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A *Pseudomonas* strain accumulating polyesters of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids

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Summary. A citronellol-utilizing bacterium was isolated that accumulated a polyester consisting of 3-hydroxybutyric acid (3HB) and of medium-chain-length 3-hydroxyalkanoic acids ($3HA_{MCL}$) from various carbon sources up to approximately 70% of the cellular dry matter if the cells were cultivated in a mineral salts medium under nitrogen limitation. In octanoate-grown cells, for instance, the polyester consisted of 87.5 mol% 3HB and 12.5 mol% 3-hydroxyoctanoic acid (3HO), whereas it consisted of 10.3 mol% 3HB, 16.7 mol% 3HO and 73.0 mol% 3-hydroxydecanoic acid (3HD) in gluconate-grown cells. However, the results of various experiments indicated that a blend rather than a copolyester was synthesized in the cell. It was the only strain among 45 different recently isolated citronellol-utilizing bacteria that accumulated such a polyester. All other citronellol-utilizing bacteria behaved like *Pseudomonas aeruginosa* with respect to their polyhydroxyalkanoic acid (PHA) biosynthetic capabilities and accumulated PHA consisting of $3HA_{MCL}$ with 3HO and 3HD as the main constituents from octanoate or gluconate, respectively, whereas 3HB was never present. None of 232 different heavy-metal-resistant bacteria was able to accumulate PHA composed of 3HB plus, for example, 3HO. Only 20.3% did not accumulate any PHA at all, 44.8% accumulated PHB from gluconate, and 34.9% behaved like *P. aeruginosa*. Many bacteria belonging to the latter group were distinguished from the other by rapid growth in nutrient broth and in gluconate mineral salts medium and by their ability to grow in the presence of a high concentration (up to 1.5%, w/v) of octanoate.

Introduction

Biosynthetic polyhydroxyalkanoic acids (PHA) occur in many bacteria as storage compounds if a carbon source is provided in excess to the cells and if growth is impaired due to the lack of one essential nutrient (Anderson and Dawes 1990; Steinbüchel 1991a). At present 40 different constituents of PHA have been already identified, including not only saturated, unsaturated, halogenated, branched and aromatic 3-hydroxyalkanoic acids (3HA) with three to fourteen carbon atoms (recently compiled by Steinbüchel 1991a, b), but also 4-hydroxyalkanoic acids (Kunioka et al. 1988; Valentin et al. 1992) and 5-hydroxyvaleric acid (Doi et al. 1987). With the exception of 3-hydroxy-4-pentenoic acid, which was detected as a constituent of PHA accumulated by *Rhodospirillum rubrum* (Lenz et al. 1990), polyesters consisting of any of the other constituents can be obtained from *Alcaligenes eutrophus* and *Pseudomonas oleovorans* only. Both bacteria rely on two different PHA-biosynthetic pathways (Steinbüchel 1991a). In addition, enzymatic, molecular, and physiological studies provided direct and indirect evidence that the PHA synthases of these two bacteria are (i) rather unspecific and (ii) distinguished by two different ranges of substrate specificities that obviously do not overlap. The PHA synthase of *A. eutrophus* is restricted to the use of coenzyme A thioesters of 3-, 4- or 5-hydroxyalkanoic acids with three to five carbon atoms, whereas the synthases of *P. oleovorans* seem to utilize only coenzyme A-thioesters of 3-HA with six or more carbon atoms as substrates.

So far, no naturally occurring bacterium has been described that accumulates a polyester consisting of both 3-hydroxybutyric acid (3HB) and 3-hydroxyoctanoic acid (3HO) at a relevant fraction. In a recent study, a recombinant strain of *P. oleovorans* was investigated that harbours and expresses the *A. eutrophus* PHA-biosynthetic pathway (Steinbüchel and Schlegel 1991) and accumulated a blend of the homopolyester PHB and of a copolyester of 3HO and 3-hydroxyhexanoic acid (3HHx) as main or minor constituents, respectively (Steinbüchel and Schubert 1989; Timm et al. 1990). Both polyesters were even deposited in separate granules (Preusting et al. 1992). In this study a bacterium was isolated that accumulated a polymer of 3HB and of 3-HA of medium-chain-length ($3HA_{MCL}$), indicating the presence of a PHA synthase with unusual properties. We investigated the culture conditions for the accumula-

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tion of PHA in this bacterium. In addition, we screened a large variety of different bacterial wild-type strains, which were either isolated from the environment for this study or were obtained from the culture collections of other laboratories, for their capability to accumulate polyesters consisting of 3HB and $3HA_{MCL}$.

