4HB to the PHA-negative mutant PHB$^-$4 of *Alcaligenes eutrophus.* This PHA synthase was therefore a promising candidate for the biosynthesis of polyesters that contain 4-hydroxyhexanoic acid (4HHx) as a consituent, a hitherto no-mentioned constituent of biosynthetic polyesters.

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Materials and methods

Bacterial strains. *A. eutrophus* H16 (DSM 428), *A. eutrophus* PHB$^-$4 (PHA-negative mutant of H16, DSM 41), *P. oleovorans* (ATCC 29347), *P. putida* GPp104 (PHA-negative mutant of *P. putida* KT2442, Huisman et al. 1991), *Rhodococcus ruber* (NCIMB 40126) and the strains listed in Table 1 were used in this study. In addition, plasmid pHP1014::E156, which harbours PHA-biosynthesis genes of *T. pfennigii* (Liebergesell et al. 1993), was used.

Cultivation of bacteria and media. All bacteria were cultivated in the mineral salts medium (MSM) described by Schlegel et al. (1961). Filter-sterilized carbon sources were added as indicated in the text. Solidified media contained 1.5% (w/v) agar.

The ability to accumulate PHA was investigated in two different types of experiments. In one-stage fed-batch cultivation experiments the cells were grown in MSM, wich contained only 0.05% (w/v) ammonium chloride as a nitrogen source in order to promote PHA accumulation. 4-HHx was used as a carbon source for growth and PHA accumulation and was obtained from the corresponding lactones by alkaline saponification (Valentin et al. 1992). These cultures were inoculated with approximately 6% (v/v) of an overnight nutrient broth culture. In two-stage batch or fed-batch cultivation experiments, the cells were first grown in nutrient broth at a concentration of 0.8% (w/v). After 15 h cultivation the cells were harvested under axenic conditions and transferred to the same volume of MSM, which contained no nitrogen source and which was supplemented with 0.5% (w/v) of the carbon source indicated in the text. All cultivation experiments were done at 30°C under aerobic conditions on a rotary shaker with approximately 300 strokes per min and in erlenmeyer flasks containing not more than 20% (v/v) medium.

Isolation of PHA. PHA were isolated from lyophilized cells by extraction with chloroform in a Soxhlet apparatus. The polyester

Table 1. Utilization of 4-hydroxyhexanoic acid (4HHx) as a carbon source for growth and polyhydroxyalkanoic acid (PHA) biosynthesis by Gram-negative bacteria

Bacterial strain	Source or reference	Growth	Type	PHA content (% of CDW)	Composition of PHA (mol%) 3HB	3HHx	4HHx	3HO	3HD
Alcaligenes eutrophus H16	DSM 428	+++	A	78.5	98.8	1.2	—	—	—
			B	76.3	99.4	0.6	—	—	—
A. eutrophus PHB$^-$4	DSM 541	+++	A	nd	—	—	—	—	—
			B	nd	—	—	—	—	—
PHB$^-$4 (pHP1014::E156)	This study	+++	A	68.9	98.7	1.3	—	—	—
			B	48.9	96.7	1.7	1.6	—	—
A. eutrophus N9A	DSM 518	+++	A	65.8	98.9	1.1	—	—	—
			B	66.2	99.2	0.8	—	—	—
A. eutrophus TF93	DSM 531	+++	A	67.2	97.8	2.2	—	—	—
			B	nd	—	—	—	—	—
A. eutrophus JMP222	Don and Pemperton (1981)	+++	A	58.4	99.6	0.4	—	—	—
			B	65.7	100.0	—	—	—	—
Chromobacterium violaceum	DSM 30191	—	A	nd	—	—	—	—	—
			B	nd	—	—	—	—	—
Pseudomonas oleovorans	ATCC 29347	±	A	nd	—	—	—	—	—
			B	Traces	—	—	—	—	Traces
P. oleovorans	DSM 1045, ATCC 8062	++	A	18.6	92.4	7.6	—	—	—
			B	nd	—	—	—	—	—
P. putida KT2440	Worsey and Williams (1975)	++	A	25.3	—	41.8	Traces	10.4	47.8
			B	29.8	—	—	Traces	25.6	74.4
P. putida	DSM 291	±	A	nd	—	—	—	—	—
			B	nd	—	—	—	—	—
Pseudomonas sp. A33	Schirmer et al. (1993)	+++	A	15.8	0.2	90.8	Traces	6.5	2.5
			B	25.7	0.3	75.3	—	8.8	15.6
P. aeruginosa PAO1	DSM 1707	++	A	4.7	—	41.0	—	9.3	49.7
			B	8.6	—	62.2	Traces	10.7	27.1
P. putida GPp104	Huisman et al. (1991)	++	A	nd	—	—	—	—	—
			B	nd	—	—	—	—	—
GPp104 (pHP1014::E156)	This study	++	A	1.0	—	100.0	—	—	—
			B	26.7	29.0	39.9	31.1	—	—

