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Microbial Assimilation of Hydrocarbons

I. The Fine-Structure of a Hydrocarbon Oxidizing *Acinetobacter* sp.

R. S. KENNEDY and W. R. FINNERTY

Department of Microbiology, University of Georgia, Athens, Georgia 30602

K. SUDARSANAN and R. A. YOUNG

Engineering Experiment Station, Georgia Institute of Technology, Atlanta, Georgia 30332

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Abstract. 1. The fine-structure analysis of the hydrocarbon oxidizing microorganism, *Acinetobacter* sp., demonstrated a cytoplasmic modification resulting from growth on paraffinic and olefinic hydrocarbons.

2. Intracytoplasmic hydrocarbon inclusions were documented by electron microscopy with chemical identifications obtained by gas chromatography and X-ray diffraction.

3. These results demonstrate the ability of a microorganism to accumulate hydrocarbon substrates intracellularly which, in turn, indicates transport across the cell membrane.

Key words: Fine-Structure – *Acinetobacter* sp. – Hydrocarbon Inclusions – Electron Microscopy – X-Ray Diffraction – Transport of Hydrocarbons.

The numbers of microorganisms capable of effecting hydrocarbon oxidations are substantial, encompassing many diverse genera and species. Recent surveys have served to review our composite knowledge concerning specific areas of hydrocarbon microbiology and to provide comprehensive overviews of the subject (Albro and Dittmer, 1970; Floodgate, 1972; Klug and Markovetz, 1971; McKenna and Kallio, 1965; van der Linden and Thijsee, 1965). Interestingly, only brief reports are available concerning the ultrastructure of a hydrocarbon-oxidizing microorganism which grows on long chain paraffinic or olefinic hydrocarbons (Ludvík *et al.*, 1968; Munk *et al.*, 1969; Bos and DeBoer, 1968; Atlas and Heinz, 1973). The fine structure of methane-oxidizers has been described with particular emphasis on the development of intracytoplasmic membrane systems (Davies and Whittenbury, 1970; Proctor *et al.*, 1969; Smith and Ribbons, 1970).

The microorganism used in these studies has been described as an *Acinetobacter* sp. (Baumann *et al.*, 1968; Juni, 1972) with the former epithet *Micrococcus cerificans* (Finnerty *et al.*, 1962). This microorganism is capable of prolific growth on a variety of alkanes and alk-1-enes greater than 10 carbons in length. The structure of compounds arising from the growth of this microorganism on specific hydrocarbons has appeared (Finnerty *et al.*, 1962; Kallio *et al.*, 1963; Stewart *et al.*, 1959, 1960; Stewart and Kallio, 1959), as well as the mechanisms involved in wax ester biosynthesis (Finnerty and Kallio, 1964). Detailed biochemical analyses have defined specific aspects of lipid metabolism in relationship to hydrocarbon metabolism (Makula and Finnerty, 1968a, b, 1970, 1971, 1972; McCaman and Finnerty, 1968). This report extends this series of studies by examining the fine-structure detail of *Acinetobacter* sp. in relationship to the physiological and structural parameters that exist during hydrocarbon metabolism.

Materials and Methods

Organism. The microorganism used in these studies, initially described as *Micrococcus cerificans* (Finnerty *et al.*, 1962), has been the subject of many investigations. This microorganism has been classified as an *Acinetobacter* sp. (Baumann *et al.*, 1968; Juni, 1972) and will henceforth be regarded as a member of this genus.

Culture Conditions. The organism was grown on a mineral medium consisting of (in grams per liter): $(NH_4)_2SO_4$, 2; KH_2PO_4, 4; Na_2HPO_4, 6; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; pH 7.5. Alkanes and alk-1-enes (Humphrey Chemical Co., New Haven, Conn.) were added to a final concentration of 1%. Cultures were also grown on nutrient broth (0.8%)-yeast extract (0.5%) NBYE, acetate (2%), and ribose (2%), respectively, for comparative studies. All hydrocarbon and non-hydrocarbon growth substrates were added to the defined mineral medium. Cultures were grown on a gyratory shaker at room temperature (25° C) to the mid-to late exponential growth phase and harvested by centrifugation at room temperature.

Electron Microscopy. Cell pellets of *Acinetobacter* sp. obtained from growth on specified carbon sources were fixed by two alternate procedures. Method 1 followed the stand-

ard procedures developed for osmium tetroxide (Kellenberger *et al.*, 1958). In method 2, a cell suspension was mixed with an equal volume of 5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.3) containing $CaCl_2$ (Glauert and Thornley, 1966). The fixed cells were dehydrated by two alternate procedures. Method 1 involved processing the samples through a graded series of ethanol-water mixtures followed by propylene oxide and infiltrated with Epon 812 (Luft, 1961) or Maraglas (Freeman and Spurlock, 1962). In method 2, the samples were dehydrated by processing through a graded series of Durcupan (Staubli, 1963) and embedded in Araldite. Ultra-thin sections were cut on a Reichert OMU-2 ultramicrotome and mounted on uncoated 300 mesh copper grids. The sections were stained with lead citrate (Reynolds, 1963) followed by uranylacetate and examined in a Philips-200 electron microscope operating at 80 Kv. Samples were negatively stained by the drop method with the use of phosphotungstic acid (PTA), pH 6.8, at a final concentration of 1%.

