ORGANIZATION of PROKARYOTIC CELL MEMBRANES

Volume III

Bijan K. Ghosh

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Organization of Prokaryotic Cell Membranes

Volume III

Editor

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FOREWORD

The pioneering investigations of Gorter and Grendel (1975) and Danielli and Davson (1935) in which cell membranes were visualized as bimolecular leaflets ushered in what might be termed the modern era of biomembrane research. Electron microscopy played a key role in establishing the universal existence and anatomical features of biological membranes in cells of animal, plant, and microbial origin, and indeed provided the necessary methodology for the isolation and characterization of biomembranes. Moreover, the ultrastructural studies pointed to the essential differences in surface organization and membranous organelles of eukaryotic and prokaryotic cells. The robust bacterial cell walls of Gram-positive organisms and the envelopes of Gram-negative bacteria became amenable to isolation in the 1950s, and soon after, the pioneering work of Weibull (1953) paved the way for the study of prokaryotic cytoplasmic membranes. Three decades of interest in the physiological and biochemical properties of bacterial plasma membranes have witnessed great advances in the state of our knowledge of their structure and functions. Dr. Bijan Ghosh is to be congratulated in bringing together so many distinguished leaders in the field of prokaryotic membrane research in three Volumes devoted to the "Organization of Prokaryotic Cell Membranes." The collection of authoritative articles covering the most active areas of prokaryotic biomembrane investigations into the several volumes has provided a great service not only to those interested in the field but also to microbiologists in general. We are deeply indebted to Dr. Bijan Ghosh for his considerable editorial efforts in assembling truly valuable contributions to our understanding of such basic aspects of bacterial membrane studies as transport functions, energizing membranes, the biochemistry and immunochemistry of membranes, and the structure-function relationships of photosynthetic membranes, gas vacuoles, and the more controversial mesosomes. The extensive reference lists will be invaluable for students and research workers in the various fields of prokaryotic membrane research especially in the "exploding" segments of the molecular and genetic aspects of Gram-negative cell membranes. These monographs will also serve to focus attention on prokaryotic membranes that are so often ignored by eukaryotic "membraneologists" and will provide an excellent reference source for many years to come.

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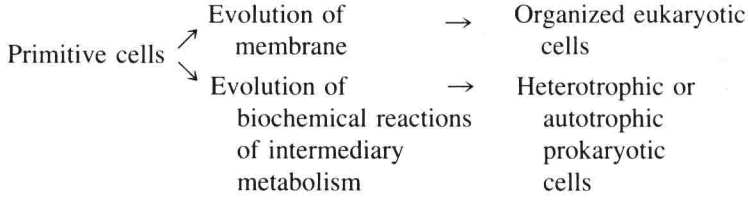
PREFACE TO VOLUME III

The hallmarks of bacterial physiology are the fast growth rate of these prokaryotes, the minuteness of their size, and a high degree of adaptability to differing growth conditions. Their fast rate of growth and colonization enable the bacterial cells to flourish on limited and transient resources. As a result of their smallness these cells have a high membrane-to-cytoplasm ratio. It is possible that this high ratio is important for the establishment of greater contact between the biochemical machinery of the cells and the biosphere.

It has been frequently suggested in the literature that eukaryotic cells have evolved from prokaryotic ancestors. These forerunners of the eukaryotes might have been basically prokaryotic, but it cannot be denied that contemporary prokaryotic cells have evolved equally as much as the eukaryotic cells. Therefore, a dual direction of cell evolution can be suggested.



