APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1993, p. 1220–1227
0099-2240/93/041220-08$02.00/0

Vol. 59, No. 4

Degradation of Poly(3-Hydroxyoctanoic Acid) [P(3HO)] by Bacteria: Purification and Properties of a P(3HO) Depolymerase from *Pseudomonas fluorescens* GK13

ANDREAS SCHIRMER, DIETER JENDROSSEK,* AND HANS G. SCHLEGEL

Institut für Mikrobiologie der Georg-August-Universität Göttingen, Grisebachstrasse 8, 3400 Göttingen, Germany

Received 23 September 1992/Accepted 5 February 1993

Twenty-five gram-negative bacteria and one gram-positive bacterium capable of growing on poly(3-hydroxyoctanoic acid) [P(3HO)] as the sole source of carbon and energy were isolated from various soils, lake water, and activated sludge. Most of the isolates degraded only P(3HO) and copolymers of medium-chain-length (MCL) hydroxyalkanoic acids (HA). Except for the gram-positive strain, which was able to hydrolyze P(3HO) and poly(3-hydroxybutyric acid) [P(3HB)], no isolate was able to degrade polymers of short-chain-length HA, such as P(3HB) or poly(3-hydroxyvalerate) [P(3HV)]. All strains utilized a large variety of monomeric substrates for growth. All gram-negative strains, but not the gram-positive strain, accumulated poly(hydroxyalkanoic acids) (PHA), consisting of MCL HA, if they were cultivated under accumulation conditions. One strain, which was identified as *Pseudomonas fluorescens* GK13 (biovar V), was selected and the extracellular P(3HO) depolymerase of this strain was purified from the culture medium of P(3HO)-grown cells by chromatography with Octyl-Sepharose CL4B and by gel filtration with Superose 12. The relative molecular weights of the native and sodium dodecyl sulfate-treated enzymes were 48,000 and 25,000, respectively. The purified enzyme hydrolyzed P(3HO), copolymers of MCL HA, and *para*-nitrophenyl esters of fatty acids. P(3HB), P(3HV), and characteristic substrates for lipases, such as Tween 80 or triolein, were not hydrolyzed. The P(3HO) depolymerase of *P. fluorescens* GK13 was insensitive to phenylmethylsulfonyl fluoride and dithioerythritol, unlike other PHA depolymerases. The dimeric ester of 3-hydroxyoctanoic acid was identified as the main product of enzymatic hydrolysis of P(3HO). The expression of the P(3HO) depolymerase by *P. fluorescens* GK13 was inducible by P(3HO), various 3- and 2-hydroxy compounds and by MCL fatty acids. However, high enzyme activity was detected only at the end of exponential growth or after cessation of growth.

Poly(3-hydroxybutyric acid) [P(3HB)] and other poly(hydroxyalkanoic acids) (PHA) are widespread bacterial storage compounds of carbon and reducing power (1, 12, 27). These polyesters are synthesized and deposited intracellularly in the form of inclusion bodies and can amount to 90% (dry weight) of cells, if the bacteria are cultivated in the presence of excess carbon and if one nutrient limits growth (28). Beside the most well-known polyester, P(3HB), various polyesters consisting of short-chain-length hydroxyalkanoic acids (SCL HA) with 3 to 5 carbon atoms or medium-chain-length (MCL) HA with 6 to 14 carbon atoms have been detected in the last decade (1, 33, 34). These polyesters are synthesized by bacteria during growth on related compounds such as fatty acids or unrelated compounds such as glucose, fructose, or gluconate (2, 11, 13, 14, 17, 19, 36, 39). Only P(3HB) and poly(3-hydroxyvaleric acid) [P(3HV)], which is synthesized by *Chromobacterium violaceum* during growth on valeric acid (35, 38a), and poly(4-hydroxybutyrate) [P(4HB)], which is synthesized by a mutant strain of *Alcaligenes eutrophus* (34a), are homopolymers. All other PHA consist of two or more HA.

The most attractive feature of PHA is their biodegradability to CO_2 and H_2O. Aerobic and anaerobic P(3HB)-degrading bacteria are widely distributed and were isolated from various ecosystems, such as soil (*Pseudomonas lemoignei* [7], molds [22], and *Comamonas* sp. [16]), activated sludge (*Alcaligenes faecalis* T_1 [16, 38]), lake water and air (16), sea water (*Comamonas testosteroni* [23]), estuarine sediment (*Ilyobacter delafieldii* [15]), and anaerobic sewage sludge (consortium [6]; *Clostridium* isolates [15a]). The extracellular P(3HB) depolymerases of *P. lemoignei*, *A. faecalis*, and *Comamonas* sp. have been isolated and characterized. The purified depolymerases were specific for P(3HB) and copolymers of 3HB which also contained 4HB, 3HV, or 3-hydroxypropionic acid. While the presence of 3HV in the polyester decreased the degradation rate, the presence of 4HB or 3-hydroxypropionic acid increased the degradation rate by the purified depolymerases of *A. faecalis* T_1, *Comamonas* sp., and *P. lemoignei* (7, 9, 10, 16, 21, 24, 25, 30, 38).

Little is known about the degradation of PHA other than P(3HB). Recently, we discovered the ability of *P. lemoignei* and a few new isolates to degrade P(3HV) homopolyester and to use the hydrolysis products for growth (16). The P(3HV) depolymerase of *P. lemoignei*, which is specifically synthesized during growth on P(3HV) or valerate, in addition to known P(3HB) depolymerases A and B of *P. lemoignei* (21, 25) was purified and characterized (24). In contrast to all other known P(3HB) depolymerases, which hydrolyze P(3HV) at less than 2% of the rate obtained with P(3HB), the purified P(3HV) depolymerase of *P. lemoignei* hydrolyzed the homopolyesters and copolymers of 3HB and 3HV at comparable rates. None of the P(3HB)- or P(3HV)-degrading bacteria tested so far were able to degrade PHA consisting of 3-hydroxyalkanoic acids with six or more carbon atoms. Since there is no evidence for accumulation of PHA in the environment, organisms capable of degrading these polymers must exist. In this study, we describe the isolation and

* Corresponding author.

characterization of poly(3-hydroxyoctanoic acid) [P(3HO)]-degrading bacteria and the purification and properties of a novel PHA depolymerase.

