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Characterization of Intracellular Inclusions Formed by *Pseudomonas oleovorans* During Growth on Octane

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The growth of *Pseudomonas oleovorans* on *n*-octane was characterized by the formation of intracellular structures. These inclusions were isolated and characterized. Morphologically, they resembled the poly-β-hydroxybutyrate granules found in *Bacillus cereus*, as shown by freeze-fracture electron microscopy. The elemental analysis of isolated granules showed, however, that they do not contain poly-β-hydroxybutyric acid. Instead, the analysis was consistent with a C_8 polyester, which interpretation was supported by the fatty acid analysis of hydrolyzed granules. From the evidence presented here, we conclude that *P. oleovorans* forms poly-β-hydroxyoctanoate granules when grown on *n*-octane.

We have been investigating the synthesis of 1,2-epoxyoctane by *Pseudomonas oleovorans* during growth on high levels of 1-octene (3) to develop procedures for the site-specific oxidation of alkanes and the stereospecific oxidation of alkenes in bioreactors utilizing *P. oleovorans* or related strains (2). In a study on the morphology of *P. oleovorans* after growth on 20 to 50% (vol/vol) *n*-octane or 1-octene, we observed the appearance of intracellular inclusions and vesicle-like structures by freeze-fracture electron microscopy (M. J. de Smet, Ph.D. thesis, University of Groningen, The Netherlands, 1982).

Many bacterial strains contain inclusion bodies, which have been classified into two major groups based on the presence or absence of a surrounding membrane. Examples include protein, polyglucoside, and polyphosphate storage granules, which are not enclosed by a membrane; and lipid deposits, like poly-β-hydroxybutyrate, sulfur globules, and gas vacuoles, which are surrounded by a non-unit membrane (15).

These inclusion bodies appear to function as nutrient reservoirs. Thus, many bacteria are known to produce poly-β-hydroxybutyrate when carbon and energy sources are in excess (9, 10, 15, 20, 23). This situation might apply to *P. oleovorans*, since its intracellular inclusions resemble the poly-β-hydroxybutyrate inclusions observed in freeze-fractured cells of *Bacillus cereus* (5).

However, hydrocarbon-utilizing bacteria, including several *Pseudomonas* species, generally do not accumulate poly-β-hydroxybutyrate. Instead, Scott and Finnerty have shown that the growth of an *Acinetobacter* species in the presence of *n*-hexadecane results in the intracellular accumulation of the unmodified hydrocarbon (13). This finding was general in that hydrocarbon pooling was characteristic of a variety of hydrocarbon-oxidizing microorganisms; these same organisms accumulated insignificant amounts of poly-β-hydroxybutyrate after growth on either hydrocarbon or nonhydrocarbon substrates (14). In addition, poly-β-hydroxybutyrate has never been observed in *P. putida*, and the absence of this polymer has been shown to be characteristic of the fluorescent pseudomonads (1, 17).

As a result, we became interested in the nature of the intracellular structures produced by *P. oleovorans* in response to hydrocarbon substrates. The present study describes the isolation and the characterization of these inclusions, which appear to consist of an aliphatic polyester. This polymer has never been found in bacteria before this study.

MATERIALS AND METHODS

Chemicals. *n*-Octane (>99% pure) was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J. A standard mixture (4-5436) of fatty acid methyl esters was purchased from Supelco, Inc., Bellefonte, Pa.; stearic methyl ester was purchased from Merck-Schuchardt A. G.; and egg white lysozyme was purchased from Boehringer Mannheim GmbH and Soehne GmbH. Rnase I (EC 3.1.4.22) was purchased from Miles-Seravac Ltd., and DNase I (EC 3.1.4.5) was purchased from Sigma Chemical Co., St. Louis, Mo. Poly-β-hydroxybutyrate was a gift from the Depart-