Materials and methods

Bacterial strains. The bacterial strains isolated and investigated in this study are listed in Table 1. *Pseudomonas* strain GP4BH1 has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, FRG) as strain DSM 5927. For comparative studies we also investigated *P. aeruginosa* PAO1 (DSM 1707) and *P. citronellolis* (DSM 50332, Seubert 1960).

Enrichment techniques. Bacteria that utilize citronellol as sole carbon source for growth were enriched and isolated by two different methods from soil, small local creeks, eutrophic lakes, potting compost, decaying leaves from conifers, deciduous trees or plants and activated sewage sludge. In method A, samples were used for inoculation of 300-ml erlenmeyer flasks containing 50 ml mineral salts medium. Citronellol was supplied in glass tubes, which were rectangularly connected to the flasks and which usually function as a cuvette for measuring the optical density of the cell suspension in a Klett-Summerson colorimeter. After 3 days of incubation at 30° C, samples were withdrawn and diluted on mineral agar plates that contained no carbon source. Approximately 0.2 ml citronellol was applied onto a filter paper disc in the lid of each petri dish, and the dishes were incubated head over at 30° C. In method B, citronellol mineral agar plates were inoculated directly with the sample or with a suspension of the sample. In most cases colonies appeared after 3–7 days of incubation. Bacteria were purified from single colonies to pure cultures by a series of spreadings on citronellol agar plates.

Twenty strains (isolate nos. 1–6, 8, 10, 13, 15a, 16–19, 30, 44, 46, 50, 51 and 53) were kindly provided as new isolates by Dr. S. Verbarg (DSM).

Growth of bacteria and pigment production. Bacteria were grown aerobically at 30° C either in nutrient broth (NB) medium (0.8%, w/v) or in mineral salts medium (MM), which was supplemented with filter-sterilized carbon sources as indicated in the text (Schlegel et al. 1961). To allow extensive accumulation of PHA, the concentration of ammonium chloride in the MM was reduced to 0.05% (w/v). To obtain solidified media, 1.5% (w/v) agar was added. Citronellol was never included directly in the medium but was provided by a filter paper disc in the lid of the petri dish. For detection of fluorescent pigments and pyocyanin, cells were cultivated on pseudomonas F and pseudomonas P agar plates, respectively, and analysed according to Ajello and Hoadley (1976).

Analysis of PHA. To determine the PHA content of the cells and the composition of the polyester, the polymer of 4 ± 1 mg freeze-dried cells was transformed to the constituent 3-hydroxycarboxylic acid methyl esters (Brandl et al. 1988). These esters were analysed by gas chromatography as described recently in detail (Brandl et al. 1988; Timm et al. 1990). This method allowed the detection of 3HA with a chain-length in the range from four to twelve carbon atoms.

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

Analysis of ammonium. The concentration of ammonium in the medium was determined after the cells had been sedimented by centrifugation. Analysis was done with a detection kit obtained from Boehringer and Soehne (Mannheim, FRG), which applies the reaction of glutamate dehydrogenase as described by da Fonseca-Wollheim et al. (1974).

Determination of protein. Protein of bacterial cell suspensions was estimated by using the Folin reaction as described by Lowry et al. (1951) with bovine serum albumin as a standard.

Plasmid analysis. For plasmid analysis, crude lysates were prepared according to the method described by Kado and Liu (1981) and separated in horizontal slab gels containing 0.8% (w/v) agarose in TBE buffer (50 mM TRIS hydrochloride, 50 mM boric acid, 1.25 mM disodium EDTA, pH 8.5).