Scanning Electron Microscopy. Cell preparations for scanning electron microscopy were either fixed in glutaraldehyde or dried unfixed onto specimen stubs, coated with gold-palladium (40:60) in a vacuum evaporator and viewed in a Cambridge Scanning Electron Microscope.

Extraction of Cells. Cell pellets obtained from the growth of *Acinetobacter* sp. on hydrocarbon substrates were washed 5 times with distilled water by repeated centrifugations. Each cell pellet was processed through a graded series of ethanol-water mixtures as used for electron microscopy (50:50, 70:30, 95:5, 100:0, 100:0, v/v). The alcohol extracts were pooled and reduced to dryness or constant volume in vacuo. The residue was dissolved in chromatopure hexane for analysis by gas chromatography.

Gas Liquid Chromatography (GLC). A Packard gas chromatograph, series 7500, consisting of a dual-column oven with coiled-glass columns (4 mm inside diameter, 1.83 m long) was used for the analysis of alcohol extracts. The detection system was an argon ionization detector with column support systems consisting of liquid phases of 10% Apiezon L and 20% diethylene glycol succinate (DEGS) on a support of 70–80 Anakrom A (AnaLabs, Inc.). Operating conditions were: column temperature 110° C or 155° C; detector temperature, 190° C; injection temperature, 180° C; outlet temperature, 205° C; argon flow rate, 60 ml/min; chart speed, 2.5 min/inch.

X-Ray Diffraction Analysis. Cultures grown on specified hydrocarbon and non-hydrocarbon substrates were washed extensively and lyophilized. These cell preparations were encapsulated in a Mylar film, cooled by sudden immersion in a dry ice and alcohol bath, and then were maintained at −10° C with a cold air stream. The sudden chilling transformed the hydrocarbon to crystalline solids with crystallites small enough so that the diffraction-pattern rings were continuous and uniform. The cold specimens were exposed to a 1 mm diameter beam of Ni-filtered X-rays from a copper anode and a flat film was set perpendicular to the beam approximately 5.3 cm behind the specimen (flat-plate forward-reflection technique). The exposed film was quantitatively analyzed by microphotometry along a diametric line.

Chemical Methods. Poly-β-hydroxy butyrate was determined by the procedure of Law and Slepecky (1961).

Results

Phase Microscopy and Scanning Electron Microscopy

A phase microscopy study of *Acinetobacter* sp. growing at the expense of hexadecane demonstrated bacteria clustering around small hydrocarbon spheres (Fig. 1 A). This phenomenon was studied in greater detail by scanning electron microscopy. A low power survey revealed multiple hydrocarbon spheres densely covered with bacteria (Fig. 1 B). Higher magnification showed the topographical distribution of bacteria over the surface of a hydrocarbon sphere (Fig. 1 C).

Fine-Structure of Non-Hydrocarbon Grown Bacteria

A detailed study was initiated into the ultrastructure of *Acinetobacter* sp. grown on hydrocarbon and non-hydrocarbon substrates. Ultra-thin sections were prepared for electron microscopy from cells grown on acetate, ribose, and nutrient broth-yeast extract (NBYE), respectively. The fine-structure details were essentially identical for each of these cell preparations. A cell envelope characteristic of Gram-negative bacteria was apparent (Fig. 2 A–C). Ribose grown cells were observed to possess extracellular filaments (Fig. 2 B) and was the only carbon source which resulted in the formation of these filamentous structures. NBYE grown cells were characterized by the presence of mesosomal-like structures localized at the cell periphery (Fig. 2 C). These specified fine-structural details represented singular differences in non-hydrocarbon grown cells.

Fine-Structure of Alkane-Grown Bacteria

In contrast, *Acinetobacter* sp. grown on hydrocarbons exhibited unique fine-structure characteristics that distinguished them from non-hydrocarbon grown bacteria. Figs. 2 D and 3 A are electron micrographs of thin-sections prepared from hexadecane- and heptadecane-grown bacteria, respectively. Multiple inclusion bodies are readily apparent and served to characterize an ultra-structure feature associated with only hydrocarbon grown bacteria. This inclusion body was a characteristic feature observed with the growth of *Acinetobacter* sp. on a homologous set of alkanes varying in chain length from 12–20 carbon atoms, respectively. The specificity of the intracellular inclusions was established by means of preparing thin-sections of colonies of *Acinetobacter* sp. which had been grown on the surface of a mineral salts-agar medium in