Colony growth was monitored on mineral salts medium (MSM) agar plates containing 0.2% (w/v) 4HHx as sole carbon source. The plates were inoculated from cell material that was obtained from nutrient broth agar plates then suspended in 0.9% (w/v) sodium chloride. Type A (one-stage fed-batch experiment): cells were grown in batch cultures in MSM containing 0.05% (w/v) ammonium chloride as the nitrogen source and 0.2% (w/v) 4HHx as carbon source. After 24 h (strains of *A. eutrophus*) or 48 h (*Pseudomonas* sp.) of cultivation an additional portion of 0.5% (w/v) 4HHx was fed. Cells were harvested after 48 h (strains of *A. eutrophus*) or 72 h (*Pseudomonas* sp.). Type B (two-stage fed-batch experiment): cells were harvested from an overnight culture in nutrient broth medium, washed and transferred to MSM containing no nitrogen source and 0.5% (w/v) 4HHx as carbon source. Cells were fed with an additional portion of 0.5% (w/v) 4HHx after 24 h incubation at 30°C and were harvested after 48 h of incubation. *Symbols:* +++, good growth; ++, medium growth; +, poor growth; ±, very poor growth; —, no growth. *Abbreviations:* ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; 3HB, 3-hydroxybutyric acid; 3HHx, 3-hydroxyhexanoic acid; 3HO, 3-hydroxyoctanoic acid; 3HD, 3-hydroxydecanoic acid; nd, not done; CDW, cellular dry weight

was precipitated from the chloroform solution by addition of 10 vol. 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.

Quantitative and qualitative analysis of PHA polyester. For quantitative determination of PHA, 3–5 mg of lyophilized cell material or isolated polyester was subjected to methanolysis in the presence of 15% (v/v) sulphuric acid, and the hydroxyacyl methylesters were analysed by gas chromatography (GC) as described in detail by Brandl et al. (1988) and Timm et al. (1990).

Nuclear magnetic resonance (NMR) spectroscopic analysis. The ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian (Palo Alto, Calif., USA) VXR-500 S or Varian VXR-200 spectrometer. The 200-MHz ^{1}H-NMR spectra were recorded at 50°C from a $CDCl_3$ solution of the polyesters (15 mg/ml) using 30 degree pulses, 3.752 s repetition time, 4,000 Hz spectral width, 32 K data points and 48 accumulations. The 125-MHz ^{13}C-NMR spectra were recorded at 50°C in a $CDCl_3$ solution of the polyesters (50 mg/ml) using Waltz-decoupling, 25 degree pulses, 1.193 s repetition time, 25,000 Hz spectral width, 64 K data points and 10,000 accumulations. The CH-Cosy spectrum was observed at 50°C on a Varian VXR 500 S spectrometer with 9,400 Hz spectral width for the ^{1}H dimension and 2,500 Hz spectral width for the ^{13}C dimension using the QUICKCH pulse sequence (Reynolds et al. 1985). ^{1}H-NMR chemical shifts were referred to $CHCl_3$ (δ=7.25 ppm), ^{13}C-NMR chemical shifts to $CDCl_3$ (δ=77.00 ppm).

Chemicals. All lactones were obtained from Aldrich (Steinheim, Germany), 4HB from Fluka (Buchs, Switzerland) and most other chemicals from Merck (Darmstadt, Germany).

Results

Growth on 4HHx

At the beginning we examined a variety of aerobic Gram-negative bacteria for their capability to use 4HHx as a sole carbon source for growth. As is obvious from Table 1, all strains of *A. eutrophus* and the species of the genus *Pseudomonas* were able to grow on mineral agar plates that contained 4HHx at a concentration of 0.2% as sole carbon source. Only *P. oleovorans* ATCC 29347, *P. putida* DSM 291 and *Chromobacterium violaceum* failed to grow. All strains listed in Table 1 were not able to utilize the corresponding lactone, i.e., 4-caprolactone, if it was provided at a concentration of 0.2% in MSM agar plates, as a carbon source for growth.