Electron microscopy. Cells that had been washed and suspended in 50 mM potassium phospate buffer (pH 6.8) were fixed with glutaraldehyde and embedded in Spurr's low-viscosity resin (Spurr 1969) as described by Walther-Mauruschat et al. (1977). Thin sections were contrasted with lead citrate and examined in a Philips EM301 electron microscope at calibrated magnifications.

Chemicals. Citronellol (3,7-dimethyl-6-octen-1-ol) was obtained from Sigma (St. Louis, Mo., USA). Pseudomonas F and P agar were obtained from Difco (Detroit, Mich., USA). Most other chemicals were from Merck (Darmstadt, FRG).

Results

Accumulation of PHA by the isolate GP4BH1

Strain GP4BH1 was obtained from a student course, in which citronellol-utilizing bacteria were enriched. It was isolated from the creek Weende near Göttingen by applying method A as described in Materials and methods. When this bacterium was analysed for the capability to accumulate PHA after the cells had been cultivated in a mineral salts medium with a limited amount of nitrogen source and with different carbon sources, the composition of the accumulated polyesters revealed an unusual pattern of constituents. 3HB appeared together with $3HA_{MCL}$ as constituents of the polyester accumulated by GP4BH1 (Table 1). Whereas 3HB was the main constituent in octanoate-grown cells, $3HA_{MCL}$ with 3-hydroxydecanoic acid (3HD) as the main constituent dominated in gluconate-grown cells. This is also obvious from Fig. 1, which shows representative growth and accumulation of PHA by GP4BH1 in mineral salts medium containing octanoic acid or gluconic acid as sole carbon source. Electron micrographs of ultrathin sections confirmed mass accumulation of PHA in both gluconate- and octanoate-grown cells (Fig. 2).

When NB-grown cells of GP4BH1 were transferred to ammonium-free MM containing 0.2% (w/v) sodium valerate as sole carbon source and were incubated at 30° C for 55 h, PHA was accumulated up to 17% of the cellular dry matter. The polyester consisted of 14 mol% 3HB and 85 mol% 3-hydroxyvalerate (3HV) in addition to trace amounts (0.8 mol%) of 3HV. If 2.0% (w/v) 4-hydroxyvalerate (4HV) was used as carbon source, only small amounts of PHA (approximately 0.5% of

Table 1. Accumulation of polyhydroxyalkanoic acid (PHA) by citronellol-utilizing and heavy-metal-resistant bacteria

Strain	Source	Growth on							Accumulation of PHA during cultivation with											
		Octanoate (%, w/v)					Citronellol (%, w/v)	Gluconate	Gluconate						Octanoate					
									Content	Composition					Content	Octanoate				
		0.1	0.4	0.75	1.0	1.5	Gas phase	0.5	% of CDW	3HB	3HHx	3HO	3HD	3HDD	% of CDW	3HB	3HHx	3HO	3HD	3HDD
Pseudomonas aeruginosa	DSM 1707	+ + +	+ + +	+ +	+	−	+ +	+ + +	60.9	−	2.4	30.5	62.4	4.7	45.7	−	3.1	90.4	6.3	0.2
P. citronellolis	DSM 50332	+ + +	+ + +	+	−	−	+ + +	+ + +	75.1	−	3.9	71.7	65.1	2.7	68.3	−	4.8	94.0	1.2	−
GP4BH1	This study	+ + +	+ + +	+ +	+ +	+	+ +	+ + +	65.0	10.3	−	16.7	73.0	−	61.3	87.5	−	12.5	−	−
GP5PS	This study	+ + +	+ +	+ +	+ +	SC	+	+ + +	24.5	−	6.5	30.7	46.5	16.3	58.3	−	8.9	87.4	3.7	−
GP6	This study	+ + +	+ +	−	−	−	+	−	13.4	−	8.5	21.3	68.7	11.5	44.5	−	8.1	82.1	8.0	1.8
KT27	Timotius and Schlegel (1987)	+ + +	+ + +	+ + +	+ +	+	nd	+ + +	66.9	−	0.6	34.5	60.5	4.4	43.2	−	8.1	82.1	8.0	1.8
M310	H. G. Schlegel (unpublished)	+ +	+ +	+	−	−	nd	+ +	7.1	−	8.4	40.7	33.1	17.8	5.2	−	9.1	90.9	−	−
40C	Schmidt and Schlegel (1989)	+ + +	+ + +	+ + +	+ + +	+ +	nd	+ + +	50.5	−	4.9	25.0	66.4	3.6	40.8	−	9.0	81.5	7.8	1.7

