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Combined Determination of Poly-β-Hydroxyalkanoic and Cellular Fatty Acids in Starved Marine Bacteria and Sewage Sludge by Gas Chromatography with Flame Ionization or Mass Spectrometry Detection

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Extraction of lipids from bacterial cells or sewage sludge samples followed by simple and rapid extraction procedures and room temperature esterification with pentafluorobenzylbromide allowed combined determinations of poly-β-hydroxyalkanoate constituents and fatty acids. Capillary gas chromatography and flame ionization or mass spectrometric detection was used. Flame ionization permitted determination with a coefficient of variation ranging from 10 to 27% at the picomolar level, whereas quantitative chemical ionization mass spectrometry afforded sensitivities for poly-β-hydroxyalkanoate constituuents in the attomolar range. The latter technique suggests the possibility of measuring such components in bacterial assemblies with as few as 10^2 cells. With the described technique using flame ionization detection, it was possible to study the rapid formation of poly-β-hydroxyalkanoate during feeding of a starved marine bacterium isolate with a complex medium or glucose and correlate the findings to changes in cell volumes. Mass spectrometric detection of short β-hydroxy acids in activated sewage sludge revealed the presence of 3-hydroxybutyric, 3-hydroxyhexanoic, and 3-hydroxyoctanoic acids in the relative proportions of 56, 5 and 39%, respectively. No odd-chain β-hydroxy acids were found.

Total biomass, community structure, metabolic activity, and nutritional status are important features that characterize the ecology of microbial communities. These parameters can be examined by studies of specific cellular components, "signatures" (25). The community structure of microbial assemblies has been defined by the fatty acids derived from the phospholipids of microorganisms (25). The nutritional status of procaryotes has been assessed by measuring the storage lipid polymer-β-hydroxyalkanoate (PHA) (6, 9, 19).

Fatty acids or monomers of PHA in environmental samples can be quantitatively analyzed by gas chromatography (GC) (see, e.g., 3, 9, 12). In principle, the lipid extracts of a sample are separated into neutral, phospho-, and glycolipids by liquid chromatography on silicic acid. After hydrolysis or acid-catalyzed transesterification, the fatty acids are analyzed by capillary GC as their methyl esters or, in case of the low-molecular-weight β-hydroxy acids, as the ethyl esters. The flame ionization detector (FID) allows determination of components down to the picomolar level, which corresponds to 10^5 to 10^6 bacterial cells the size of *Escherichia coli* (25).

Pentafluorobenzyl (PFB) derivatives of carboxylic acids offer certain advantages over the traditional methyl esters in GC work (7, 8). First, the PFB reagent forms esters of significantly higher boiling points than those of the methyl esters. This is an advantage when dealing with low-molecular-weight acids for which the methyl esters might be very volatile, leading to poor resolution because of short retention times. Second, these derivatives show strong electron affinity, making them suitable for the extremely sensitive electron capture detection (7, 8) or chemical ionization (CI)-mass spectrometry (MS) detection of negative ions (22). Recent studies indicated that this technique operating in the selected ion monitoring (SIM) mode allowed determinations of bacterial fatty acids down to the low femtomolar levels, corresponding to a few hundreds of bacteria the size of *E. coli* (20).

Our experience with GC of PFB esters of bacterial fatty acids led us to investigate whether these esters could be utilized in an improved procedure allowing combined estimations of cellular fatty acids and the monomers of PHA. The developed method omits, in particular, the rather tedious liquid chromatographic steps previously used and involves only extractions, simple derivatization, and GC analyses. For routine analysis the FID was used. However, the inherent features of the procedure also allowed highly sensitive determinations, using CI-MS detection.

The method was used to study the recovery of PHA synthesis after feeding of a starved marine bacterium. Previous studies at our laboratories have shown that this bacterium synthesizes PHA during growth and that the polymer is utilized during starvation conditions (14, 15). The MS technique was used in an investigation of the PHA monomer composition in activated sewage sludge.