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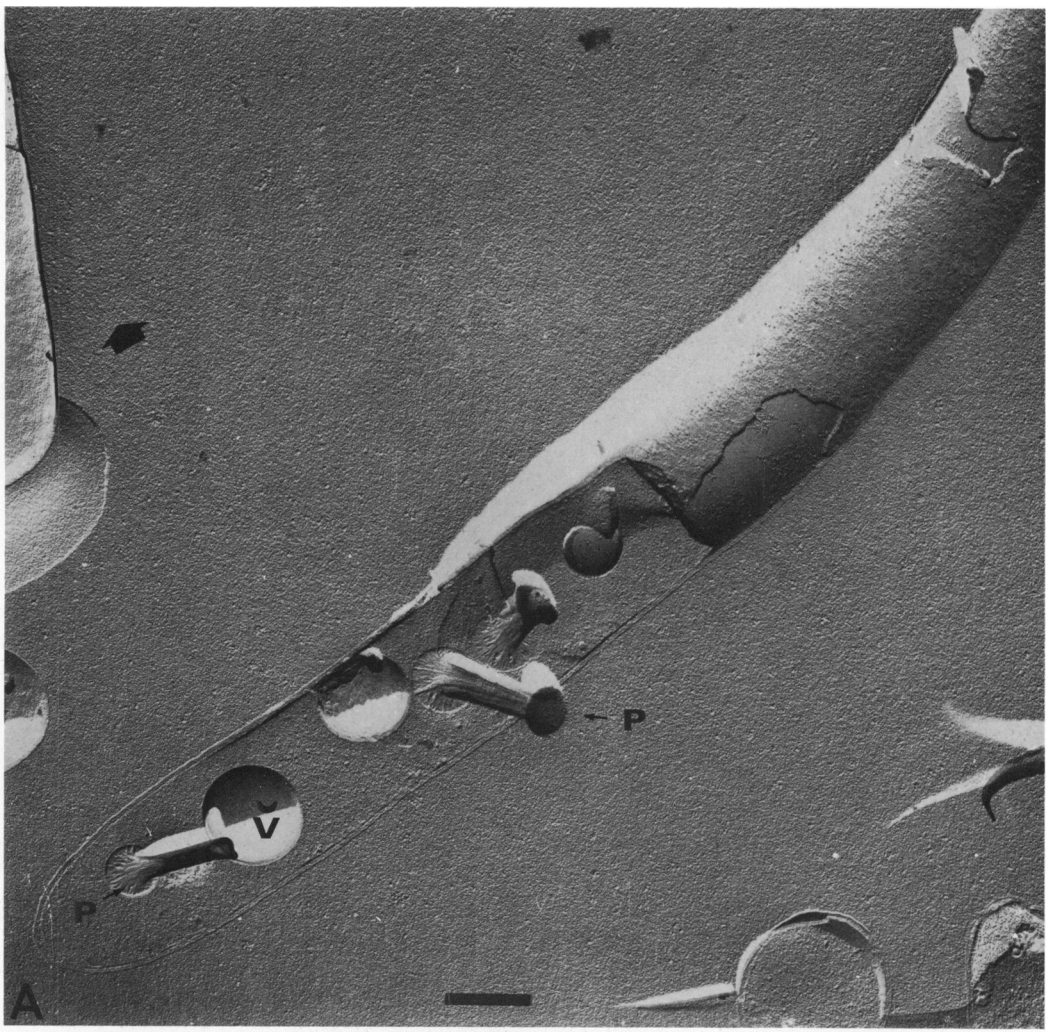


FIG. 1. Freeze-fractured cells of *P. oleovorans* grown on *n*-octane for 22 h. The direction of shadowing has been marked with an arrow. The shadows are white. (A) Concave face of a vesicle (V) left by the removal of the contents of the inclusions and protruding structures (P). The bar represents 0.5 μm. (B) Mushroom-like extrusion. The bar represents 0.2 μm.

ment of Microbiology, University of Groningen, The Netherlands. All other chemicals were of the highest grade commercially available.

Bacterial strain and growth conditions. *P. oleovorans* TF4-1L (ATCC 29347) was used throughout the experiments. This strain was a gift from C. J. McCoy (Corporate Research Laboratories, Exxon Research and Engineering, Linden, N.J.). Details on the growth conditions and the determination of the cell density have been described elsewhere (3, 12, 21).

Isolation of intracellular inclusions. Cells grown on 50% *n*-octane for 45 to 50 h were centrifuged (5,000 × *g*, 10 min, 0°C) and washed once in 0.1 M sodium phosphate buffer (pH 7.0). The cells were converted into spheroplasts as described previously (22). The spheroplasts were suspended in 10 mM Tris-hydrochloride (pH 8.0) containing 10 μg of RNase per ml and 10 μg of DNase per ml and disrupted by sonication for 5 min with a Branson B-12 Sonifier (Branson Sonic Power Co., Danbury, Conn.). The temperature never exceeded 20°C. After centrifugation (5,000 × *g*, 10 min) to remove unlysed cells, 10 ml of the supernatant was layered onto 15 ml of 15% (wt/wt) sucrose in 10 mM Tris-hydrochloride–1 mM EDTA (pH 8.0), and the tubes were centrifuged in a 60 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 4°C (140,000 × *g*, 2.5 h). After centrifugation, membranes were present in the pellet, and the inclusions banded as a broad white band at the top of the 15% (wt/wt) sucrose solution. The inclusions were collected, centrifuged (40,000 × *g*, 30 min, 0°C), and washed twice in 10 mM Tris-hydrochloride–1 mM EDTA (pH 8.0). They were then suspended in 10 mM Tris-hydrochloride–1 mM EDTA (pH 8.0) or in distilled water and stored at −20°C. EDTA was added as a standard precaution to prevent the aggregation of the inclusions.