MATERIALS AND METHODS

Bacterial strains and isolates. The following bacteria were used in this study: *A. eutrophus* H16 (DSM 428), *C. violaceum* DSM 30191, *Pseudomonas oleovorans* (ATCC 29347), *Pseudomonas citronellolis* DSM 50332, *Pseudomonas fluorescens* (biovar I) (DSM 50090), and *P. fluorescens* GK13 (biovar V) (this study; DSM 7139). All other strains and isolates are listed in Table 1.

Cultivation of polymer-degrading bacteria. Polymer-degrading bacteria were routinely grown in mineral medium (29) with carbon sources as indicated in the text. Solid medium contained 1.5% (wt/vol) agar. Growth of bacteria was monitored by measuring the turbidity with a Klett-Summerson colorimeter equipped with a green filter.

Preparation of PHA. Denatured granules of P(3HB) and P(3HV) were isolated from *A. eutrophus* H16 or *C. violaceum*, respectively, as described previously (16). P(3HO) was isolated from *P. oleovorans* grown in mineral medium with sodium octanoate (0.75% [wt/vol]; applied in two aliquots of 0.5 and 0.25%, respectively) as the carbon source (8), as described by Timm and Steinbüchel (39), and finally dissolved in chloroform at a concentration of 2.5% (wt/vol). This polyester usually contained approximately 6 to 10 mol% 3-hydroxyhexanoic acid (3HHX). In this study we refer to this copolyester as P(3HO). The weight averages (M_w) and the dispersity values (M_w/M_n) of the isolated polyesters were determined by gel filtration with polystyrenes as standards and calculated to be 2.3×10^6 (M_w/M_n, 2.1), 8.2×10^5 (M_w/M_n, 68), and 4.6×10^5 (M_w/M_n, 2.4) for P(3HB), P(3HV), and P(3HO), respectively. Another copolymer [P(3HD-*co*-3HO)] was isolated from sodium gluconate-grown cells (1.5% [wt/vol]) of *P. citronellolis* by the same procedure as for P(3HO). The isolated polyester consisted of 3 mol% 3HHX, 28 mol% 3HO, 64 mol% 3-hydroxydecanoic acid (3HD), and 5 mol% 3-hydroxydodecanoic acid (3HDD). In this study we refer to this polyester as P(3HD-*co*-3HO). The monomeric compositions of all polyesters were determined gas chromatographically by the method of Brandl et al. (5) under the conditions described in detail recently (39).

P(3HO) depolymerase assay. P(3HO) depolymerase was routinely assayed as *para*-nitrophenyloctanoate (PNPO) esterase activity at 30°C. The reaction mixture contained 30 μl of a 10 mM solution of PNPO in ethanol, 10 to 50 μl of enzyme, and 50 mM potassium phosphate buffer (pH 7.9) to a total volume of 1 ml. One unit of PNPO esterase activity is defined as the hydrolysis of 1 μmol of PNPO in 1 min at 30°C. The extinction coefficient, ε, for the *para*-nitrophenylate ion at pH 7.9 was assumed to be 14.93 mM^{-1} cm^{-1}. P(3HO) depolymerase could be also assayed in a drop test on P(3HO) depolymerase activity plates: 3 ml of a 2.5% (wt/vol) solution of P(3HO) in chloroform, which contained traces of Sudan red as a marker, were poured on a solid medium (1.5% [wt/vol] agar in 100 mM Tris-HCl buffer, pH 8.5), and the solvent was evaporated. Solutions (5 to 50 μl) containing P(3HO) depolymerase were applied as drops onto the surface of the polymer and were incubated at 30°C for about 1 day. The diameter of the resulting clear zone semiquantitatively indicated the activity of the enzyme. A similar procedure was performed for activity staining of P(3HO) depolymerase separated in native polyacrylamide gels: after electrophoresis, the gels were layered onto an activity plate and were incubated at 30°C for several hours. Hydrolysis of the polymer indicated the position of P(3HO) depolymerase in the gel.

Isolation of P(3HO) depolymerase. Ten 2-liter Erlenmeyer flasks, with each flask containing 500 ml of mineral medium and 0.15% (wt/vol) P(3HO) as a thin polymer layer at the bottom, were each inoculated with a 10-h seed culture of *P. fluorescens* GK13 on 0.15% (wt/vol) sodium octanoate and shaken on a rotary shaker at 150 rpm for approximately 36 h at 30°C. All subsequent steps were performed at 0 to 4°C. The proteins of the culture supernatant (5 liters) were concentrated about 120-fold by passage through a YM10 ultrafiltration membrane (Amicon, Witten, Germany), dialyzed against equilibration buffer (50 mM potassium phosphate, pH 8.2), and applied onto an Octyl-Sepharose CL4B column (40-ml bed volume; diameter, 26 mm). P(3HO) depolymerase was eluted by subsequent passage of 80 ml of equilibration buffer, 80 ml of 5 mM potassium phosphate (pH 8.2), and an ethanediol gradient (0 to 80% [vol/vol]; 160 ml in water) through the column. Fractions (5 ml each) containing P(3HO) depolymerase activity were combined, concentrated 10- to 20-fold by passage through a YM10 membrane, centrifuged at $5,000 \times g$ for 15 min, and finally run through a Superose 12 HR 10/30 column (23.5-ml bed volume; diameter, 10 mm) equilibrated with equilibration buffer. Fractions (0.5 ml each) containing the purified P(3HO) depolymerase activity were pooled, concentrated with a YM10 membrane, and stored at 4 or −20°C.

Purification and identification of hydrolysis products. After hydrolysis of P(3HO) by the purified P(3HO) depolymerase, the protein was precipitated with trichloroacetic acid (235 mM) and removed by centrifugation. The hydrolysis products were loaded on a Chromabond C_{18ec} column (Macherey-Nagel, Düren, Germany; 500-mg sorbent weight, 3-ml bed volume, equilibrated with 2 bed volumes of methanol and 2 bed volumes of water), washed with 3 bed volumes of water, and finally eluted with 2 ml of chloroform. The hydrolysis products were identified by direct chemical ionization in the presence of ammonia (DCI-NH_3-mass spectrometry) (3) with a type 95 model (Finnigan MAT, Bremen, Germany) at 200 eV. 3HHX [M_w + 18, 150] and 3HO [M_w + 18, 178] served as standards.