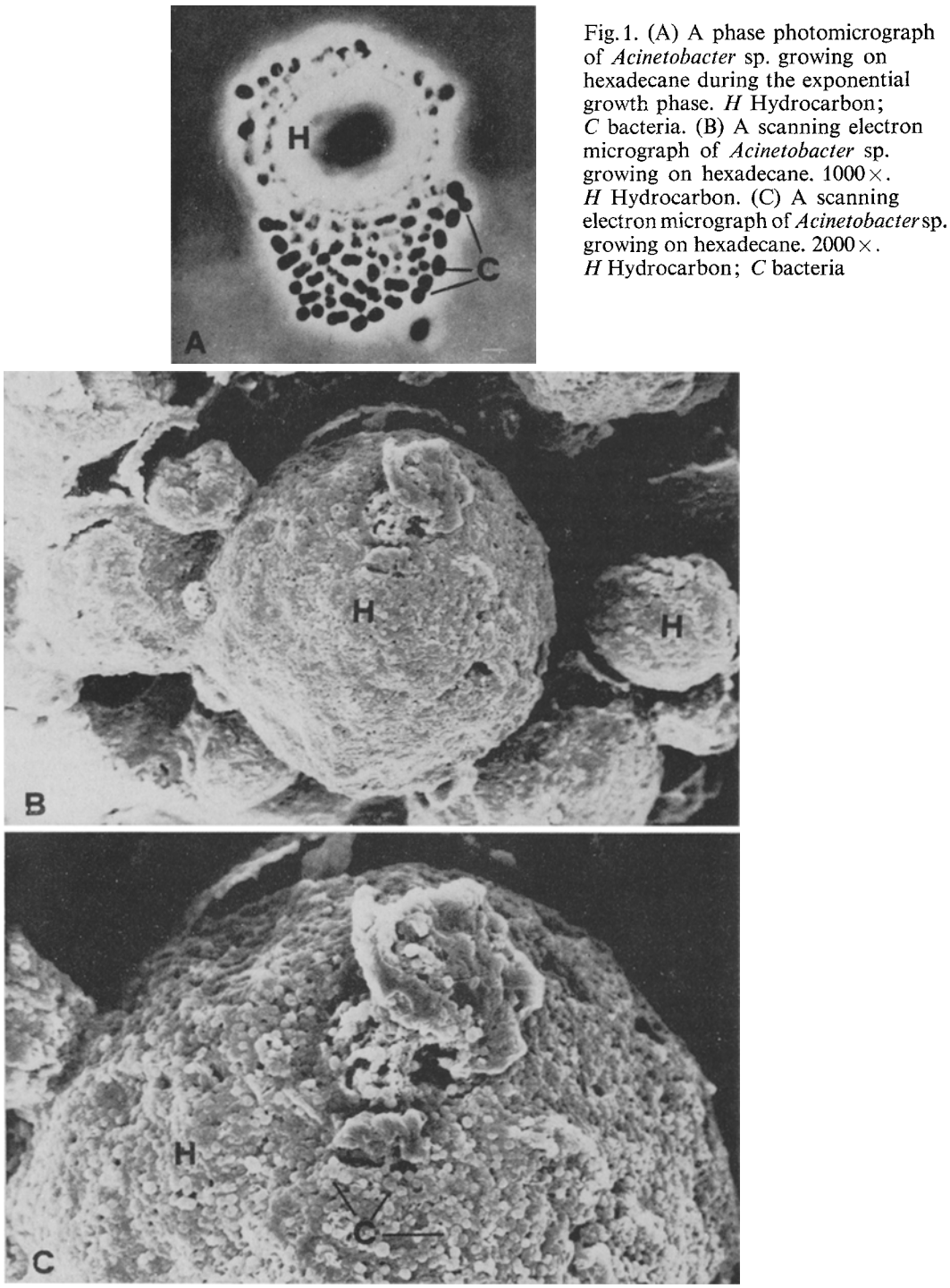

Fig. 1. (A) A phase photomicrograph of *Acinetobacter* sp. growing on hexadecane during the exponential growth phase. *H* Hydrocarbon; *C* bacteria. (B) A scanning electron micrograph of *Acinetobacter* sp. growing on hexadecane. 1000×. *H* Hydrocarbon. (C) A scanning electron micrograph of *Acinetobacter* sp. growing on hexadecane. 2000×. *H* Hydrocarbon; *C* bacteria

the presence of hexadecane vapors. The bacterial colonies were never in direct physical contact with liquid hexadecane. This study demonstrated the presence of multiple inclusion bodies (Fig. 3B). Whole cell negative staining with phosphotungstic acid revealed the presence of intracellular inclusions in hydrocarbon grown bacteria (Fig. 3C) which were not present in non-hydrocarbon grown bacteria (Fig. 3D).