Growth on citronellol, gluconate and octanoate was determined on solid mineral salts medium containing 0.1% (w/v) ammonium chloride and the carbon source as indicated. Citronellol (200 µl) was provided on a cellulose filter disc in the lid of the petri dish: + + +, good growth; + +, medium growth; +, poor growth; −, no growth; SC, only single colonies appeared. Cells were also cultivated in liquid mineral salts medium under nitrogen-limiting conditions (0.05% ammonium chloride, w/v) and in the presence of 1.5% sodium gluconate or 0.5% sodium octanoate as carbon source. For cultivation of strain GP6 1.5% (w/v) fructose was used instead of gluconate as carbon source. After 72–96 h cells were harvested by centrifugation, washed with 100 mM potassium phosphate buffer, pH 7.0, lyophilized and analysed for PHA content and composition as described in Materials and methods: CDW, cellular dry weight; 3HB, 3-hydroxybutyric acid; 3HHx, 3-hydroxyhexanoic acid; 3HO, 3-hydroxyoctanoic acid; 3HD, 3-hydroxydecanoic acid; 3HDD, 3-hydroxydodecanoic acid; nd, not determined

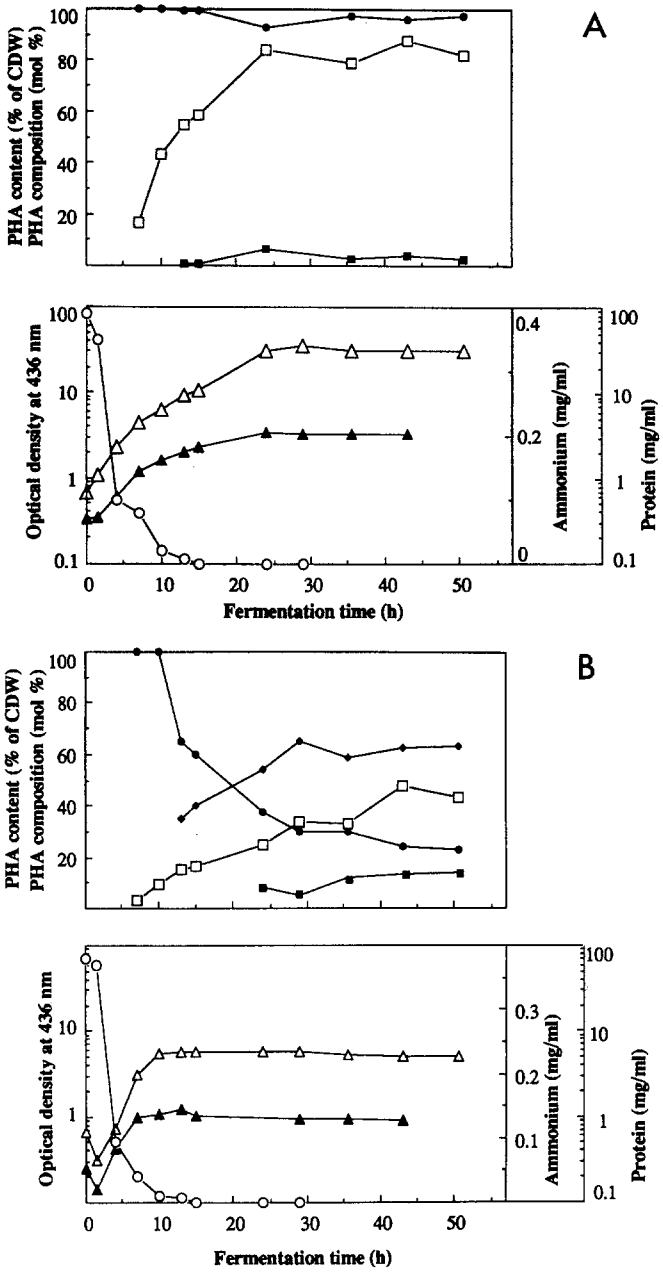

Fig. 1A, B. Accumulation of polyhydroxyalkanoic acids (PHA) by *Pseudomonas* strain GP4BH1. Cells were cultivated in a mineral salts medium with octanoate **A** or gluconate **B** as sole carbon sources as described