Gel electrophoresis. Proteins were separated by electrophoresis either in native 5 to 7.5% (wt/vol) vertical polyacrylamide gels (104 by 83 by 1 mm), as described by Stegemann et al. (32), or in sodium dodecyl sulfate (SDS)-polyacrylamide gels (12% polyacrylamide [wt/vol]) by the method of Laemmli (18). Phosphorylase *b* (M_r, 94,000), albumin (M_r, 67,000), ovalbumin (M_r, 43,000), carbonic anhydrase (M_r, 30,000), trypsin inhibitor (M_r, 20,100), and α-lactalbumin (M_r, 14,400) were used as molecular weight standard proteins. Two-dimensional electrophoresis was performed by isoelectric focusing (first dimension) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (second dimension) by the method of O'Farrell (26). Proteins were visualized by silver staining (4) or were subjected to activity staining for P(3HO) depolymerase.

Chemicals. Chromatography media and relative molecular weight standard proteins were obtained from Pharmacia-LKB (Uppsala, Sweden). Acrylamide was from Serva (Heidelberg, Germany). Enzymes and most biochemicals were obtained from Boehringer (Mannheim, Germany); all other chemicals were from Merck (Darmstadt, Germany), Fluka (Neu-Ulm, Germany), Riedel de Haen (Seelze, Germany), or Sigma (St. Louis, Mo.).

RESULTS

Identification and isolation of aerobic P(3HO)-utilizing bacteria. The identification and isolation of P(3HB)- and P(3HV)-degrading bacteria are very simple because of the appearance of translucent halos during growth on mineral agar medium containing an opaque suspension of a polymer as the sole source of carbon and energy. Because of the rubber-like consistence of P(3HO), liquid enrichment cultures contained a thin layer of P(3HO) at the bottom of an Erlenmeyer flask. After incubation at 30°C for 1 to 3 weeks, the polymer was hydrolyzed almost completely, and the turbidity of the medium had increased. Dilutions of these suspensions were streaked onto solid mineral medium which contained 0.15% (wt/vol) sodium octanoate as the sole source of carbon and energy. Colonies of octanoate-utilizing bacteria were purified and finally tested for their ability to hydrolyze P(3HO) in liquid culture with P(3HO) as the sole source of carbon. Altogether, 26 P(3HO)-degrading bacteria were isolated independently from various soils, lake water, and activated sludge [first letter(s) in isolate designations, A or K, BG, and GK, respectively] (Table 1). Among 300 strains from culture collections, including 20 strains of *Pseudomonas aeruginosa*, 11 strains of *P. fluorescens*, and 30 other *Pseudomonas* strains, no strain was able to hydrolyze P(3HO).

Characterization of P(3HO)-degrading bacteria. Among 26 isolates of aerobic P(3HO)-degrading bacteria, there were 25 gram-negative, aerobic, rod-shaped bacteria and one gram-positive isolate (Table 1). All gram-negative bacteria utilized a large variety of organic acids, except for gluconate, and complex media or sugars as carbon sources for growth. Nineteen gram-negative bacteria were able to hydrolyze P(3HD-*co*-3HO), produced yellow-green fluorescent pigments, and shared other physiological properties. These bacteria were tentatively assigned to the fluorescent pseudomonads (31). Seven of the fluorescent bacteria, which were isolated from various ecosystems, resembled *P. fluorescens* GK13 (Table 1). None of the gram-negative isolates were able to hydrolyze P(3HB) or P(3HV). However, the gram-positive isolate K10 hydrolyzed P(3HB) and P(3HO).

Representative bacteria of the gram-negative isolates were cultivated in liquid culture on various carbon sources, and the doubling times were determined: the shortest doubling times (t_d, 1.0 to 2.0 h) occurred with organic acids (succinate, octanoate, 3-hydroxybutyrate, hexanoate, pyruvate, or acetate). Medium doubling times (t_d, 2 to 10 h) were measured with P(3HO) or glucose as the substrate. With P(3HD-*co*-3HO) as the carbon source, growth was poor and slow (t_d, 1 to 4 days). While growth on monomeric substrates started immediately, a lag phase of 8 to 12 h was observed during growth on P(3HO) or P(3HD-*co*-3HO). To determine the amount of degraded polyester at the end of growth, the remaining polymer was extracted with chloroform and assayed by gas chromatography (Table 2). The fluorescent bacteria were the most efficient ones: 78 to 95% of P(3HO) and 24 to 88% of P(3HD-*co*-3HO) were decomposed after 38 h or 5 days, respectively. The gram-positive bacterium and the nonfluorescent bacteria utilized only 14 to 52% of P(3HO). When the remaining polymers were analyzed, no significant change in the monomer composition was observed compared with that of the original polymer. No degradation of the polymer was measured in control experiments with non-P(3HO)-degrading bacteria, such as *A. eutrophus* H16 or *P. fluorescens* biovar I.

Accumulation of PHA by P(3HO)-degrading bacteria. Representative strains of this study were tested for their ability to accumulate PHA during growth on nitrogen-limited mineral medium. All gram-negative strains accumulated PHA up to 70% (dry weight) of cells. During growth on most even-numbered substrates or substrates which are metabolized via even-numbered compounds, the polymer formed contained mainly 3HO, 3HD, and 3HDD and minor amounts of 3HHX. On valerate, the polymer mainly consisted of 3HV, and 3-hydroxyheptanoate, 3HO, 3-hydroxynonanoate, and 3HD were minor constituents.