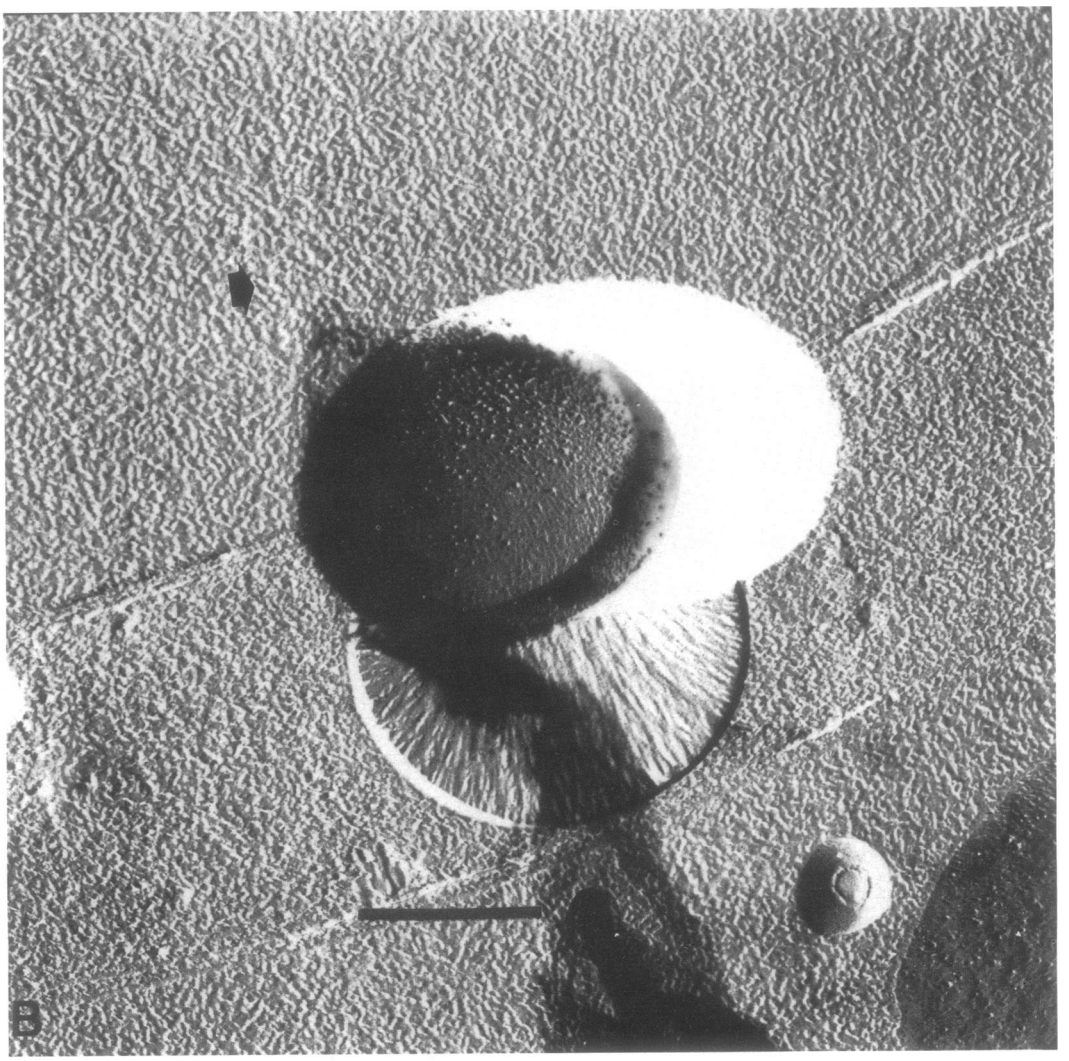


FIG. 1—*Continued*

Lipid extraction and analysis. Phospholipids and neutral lipids were extracted with methanol-chloroform (2:1 [vol/vol]) and analyzed by thin-layer chromatography as described previously (6). The analytical thin-layer chromatographic studies were conducted with commercially prepared plates with Silica Gel 60 (Merck). The solvent systems used were (i) chloroform-methanol-acetic acid (65:25:6 [vol/vol/vol]) and (ii) hexane-diethylether-acetic acid (80:20:1 [vol/vol/vol]). The compounds were visualized with iodine vapor and phosphate stain (6).

Fatty acid analysis. Samples were hydrolyzed in 5.7 N twice-distilled hydrochloric acid in vacuo at about 110°C for 20 h; the free fatty acids were esterified by heating in 1 N methanolic sulfuric acid under reflux for 2 h. The resulting methyl esters were extracted with *n*-pentane and analyzed by gas-liquid chromatography. The methyl esters were separated isothermally (160°C) on a DEGS stainless steel column (12 ft by 1/8 in. [3.7 m by 0.32 cm]; packed with 10% diethyleneglycol succinate on 80/100 Chromosorb WAW) with helium as the carrier gas. The column was fitted to a Hewlett-Packard gas chromatograph (model F & M 5750), which was equipped with a flame ionization detector and connected to an electronic integrator (Chromatronix/autolab, model 22000-010; Spectra-Physics Inc.). The identification of fatty acids was based on the retention times of standards (standard mixture 4-5436; Supelco, Inc.) whenever possible. When standards were not available, experimental retention times were compared with those predicted from curves in which the log (retention time) was plotted against the carbon number for a series of related fatty acids. For quantitative analyses, stearic acid (methyl ester) was used as an internal standard.