PHA accumulated by a recombinant strain of P. putida from 4HHx as revealed by GC analysis

When lyophilized material of whole cells of *P. putida* GPp104 (pHP1014::E156), which were cultivated in two-stage batch cultures in mineral salts medium without nitrogen and with 0.5% (w/v) 4HHx as carbon source, were subjected to methanolysis, and when the products were analysed by the standard GC method, not only 3HB and 3HHx methyl ester (retention times 7.45 and 10.55 min, respectively) occurred as derivatives. In addition, two major peaks exhibiting retention

Poly (3HB-*co*-3HHx-*co*-4HHx)

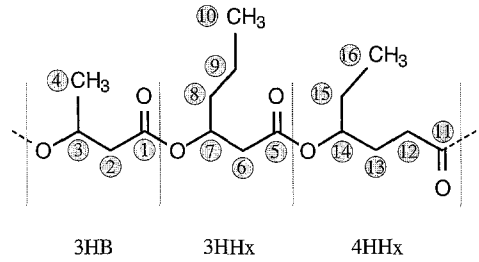

Fig. 1. Structural formula of poly(3-hydroxybutyric acid-*co*-3-hydroxyhexanoic acid-*co*-4-hydroxyhexanoic acid) [poly3HB-*co*-3HHx-*co*-4HHx] co-polyester

times of 6.76 min and 11.81 min occurred. Since 4HHx subjected to methanolysis gave peaks with identical retention times, and since 4-caprolactone directly injected onto the column gave a peak with a retention time of 11.79 min, it was concluded that the peaks at 6.76 min and 11.81 min represented the 4HHx methyl ester and 4-caprolactone, respectively. This indicated that 4HHx is a constituent of the PHA synthesized by the recombinant *P. putida* strain from 4HHx (Fig. 1).

Analysis of the polyester isolated from cells of recombinant P. putida and A. eutrophus

In order to confirm the composition of the polyester accumulated by the recombinant strain of *P. putida,* we isolated the polyester from the cells. From three 200-ml cultures of *P. putida* GPp104 (pHP1014::E156) in MSM with 0.5% 4HHx as carbon source, we obtained 230 mg lyophilized cells. Chloroform extraction of the latter followed by ethanol precipitation gave 58.5 mg PHA, which according to the GC analysis had almost the same composition as that obtained from the analysis of whole cells. *P. putida* GPp104 (pHP1014::E156) was also grown in 10 l MSM containing 0.05% (w/v) ammonium chloride as the nitrogen source and 0.2% octanoic acid as a carbon source at the growth phase and 0.5% (w/v) 4HHx as carbon source in the stationary phase when the nitrogen source was exhausted. Under these conditions, the cells accumulated a terpolyester of 3HB, 3HHx and 4HHx with an increased molar fraction of 4HHx (approximately 47 mol%).

A terpolyester of 3HB, 3HHx and 4HHx could be also obtained with *A. eutrophus* PHB^{-}4 (pHP1014::E156) if this recombinant strain was grown in 10 l MSM that contained 0.05% (w/v) ammonium chloride as the nitrogen source and 0.35% (w/v) sodium gluconate in the exponential growth phase, and if 0.5% (w/v) 4HHx was added in the stationary phase after the nitrogen source was exhausted. From 38.6 g lyophilized cells we isolated 10.5 g poly(3HB-*co*-3HHx-*co*-4HHx), which contained 3HB as the main constituent (97.1 mol%) and 3HHx and 4HHx as minor constituents (1.5 or 1.4 mol%, respectively). Although many efforts were made, the molar fraction of 4HHx could not be increased.