Fine-Structure of Alk-1-ene-Grown Bacteria

Alk-1-enes as sole carbon sources exhibited a different but characteristic fine-structure pattern. Fig. 4A shows

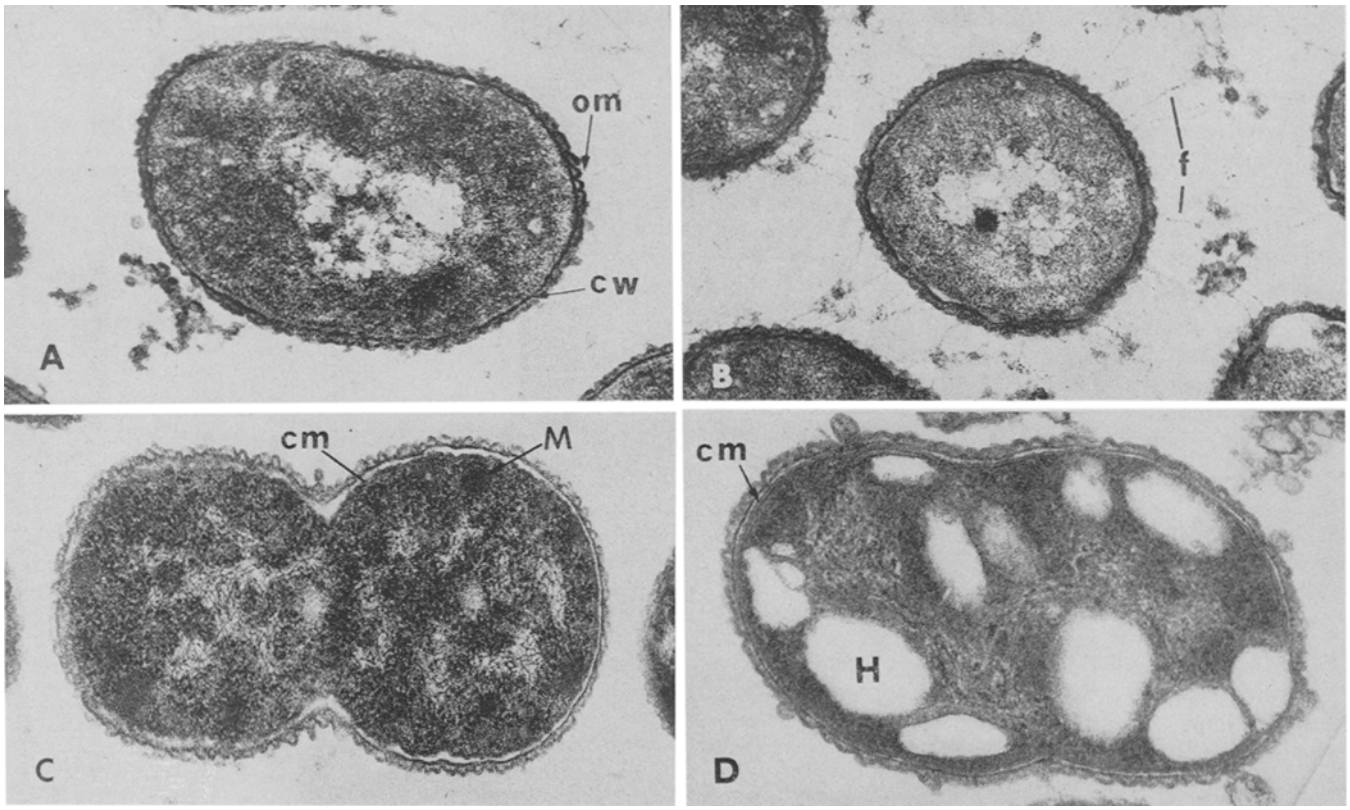

Fig. 2. (A) Thin-section of *Acinetobacter* sp. grown on acetate. 48300×. Fixation by method 1, dehydration by method 1, and embedded in Epon 812. *CW* Cell wall; *OM* outer membrane. (B) Thin-section of *Acinetobacter* sp. grown on ribose. 51850×. Fixation by method 1, dehydration by method 1, and embedded in Epon 812. *f* Filaments. (C) Thin-section of *Acinetobacter* sp. grown on nutrient broth-yeast extract. 42500×. Fixation by method 1, dehydration by method 1, and embedded in Epon 812. *M* Mesosomal-like structures; *cm* cytoplasmic membrane. (D) Thin-section of *Acinetobacter* sp. grown on hexadecane. 48300×. Fixation by method 1, dehydration by method 1, and embedded in Epon 812. *H* Hexadecane inclusion; *cm* cytoplasmic membrane

an electron micrograph of a thin-section of hexadec-1-ene grown cells with two osmiophilic inclusion bodies. These inclusions are identified with only alk-1-ene grown bacteria and additionally appear as strongly osmiophilic bodies only when water-soluble dehydration procedures are used. The use of conventional procedures, which requires organic solvents, removes the osmiophilic inclusions (Fig. 4B). The osmiophilic inclusions were extracted from thin-sections by "floating" the section on organic solvents (*e.g.*, ethanol, chloroform, hexane).