in detail to the legend of Table 1. Samples were withdrawn at the indicated times. Cells were analysed for PHA content, and cell-free supernatants were analysed for ammonium as described in Materials and methods: △, optical density; ▲, protein; □, PHA; ○, ammonium; ●, 3-hydroxybutyrate (3HB); ■, 3-hydroxyoctanoate (3HO); ◆, 3-hydroxydecanoate (3HD)

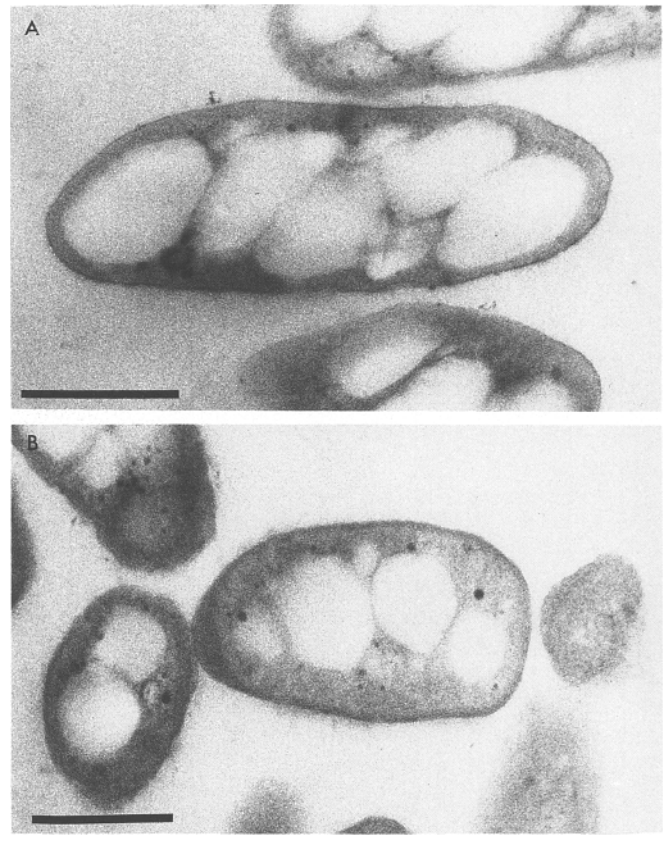

Fig. 2A, B. Electron micrographs of thin sections of *Pseudomonas* strain GP4BH1. Cells of the new isolate were cultivated aerobically in a mineral salts medium that contained a limited amount of ammonium chloride (0.05%, w/v); 0.5% (w/v) sodium octanoate **A** or 1.5% w/v sodium gluconate **B** were provided as sole carbon sources. After approximately 48 h incubation on a revolving shaker at an agitation rate of 150 rpm, the cells were harvested and analysed. Thin sections were prepared as described in Materials and methods. Gas chromatographic analysis revealed that PHA contributed to approximately 70% of the cellular dry matter. PHA of octanoate-grown cells was composed of 87.5 mol% 3HB and 12.5 mol% 3HO, whereas that of gluconate-grown cells was composed of 10.3 mol% 3HB, 16.7 mol% 3HO and 73 mol% 3HD. The *bar* represents 0.5 μm

cellular dry matter), consisting of 81 mol% 4HV and 19 mol% 3HB, were accumulated.