Chemical analysis. Total carbohydrate was determined by the phenol-sulfuric acid technique (4) with glucose as the standard. 3-Deoxy-D-manno-octulosonic acid was estimated by the method of Osborn et al. (11). Elemental analyses were performed in the

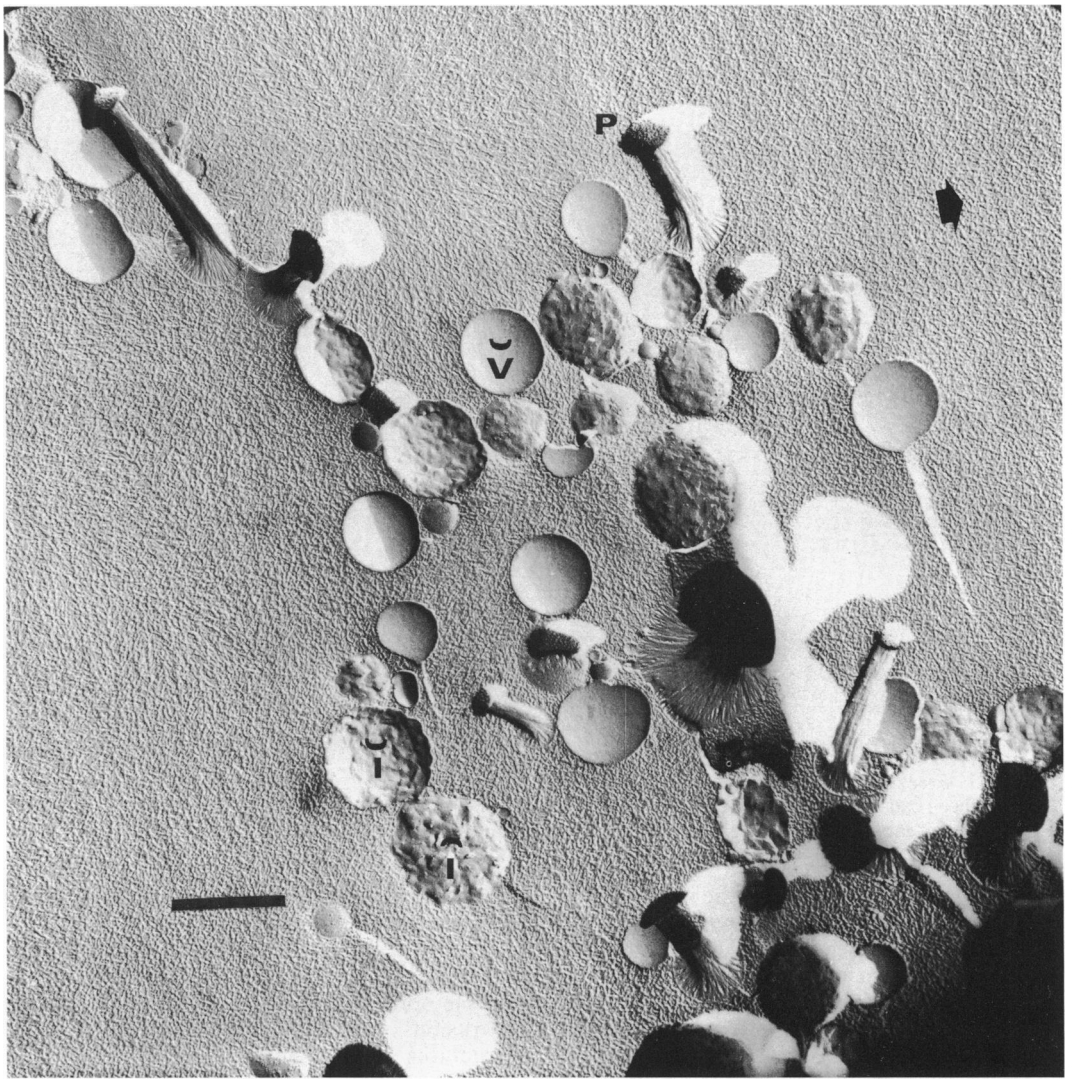


FIG. 2. Freeze-fractured inclusions isolated from *P. oleovorans* cells. The direction of shadowing has been marked by an arrow. The shadows are white. The bar represents 0.5 μm. V̆, Concave face of a vesicle, left by the removal of the contents of the inclusion; Ĭ, concave face of the inclusion; Î, convex face of the inclusion; P, protruding structures having a horn- or mushroom-like shape.

microanalytical department of this laboratory. A Perkin-Elmer elemental analyzer was used to determine the amounts of carbon, hydrogen, and nitrogen. Oxygen was determined by the method of Unterzaucher (18). Infrared spectra were recorded on a Unicam SP-200 infrared spectrophotometer.