Characterization of *P. fluorescens* GK13. The P(3HO)-degrading isolate GK13, which was isolated from activated sludge, was selected to study the degradation of P(3HO) in detail. GK13 is a nonsporulating rod with a size of 0.5 to 0.7 μm by 1.2 to 2.0 μm which was highly motile by one or a few polar flagella. In addition to growing on P(3HO) and on P(3HD-*co*-3HO), GK13 grew well on alcohols, complex media, sugars, and most organic acids, except for gluconate. Isolate GK13 produced yellow-green fluorescent pigments but no pyocyanin. Isolate GK13 was identified as *P. fluorescens* GK13 (biovar V) by the identification service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

Strain GK13 grew on P(3HO) at a relatively high rate (t_d, 2 h) and on P(3HD-*co*-3HO) at a rather low rate (t_d, 24 h; Fig. 1). During growth on both polymers, a P(3HO) depolymerase was secreted into the culture medium (Fig. 2). The enzyme activity was measured qualitatively by activity staining or quantitatively by measuring the esterase activity with PNPO as the substrate. Secretion of active P(3HO) depolymerase was substrate dependent: no or little activity (<0.01 U/ml) was detected in the culture medium during growth on glucose, alanine, acetate, succinate, or acetate plus P(3HO); low activities (<0.05 U/ml) were measured after cessation of growth on MCL fatty acids, such as hexanoate, octanoate, 2-hydroxyoctanoate, and decanoate; high activities (>0.05 U/ml) were found at the end of the exponential-growth phase on lactate, pyruvate, and on 3-hydroxy acids or PHA such as 3HB, 3HO, 3HD, P(3HO), or P(3HD-*co*-3HO).

Purification of the P(3HO) depolymerase. To determine the localization of the P(3HO) depolymerase, the proteins of the concentrated culture supernatant, the membrane fraction, and the soluble fraction of P(3HO)-grown cells of strain GK13 were separated in native polyacrylamide gels and subjected to activity staining. All three fractions exhibited P(3HO) depolymerase activities (Fig. 3). However, the P(3HO) depolymerase of the soluble fraction and of the membrane fraction migrated with R_f values of 0.26 and 0.28, respectively, whereas the P(3HO) depolymerase of the culture supernatant revealed an R_f value of 0.37.

The proteins from 5 liters of supernatant fluid of a P(3HO)-grown culture of *P. fluorescens* GK13 were separated by hydrophobic interaction chromatography on Octyl-Sepharose. PNPO esterase was completely bound and was eluted in the presence of 15% (vol/vol) ethanediol in one peak (Fig. 4A). This purification step was very efficient, as revealed by denaturing SDS-PAGE (Fig. 5). Remaining contaminating proteins were removed by gel filtration (Fig. 4B). Interestingly, two peaks of PNPO esterase were identified. Only the second, major protein peak exhibited P(3HO) depolymerase activity. Table 3 summarizes details of the purification procedure. Unfortunately, the enzyme tended to bind to YM10 membranes during the concentration steps, and a large portion of the enzyme activity was lost during chromatography on Octyl-Sepharose. The whole purification

TABLE 1. Physiological properties of P(3HO)-degrading bacteria[a]

Physiological property	Strain or isolate																	
	A. faecalis T_1	*Comamonas* sp. DSM 6781	*P. lemoignei* LMG 2207	*P. fluorescens* DSM 50090	*P. fluorescens* GK13, DSM7139, GK10, GK11, GK12, GK14, BG11, BG12, and K11	GK16 and GK17	GK18 and GK19	A30, A31, and A32	A33	A34	A35	GK15	A38	BG13	BG14	K12	A40 and A41	K10
Gram stain	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND
Pyocyanin	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Fluorescein	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
P(3HB)	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
P(3HV)	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
P(3HO)	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P(3HD-*co*-3HO)	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Glucose	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+
Fructose	+	−	−	+	+	+	+	+	+	+	+	±	−	−	−	−	−	±
Trehalose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Inositol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±
Gluconate	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Succinate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pyruvate	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactate	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3-Hydroxybutyrate	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+
Valerate	±	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+
Hexanoate	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Octanoate	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±
Benzoate	+	ND	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	±
Ethanol	+	−	−	+	+	+	+	+	+	+	+	+	−	−	−	+	+	±
Sucrose	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	±
Starch	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Gelatin	−	−	−	+	+	+	+	+	+	+	−	+	+	±	−	−	−	+
Lecithin	−	−	ND	+	−	±	−	+	±	±	−	−	±	−	±	+	−	+
Tween 80	−	−	ND	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Autotroph[b]	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±
Denitrification	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	ND
NB																		
4°C	−	ND	ND	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−
30°C	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
41°C	+	−	ND	+	−	−	−	−	−	−	+	−	+	−	−	+	−	−
NB																		
TC	−	−	ND	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+
SM	+	+	ND	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
KM	+	±	ND	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
AP	+	+	ND	+	+	+	+	+	+	+	+	+	−	+	+	−	+	+
CM	+	−	ND	+	+	+	+	+	+	+	+	+	−	+	−	−	+	−

[a] Carbon sources and antibiotics at the indicated concentrations were applied as follows: glucose, fructose, gluconate, and succinate, 0.4% (wt/vol); P(3HB), P(3HV), pyruvate, lactate, acetate, and 3-hydroxybutyrate, 0.3% (wt/vol); P(3HO), P(3HD-*co*-3HO), trehalose, inositol, valerate, hexanoate, octanoate, and benzoate, 0.15% (wt/vol); nutrient broth (NB), 0.8% (wt/vol); ethanol, 100 μl per plate; tetracycline (TC), 15 μg/ml; streptomycin (SM), 100 μg/ml; kanamycin (KM), 50 μg/ml; ampicillin (AP), 50 μg/ml; and chloramphenicol (CM), 34 μg/ml. Production of pigments and extracellular enzymes was tested by the methods of Stanier et al. (31). +, growth or hydrolysis; ±, poor growth or hydrolysis; −, no growth or hydrolysis; ND, not determined.

[b] 80% H_2–10% O_2–10% CO_2.

TABLE 2. Polymer degradation of various strains and isolates[a]

Strain or isolate	Fluorescent pigments	Polymer degradation (%)	
		P(3HO)	P(3HD-*co*-3HO)
Strains			
A. eutrophus H16[b]	−	0.5	ND[d]
P. fluorescens DSM 50090[c]	+	0.4	3
P. fluorescens GK13 DSM 7139	+	92	82
Isolates			
GK10	+	82	ND
GK11	+	87	ND
GK12	+	78	ND
GK14	+	82	ND
GK17	+	95	24
GK18	+	95	ND
GK19	+	ND	64
K11	+	88	88
A31	+	ND	34
A33	+	96	76
A35	+	92	31
A38	−	18	NG[e]
BG13	−	22	NG
BG14	−	26	NG
K12	−	14	NG
K10	−	52	NG

[a] The polymer, P(3HO) or P(3HD-*co*-3HO) (0.25% [wt/vol]) was applied to 10 ml of mineral medium as the sole source of carbon and energy. After aerobic cultivation at 30°C for 38 h [P(3HO)] or 5 days [P(3HD-*co*-3HO)], the remaining polymer was extracted with solvents and analyzed by gas chromatography.
[b] 0.4% (wt/vol) fructose was added as an additional substrate.
[c] 0.15% (wt/vol) octanoate was added as an additional substrate.
[d] ND, not determined.
[e] NG, no growth.

procedure yielded 1.8 mg of electrophoretically homogeneous P(3HO) depolymerase (Fig. 5).