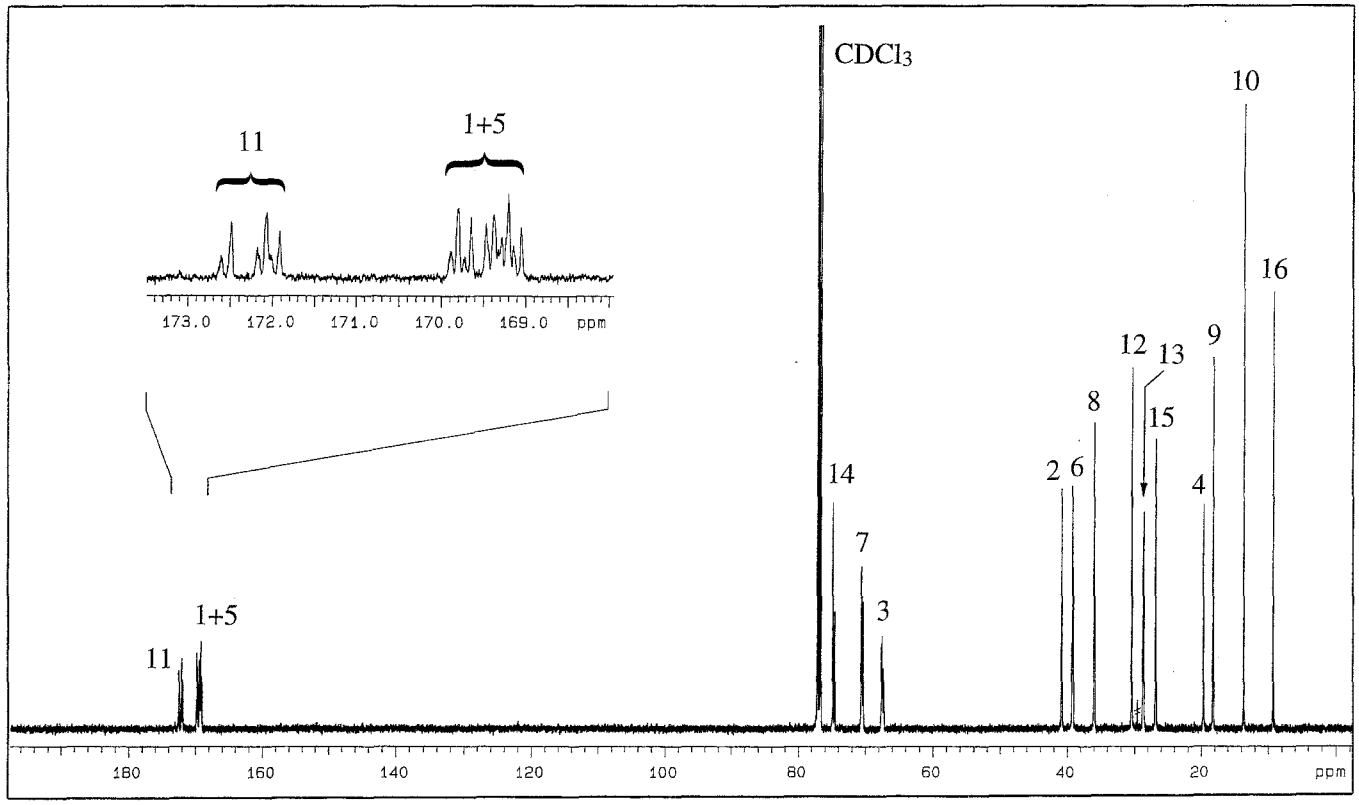

Fig. 2. ^{13}C-Nuclear magnetic resonance spectrum of poly(3HB-*co*-3HHx-*co*-4HHx). The spectrum was recorded from the polyester isolated from cells of *Pseudomonas putida* GPp104 (pHP1014::E156) cultivated with 4HHx as sole carbon source. The numbers refer to those in Fig. 1. The spectrum ranging from 168.0 ppm to 173.5 ppm was spread and is shown as an *insert*

NMR spectroscopic analysis of poly(3HB-co-3HHx-co-4HHx)

To verify the previous results obtained by GC analysis, the isolated poly(3HB-*co*-3HHx-*co*-4HHx) terpolyester was subjected to NMR spectroscopy analysis. Figure 2 shows the 125 MHz ^{13}C-NMR spectrum of the polymer isolated from cells of a two-stage fed-batch culture of *P. putida* GPp104 (pHP104::E165), which was incubated for 48 h in nitrogen-free MSM and fed twice with 0.5% 4HHx. All signals are split into several peaks, indicating a statistically random sequence distribution of the different monomers, as already reported for other co- and terpolymers (Doi et al. 1986; Bluhm et al. 1986; Valentin et al. 1992). The split of signals is clearly demonstrated by the stretch of the ^{13}C spectrum from 168.0 to 173.5 ppm including the signals of the carbonyl carbons (insert in Fig. 2). Since the constituents of the polymer occurred at almost equimolar concentrations (28 mol% 3HB, 40 mol% 3HHx plus 32 mol% 4HHx as determined by ^{1}H-NMR spectroscopic analysis), and since almost all ^{13}C-signals are split into several peaks, the ranges of the chemical shifts are reported in Table 2.