MATERIALS AND METHODS

Solvents, reagents, and standards. Solvents were glass-distilled, reagent-grade commercial products used without further purification. 2,3,4,5,6-Pentafluorobenzylbromide (purity > 99%), heptafluorobutyric anhydride (HFBA) (purity > 99%), and rac-3-hydroxybutyric acid (purity > 97%) were from Fluka AG, Buchs, Switzerland. 3*R*-Hydroxynonanoic acid, prepared from ricinoleic acid (21), was from our laboratory collection, and the bacterial fatty acid mixture was from Supelco Inc. (Bellefonte, Pa.) (lot LA 11091).

The fatty acids in this study are designated by the number of total carbon atoms:number of unsaturations, with the

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position of the double bond closest to the aliphatic end (ω) of the molecule and the configuration "c" for *cis* or "t" for *trans* indicated. Cyclopropane rings are designated by "cyc" and hydroxyl groups are designated with the prefix OH and position indicated.

Sludge samples. Sludge from an activated sewage treatment plant (Veberöd, Sweden) was freeze-dried, and 10.0-mg portions were extracted as described below.

Feeding of marine bacteria during starvation. In this experiment an unidentified marine isolate, S14, was used (10). The strain is a gram-negative, motile, short rod. The organisms were grown on V medium (26) with a nine-salt solution (NSS) as diluent (4). During growth in this medium S14 cells synthesize large amounts of PHA (14, 18).

S14 cells were harvested at the end of the log phase by centrifugation at 6,000 × *g*, washed twice in NSS, and suspended at a concentration of 3×10^7 cells per ml in pure NSS. After 5 or 24 h of starvation in NSS, the cells were fed by adding either V medium or glucose at final concentrations corresponding to the initial growth medium or to 2 g of glucose per liter. Samples were taken at various times for determination of total counts, cell volumes, viable counts, and PHA content. Measurements of cell numbers, cell volumes, and viable counts were made according to Mårdén et al. (15). For lipid analysis 10-ml samples were centrifuged at 6,000 × *g* for 10 min. The pellet was suspended in 1 ml of NSS, transferred to a test tube, and immediately freeze-dried.

Lipid extraction. Freeze-dried samples were extracted with 3 ml of a single-phase mixture consisting of chloroform-methanol-water (1:2:0:8, vol/vol/vol) according to Bligh and Dyer (2) in 10-ml Teflon-lined screw-cap test tubes. After sonication for 15 min, the tubes were left overnight and centrifuged at 4,000 × *g*, and the extract was transferred to a different set of test tubes. The cellular residue was washed twice with 0.4 ml of solvent, and the combined solutions were split into two phases by the addition of 1 ml of water and 1 ml of chloroform. After centrifugation (4,000 × *g*) the aqueous layer was discarded and the chloroform phase was supplemented with internal standards, typically 5 to 20 μg of 3-OH 9:0 and 19:0. The solvents were removed under a stream of nitrogen.

Acid recovery. The residue was subjected to mild alkaline hydrolysis by the addition of 1 ml of 15% NaOH in a 1:1 (vol/vol) mixture of methanol-water followed by heating at 80°C for 30 min (17). A total of 0.5 ml of water and 1.5 ml of hexane were added, the two-phase system was vigorously shaken and then centrifuged, and the hexane phase was discarded. The aqueous phase was washed a second time with hexane to remove traces of neutral material. Acidification with 0.5 ml of 25% HCl in water afforded the free acids. The aqueous samples were freeze-dried and the residues were triturated and sonicated twice with 1.5-ml portions of methylene chloride. After centrifugation (4,000 × *g*) the organic solutions were combined in 7-ml screw-cap test tubes and evaporated to dryness under a slow stream of nitrogen at room temperature to yield the free acids.

Derivatization. The acids were dissolved in 30 μl of acetonitrile. A 10-μl portion of 35% PFB-bromide in acetonitrile was added followed by 10 μl of triethylamine as a catalyst (20, 22). After 10 min at room temperature, 20 μl of HFBA was added to acylate any hydroxy acids present, followed by 250 μl of hexane. The hexane solution was sampled directly for GC analysis.

Alternatively, in the case of small samples, the contents in the test tube were evaporated under a gentle stream of nitrogen and then transferred with 2 × 100 μl of hexane to a capillary tube (inside diameter, 4 mm), evaporated, and redissolved in 10 to 50 μl of hexane.