In general, the prokaryotic cell membrane lacks plasticity and ability to differentiate; however, these properties are inherent in the eukaryotic cell membranes. It is possible that the physiological functions related to membrane plasticity and differentiation, e.g., phargocytosis, pinocytosis, organelle formation, etc., did not develop in the prokaryotic cells. Evolution in prokaryotic cells may have progressed towards the diversification of the biochemical reactions for intermediary metabolism. Because of this metabolic diversity the habitat for prokaryotic cells varies widely. The following concepts are consistent with the properties of the prokaryotic membranes: (1) because of the lack of differentiation, the characteristics of the primitive ancestral cell membrane might have been conserved through evolution in prokaryotic cells; (2) the diversity of the intermediary metabolism in different groups of prokaryotic cells is likely to be accompanied by biochemical differences of the membranes. Due to this extreme variability, it is difficult to form a general concept of the structure and function of the prokaryotic cell membrane.

One group of eukaryotic cells (i.e., fungi) does not fit in this simplistic model of cell evolution. Wide diversity of intermediary metabolism is well known in fungal organisms. As regards subcellular morphology, some fungal cells may be richly endowed with a variety of organelles commonly found in plant or animal cells, whereas the others may show such paucity of organelles that hardly any subcellular body could be demonstrated besides the nucleus. This notable variation in subcellular organelle content of fungal cells may be frequently correlated with subtle differences in their growth conditions. One may speculate from these observations that the organelle formation, presumably dependent upon membrane differentiation, is at an intermediate stage of development in fungal cells. This possibility, when taken together with the present view that fungal cells are highly evolved, suggests that membrane differentiation may have failed to progress enough in the ancestral cells of fungal organisms. Hence, both differentiability and primitive property of the membrane has been conserved in fungi. The membrane system in fungal cells may have retained the potential to revert back to its primitive undifferentiated character. Current views on evolution of membrane and intermediary metabolism may be explained by one general model as described above. A critical study of membrane evolution is a rewarding area of research in cell biology.

The above discussion shows the difficulty in organizing a comprehensive properties of diversity of prokaryotic cell membranes. Hence, both general and specialized properties of prokaryotic membranes have been discussed. A general feeling has been expressed by

colleagues in the field of membrane research that the work in this area is progressing at a very fast rate. Therefore, the scientific material in a manuscript becomes largely out of date because of the delay inherent in the publication of the book. Hence, the editor's aim is to organize a general text written by experts in the field. The authors have presented a thorough review of the available scientific material within a general conceptual framework and indicated the future direction of research. In addition, an attempt has been made to provide an extensive bibliography.

Frequently, investigators working with a specific organism lose appreciation for the diversity of the prokaryotic membrane. Consequently, discussions on the flow of information from researches on membranes originating from a variety of prokaryotic cells are helpful in formulating a unified approach to study prokaryotic cell membranes.

Bacterial cytoplasm directly interacts with the external environment. Membrane is the interface of this interaction. Therefore, the information exchange between the cytoplasmic material (enzymes, genetic material, and other factors) and the extracellular environment is mediated through the membrane. There is a strong possibility that the prokaryotic cell membrane receives information input from the biosphere and regulates physiological activities accordingly. Vigorous research activity will develop in this area of coupled receptor regulator activity of membranes. A thorough understanding of the regulatory role of membranes will stimulate the development of technology for programming bacterial cells for the production and secretion of industrially important substances into the growth (fermentation) medium.

In fact, uses of bacterial cells are steadily increasing in the field of bioorganic industry. The future holds the possibility of extensive use of bacterial cells in the production of biomedical and agricultural material and biomass utilization. The production of enzymes by microorganisms is already a several hundred million dollar industry.

Recent advances in recombinant DNA technology show the promise that through genetic engineering, strains of bacterial cells, targeted for the synthesis of a wide range of biomedical and agricultural substances, may be constructed. However, uses of these constructed organisms in industrial fermentation are limited because of a variety of problems. One of these is the recovery of material from cell-free fermentation medium. Secretory activity, which is a complex membrane phenomenon, regulates the accumulation of material synthesized inside bacterial cells in the growth medium.

Thus, the study of the prokaryotic cell membrane is rewarding, both for the understanding of basic biological phenomena and for the development of a technology to use prokaryotic cells in industry. I hope the material in these three volumes will stimulate research and help students engaged in the studies on prokaryotic cell physiology.