Cell Surface Orientation of Hydrocarbons

Cells were grown on hexadecane containing 0.01% nickel napthenate in an attempt to determine localization of hydrocarbon at the cell membrane surface. Fig. 4C is an electron micrograph demonstrating that nickel napthenate accumulates at the outer membrane surface and multiple inclusion bodies accumulate within the cell. No indication of hydrocarbon accumulation at the cytoplasmic membrane surface was detected. Cells grown on hexadec-1-ene were studied taking advantage of the double bond reactivity to osmium tetroxide. If either cell wall and/or cell membrane surface localization of hexadec-1-ene occurred then exposure to osmium tetroxide vapors would effect an electron-dense surface to the cell. The examination of hexadec-1-ene grown cells by treating with osmium tetroxide vapors followed by negative staining with phosphotungstic acid provided no indication that such a surface orientation of hexadec-1-ene occurred (Fig. 4D).

Gas-Liquid Chromatography

The chemical identification of these inclusion bodies was established with gas liquid chromatography (GLC) and X-ray diffraction. GLC identifications of pentadecane, hexadecane, hexadec-1-ene, heptadecane, and

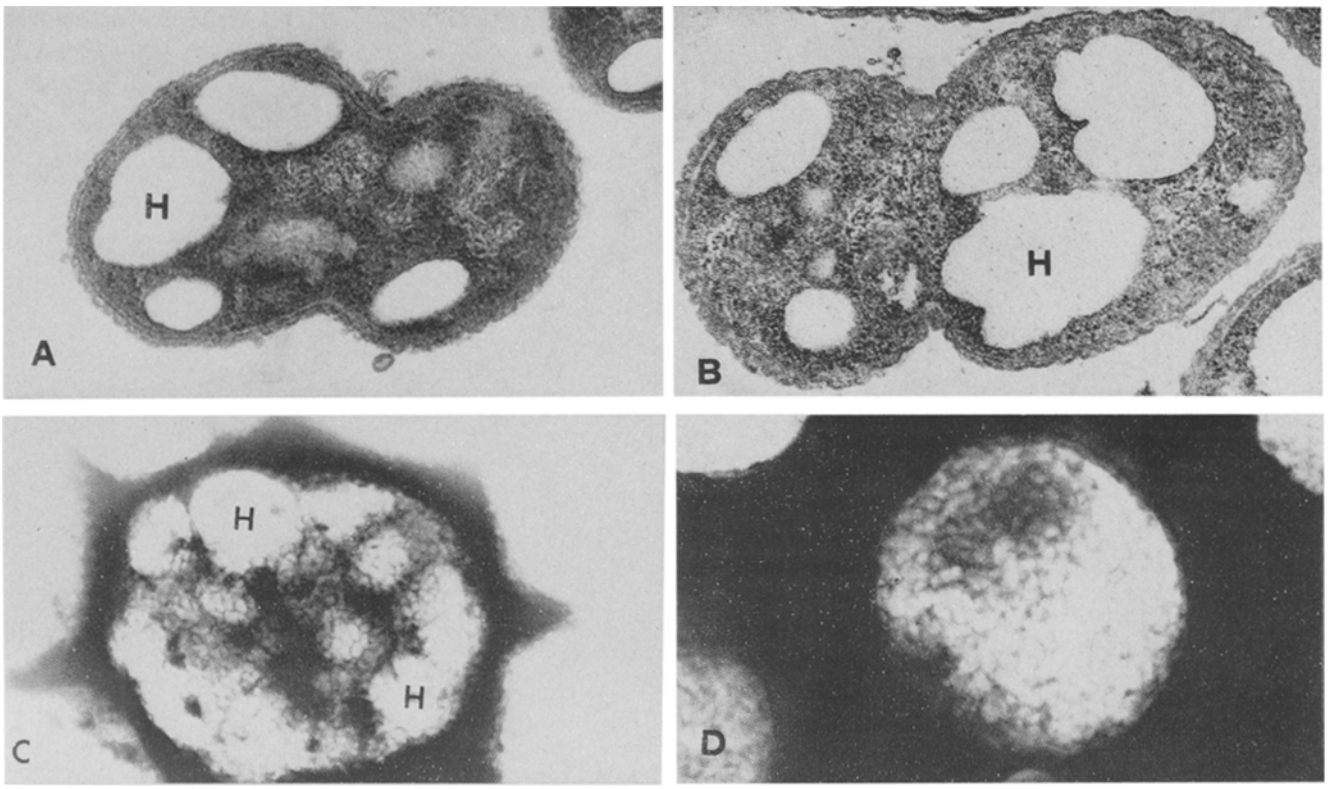

Fig. 3. (A) Thin-section of *Acinetobacter* sp. grown on heptadecane. 66000×. Fixation by method 1, dehydration by method 1, and embedded in Maraglas. *H* Heptadecane inclusion. (B) Thin-section of *Acinetobacter* sp. grown on the vapors of hexadecane. 57400×. Fixation by method 1, dehydration by method 1, and embedded in Epon 812. *H* Hexadecane inclusions. (C) Whole cells of *Acinetobacter* sp. grown on hexadecane and negatively stained with phosphotungstic acid. 46000×. *H* Hydrocarbon inclusion. (D) Whole cells of *Acinetobacter* sp. grown on nutrient broth-yeast extract and negatively stained with phosphotungstic acid. 57000×

octadecane were substantiated from the alcohol extracts of cells grown individually on these respective hydrocarbons. A composite GLC chromatogram of the alcohol extracts is shown in Fig. 5. This type of analysis provides for structure identification but does not aid in establishing the intracellular localization of hydrocarbon inclusions. Chemical analyses for poly-β-hydroxybutyrate were routinely negative in hydrocarbon and non-hydrocarbon grown cells.