GC. The GC analyses were performed on a Varian 3700 instrument equipped with FID. A 25-m fused silica capillary column (inside diameter, 0.2 mm) statically coated with SE-54 as stationary phase on a D4 deactivated surface was used. The injector temperature was 250°C and the detector temperature was 270°C. The oven temperature was initially 110°C and after 2 min it was linearily increased at 4°C/min to 270°C. The injections (1 μl) were made in the splitless mode; the splitter was opened 1 min after injection. Quantification was performed with a Hewlett-Packard model 3390A electronic integrator.

Calibration curve and precision measurements for GC/FID. Chloroform phases obtained after splitting the one-phase extract (Bligh-Dyer) of 10.0-mg samples of freeze-dried sewage sludge were supplemented with 21.0 g of 3-OH 9:0 as internal standard. To six extracts were added, respectively, 0, 9.1, 36.4, 91.0, 182.0, and 364.0 μg of 3-OH 4:0. The fatty acids were recovered, esterified, and quantified as described above.

The precision of the GC/FID method was evaluated by performing five parallel analyses (complete procedure) of the marine bacterium isolate S14. The samples contained about 5 mg (dry weight) of log-phase cells.

MS. The GC/MS data acquisition system used was a Ribermag R10-10c quadropole instrument equipped with a Carlo Erba model 4160 capillary GC. The GC conditions were those described above. Helium at a flow rate of 1 ml/min served as carrier gas.

For the negative-ion CI measurements, methane (purity > 99.95%) at 0.07 torr (1 torr = 133.3 Pa) was used as reagent gas, whereas in the positive-ion CI measurements ammonia (purity > 99.90%) at 0.10 torr was used. Ion source temperature was 80 (CH_4) or 110°C (NH_3) and electron energy was 94 eV.

RESULTS

GC. The initial experimants comprised studies of the chromatographic conditions to obtain optimum combined separation of the PFB-HFBA esters of 3-OH 4:0, 3-OH 9:0 (internal standard), and other higher bacterial fatty acids. With hydrogen at 4 ml/min as carrier gas, the capillary GC conditions afforded approximate retention times of 10 and 20 min for 3-OH 4:0 and 3-OH 9:0, respectively (Fig. 1). The analysis was completed after 45 min.

Quantification by FID. The linearity of the calibration curve for the PFB-HFBA ester of 3-OH 4:0, using 3-OH 9:0 as internal standard, was satisfactory ($r = 0.95$). The extrapolated intercept of the x axis gave the amount of 3-OH 4:0 in the sludge sample (30 μg) corresponding to 3 μg per mg (dry weight) of sludge.

The precision of the GC/FID method for analysis of 3-OH 4:0 and fatty acids was evaluated by analysis of samples of S14 cells (Table 1). The coefficient of variation ranged from 27% for 3-OH 4:0 to about 10% for 18:0.

MS. Mass spectra of PFB derivatives of 3-OH 4:0 were recorded by three different ionization methods (Table 2).

In positive-ion electron ionization, the molecular radical ion is absent and the most abundant fragments are those corresponding to the PFB ions (m/z 181). In positive-ion CI with ammonia as reagent gas, however, abundant adduct ions at m/z 302 (=M + 18) related to the structure of 3-OH 4:0 are formed. Likewise, structurally related ions forming