Characterization of isolate GP4BH1

On many substrates tested strain GP4HB1 formed characteristic colonies with a curled surface, and the cells were held together by a pellicle-like substance. This bacterium grew well in mineral salts medium with, for example, gluconate, octanoate, γ-valerolactone, 4HV and 3-hydroxypropionic acid as carbon source; however, the cells flocculated in liquid media. Strain GP4BH1 was a Gram-negative, oxidase- and catalase-positive bacterium that formed non-spore-forming rod-shaped cells (width 0.5–0.7 μm, length 1.2–2.5 μm) with one polar flagellum. The identification service of the Deutsche Sammlung für Mikroorganismen und Zellkulturen revealed the following additional properties of the new isolate: lysis of the cells in the presence of 3% KOH; non-denitrifying but reducing nitrate to nitrite; no anaerobic growth; growth at 37 and 41° C; prototrophic; pigments including pyocyanin are not synthesized; no hydrolysis of starch, gelatin, casein, DNA, Tween 80, esculin or urea; absence of phenylalanine deaminase; no levane formation from sucrose; substrates utilized as

sole carbon source for growth: acetamide, acetic acid, adipic acid, alanine, aminobutyric acid, DL-arginine, azelaic acid, betaine, caproic acid, citric acid, geraniol, glucose, gluconic acid, glycolic acid, 3HB, itaconic acid, 2-ketogluconic acid, lactic acid, levulinic acid, malic acid, propionic acid, sarcosine, sebacic acid, L-valine; substrates that are not utilized as sole carbon source for growth; L-arabinose, fructose, glycine, L-histidine, inositol, malonic acid, mannose, maltose, mannitol, muconic acid, L-serine, suberic acid, trehalose, xylose. Due to the phenotypic properties and due to the pattern of fatty acids, strain GP4BH1 clearly belongs to the rRNA homology group I of the genus *Pseudomonas*. No clear reference to one particular species of the genus could be made; however, strain GP4HB1 is closely related to *P. aeruginosa*, *P. mendocina* and *P. citronellolis*.

Gel electrophoretic analysis of crude lysates did not reveal the presence of plasmid DNA. Hybridization of genomic, *Eco*RI-digested DNA with a DNA fragment encoding for the *A. eutrophus* PHB synthase structural gene was also negative. These results did not support our initial assumption that the PHA-biosynthetic capabilities of GP4BH1 might result partially from the genetic transfer of genes for the *A. eutrophus* PHB-biosynthetic apparatus, which were at that time being investigated in our laboratory.

Characterization of the polyester accumulated by GP4BH1

In contrast to a recent study (Timm et al. 1990), we were not able to demonstrate a clear differential extraction of polyhydroxybutyric acid (PHB) and PHA from lyophilized octanoate-grown cells of GP4BH1 with chloroform in a Soxhlet apparatus. However, upon ethanol precipitation of the extracted polyester and air-drying of the filtered polymers, visual examination revealed that the polymer did not appear homogeneous, because it had obviously separated into two different materials. One was a white, fluffy, opaque and fibrous material, whereas the other was translucent and rubberlike. Gas chromatographic analysis of samples withdrawn from either region revealed the presence of 3HB or 3HD plus small amounts of 3-hydroxydodecanoic acid (3HDD) and 3HO in the opaque or translucent material, respectively. Therefore, a blend of two different polyesters was presumably synthesized by GP4BH1. ^{13}C-Nuclear magnetic resonance (NMR)-spectroscopic analysis of the isolated PHA (data not shown) confirmed the presence of two different polyesters.

PHA accumulation in other citronellol-utilizing bacteria

The detection of polyesters consisting of 3HB, 3HO and 3HD in gluconate-grown cells or of 3HB and 3HO in octanoate-grown cells of GP4BH1 prompted us to screen related bacteria for their capability to accumulate these types of polyesters. As all 16 recently investigated strains of *P. aeruginosa* and also the strains of *P. citronellolis* and *P. mendocina* available from the DSM did not accumulate these polyesters (Timm and Steinbüchel 1990), we isolated 25 citronellol-utilizing bacteria from different natural environments. In addition, we obtained citronellol-utilizing bacteria from Dr. Verbarg. In total, we investigated 45 newly isolated citronellol-utilizing bacteria, which were distinquished by the pattern of carbon sources utilized for growth and/or by their colony morphology or by other properties. With no exception, all these bacteria behaved like the type strains of *P. aeruginosa* or *P. citronellolis* with respect to PHA biosynthesis. When these bacteria were cultivated under nitrogen-limiting conditions for 72 h at 30° C in mineral salts medium that contained 1.5% (w/v) sodium gluconate or 0.5% sodium octanoate, they accumulated PHA, which consisted of 3HD, 3HO and 3HDD or of 3HHx and 3HO, respectively. None of these strains incorporated 3HB, even in small amounts, into the polyester.