X-Ray Diffraction

Low temperature X-ray diffraction was employed to aid in establishing that the intracellular inclusion bodies were hydrocarbon pools. The densitometric tracings of the X-ray diffraction patterns obtained from the analyses of lyophilized preparations of *Acinetobacter* sp. grown on specified hydrocarbon and non-hydrocarbon substrates are shown in Fig. 6. The results demonstrate an exact correlation between the standard hydrocarbon and the organisms grown on the hydrocarbon. The X-ray diffraction pattern of control cells which had been grown on nutrient broth-yeast extract did not agree with the standard hydrocarbon patterns.

By knowing the diffraction pattern of the standard hydrocarbon it is possible to identify the hydrocarbon substrate which yielded a particular cell mass. This technique is applicable only when the concentration of hydrocarbon is large enough so that a characteristic crystalline lattice structure is formed upon super cooling. Heterogeneous crystals (2 or more components) would possess a different diffraction pattern than a homogeneous crystal (1 component). A summary of D values and relative intensities are shown in Table 1.

Discussion

These studies have demonstrated a fine-structure modification of *Acinetobacter* sp. grown on paraffinic and olefinic hydrocarbons. An orientation of this microorganism to the surface of hydrocarbon spheres was noted with both phase and scanning electron microscopy. This surface attraction was not observed for bacteria unable to grow on hydrocarbons (*e.g.*