Electron microscopy. Freeze-fracturing, the preparation of replicas, and electron microscopy have been described elsewhere (19). Briefly, the material to be examined was suspended in buffer containing 30% glycerol and frozen by dipping in liquid Freon 22. The fracture process was carried out at −100°C on a Balzers BAF 301 instrument (Balzers, Fürstentum, Liechtenstein). Replicas were cleaned in commercial bleach and examined with a Philips EM 200 or EM 300 electron microscope.

RESULTS

Isolation of the intracellular structures. The intracellular structures were isolated from cells of *P. oleovorans* which had been grown for 45 to 50 h in a culture containing 50% (vol/vol) *n*-octane. The cells were first converted to spheroplasts, which were subsequently disrupted by sonication. The sonic extract, which contained the cell membranes and the inclusion bodies, was layered onto a 15% (wt/wt) sucrose solution. After centrifugation, the membranes were present in the pellet, whereas the inclusions banded as a broad white band at the top of the 15% sucrose layer. This band appeared to be

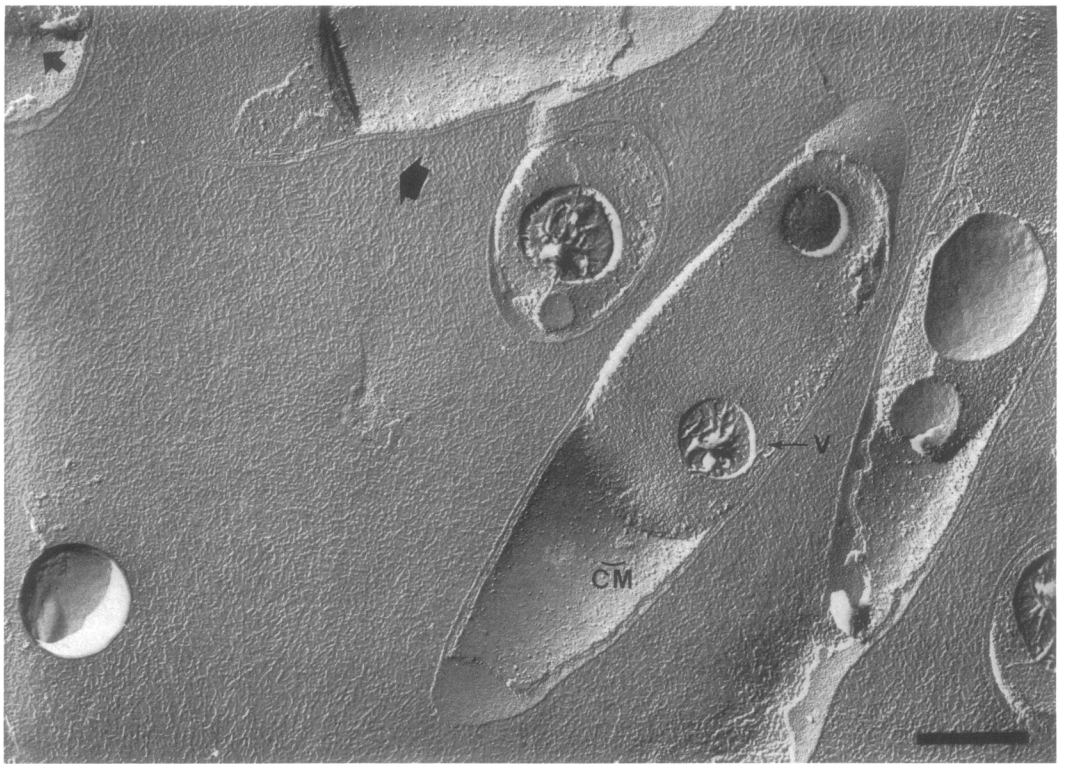


FIG. 3. Freeze-fractured cells of *P. oleovorans* grown on *n*-octane for 72 h. Inclusions with irregular internal fracture faces can be seen. The direction of shadowing has been marked with an arrow. The shadows are white. The bar represents 0.5 μm. V, Vesicle; CM, concave cytoplasmic membrane.

heterogeneous, indicating that the inclusions differed with respect to buoyant density or size or both, as was also clear from electron microscopy. The isolated inclusions were not fractionated further.

Freeze-fracture electron microscopy. Previous studies have shown that intracellular structures occur in cells of *P. oleovorans* grown on *n*-octane or 1-octene (5 to 50% [vol/vol]), but they are not present in cells grown on citrate (0.2 to 1% [wt/vol]) (de Smet, Ph.D. thesis).