The ^{13}C resonances of 3HB (C-1, C-2, C-3, and C-4) and 3HHx (C-5, C-6, C-7, C-8, C-9 and C-10) were assigned by data comparison with previous reports (Doi et al. 1986; Gross et al. 1989). The remaining signals (C-11, C-12, C-13, C-14, C-15 and C-16) belonged to 4HHx: the ^{13}C signals of C-11, C-14 and C-16 were confirmed by an attached proton test spectrum (Patt and Shoolery 1982). To distinguish the ^{13}C resonances of C-12, C-13 and C-15 signals, a CH-Cosy spectrum was recorded (Fig. 3). The ^{1}H resonances of the CH-Cosy spectrum were also assigned by data comparism with Doi et al. 1986 (3HB) and Gross et al. 1989

Table 2. Chemical shift data from the ^{13}C-NMR spectra of poly(3HB-*co*-3HHx-*co*-4HHx)

Repeating unit	Carbon atom	Chemical shifts [in ppm]	
		From	To
3HB	1	169.05	169.89
	2	40.84	40.91
	3	67.32	67.78
	4	19.70	19.83
3HHx	5	169.05	169.89
	6	39.18	39.41
	7	70.34	70.77
	8	35.96	36.12
	9	18.30	18.34
	10	13.73	
4HHx	11	171.91	172.61
	12	30.46	30.50
	13	28.61	28.86
	14	74.66	74.95
	15	26.80	26.95
	16	9.38	9.42