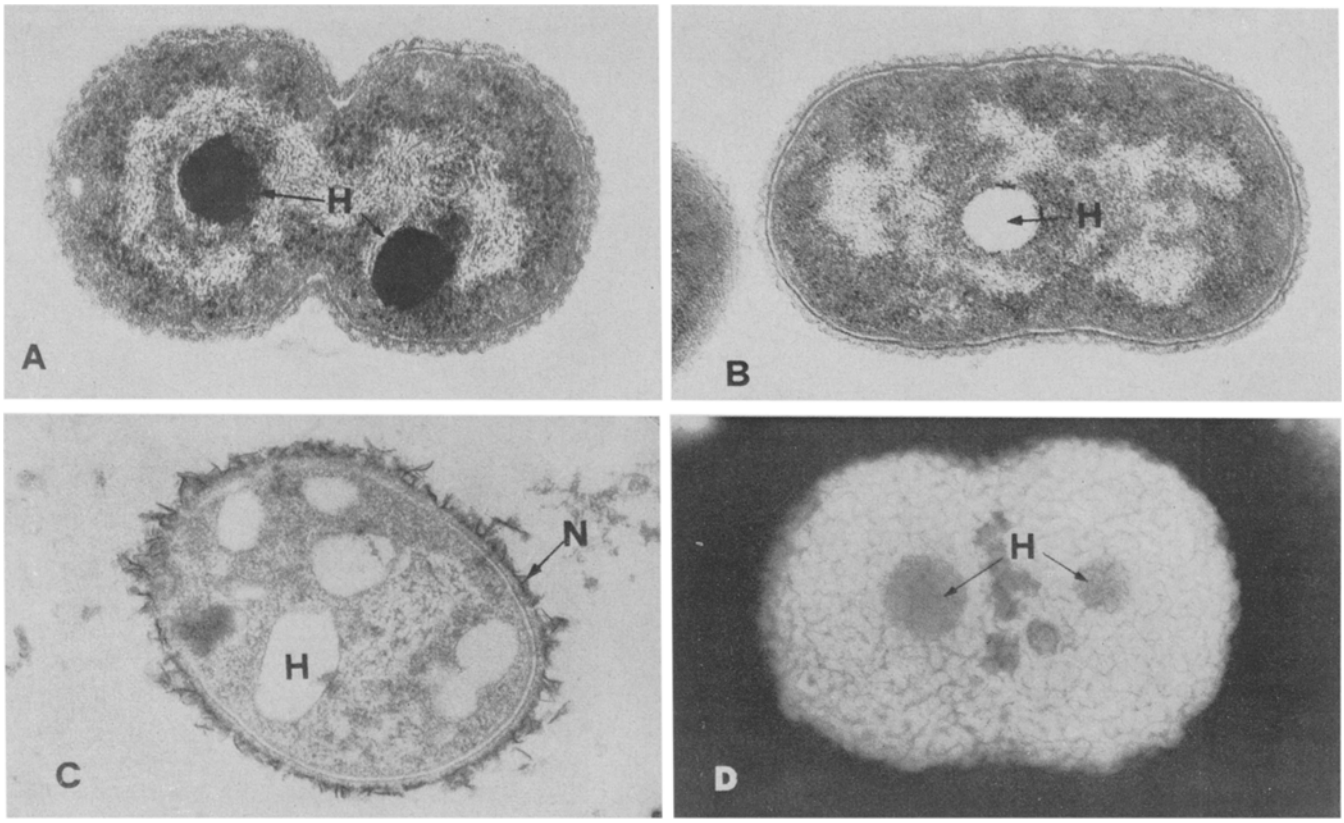

Fig. 4. (A) Thin-section of *Acinetobacter* sp. grown on hexadec-1-ene. 69700×. Fixation by method 2, dehydration by method 2, and embedded in Maraglas. *H* Hexadec-1-ene inclusion. (B) Thin-section of *Acinetobacter* sp. grown on hexadec-1-ene. 86100×. Fixation by method 2, dehydration by method 1, and embedded in Maraglas. *H* Hexadec-1-ene inclusion. (C) Thin-section of *Acinetobacter* sp. grown with hexadecane containing 0.01% nickel napthenate. 49200×. Fixation by method 2, dehydration by method 2, and embedded in Araldite. *N* Nickel napthenate; *H* hexadecane inclusion. (D) Whole cells of *Acinetobacter* sp. grown on hexadec-1-ene, fixed with osmium tetroxide, and negatively stained with phosphotungstic acid. 69700×. *H* Hexadec-1-ene inclusions

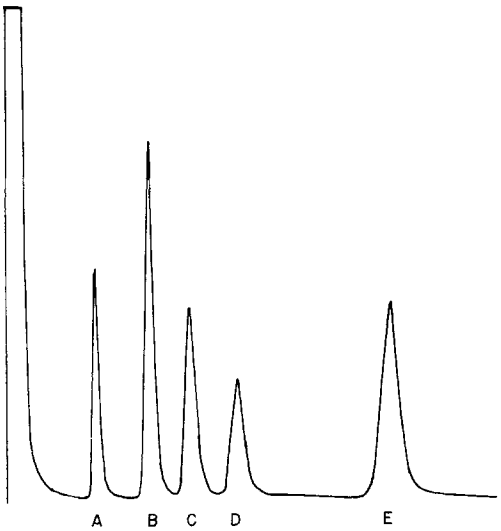

Fig. 5. A composite gas chromatograph profile of the hydrocarbons extracted from cells grown on each hydrocarbon individually. Samples analyzed on 20% DEGS, column temperature 110°C; and 10% Apiezon L, column temperature 155°C. The results shown were obtained by analysis on 20% DEGS. *A* Pentadecane; *B* hexadecane; *C* hexadec-1-ene; *D* heptadecane; *E* octadecane

Escherichia coli, Aerobacter aerogenes, Bacillus subtilis). This phenomenon has been studied in greater detail with *Torulopsis* sp. growing on alkanes (McLee and Davies, 1972). Scanning electron microscopy has not been heretofore used to study a hydrocarbon utilizing microorganism. These results suggest that a specific attraction of the microorganism to the hydrocarbon occurs. The chemical nature and structural specificity of this interaction between the microorganism and the hydrocarbon is presently undetermined.

Electron microscopy demonstrated characteristic differences in the fine-structure of hydrocarbon-grown bacteria. These cultures possessed intracellular inclusion bodies that were not present in non-hydrocarbon cultures. Identification of these inclusion bodies was established indirectly by electron microscopy and directly by gas liquid chromatography and X-ray diffraction. The latter procedure enabled an *in situ* analysis of hydrocarbon pools localized intracellularly. Previous studies with *Candida lipolytica* (Ludvík *et al.*,