The publication of the third volume has been delayed for reasons beyond control. The structure of this volume, however, has not been changed. Discussions on the membrane of a highly specialized organism, i.e., hydrocarbon-utilizing bacteria, and general discussions on membrane organization and function, i.e., biosynthesis of outer membrane proteins, functional organization of membrane proteins, and protein secretion have been included in this volume. Through the discussion of twelve topics in these three volumes it has become apparent that the membrane is the hub of physiological and regulatory activity in prokaryotic cells. Within the wide range of microorganisms, there is both unity and diversity in these activities. The growth of information on the bacterial membrane is fast, and new ground is being broken. Hence, a project which started as small requires further expansion. We contemplate, as a consequence, discussions of new topics such as (1) Role of Membrane in Chemotaxis; (2) Sporulation; (3) Organization of Chemoautotrophic Bacterial Membrane; and (4) Membrane Genetics, in a possible future volume.

I must thank all the contributors for their valuable articles. In spite of their extremely busy schedules, they sympathetically considered my proposal and gave their time. With their help, we are making a steady progress in this ambitious project.

THE EDITOR

Bijan K. Ghosh, D.Sc., is Professor in Physiology and Biophysics in the Department of Physiology and Biophysics at the University of Medicine and Dentistry of New Jersey—Rutgers Medical School, Piscataway, and Honorary Professor in Microbiology at the Waksman Institute of Microbiology, Rutgers State University, N.J.

Dr. Ghosh received the B.Sc. and the M.Sc. degrees in physiology from Presidency College, Calcutta University. His doctorate was awarded by Calcutta University while he was working in the Indian Institute of Experimental Medicine in 1963. He engaged in postgraduate study at various institutions including the Woods Hole Oceanographic Institute, Woods Hole, Massachusetts, and the Anatomy Institute of the University of Bern, Switzerland.

Dr. Ghosh was an instructor and subsequently, a junior research fellow, at Presidency College during 1958 and 1959. At the Indian Institute of Experimental Medicine Dr. Ghosh was a Junior Research Fellow from 1959 to 1961, and a Senior Scientific Assistant until 1964. From July 1964 until November 1966 he was a Medical Research Council, Canada, Postdoctoral Fellow at the Department of Bacteriology and Immunology of the University of Western Ontario. He became associated with Rutgers University as Waksman-Merck Postdoctoral Fellow at Waksman Institute of Microbiology in November 1966, and served there as an assistant professor from 1967 to 1973. He was a visiting Professor at the University of Amsterdam, Netherlands in 1973. He moved to the Rutgers Medical School at the end of 1973. He visited China as a visiting Professor on an invitation from the Chinese Academy of Sciences and taught a course on "Cell Ultrastructure and Electron Microscopy" in 1981. He received an International Travel Award in 1984 from the National Science Foundation and visited India on an invitation from the Council of Scientific and Industrial Research of the Government of India, to present a lecture series on cell biology and to initiate a joint U.S.—India research program.

Dr. Ghosh has been a member of the Editorial Board of the *Journal of Bacteriology*, and he was very active in the Morphology and Ultrastructure Division of the American Society for Microbiology. He has organized several symposia in the general area of structure/function interrelationship in microorganisms.

Dr. Ghosh is a member of the Canadian Society of Biochemistry, the American Society for Microbiology, the Electron Microscopic Society of America, and the American Association for the Advancement of Science. He is a fellow of the American Institute of Chemists and a member of the New York Academy of Sciences. He is author or co-author of many original papers including some reviews and chapters on Bacterial and Fungal Ultrastructure in the *CRC Handbook of Microbiology*.

Among Dr. Ghosh's awards are a University Gold Metal from the Calcutta University, the Medical Research Council of Canada Postdoctoral Fellowship, a Waksman-Merck Postdoctoral Fellowship at the Waksman Institute of Microbiology of Rutgers University, and the Research Career Development Award from the National Institute of General Medical Sciences of the National Institutes of Health.