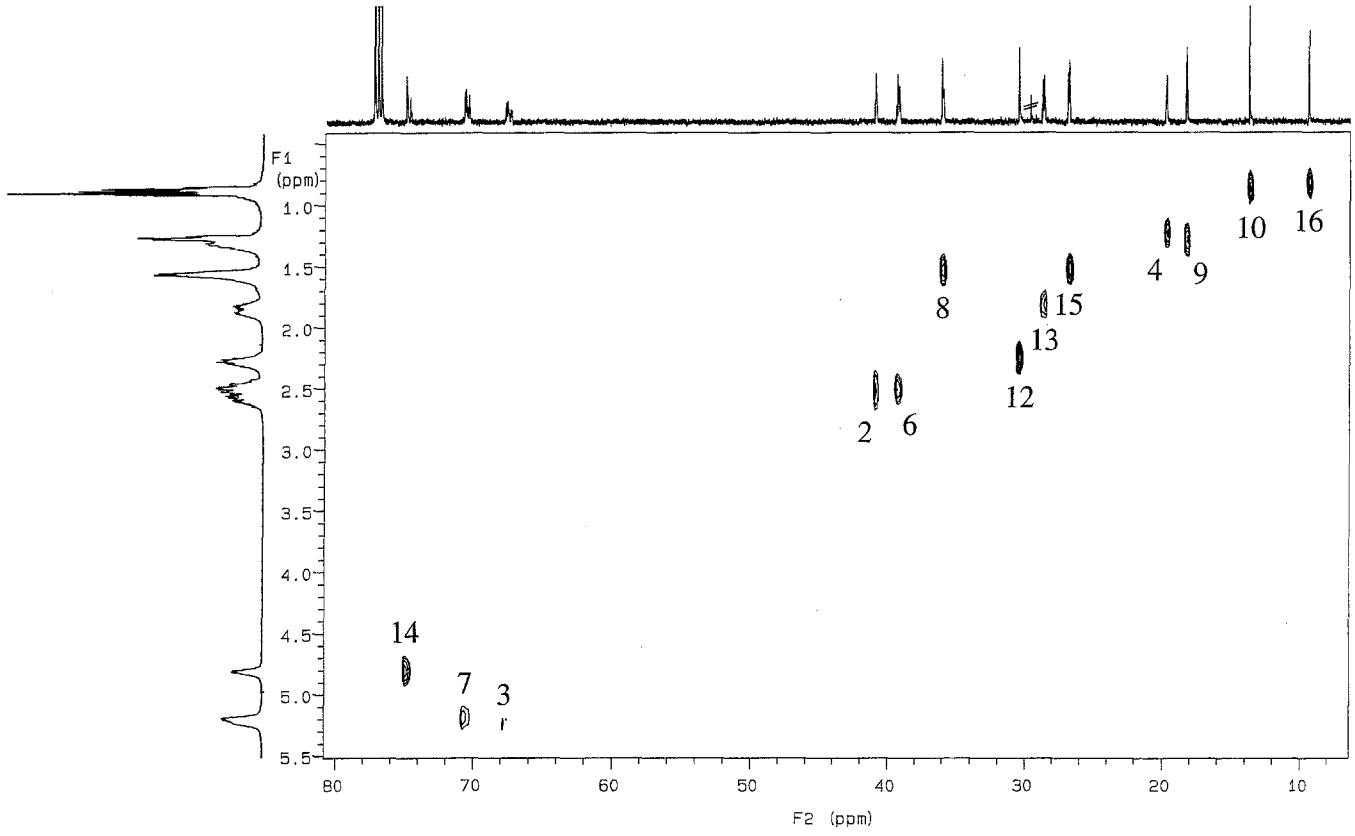

Fig. 3. CH-Cosy spectrum of poly(3HB-*co*-3HHx-*co*-4HHx). The ^{13}C data are shown on the x-axis (F2), and ^{1}H data are shown on the y-axis (F1). The spectrum was recorded from the same polyester shown in Fig. 2

(3HHx). The ^{1}H-NMR signals of 14-CH as well as of 12-CH_2 and 13-CH_2 of 4HHx were in close relation to the corresponding signals of 4HV in poly(3HB-*co*-3HV-*co*-4HV) (Valentin et al. 1992). The chemical shifts of ^{1}H-NMR signals from 12- and 13-methylene groups were also closely related to the corresponding signals of 4HB in poly(3HB-*co*-4HB) (Doi et al. 1988).

PHA accumulated by R. ruber from 4HHx

R. ruber was another suitable candidate for incorporation of 4HHx into PHA since this Gram-positive bacterium accumulated a copolyester of 3HB, 3HV and 3HHx from hexanoic acid or from 2-hexenoic acid as carbon sources, and since it accumulated a copolyester of 3HB, 3HV and 4HB from 1,4-butanediol or 4HB as carbon sources (Haywood et al. 1991). When this Gram-positive bacterium was cultivated for 96 h in a one-stage fed-batch experiment (four times 0.2% 4HHx) or in a two-stage fed-batch experiment (0.8% nutrient broth in the first stage, four times 0.2%, v/v, 4HHx in the second stage), a complex copolyester was accumulated that contained, in addition to 3HB, 3HV and 3HHx, also small amounts of 4HHx (Table 3) as revealed by GC analysis of whole cells as well as of the isolated polyester.

4HHx as a carbon source for PHA biosynthesis in other bacteria

We also investigated some other bacteria for their capability to incorporate 4HHx into PHA. However, no srain mentioned in Table 1 except the recombinant

Table 3. Accumulation of PHA by *Rhodococcus ruber* from 4HHx

Type of experiment	PHA content (% of CDW)	Composition of accumulated PHA (mol%) 3HB	3HV	3HHx	4HHx
A	41.2	50.2	15.1	33.4	1.3
B	42.8	58.8	18.1	21.9	1.2

Type A and type B experiment represented one or two-stage fed-batch fermentation experiments done on a 500-ml scale in 2-l erlenmeyer flasks. Cells were cultivated for 96 h, and 4HHx was provided at portions of 0.2% (w/v) at the beginning and after 24, 48 and 72 h cultivation at 30°C: 3HV, 3-hydroxyvaleric acid; for other abbreviations see legend of Table 1

strains mentioned above accumulated PHA containing 4HHx in either one-stage cultivation experiments or in two-stage cultivation experiments as revealed by GC analysis. Trace amounts of 4HHx, which were close to the limit of detection (0.1 mol%), were detected in the cell material of some pseudomonads (Table 1). Whereas all wild types of pseudomonads except *P. oleovorans* DSM 1045 synthesized PHA, which consisted mainly of 3-hydroxyalkanoic acids of medium chain length, all strains of *A. eutrophus* and *P. oleovorans* DSM 1045 synthesized a copolyester consisting of 3HB as the main constituent and of 3HHx as a minor constituent, from 4HHx.