In intact cells, both cavities, ranging in diameter from 240 to 700 nm, and protruding structures were observed (Fig. 1A). In most cases, the extrusions were found to have a mushroom-like shape, of which the outer surface was covered with particles (Fig. 1B). The extrusions appeared to be stretched from a central region to reveal parallel strands in the stretched part. The cavities were presumably formed by the removal of the inclusion contents and generally showed a smooth inner surface (Fig. 1A).

Freeze-fractured preparations of isolated inclusions revealed structures similar to those seen in intact cells (Fig. 2). In addition, we observed inclusions which were neither stretched nor scooped out. The convex and concave fracture faces of these inclusions have an irregular plied structure.

It is interesting to note that in cells which were incubated with 50% (vol/vol) *n*-octane or 1-octene for 72 h the vesicular structures and extrusions were no longer seen (Fig. 3). Only structures which resemble the hexadecane inclusions in *Acinetobacter* sp. as studied by Scott and Finnerty (13) were observed.

Characterization of the isolated inclusions. The intracellular material, which aggregated very easily, was insoluble in most organic solvents, but it was soluble in boiling chloroform. Freeze-fracture electron microscopy of both cells and isolated inclusions revealed structures which resembled the freeze-fracture morphology of poly-β-hydroxybutyrate granules (5, 16). These observations led us to consider the possibility that the inclusions were composed of poly-β-hydroxybutyrate. Therefore, the isolated material was subjected to elemental analysis. A comparison of the analytic data with those found and calculated for poly-β-hydroxybutyrate (3-OH-4:0) shows that there are significant differences in the elemental composition (Table 1). Instead, the data found for the inclusions correspond exactly to a compound with the empirical

TABLE 1. Comparison of the elemental analyses of the inclusions isolated from *P. oleovorans* and poly-β-hydroxybutyric acid

Substance analyzed	% of dry wt that was:				Total
	C	H	O	N	
Inclusions	66.27	9.76	23.40	0.20	99.63
$C_8H_{14}O_2$ (calculated)	67.57	9.97	22.51	—[a]	100
Poly-β-hydroxybutyric acid[b]	55.01	7.67	ND[c]	ND	ND
$C_4H_6O_2$ (calculated)	55.81	7.03	37.16	—	100

[a] —, None.
[b] From reference 8.
[c] ND, Not determined.

formula $C_8H_{14}O_2$. Very little nitrogen (0.2%) and residual ash (0.4%) were detected.

The inclusions contained no carbohydrate as determined by the phenol-sulfuric acid assay and no 3-deoxy-D-manno-octulosonic acid. Approximately 1% (wt/wt) phospholipids and neutral lipids could be detected. The lipids were analyzed by thin-layer chromatography and appeared to have the same composition as the lipids extracted from the cell membranes of *P. oleovorans* (data not shown).

The intracellular material was hydrolyzed and subsequently esterified. For comparison, poly-β-hydroxybutyrate isolated from *Bacillus megaterium* was treated in the same way. Although hydrolysis in 5.7 N HCl is a drastic method, the hydrolysis of the intracellular material, as well as that of the poly-β-hydroxybutyrate, was not complete. Approximately 15% of the dry weight was converted into free fatty acids in both cases. Table 2 shows the composition of the resulting methyl esters. In addition to the traces of fatty acids, which were also found in membrane lipids, two unknown fatty acids were liberated from the inclusions. The major fatty acid was identified as 3-hydroxy-octanoic acid (3-OH-8:0) based on a comparison of its retention time on gas-liquid chromatography with that of a series of 3-hydroxy fatty acids. Since hydroxy fatty acids are partly degraded during hydrolysis in 5.7 N HCl (unpublished data), the minor fatty acid was probably a degradation product of 3-OH-8:0.

Figure 4 shows the infrared spectrum of the intracellular inclusions. For comparison, the spectrum of poly-β-hydroxybutyrate isolated from *B. megaterium* is also shown. Both spectra demonstrate a strong absorption in the 1,720 cm^{-1} region due to the C=O stretching vibration, a band characteristic of the C-O stretch at 1,180 cm^{-1}, and absorption bands at about 3,000 and 1,460 cm^{-1}, resulting from the C-H stretching and bending, respectively. A band at 1,380 cm^{-1} is also found for both compounds, which indicates the presence of a methyl group. In addition to the fingerprint region, the most striking difference between the two spectra is the intensity of the absorption band at 3,000 cm^{-1} in relation to the intensity of the band at 1,720 cm^{-1}. Although the peak at 3,000 cm^{-1} is weak for poly-β-hydroxybutyrate, the absorption is strong at this frequency for the intracellular material, indicating a longer aliphatic side chain in the *P. oleovorans* polymer.