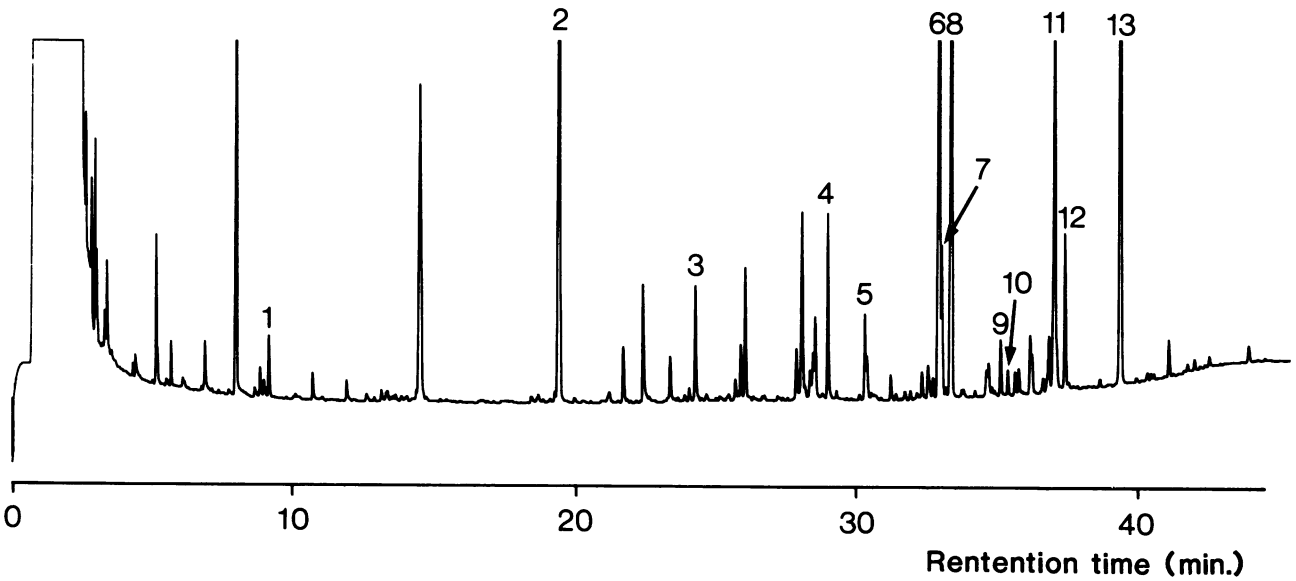

FIG. 1. GC of PFB-HFBA derivatives of PHA constituents and fatty acids of the marine bacterium S14, using FID. 1, 3-OH 4:0, 2, 3-OH 9:0 (IS); 3, 12:0; 4, 14:0, 5, 15:0; 6, 16:1ω7c; 7, 16:1ω7t; 8, 16:0; 9, 17:0cyc; 10, 17:0; 11, 18:1ω9c; 12, 18:0; 13, 19:0 (IS).

the base peak, consisting of the carboxylate ions (*m/z* 103), are produced in negative-ion CI with methane as reagent gas.

A different situation was observed in negative-ion CI (Table 2) with methane as the reagent gas of the PFB-HFBA ester of 3-OH 4:0. In this case, the base peak at *m/z* 213 corresponds to fragments with negative charge retention on the HFB anions. Only about 20% of 3-OH 4:0-related fragments (carboxylate ions) carry negative charge and, furthermore, they expel a molecule of water (*m/z* 85).

Quantification by SIM. The PFB and PFB-HFBA derivatives of 3-OH 4:0 were analyzed at a femtomolar level with SIM, using negative-ion CI with methane as reagent gas, monitoring the ions at *m/z* 103.1 and 213.2, respectively. A dose-response curve of the PFB ester of 3-OH 4:0 was constructed in the range of 50 to 700 fmol. The correlation coefficient (*r*) of the regression line for this curve was 0.990. The sensitivity for SIM analyses of the PFB-HFBA derivative was about 50 times higher than that of the PFB ester. The dose-response curve obtained in the range of 0.8 to 8 fmol of this derivative had a correlation coefficient of 0.988. The detection limit for the PFB-HFBA derivative was approximately 0.5 fmol at a signal/noise ratio of 2.

Composition of PHA in sewage plant sludge. The PFB esters of the short-chain β-hydroxy acids recovered from the sludge were analyzed by SIM, using two different ionization methods. In positive-ion CI-MS (ammonia) the ions of *m/z* 302, 316, 330, 344, and 358 corresponding to the molecular adduct ions (M + 18) of 3-OH 4:0, 3-OH 5:0, 3-OH 6:0, 3-OH 7:0, and 3-OH 8:0, respectively, were monitored. Negative-ion CI-MS (methane) was performed by recording the corresponding carboxylate ions of *m/z* 103, 117, 131, 145, and 159. The fragmentograms indicated signals for 3-OH 4:0, 3-OH 6:0, and 3-OH 8:0, equivalent to the relative proportions of 56, 5, and 39%, respectively. No short odd-carbon chain β-hydroxy acids were detected in the sample.