PHA accumulation in heavy-metal-resistant bacteria

In addition to the species of the genus *Pseudomonas* (Timm and Steinbüchel 1990) and to citronellol-utilizing bacteria (this study), we analysed in total 232 new isolates of soil bacteria that exhibited resistance to nickel, cadmium or cobalt, which were kindly provided by Prof. Dr. H. G. Schlegel, for their ability to synthesize and accumulate PHA. Again, the bacteria were cultivated under nitrogen-limiting conditions for 72 h at 30° C in mineral salts medium that contained 1.5% (w/v) sodium gluconate. Only very few bacteria were unable to grow on gluconate; for these bacteria fructose or glucose (each 1.5%, w/v) instead of gluconate was used as carbon source. Approximately 50% of all heavy-metal-resistant bacteria investigated in this study were able to utilize octanoate as sole carbon source for growth. These bacteria were, in addition, also cultivated in mineral salts medium which contained 0.5% (w/v) sodium octanoate (Table 1).

With respect to the ability to accumulate PHA, three groups of bacteria could be distinguished. A first group of bacteria, which comprised 104 strains (44.8%), behaved like *A. eutrophus* and accumulated appreciable amounts of PHB homopolyester with gluconate or with octanoate as carbon source. None of these isolates accumulated a copolyester of 3HB and 3HV from gluconate as for example, *Rhodococcus ruber* and some other Gram-positive bacteria (Haywood et al. 1991) or mutants of *A. eutrophus* altered in the metabolism of branched-chain amino acids (Steinbüchel and Pieper 1991) do. A second group of bacteria, which comprised 81 strains (34.9%), behaved like *P. aeruginosa* and accumulated a polyester consisting of 3HO and 3HD from gluconate or of 3HHx and 3HO from octanoate. However, only approximately 35% of the strains belonging to this group accumulated appreciable amounts of PHA. Most of them were distinquished from the others by a much more rapid growth on NB, gluconate and oc-

tanoate. Citronellol-utilizing strains were only detected in this group of heavy-metal-resistant bacteria, and at only a very low frequency: only one strain (isolate 80) grew well on citronellol, whereas citronellol was a poor subtrate for five additional isolates. In addition, most of these strains grew in the presence of 0.5% (w/v) sodium octanoate, which most other bacteria did not tolerate (this study and Liebergesell et al. 1991). Some bacteria exhibited an extraordinary tolerance for high concentrations of octanoic acid and grew even in the presence of 1.5% (wt/vol) sodium octanoate (Table 1). PHA contributed only marginally (0.5–2%) to the cellular dry matter in approximately 65% of the strains belonging to this group. The third group of bacteria comprised only 47 strains (20.3%), which were unable to accumulate any detectable PHA under the conditions tested. None of the 232 heavy-metal-resistant isolates behaved like GP4BH1 and accumulated a polyester consisting of 3HB and of $3HA_{MCL}$. These results indicate, however, that (i) presumably a high number of the heavy-metal resistant bacteria and (ii) most probably all citronellol-utilizing bacteria investigated in this study belong taxonomically the rRNA homology group I of the genus *Pseudomonas*.