DISCUSSION

The growth of *P. oleovorans* on hydrocarbons resulted in the formation of inclusion bodies. These inclusions did not contain unmodified hydrocarbon, such as appears to be the case for the inclusions of a variety of other hydrocarbon-degrading bacteria (14). Instead, our results strongly indicate that the inclusions consisted of an aliphatic polyester.

Both in isolated preparations and in intact cells, the inclusion bodies were partly plastically deformed as a consequence of the freeze-fracture procedure (Fig. 1 and 2). This plastic deformation, which has been recognized in non-biological polymers such as polystyrene and polyacrylate latex spheres, as well as in many biological macromolecules, was similar to what has been demonstrated for poly-β-hydroxybutyric acid granules (5, 16). The stretched surfaces of the deformed inclusion bodies exhibit parallel strands that can be interpreted as oriented polymer fibrils (16).

The isolated preparations showed fracture faces through two types of surfaces, in addition to plastically deformed inclusions (Fig. 2). Smooth surfaces occurred either as concave fracture faces through vesicle-like membrane structures or as convex fracture faces at the tips of protrusions, suggesting that protrusion tips

TABLE 2. Quantitative analyses of fatty acids present in inclusions isolated from *P. oleovorans* and in poly-β-hydroxybutyric acid

Fatty acid methyl ester	% of total fatty acids in:	
	Inclusions	Poly-β-hydroxybutyric acid
3-OH-4:0	0	100
Unknown	24	0
12:0	0	0
3-OH-8:0	76	0
14:0	0	0
16:0	Tr	0
16:1	Tr	0
17:cy	Tr	0
18:1	Tr	0

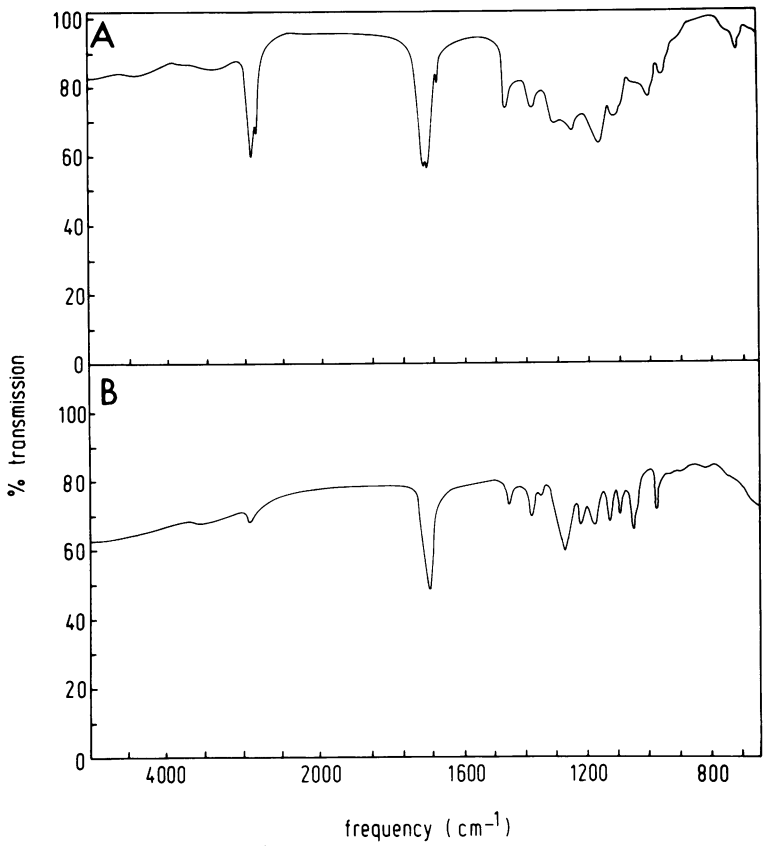


FIG. 4. Infrared spectra of (A) intracellular polymer isolated from *P. oleovorans* cells and (B) poly-β-hydroxybutyric acid isolated from *B. megaterium*.

and concave vesicles have complementary surfaces, as indicated in Fig. 5a through d.

A second type of structure, not seen in intact cells, shows convex and concave convoluted fracture faces, indicating a polymer granule with a plied or convoluted surface. The fracture might occur within a bilayer membrane surrounding a polymer granule (Fig. 5f through h), or it might represent the hydrophilic fracture face between a polymer granule and the surrounding medium (Fig. 5i through k). This structure is not stretched during fracturing, indicating that either the forces holding the fracture faces together are weak or that this structure is not easily deformed. Since both structures presumably derive from the same intracellular structure, they must be related.

One possibility is that both fracture planes occur within the same structure, as is the case for the structure postulated for poly-β-hydroxybutyric acid granules by Dunlop and Robards (5). An alternative and more likely explanation is that a fraction of the intracellular granules is altered during or after cell growth and isolation to yield a more easily fractured bilayer or a polymer of lower plasticity (cf. Fig. 3 and Fig. 1).