Discussion

GC and NMR-spectroscopic analysis had revealed the presence of 4HHx as a hitherto unknown constituent of the bacterial storage compound PHA. Polyesters containing this new constituent were only synthesized and accumulated if the cells were cultivated in the presence of 4HHx as carbon source; these polyesters were not synthesized from non-related carbon sources. The capability to incorporate 4HHx into PHA was restricted to recombinant strains of *P. putida* and *A. eutrophus* harbouring the PHA-synthase gene from *T. pfennigii* and to *R. ruber*. However, the latter bacterium incorporated 4HHx only as a minor fraction, although it utilized 4HHx readily as a carbon source for PHA synthesis as indicated by the large amounts of PHA that accumulated in the cells and consisted mainly of 3HB, 3HV and 3HHx. All other bacteria investigated in this study did not incorporate 4HHx into PHA. We did not analyse recombinant strains of *A. eutrophus* or *P. putida,* which harbour the *R. ruber* PHA-biosynthesis genes, for their capability to confer synthesis of PHA containing 4HHx as a constituent, since the PHA synthase of this Gram-positive bacterium was only very weakly experessed in other bacteria most probably due to the rare translational start codon TTG of the PHA-synthase structural gene (Pieper and Steinbüchel 1992).

After having demonstrated synthesis of PHA consisting of 3HB, 3HHx and 3HO from octanoic acid by a recombinant strain of the PHA-negative mutant GPp104 of *P. putida* harbouring the PHA-synthase gene of *T. pfennigii* (Liebergesell et al. 1993), for a second time indirect evidence was obtained that the PHA synthase of *T. pfennigii* exhibits a rather unusual substrate specificity. As revealed by the composition of PHA accumulated by cells of the recombinant strains in this and in the previous study, this enzyme seems to accept readily 3-hydroxyhexanoyl-coenzyme A (CoA) and 4-hydroxyhexanoyl-CoA as substrates in addition to 3-hydroxybutyryl-CoA and 3-hydroxyvaleryl-CoA, which are preferably used as substrates by PHA_{SCL} synthases of other bacteria. It will be interesting to know how unrestricted the substrate specificity of the PHA synthase of *T. pfennigii* really is and whether the enzyme allows the incorporation of additional different constituents. Fermentation studies will be done in order to increase the yield and amount of the new polyesters and to investigate some of their physical properties and their biodegradability. This and the previous study (Liebergesell et al. 1993) also clearly demonstrate that the physiological background, i.e. the capability of a bacterium to provide hydroxyacyl CoA thioesters as substrates for the PHA synthase, is very important in order to demonstrate the unspecificity of a given synthase. The unspecificity of the *T. pfennigii* PHA synthase only became obvious after expression in *A. eutrophus* or *P. putida* (Liebergesell et al. 1993, this study) but was not obvious from the studies done with *T. pfennigii* itself (Liebergesell et al. 1991).

Acknowledgements. The authors thank B. Witholt (Zürich) and M. Liebergesell (Göttingen) for providing mutant GPp104 or plasmid pHP1014::E156, respectively, and Dipl.-Chem. R. Machinek for recording the NMR spectra. This study was supported by a grant provided by the Max Buchner-Forschungsstiftung.

References

Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54:450–472

Bluhm TL, Hamer GK, Marchessault RH, Fyfe CA, Veregin RP (1986) Isodimorphism in bacterial poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate). Macromolecules 19:2871–2876

Brandl H, Gross RA, Lenz RW, Fuller RC (1988) *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl Environ Microbiol 54:1977–1982

Doi Y, Kunioka M, Nakamura Y, Soga K (1986) Nuclear magnetic resonance studies on poly(β-hydroxybutyrate) and a copolyester of β-hydroxybutyrate and β-hydroxyvalerate isolated from *Alcaligenes eutrophus* H16. Macromolecules 19:2860–2864

Doi Y, Tamaki A, Kunioka M, Soga K (1987) Biosynthesis of terpolyesters of 3-hydroxybutyrate, 3-hydroxyvalerate and 5-hydroxyvalerate in *Alcaligenes eutrophus* from 5-chloropentanoic and pentanoic acids. Makromol Chem Rapid Commun 8:631–635

Doi Y, Kunioka M, Nakamura Y, Soga K (1988) Nuclear magnetic resonance studies on unusual bacterial copolyesters of 3-hydroxybutyrate and 4-hydroxybutyrate. Macromolecules 21:2722–2727