Discussion

Recent studies have provided cumulative evidence for the existence of two different types of bacterial PHA synthases, which are distinguished by their substrate specificities. The PHA-biosynthetic apparatus of *A. eutrophus* allows the incorporation of saturated 3-, 4-, and 5-hydroxyalkanoic acids with up to five carbon atoms; hydroxyalkanoic acids with six or more carbon atoms were not detected in PHA accumulated by this bacterium (e.g. Liebergesell et al. 1991). Enzymatic studies have clearly demonstrated that the PHB synthase from *A. eutrophus* is active with 3-hydroxybutyryl-CoA and 3-hydroxyvaleryl-CoA but is inactive with 3-hydroxyhexanoyl-CoA (Haywood et al. 1989a). In contrast, the PHA-biosynthetic apparatus of *P. oleovorans* allows the synthesis of PHA consisting of saturated, unsaturated, halogenated, branched and aromatic 3-hydroxyalkanoic acids with six to 14 carbon atoms; 3HB was never detected as a constituent of PHA accumulated by these bacteria (Brandl et al. 1988; Lageveen et al. 1988; Haywood et al. 1989b; Huisman et al. 1989). Enzymatic studies revealed that the PHA synthases of pseudomonads belonging to the rRNA homology group I do not utilize 3-hydroxybutyryl-CoA as a substrate (A. Timm and A. Steinbüchel, unpublished results).

Physiological studies provided indirect evidence that the PHA synthases of most other bacteria share a very similar substrate specifity either with the synthases of *A. eutrophus* or of *P. oleovorans*. Only minor exceptions exist, such as the PHA-synthases from *P. aeruginosa* and other pseudomonads belonging to the rRNA homology group I, which in contrast to the synthase of *P. oleovorans* incorporated also 3HV into PHA if the cells were cultivated with valeric acid as sole carbon source (Timm and Steinbüchel 1990). Only recently small amounts of 3HB were detected in a PHA consisting mainly of $3HA_{MCL}$ in *P. resinovorans* (Ramsay et al. 1992). The PHA synthases of *Rhodospirillum rubrum* and *Rhodocyclus gelatinosus* seem to use also 3-hydroxyhexanoyl-CoA in addition to thioesters of 3HB and 3HV (Brandl et al. 1989; Liebergesell et al. 1991) as substrate. *Pseudomonas* sp. GP4BH1 is the first naturally occurring bacterium that accumulates PHA consisting of 3HB and of $3HA_{MCL}$, both in significant fractions and in variable amounts.

In gluconate-grown cells of GP4BH1, 3HD and 3HB were the main constituents of PHA. Incorporation of 3HD from gluconate indicated that GP4BH1 belongs to those pseudomonads, which are able to synthesize PHA from unrelated substrates (Haywood et al. 1990; Timm and Steinbüchel 1990), and that GP4BH1 possesses the *P. aeruginosa* PHA-biosynthetic pathway. Relevant phenotypic markers of the new isolate indicated that GP4BH1 is taxonomically related to the rRNA homology group I of the genus *Pseudomonas*, which are the only known bacteria relying on this pathway. Incorporation of 3HO as a minor constituent from octanoate provided evidence for the presence of the *P. oleovorans* PHA-biosynthetic pathway in GP4BH1. Both the *P. oleovorans* and the *P. aeruginosa* PHA-biosynthetic pathway rely on two similar PHA synthases, which are closely linked on the genome (Huisman et al. 1991, A. Timm and A. Steinbüchel, unpublished results). Incorporation of 3HB from gluconate provided evidence for the presence of also the *A. eutrophus* PHA-biosynthetic pathway in GP4BH1 and, since the cells obviously did not synthesize a copolyester of 3HB and of $3HA_{MCL}$, GP4BH1 will most probably possess an additional PHA synthase, which prefers 3-hydroxybutyryl-CoA as a substrate. Therefore, it seems very unlikely that only one single rather unspecific PHA synthase exists in GP4BH1.

Irrespective of the type of polyester synthesized by GP4BH1, the composition of PHA in this naturally occurring bacterium is unique and the analysis of the molecular basis for the accumulation of PHA in strain GP4BH1 will therefore be very interesting. There is no, even weak, evidence that GP4BH1 might represent a fluorescent species of the genus *Pseudomonas* that has acquired the ability to synthesize PHB by genetic transfer of the *A. eutrophus* PHB synthase gene. The PHB-biosynthetic genes of the latter bacterium have been studied intensively in our laboratory (Schubert et al. 1988, 1991; Steinbüchel and Schlegel 1991).

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