Since the inclusions of *P. oleovorans* exhibited properties which were characteristic of poly-β-hydroxybutyric acid, it seemed possible that the inclusions were identical with this polymer. However, fatty acid analysis showed the absence of 3-hydroxybutyric acid. In addition, both the infrared spectrum and the quantitative elemental composition were significantly different from that of poly-β-hydroxybutyric acid. Thus, the inclusions do not contain poly-β-hydroxybutyric acid. Instead, the results strongly suggest that the inclusions are composed of poly-β-hydroxyoctanoic acid (Fig. 6B): first, the quantitative elemental analysis corresponds to an empirical formula of $C_8H_{14}O_2$; second, fatty acid analysis showed the presence of only two fatty acids, of which the major one was concluded to be 3-hydroxyoctanoic acid; and third, the infrared spectrum indicates a longer aliphatic side chain in comparison with poly-β-hydroxybutyric acid.

The inclusion bodies disappeared after prolonged incubation of the cells with *n*-octane or 1-

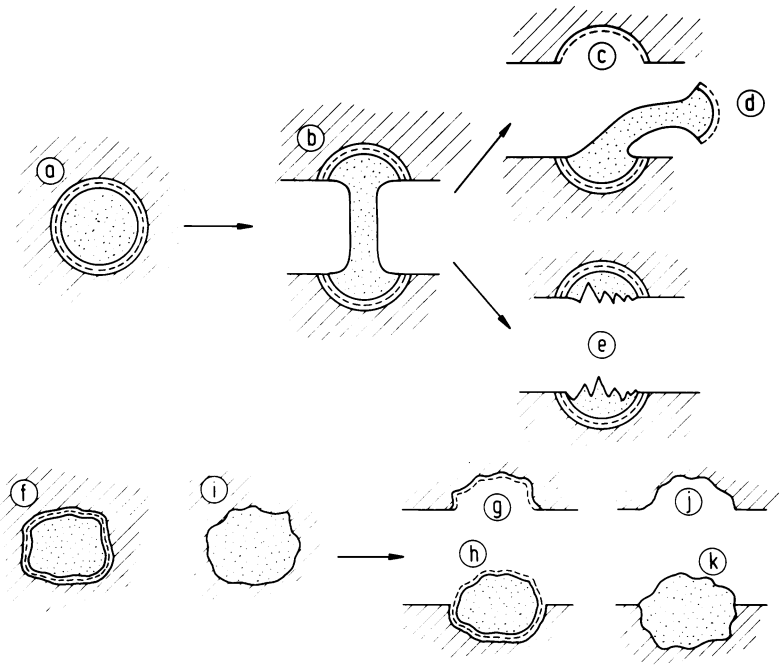
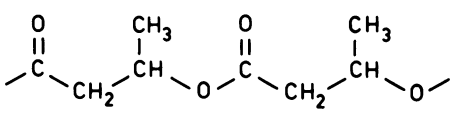


FIG. 5. Diagrammatic representation of the likely origin of the fracture faces observed for isolated and intracellular *P. oleovorans* granules. A polymer granule, surrounded by a bilayer membrane (a), is stretched during freeze-fracturing (b). Eventually, the inner monolayer is split away from the outer monolayer, leaving a smooth, concave fracture face (c) and a protrusion covered by a particle-rich convex fracture face (d). As the cells age, the polymer may be weakened and snap, leaving jagged polymer remnants in membrane cavities (e), such as seen in Fig. 3. Isolated granules may be heterogeneous, since they often reveal convoluted surfaces, besides the smooth surfaces also seen in intact cells. Convoluted granules (f) do not show any stretching. Freeze-fracturing results in complementary concave (g) and convex (h) fracture faces. It is conceivable that these granules are not surrounded by a membrane (i), in which case the fracture faces represent the interface between the granules and the surrounding medium (j and k).

octene. Although there is no ready explanation for this phenomenon, it may be due to the metabolism of the inclusion body in the stationary phase, as is the case with poly-β-hydroxybutyrate granules (15).



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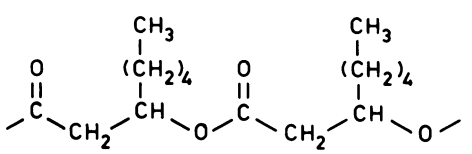

B

FIG. 6. Structure of (A) poly-β-hydroxybutyric acid and (B) poly-β-hydroxyoctanoic acid.

To our knowledge, the accumulation of aliphatic polyesters other than poly-β-hydroxybutyric acid by bacteria has not been reported previously. However, further studies are necessary to conclusively prove the structure and to examine the physical properties of the intracellular polymer, as well as its synthesis and degradation. It is interesting to note that poly-β-hydroxybutyric acid is currently being studied extensively in view of its potential as a thermoplastic (7). Thus, *P. oleovorans* might be a welcome additional candidate in the microbial synthesis of aliphatic polyesters.

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