Recovery of PHA after starvation. Starvation of S14 cells in energy- and nutrient-free salt solution induced an increase in cell numbers but a decrease in cell volume and PHA content (Fig. 2A to C). These changes were most pronounced within the first 5 h of starvation. Feeding of the starved cells at time 5 and 24 h with the complex V medium initiated a rapid and marked increase in cell volumes as well as a synthesis of PHA. However, 3 h after addition of the medium the amount of PHA was again back to the level at the onset of the feeding. The corresponding addition of glucose was followed by a smaller increase in cell volumes and PHA content. In this case, the start of the synthesis of PHA was about 1 h delayed. The cell numbers did not change as markedly as the cell volumes and PHA content after addition of substrates. The cells were 100% viable throughout the entire period of the experiment.

DISCUSSION

Extraction and fatty acid recovery. The one-phase extraction solution consisting of methanol-chloroform-water was chosen as it removes quantitatively neutral lipids, phospholipids, glycolipids, and PHA from the cellular material (12). As free acids are required for the PFB esterification, hydrolysis of the complex extractable lipids was preferred rather than transesterification. Alkaline hydrolysis is rapid and selective, but care must be taken to avoid dehydration of, in particular, β-hydroxy acids. Moss et al. (18) compared four commonly used methods and they suggested 15% NaOH in

TABLE 1. Precision of GC method (FID) for analysis of PFB-HFBA derivatives of fatty acids in S14 cells

Parameter	Fatty acid											
	3-OH 4:0	12:0	14:0	15:0	16:1ω7c	16:1ω7t	16:0	17:0cyc	17:0	18:1ω9c	18:0	Total
Amt (μg/mg [dry wt])	3.08	0.79	1.59	0.07	29.17	0.07	17.25	0.46	0.05	5.76	0.37	55.61
SD (n = 5)	0.83	0.22	0.30	0.01	6.13	0.01	3.41	0.08	0.01	0.66	0.04	10.75
Coefficient of variation (%)	26.9	27.4	18.6	17.2	21.0	19.9	19.8	16.8	12.9	11.4	9.52	19.3

TABLE 2. Significant ions in mass spectra of PFB and PFB-HFBA derivatives of 3-OH 4:0

Ionization	Most abundant ion		Other significant ions	
	m/z	%	*m/z*	%
PFB derivative				
Positive EI	181	100	87	51
Positive CI (NH_3)[a]	50	100	302	57
Negative CI (CH_4)[a]	103	100		
PFB-HFBA derivative				
Negative CI (CH_4)[a]	213	100	85	20

[a] Reagent gas.

50% aqueous methanol as hydrolysis reagent with heating at 80°C for 30 min. Under these conditions breakdown of sensitive acid constituents is reduced to a minimum.

The recovery of short-chain β-hydroxy acids from the aqueous, acidified hydrolysis mixture deserves comment. In particular, 3-OH 4:0 distributes unfavorably between water and organic solvents and is, therefore, not quantitatively extracted from the aqueous solution with small amounts of organic solvents such as methylene chloride. This problem was overcome by removing the water before extraction. We found freeze-drying to be the most convenient since it was applicable to a large number of samples.

Derivatization. The peak of the PFB esters of the hydroxy acids showed increased tailing after several chromatographic runs. By adding HFBA to the esterification mixture the polar hydroxyl group was protected by fluoroacylation. The peak shape of the hydroxy esters improved markedly with only a slight increase of the GC retention times. As expected, fluoroacylation did not affect nonsubstituted PFB esters.

The PFB esters prepared as described appeared stable during long periods of time at room temperature. It was, however, observed that the PFB-HFBA derivatives of 3-OH 4:0 and 3-OH 9:0 slowly changed, resulting in decreased GC peaks during 24 h at room temperature if triethylamine was used in excess as compared with the HFBA reagent. By adding more HFBA reagent, the derivatives reformed as indicated by GC, and it was found that a 100% excess of acylating agent versus base resulted in derivatives that were stable for at least several weeks.