Characterization of the purified P(3HO) depolymerase. (i) Molecular mass and quaternary structure. The M_r of the purified P(3HO) depolymerase of strain GK13 was determined in sucrose gradients and in native polyacrylamide gradient gels as 45,500 ± 3,500 or 50,000 ± 2,000, respectively. Determination of M_r by gel filtration with Superose 12 resulted in an apparent value of 6,000. Obviously the protein had been retarded by interaction with the column matrix. Similar observations were obtained for the determination of M_r of the P(3HV) depolymerase of *P. lemoignei* by gel filtration (24).

Separation of the purified P(3HO) depolymerase in denaturing SDS-polyacrylamide gels sometimes revealed a double band with an M_r of 25,000 ± 1,000. To determine whether the double band represented an experimental artifact or whether the purified P(3HO) depolymerase consisted of two different subunits with almost identical M_rs, the protein was separated (i) by denaturing SDS-PAGE in the presence of 5 M urea and (ii) in two-dimensional gels by isoelectric focusing and denaturing SDS-PAGE. In SDS-urea gels, the protein migrated as one protein band with an M_r of 24,000 ± 1,000, and in the two-dimensional gels, only one circular spot in the basic area was detected. We conclude that the native P(3HO) depolymerase consists of two identical polypeptide chains with an M_r of 24,000 to 25,000 ± 1,000.

(ii) Optimal assay conditions. P(3HO) depolymerase was active in a pH range from 6.5 to 9.3. The highest activity was

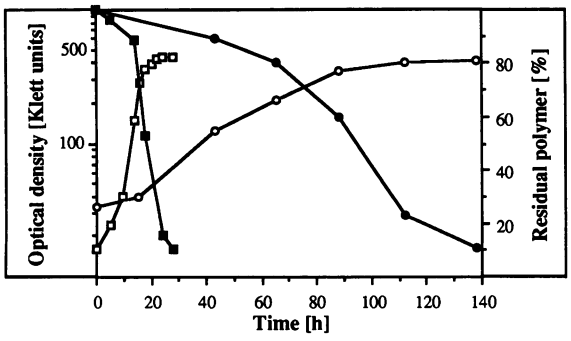

FIG. 1. Extracellular polymer degradation of *P. fluorescens* GK13 during cultivation on P(3HO) and P(3HD-*co*-3HO). Polymer (0.25% [wt/vol]) was applied to 10 ml of mineral medium as the sole source of carbon and energy. At different times, simultaneously growing cultures were extracted with solvents and analyzed by gas chromatography. Symbols: □, optical density of P(3HO); ○, optical density of P(3HD-*co*-3HO); ■, residual P(3HO); ●, residual P(3HD-*co*-3HO).

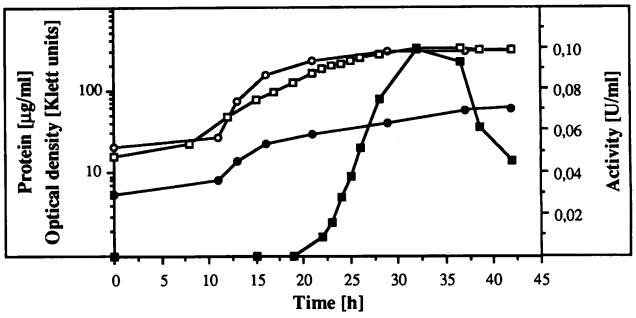

FIG. 2. Extracellular enzyme secretion by *P. fluorescens* GK13 during growth on P(3HO). Secretion of active P(3HO) depolymerase was measured as PNPO esterase activity. Symbols: □, optical density; ○, intracellular protein; ●, extracellular protein; ■, PNPO esterase activity in the culture supernatant.

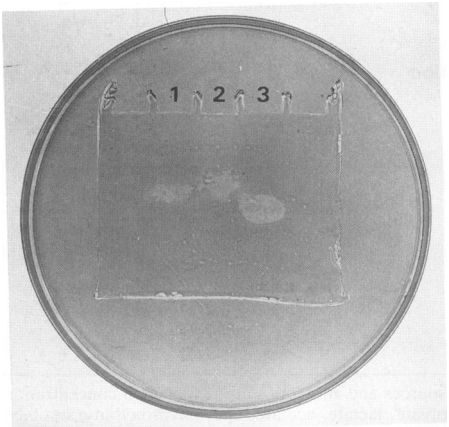

FIG. 3. P(3HO) depolymerase activity staining of electrophoretically separated native proteins of P(3HO)-grown cultures of *P. fluorescens* GK13. Lane 1, 85 µg of protein of soluble intracellular fraction; lane 2, 280 µg of protein of membrane fraction; lane 3, 5 µg of protein of concentrated culture fluid.