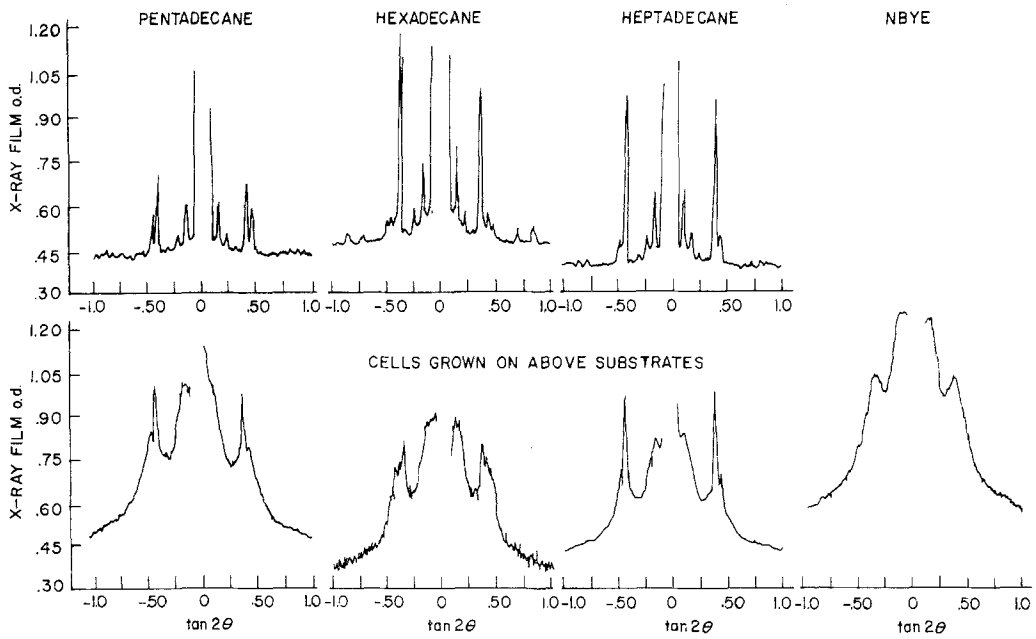

Fig. 6. Diametric densitomer tracings of X-ray diffraction photographs made with cell samples cooled to −10° C. The Bragg diffraction angle is θ. CuKα radiation ($\lambda = 1.54$ Å) was used. The optical densities (*o. d.*) of the film should not be compared from pattern to pattern because differing exposure times were used to optimize overall film exposure. The cell sample grown on NBYE served as the control

Table 1. X-ray powder diffraction spacings (d) and intensities (I) for standard hydrocarbons and for hydrocarbon-grown cells [a]

Pentadecane				Hexadecane				Heptadecane			
Standard		Cells		Standard		Cells		Standard		Cells	
d	I	d	I	d	I	d	I	d	I	d	I
10.36	62	10.45	8	10.24	47	10.43	7	11.58	36	11.58	10
7.01	24			6.88	14			7.74	15		
5.23	10			5.13	4			5.84	4		
4.06	100	4.09	100	4.49	100	4.57	100	4.61	2		
3.64	67	3.68	60	4.10	12	4.12	18	4.06	100	4.03	100
2.47	10			3.73	10	3.81	18	3.66	15	3.62	63
2.30	10			3.48	2	3.59	18	2.46	2	2.43	3
2.20	10			2.57	8			2.28	4		
				2.25	14			2.19	2		
				2.13	2			2.11	2		

[a] Estimated error in d is 2−3%.

1968) suggested hexadecane was concentrated at the external surface of the cell membrane. This interpretation was based on the localization of an electron-dense marker, vanadium napthenate, at this site. Bos and De Boer (1968) studying *C. lipolytica* could find no evidence to support hydrocarbon localization at the membrane surface although a cell wall with increased electron density was noted for hexadecane grown yeasts, presumably from increased lipid synthesis. Our results indicate that nickel napthenate was indeed localized at the outer membrane surface but for reasons of impermeability. Further, mechanisms for transport of hydrocarbons across the cell membrane are indicated due to the fact that cytoplasmic "pools" of hydrocarbon accumulate against a concentration gradient into masses sufficient for discrimination by X-ray diffraction.

It is an accepted fact that not all microorganisms are capable of hydrocarbon oxidations, presumably lacking the requisite enzymatic complement to effect

such conversions. Hydrocarbons as hydrophobic, water-insoluble compounds have circumstantially been considered as refractory to active transport processes. Mechanisms for the accommodation of hydrocarbons have been reviewed by Klug and Markovetz (1971) with these considerations necessitating an extracellular modification of the hydrocarbon at the membrane surface. Our findings that hydrocarbons are pooled indicates transport, for which the component parts and requirements are unknown. Further studies are in progress to determine the mechanisms of hydrocarbon transport and the role of known cellular and extracellular lipids as specific surfactants for effecting macro- or microemulsions of water-insoluble hydrocarbons.

The ultrastructural organization of the cell envelope appears identical to that reported for other Gram-negative bacteria (DePetris, 1967; Murray *et al.*, 1965). The irregular, undulating outer membrane layer of the cell envelope was a prominent feature in both hydrocarbon and non-hydrocarbon grown cells. Preliminary attempts to remove this outer layer by the use of various preparative techniques have been unsuccessful so that it appears to represent a tightly bound component of the cell envelope complex. The cell wall (middle layer) appears as a rigid electron dense structure well delineated in thin-sections. Evidence indicating lipopolysaccharide in cell residue fractions was demonstrated by the identification of β-hydroxy fatty acids (Makula and Finnerty, 1972). Further studies are, however, necessary to establish the presence and cell envelope localization of lipopolysaccharide.

Further studies are in progress to establish the generality of hydrocarbon sequestering by microorganisms. Studies employing diverse species of bacteria, yeasts, and fungi which grown on various classes of hydrocarbons will provide for greater insight into this aspect of hydrocarbon microbiology.

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Prof. Dr. W. R. Finnerty
Department of Microbiology, University of Georgia
Athens, Georgia 30602, U.S.A.