Dr. Ghosh has done extensive research on bacterial fine structure (structure and function of mesosomes), the evolution of subcellular organelles (subcellular organization of minute fungi), and membrane phenomena of enzyme secretion in microorganisms.

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The Role of the Membrane in the Bioenergetics of Bacterial Cells
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Chapter 1

MEMBRANES OF HYDROCARBON-UTILIZING MICROORGANISMS

W. R. Finnerty and M. E. Singer

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I. INTRODUCTION

Hydrocarbons are organic molecules that contain only the elements of carbon and hydrogen. Such molecules range in size from methane to large polycyclic aromatic molecules of undetermined weight and structure. Most hydrocarbons are, at best, only sparingly soluble in an aqueous environment.

The ability of diverse genera of microorganisms to degrade a wide variety of hydrocarbons is ubiquitous in nature, with microbial degradation of hydrocarbons receiving extensive study over the last three decades.[1] Such studies have been mainly limited to investigations concerning the metabolic interaction between a single, simple hydrocarbon and a single microorganism. Research efforts in microbial hydrocarbon metabolism have largely concentrated on whole cell physiology, primarily describing various substrate-product interrelationships. Several biochemical pathways of hydrocarbon dissimilation and mechanisms of hydrocarbon oxidation have been formulated, although significant gaps in our knowledge remain. Information at the molecular level is notably lacking in hydrocarbon microbiology. The ability of microorganisms to metabolize such hydrophobic molecules poses questions of basic interest regarding the evolution of the genetic information encoding alkane oxidation in some genera but not in others. Further, the organization and regulation of genetic information specifying alkane oxidations is poorly understood, with these genes being plasmid-encoded in *Pseudomonas* species and chromosome-encoded in *Acinetobacter* species.[2] To date, the genetics and regulation of alkane metabolism have been examined only in *Pseudomonas putida*, a bacterium capable of utilizing low-molecular-weight, simple hydrocarbons.

Major questions remain unanswered and often unrecognized concerning the growth of microorganisms at the expense of these water-insoluble substrates:

1. How do hydrocarbons enter the microbial cell?
2. How do microorganisms respond metabolically and biochemically to such physiologically unusual hydrophobic substrates for purposes of energy production and carbon assimilation?
3. What are the mechanisms of genetic regulation of microbial hydrocarbon metabolism?

Accordingly, it is important to recognize that microbial hydrocarbon metabolism requires further research at more sophisticated levels of analysis to resolve these fundamental questions.

Hydrocarbon microbiology has largely focused on studies relating to the cellular physiology of alkane metabolism. A number of basic and fundamental generalizations have emerged which collectively serve to characterize those prokaryotic and eukaryotic microorganisms exhibiting the ability to grow at the expense of alkanes as a sole source of carbon and energy.

First, a large number of bacteria, yeast, and fungi grow at the expense of alkanes varying in carbon number from 1 to 40 carbon atoms. A few representative genera

capable of alkane metabolism are *Acinetobacter, Pseudomonas, Brevibacterium, Arthrobacter, Mycobacterium, Nocardia, Corynebacterium, Candida, Cladosporium, Penicillium, Cunninghamella, Fusarium, Mucor, and Aspergillus.* These microorganisms are natural soil inhabitants of ubiquitous distribution. Such alkane-utilizing microorganisms are easily isolated from soil by appropriate selective enrichment procedures.