Quantification by FID. The straight regression line of the calibration curve and the precision study (Table 1) indicate straightforward quantification with 3-OH 9:0 as internal standard for 3-OH 4:0 and 19:0 for the fatty acids 12:0 through 18:0. The coefficient of variations is somewhat high, especially for the short-chain fatty acids. This is, however, not unusual in analysis of biological samples.

MS. The electron ionization spectrum of the PFB esters of 3-OH 4:0 resembled the corresponding esters of long-chain fatty acids (22). It was previously shown (20) that negative-ion CI-MS (methane) produced spectra of the PFB esters consisting almost exclusively of the carboxylate ions formed by loss of the PFB radical. The only exception was the 3-OH-substituted PFB esters which lost an additional water molecule, giving rise to ions of $m/z = [M - (H_2CC_6F_5 + 18)]$. Systematic investigations of the mass spectrometric behavior of the PFB ester of 3-OH 4:0 in this study revealed that the water expulsion to a marked extent was governed by the thermal conditions of the GC/MS system, in particular, those of the ion source. By lowering the temperature of the GC/MS interface to about 200°C and that of the ion source to 80°C, it was possible to record spectra with intact carboxylate ions (*m/z* 103). At higher temperatures spectra containing ions of both *m/z* 103 and 85 (103 − 18) were formed.

The increased sensitivity of the PFB-HFBA derivatives compared with the PFB esters encountered in SIM depends