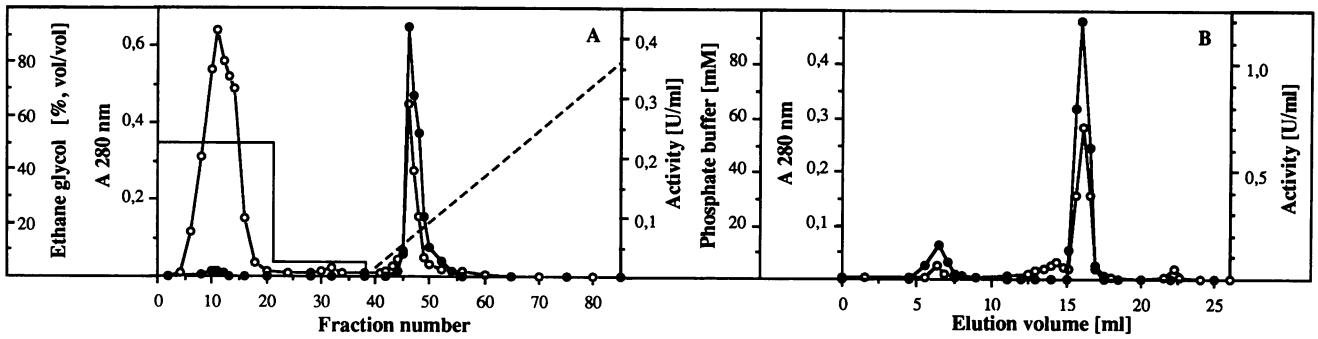

FIG. 4. Purification of extracellular P(3HO) depolymerase by chromatography on Octyl-Sepharose (A) and Superose 12 (B). Samples (20 mg) of protein from the concentrated supernatants of P(3HO)-grown cultures were applied onto an Octyl-Sepharose CL4B column (40-ml bed volume; diameter, 26 mm) which had been equilibrated with 50 mM potassium phosphate buffer (pH 8.2) (A). Samples (1 mg) of protein of the concentrated Octyl-Sepharose pool were applied onto a Superose 12 HR 10/30 column which had been washed with equilibration buffer (B). Symbols: ○, A_{280}; ●, PNPO esterase activity; – – –, ethanediol; ——, phosphate buffer.

measured in 100 mM Tris-HCl buffer, pH 8.5. If the purified P(3HO) depolymerase was assayed by a drop test on activity plates, the enzyme was active in a range from 4 to about 45°C, with a maximum at 30 to 32°C. Measurements at temperatures above 45°C were difficult because of melting of the substrate. If the PNPO esterase activity of the P(3HO) depolymerase was measured at a temperature range from 5 to 60°C, the maximum was found at 45°C. Above 52°C the protein was inactivated within minutes, and at 60°C the enzyme was completely inactive. The purified enzyme was stable at 4, −20, −70, and −196°C for at least 2 months. For routine use, the enzyme was stored at −70°C.

(iii) Substrate specificity. The enzyme was specific for the hydrolysis of P(3HO) and P(3HD-*co*-3HO). P(3HB), P(3HV), and copolymers of 3HB and 3HV were not significantly hydrolyzed by the purified P(3HO) depolymerase. No hydrolytic activity was detected with DNA, casein, or substrates for lipases, such as Tween 80 or triolein. However, esterase activity with *p*-nitrophenylacetic acid (0.03 U/mg of protein), *p*-nitrophenylbutyric acid (0.07 U/mg), *p*-nitrophenylhexanoic acid (1.0 U/mg), PNPO (4.4 U/mg), *p*-nitrophenyldecanoic acid (4.6 U/mg), and *p*-nitrophenylhexadecanoic acid (4.8 U/mg) was measured. The apparent K_m value for PNPO was 60 μM.

(iv) Inhibitors. Neither the P(3HO) depolymerase activity nor the PNPO esterase activity of the purified enzyme was significantly inhibited by 1 mM potassium cyanide, sodium azide, *p*-hydroxymercurybenzoate, monoiodoacetate, sodium EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) or 4 mM dithioerythritol. Therefore, a dependence on metal ions, reduced thiol groups, active serine residues, or essential disulfide bonds is unlikely.

(v) Identification of the hydrolysis products. Five milligrams of P(3HO) consisting of about 8 mol% 3HHX and 92 mol% 3HO, were hydrolyzed in 1.5 ml of 100 mM Tris-HCl buffer, pH 8.5, containing 0.5 mg of purified P(3HO) depolymerase at 30°C for 3 to 5 days. After trichloroacetic acid precipitation of the protein, the products were purified by solid-phase extraction on Chromabond C_{18ec} and subjected to DCI-mass spectrometry in the presence of ammonia. The dimers 3HHX-3HO [M_w + 18, 292] and 3HO-3HO [M_w + 18, 320] were identified as the main products. Additionally, minor amounts of the dimer 3HHX-3HHX [M_w + 18, 264] and of both monomers, 3HHX [M_w + 18, 150] and 3HO [M_w + 18, 178], were also identified. We concluded that the dimer is the main product of P(3HO) hydrolysis by the depolymerase of *P. fluorescens* GK13.

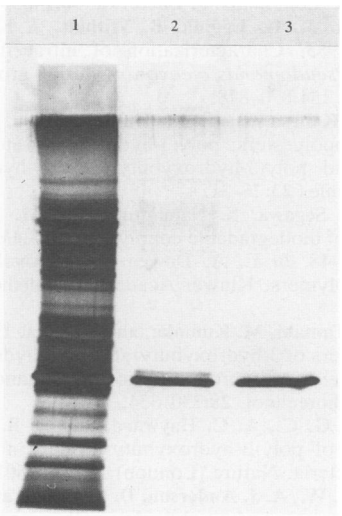

FIG. 5. Denaturing SDS-PAGE of samples of P(3HO) depolymerase at various steps of purification. Proteins were separated in an SDS–12% polyacrylamide gel and silver stained. Lane 1, 7.5 μg of protein of concentrated supernatant; lane 2, 0.5 μg of protein of Octyl-Sepharose pool; lane 3, 0.5 μg of protein of Superose pool II.

TABLE 3. Purification of the PHO depolymerase from *P. fluorescens* biovar V (GK13)

Purification step	Total amt (mg) of protein	PNPO esterase	
		Specific activity (U/mg)	Total activity (U)
Concentrated supernatant	20	3.0	61
Octyl-Sepharose CL4B	4.2	0.8	3.2
Superose 12 pool I	0.05	10.8	0.54
Superose 12 pool II[a]	1.8	1.7	2.5

[a] Pool II exhibited P(3HO) depolymerase activity.