Don RA, Pemperton JM (1981) Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus.* J Bacteriol 145:681–686

Gross RA, De Mello C, Lenz RW, Brandl H, Fuller RC (1989) Biosynthesis and characterization of poly(β-hydroxyalkanoates) produced by *Pseudomonas oleovorans.* Macromolecules 22:1106–1115

Haywood GW, Anderson AJ, Williams DR, Dawes EA, Ewing DF (1991) The accumulation of a polyhydroxyalkanoate copolymer containing primarily 3-hydroxyvalerate from simple carbohydrate substrates by *Rhodococcus* sp. NCIMB 40126. Int J Biol Macromol 13:83–88

Huisman GW, Wonink E, Meinma R, Kazemier B, Terpstra P, Witholt B (1991) Metabolism of poly(3-hydroxyalkanoates) by *Pseudomonas oleovorans:* identification and sequences of genes and function of the encoded proteins in the synthesis and degradation of PHA. J Biol Chem 266:2191–2198

Kunioka M, Nakamura Y, Doi Y (1988) New bacterial copolyesters produced in *Alcaligenes eutrophus* from organic acids. Polymer Commun 29:174–176

Liebergesell M, Hustede E, Timm A, Steinbüchel A, Fuller RC, Lenz RW, Schlegel HG (1991) Formation of poly(3-hydroxyalkanoates) by Phototrophic and chemolithotrophic bacteria. Arch Microbiol 155:415–421

Liebergesell M, Mayer F, Steinbüchel A (1993) Analysis of polyhydroxyalkanoic acid-biosynthesis genes of anoxygenic phototrophic bacteria reveals synthesis of a polyester exhibiting an unusual composition. Appl Microbiol Biotechnol, in press

Patt SL, Shoolery JN (1982) Attached proton test for carbon-13 NMR. J Magn Reson 46:535–539

Pieper U, Steinbüchel A (1992) Identification, cloning and sequence analysis of the poly(3-hydroxyalkanoic acid) synthase gene of the Gram-positive bacterium *Rhodococcus ruber*. FEMS Microbiol Lett 96:73–80

Preusting H, Nijenhuis A, Witholt B (1990) Physical characteristics of poly(3-hydroxyalkanoates) and poly(3-hydroxyalkenoates) produced by *Pseudomonas oleovorans* grown on aliphatic hydrocarbons. Macromolecules 23:4220–4224

Reynolds WF, Hughes DW, Perpick-Dumont M (1985) A pulse sequence which provides rapid, routine ^{1}H–^{13}C shift-correlated spectra. J Magn Reson 64:304–311

Schirmer A, Jendrossek D, Schlegel HG (1993) Degradation of poly(3-hydroxyoctanoic acid) [P(3HO)] by bacteria: purification and properties of a P(3HO) depolymerase from *Pseudomonas fluorescens* GK13. Appl Environ Microbiol 59:1220–1227

Schlegel HG, Kaltwasser H, Gottschalk G (1961) Ein Submersverfahren zur Kultur wasserstoffoxydierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch Mikrobiol 38:209–222

Steinbüchel A (1991a) Polyhydroxyalkanoic acids: In: D Byrom (ed) Biomaterials. Macmillan, New York, pp 123–213

Steinbüchel A (1991b) Polyhydroxyfettsäuren – thermoplastisch verformbare Polyester aus Bakterien. Nachrichtenbl Chem Tech Labor 39:1112–1124

Steinbüchel A, Hustede E, Liebergesell M, Pieper U, Timm A, Valentin H (1992) Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS Microbiol Rev 103:217–230

Timm A, Byrom D, Steinbüchel A (1990) Formation of blends of various poly(3-hydroxyalkanoic acids) by a recombinant strain of *Pseudomonas oleovorans*. Appl Microbiol Biotechnol 33:296–301

Valentin HE, Schönebaum A, Steinbüchel A (1992) Identification of 4-hydroxyvaleric acid as a constituent of biosynthetic polyhydroxyalkanoic acids from bacteria. Appl Microbiol Biotechnol 36:507–514

Worsey MJ, Williams PA (1975) Metabolism of toluene and the xylenes by *Pseudomonas putida* (arvilla) mt-2: evidence for a new function of the TOL plasmid. J Bacteriol 124:7–13