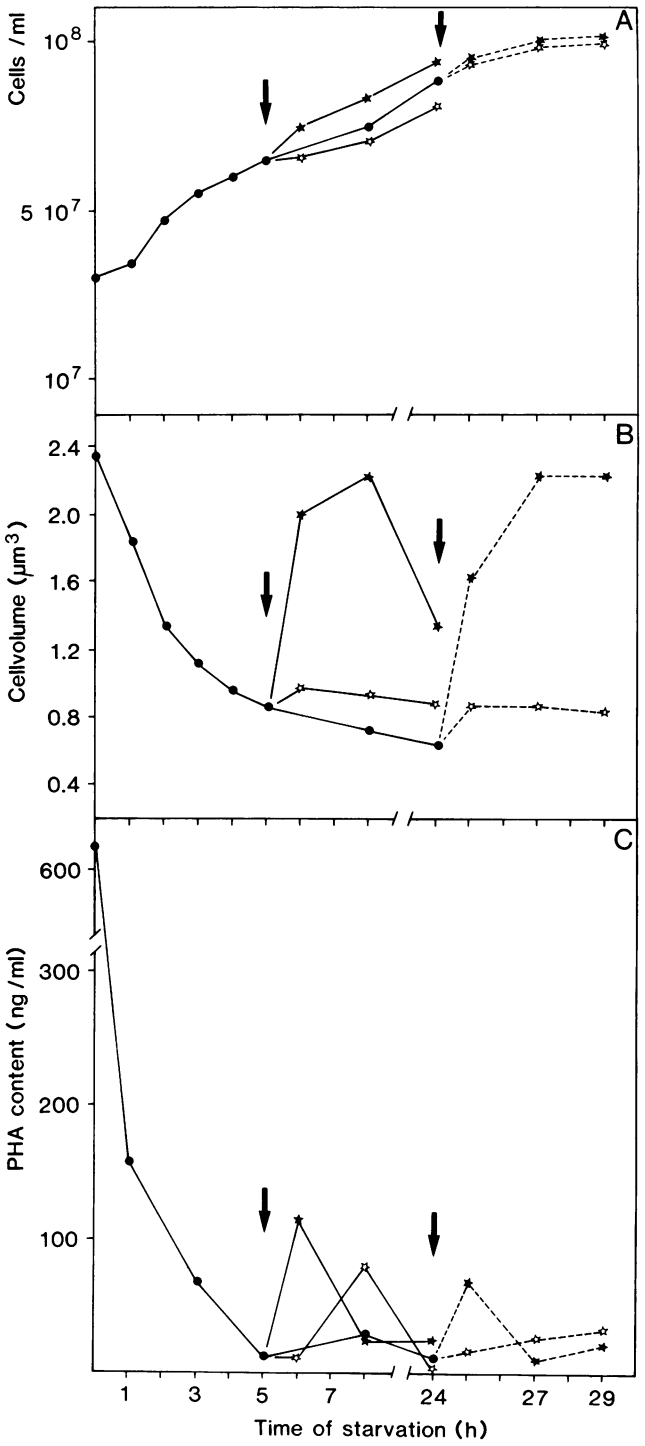

FIG. 2. Changes (●) in (A) total counts, (B) cell volume, and (C) PHA content during starvation of the marine bacterium S14 in a complete energy- and nutrient-free medium. Arrows indicate additions of a complex medium (★) or glucose (☆) after 5 (solid lines) and 24 (dashed lines) h of starvation.

on both the increased number of fluorine atoms of the former derivative and the superior peak shape. Preliminary experiments with polar capillary columns (polyethylene glycol or cyanopropyl-methyl-phenyl-polysiloxane as stationary phase) indicated, however, excellent peak shape also for the PFB esters and hence increased sensitivity. Different certainty as to the identity of the recorded 3-OH 4:0 signal is encountered for the two derivatives. The 3-OH 4:0 PFB ester peak is ensured by both the GC retention time and the mass number of the structurally related fragment, whereas for the PFB-HFBA derivative only the retention time defines the analyte.

In the S14 cells used for Table 1, the content of 3-OH 4:0 was 3.08×10^{-6} (dry weight) corresponding to 5.2×10^{-16} g per cell assuming 5.9×10^{12} bacteria/g (dry weight) (20). Therefore, at the observed MS detection limit of 500×10^{-18} mol, it should be possible to detect about 100 bacteria with the same PHA content. The high sensitivites for analyzing bacteria can be of great importance in defining the critical initial phases of contamination of air and water supplies, of infection of plants and animals, and in studies of biodeterioration processes such as biofouling and corrosions (cf. reference 20).

Composition of PHA in sewage sludge. There seems to be some controversy about the monomer composition of PHA. Wallen and Rohwedder (24) concluded from GC analysis of PHA in activated sludge that it was composed essentially of 3-OH 4:0 and 3-OH 5:0 acids with traces of C_6 and C_7 homologs. Findlay and White (9), on the other hand, showed the presence of at least 11 short-chain β-hydroxy acids in polymers extracted from marine sediments, using a combination of GC retention time data and MS. Among the components, the β-hydroxy acids with four to seven carbon atoms in the chain dominated. In contrast, our studies of activated sludge based on SIM measurements, using two independent ionization techniques, revealed only even-chain β-hydroxy acids, with acids possessing four and eight carbon atoms dominating. Since 3-OH 4:0 is synthesized via two-carbon units (acetate) (16), it seems plausible to expect essentially even-carbon chain β-hydroxy acids as constituents of PHA. This assumption agrees with the fatty acid composition in the lipopolysaccharides where 12:0, 14:0, and 16:0 β-hydroxy acids are by far the predominant hydroxy acid constituents (13). However, the discrepancies in results between the different investigations may also be due to the use of different samples and extraction procedures.

Feeding of starved marine bacteria. PHA is synthesized during unbalanced growth and is subsequently utilized during carbon- or energy-depleted conditions or both (e.g., see references 5, 14). A correlation between the amount of PHA and viability during starvation of bacteria has been demonstrated (11, 16, 23). The rapid consumption of PHA during the starvation in energy- and nutrient-free salt solutions (Fig. 2C) can enhance survival by providing energy for major cellular reorganization, including fragmentation of cells, occurring within the first few hours of starvation (14).

The addition of a complex substrate to starved cells immediately initiated formation of new PHA (Fig. 2C). Even the supplement of glucose stimulated the synthesis of PHA, although the response was somewhat delayed and lower. These results show that S14 cells are competent to synthesize PHA during different growth conditions. Furthermore, the close correlation between changes in cell volumes and in PHA content suggests that PHA probably represents a significant proportion of the cell size, as has been found for a *Vibrio* sp. (1).

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