DISCUSSION

Compared with the efforts made to explore the physiological and molecular bases for the synthesis and accumulation of PHA (reviewed by Anderson and Dawes [1] and Steinbüchel [33, 34]), investigations on the degradation of PHA are rare. Nothing is known about the degradation of polyesters of MCL HA. The present report describes the isolation and characterization of bacteria that utilize extracellular P(3HO) as the sole source of carbon and energy. Because no P(3HO)-degrading strain was identified by screening of more than 300 strains of a culture collection, this ability seems not to be widely distributed among bacteria. None of the gram-negative P(3HO)-degrading isolates was able to hydrolyze P(3HB) or P(3HV). However, the gram-positive strain, K10, hydrolyzed both P(3HB) and P(3HO) but not P(3HV). Therefore, K10 probably possesses two PHA depolymerases with substrate specificities for either P(3HB) or P(3HO). However, we cannot exclude the presence of only one PHA depolymerase of low specificity. The most efficient P(3HO)-degrading bacteria belonged to the fluorescent pseudomonads; they hydrolyzed P(3HO) and P(3HD-*co*-3HO) extracellularly by the substrate-dependent secretion of a P(3HO) depolymerase. P(3HO) depolymerase activity was measured in the culture supernatant of *P. fluorescens* GK13 grown on MCL alkanoates, various 3HA and their polyesters but not during growth on glucose or short-chain-length alkanoates. The presence of noninducing substrates such as acetate in addition to P(3HO) repressed the synthesis of active P(3HO) depolymerase. Therefore, we suppose that high expression of the P(3HO) depolymerase depends on (i) the presence of 3HA or similar compounds (inducer) or (ii) starvation of the cells for carbon. A similar regulation was found for the expression of P(3HB) depolymerase A and P(3HV) depolymerase of *P. lemoignei* (24, 37) and the P(3HB) depolymerases of most P(3HB)-degrading bacteria (16). However, it remains obscure why high activities were found at the end of growth on pyruvate and lactate. Growth on alanine or glucose, which were also metabolized via pyruvate, did not stimulate the expression of P(3HO) depolymerase.

All P(3HB)-degrading bacteria analyzed so far (7, 16) were also able to accumulate P(3HB). Accumulation of PHA was also found in the gram-negative strains of this study. If the bacteria were grown on related or unrelated substrates, the accumulated PHA consisted of MCL-HA as has been described recently elsewhere (13, 39).

The purified P(3HO) depolymerase of *P. fluorescens* GK13 resembled the PHB depolymerases of *A. faecalis* T_1 (27, 37), a *Comamonas* sp. (16), and the three PHA depolymerases of *P. lemoignei* [P(3HB) depolymerase A and B and P(3HV) depolymerase] (21, 24) with respect to the molecular weight (48,000 in comparison to 49,000, 45,000, 53,000, 54,000, and 67,000, respectively), alkaline pH optimum (8.0 to 8.6 in comparison to 7.5, 9.4, 8.0, 8.0, and 8.0, respectively), and independence from metal ions and reduced thiol groups. However, in contrast to the known PHA depolymerases, which consist of only a single polypeptide chain and which are sensitive to dithioerythritol and PMSF (except for the *Comamonas* sp.), the depolymerase of strain GK13 consists of two identical polypeptide chains (M_r, 25,000) and was not inhibited by dithioerythritol or by PMSF. These differences indicate different protein structures and reaction mechanisms.

P(3HO) depolymerase activity was found not only in the culture supernatant but also in the soluble fraction and in the membrane fractions of P(3HO)-grown cells. The low R_f value of the intracellular and membrane-bound enzyme in comparison to the extracellular P(3HO) depolymerase points to the existence of a precursor peptide of the extracellular P(3HO) depolymerase which is processed during transport across the cytoplasmic membrane. The presence of intracellular precursor peptides is a phenomenon common to extracellular proteins (reviewed by Lory [20]). It remains to be eludicated whether the second protein exhibiting PNPO esterase activity represents a 3-hydroxyoctanoic acid dimer hydrolase.

ACKNOWLEDGMENTS

We thank G. Remberg for performing mass spectrometry, R. Brinkmann for assistance during fermentation, R. J. Müller for determination of the molecular masses of P(3HO), and T. Saito and A. Steinbüchel for providing us with *A. faecalis* and *C. violaceum*.

This work was supported by the Deutsche Forschungsgemeinschaft and the Max Buchner Forschungsstiftung.

REFERENCES

1. **Anderson, A. J., and E. A. Dawes.** 1990. Occurrence, metabolism, metabolic role, and industrial use of bacterial polyhydroxyalkanoates. Microbiol. Rev. **54:**450–472.
2. **Anderson, A. J., G. W. Haywood, D. R. Williams, and E. A. Dawes.** 1990. The production of polyhydroxyalkanoates from unrelated carbon sources, p. 119–129. *In* E. A. Dawes (ed.), Novel biodegradable microbial polymers. Kluwer Academic Publishers, Dordrecht, The Netherlands.
3. **Baldwin, M., and F. McLafferty.** 1973. Direct chemical ionization of relatively involatile samples. Application to underivatized oligopeptides. Org. Mass. Spectrom. **7:**1353–1356.
4. **Blum, H., H. Beier, and H. J. Gross.** 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:**93–99.
5. **Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller.** 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. **54:**1977–1982.
6. **Budwill, K. P., M. Fedorak, and W. J. Page.** 1992. Methanogenic degradation of poly(3-hydroxyalkanoates). Appl. Environ. Microbiol. **58:**1398–1401.
7. **Delafield, F. P., M. Doudoroff, N. J. Palleroni, C. J. Lusty, and R. Contopoulos.** 1965. Decomposition of poly-β-hydroxybutyrate by pseudomonads. J. Bacteriol. **90:**1455–1466.
8. **De Smet, M. J., G. Eggink, B. Witholt, J. Kingma, and H. Wynberg.** 1983. Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J. Bacteriol. **154:**870–878.
9. **Doi, Y., Y. Kanesawa, and M. Kunioka.** 1990. Degradation of microbial copolyesters: poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate). Macromolecules **23:**26–31.
10. **Doi, Y., A. Segawa, S. Nakamura, and M. Kunioka.** 1990. Production of biodegradable copolyesters by *Alcaligenes eutrophus*, p. 37–48. *In* E. A. Dawes (ed.), Novel biodegradable microbial polymers. Kluwer Academic Publishers, Dordrecht, Germany.
11. **Doi, Y., A. Tamaki, M. Kunioka, and K. Soga.** 1988. Production of copolyesters of 3-hydroxybutyrate and 3-hydroxyvalerate by *Alcaligenes eutrophus* from butyric and pentanoic acids. Appl. Microbiol. Biotechnol. **28:**330–334.
12. **Forsyth, W. G. C., A. C. Hayward, and R. B. Roberts.** 1958. Occurrence of poly-β-hydroxybutyric acid in aerobic Gram-negative bacteria. Nature (London) **182:**800–801.
13. **Haywood, G. W., A. J. Anderson, D. F. Ewing, and E. A. Dawes.** 1990. Accumulation of a polyhydroxyalkanoate containing primarily 3-hydroxydecanoate from simple carbohydrate substrates by *Pseudomonas* sp. strain NCIMB 40135. Appl. Environ. Microbiol. **56:**3354–3359.
14. **Haywood, G. W., A. J. Anderson, D. R. Williams, and E. A. Dawes.** 1991. The accumulation of polyhydroxyalkanoate co-

polymer containing primarily 3-hydroxyvalerate from simple carbohydrate substrates by *Rhodococcus* sp. strain NCIMB 40126. Int. J. Biol. Macromol. **13:**83–88.

15. **Jansen, P. H., and C. G. Harfoot.** 1990. *Ilyobacter delafieldii* sp. nov., a metabolically restricted anaerobic bacterium fermenting PHB. Arch. Microbiol. **154:**253–259.

15a. **Jendrossek, D.** Unpublished data.

16. **Jendrossek, D., I. Knoke, R. B. Habibian, A. Steinbüchel, and H. G. Schlegel.** 1993. Degradation of poly(3-hydroxybutyrate), PHB, by bacteria and purification of a novel PHB depolymerase from *Comamonas* sp. J. Environ. Polymer Degrad. **1:**53–63.

17. **Kim, G. J., K. Y. Yun, K. S. Bae, and Y. H. Rhee.** 1992. Accumulation of copolyesters consisting of 3-hydroxybutyrate and 3-hydroxyvalerate by *Alcaligenes* sp. SH-69 in batch culture. Biotechnol. Lett. **14:**27–32.

18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.

19. **Lageveen, R. G., G. W. Huisman, H. Preusting, P. Ketelaar, G. Eggink, and B. Witholt.** 1988. Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates. Appl. Environ. Microbiol. **54:**2924–2932.

20. **Lory, S.** 1992. Determinants of extracellular protein secretion in gram-negative bacteria. J. Bacteriol. **174:**3423–3428.

21. **Lusty, C. J., and M. Doudoroff.** 1966. Poly-β-hydroxybutyrate depolymerases of *Pseudomonas lemoignei*. Proc. Natl. Acad. Sci. USA **56:**960–965.

22. **McLellan, D. W., and P. J. Halling.** 1988. Acid-tolerant poly(3-hydroxybutyrate) hydrolases from moulds. FEMS Microbiol. Lett. **52:**215–218.

23. **Mukai, K., and Y. Doi.** 1993. Enzymatic degradation of poly(3-hydroxybutyrate) by marine microorganisms. *In* A. Steinbüchel and H. G. Schlegel (ed.), Proceedings of the International Symposium on Bacterial Polyhydroxyalkanoates, Göttingen.

24. **Müller, B., and D. Jendrossek.** 1993. Purification and properties of a poly(3-hydroxyvalerate) depolymerase from *Pseudomonas lemoignei*. Appl. Microbiol. Biotechnol. **38:**487–492.

25. **Nakayama, K., T. Saito, T. Fukui, Y. Shirakura, and K. Tomita.** 1985. Purification and properties of extracellular poly(3-hydroxybutyrate) depolymerases from *Pseudomonas lemoignei*. Biochim. Biophys. Acta **827:**63–72.

26. **O'Farrell, P. H.** 1975. High resolution two dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.

27. **Schlegel, H. G., and G. Gottschalk.** 1962. Poly-β-hydroxybuttersäure, ihre Verbreitung, Funktion und Biosynthese. Angew. Chem. **74:**342–347.

28. **Schlegel, H. G., G. Gottschalk, and R. V. Bartha.** 1961. Formation and utilization of poly-β-hydroxybutyric acid by knallgas bacteria (Hydrogenomonas). Nature (London) **191:**463–465.

29. **Schlegel, H. G., H. Kaltwasser, and G. Gottschalk.** 1961. Ein Submersverfahren zur Kultur wasserstoffoxydierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. **38:**209–222.

30. **Shirakura, Y., T. Fukui, T. Saito, Y. Okamoto, T. Narikawa, K. Koide, K. Tomita, T. Takemasa, and S. Masamune.** 1986. Degradation of poly(3-hydroxybutyrate) by poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis* T_1. Biochim. Biophys. Acta **880:**46–53.

31. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.

32. **Stegemann, H., H. Francksen, and V. Macko.** 1973. Potato proteins: genetic and physiological changes, evaluated by one or two dimensional PAA-gel-techniques. Z. Naturforsch. Teil C **28:**722–732.

33. **Steinbüchel, A.** 1991. Polyhydroxyalkanoic acids, p. 123–213. *In* D. Byrom (ed.), Biomaterials, Macmillan Press, London.

34. **Steinbüchel, A.** 1991. Polyhydroxyfettsäuren—thermoplastisch verformbare Polyester aus Bakterien. Nachr. Chem. Tech. Lab. **39:**1112–1124.

34a. **Steinbüchel, A.** Personal communication.

35. **Steinbüchel, A., K. A. Malik, and D. Jendrossek.** PHB and other polyhydroxyalkanoic acids as a basis for biodegradable plastics. Proceedings of the Pacific Biotechnology Congress in Los Banos/Phillippines, in press.

36. **Steinbüchel, A., and U. Pieper.** 1992. Production of a copolyester of 3-hydroxybutyric acid and 3-hydroxyvaleric acid from single unrelated carbon sources by a mutant of *Alcaligenes eutrophus*. Appl. Microbiol. Biotechnol. **37:**1–6.

37. **Stinson, M. W., and J. M. Merrick.** 1974. Extracellular enzyme secretion by *Pseudomonas lemoignei*. J. Bacteriol. **119:**152–161.

38. **Tanio, T., T. Fukui, Y. Shirakura, T. Saito, K. Tomita, T. Kaiho, and S. Masamune.** 1982. An extracellular poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. Eur. J. Biochem. **124:**71–77.

38a. **Timm, A.** Personal communication.

39. **Timm, A., and A. Steinbüchel.** 1990. Formation of polyesters of medium-chain-length 3-hydroxyalkanoic acids from gluconate by *Pseudomonas aeruginosa* and other fluorescent pseudomonads. Appl. Environ. Microbiol. **56:**3360–3367.