Second, the mechanism(s) of oxidation employed by these various microorganisms are obligately oxygen-dependent processes involving mono-, di-, and subterminal oxidation of the alkane molecule:

$$RCH_3 \xrightarrow{O_2} RCH_2OH \rightarrow RCHO \rightarrow RCHOOH$$

$$CH_3(CH_2)_nCH_3 \xrightarrow{O_2} CH_3(CH_2)_n\text{–}COOH \xrightarrow{O_2} HOOCH(CH_2)_n\text{–}COOH$$

$$R\text{–}CH_2\text{–}CH_3 \xrightarrow{O_2} R\text{–}CH(OH)\text{–}CH_3 \rightarrow R\text{–}\overset{\overset{O}{\|}}{C}\text{–}CH_3$$

The initial oxidative enzymes are membrane-bound dioxygenases which catalyze the insertion of molecular oxygen into the alkane molecule.[1] This *n*-alkyl hydroperoxide is reduced to fatty alcohol which is oxidized to fatty acid by way of alcohol and aldehyde dehydrogenases.

$$R\text{–}CH_3 \xrightarrow{O_2} RCH_2OOH \rightarrow RCH_2OH \rightarrow RCHO \rightarrow RCOOH$$

Finally, the oxidation of alkanes yield a variety of products, many of which accumulate in the culture broth. Products characterizing alkane oxidations are fatty acids, fatty alcohols, fatty aldehydes, wax esters, ketones, dicarboxylic acids, and hydroxy fatty acids.

This chapter discusses the membrane biochemistry and unique ultrastructural features of alkane-utilizing microorganisms with special attention to *Acinetobacter. Acinetobacter* represents an environmentally ubiquitous and nutritionally diverse genus of Gram-negative bacteria capable of growth on n-alkanes (C10 to C30), fatty alcohols and aldehydes, fatty acids, and dicarboxylic acids, as well as certain amino acids and a few carbohydrates.The physiological attributes exhibited by hexadecane-grown *Acinetobacter* sp. H01-N provide a unique model system for studying the metabolism of hydrophobic, water-insoluble compounds. Physiological changes resulting from the growth of this organism on alkane involve the intracellular sequestering and membranous packaging of alkane, specific synthesis of intracytoplasmic membrane systems, the increase of cellular phospholipid and related membrane-bound phospholipid biosynthetic enzymes, repression of *de novo* fatty acid biosynthesis and quantitative changes in the cellular and extracellular lipid composition during growth on alkane.

II. ULTRASTRUCTURE OF HYDROCARBON-UTILIZING MICROORGANISMS

In *Acinetobacter* sp. H01-N unique ultrastructural modifications result from growth on hydrocarbons: (1) the intracellular pooling of hydrocarbons in membrane-bound cytoplasmic inclusions termed "hydrocarbon inclusions" and (2) the induction of an extensive intracytoplasmic membrane system.[3,4] With the exception of the hydrocarbon

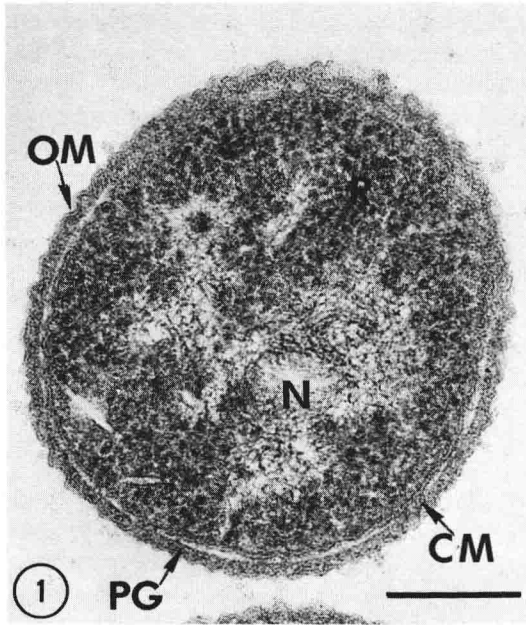

FIGURE 1. Thin section of *Acinetobacter* grown on nutrient broth medium. OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane; R, ribosomes; N, nuclear material. Bar represents 0.5 μm. (From Scott, C. C. L. and Finnerty, W. R., *J. Bacteriol.*, 127, 481, 1976. With permission.)

inclusions and the intracytoplasmic membranes, hexadecane- and nonalkane-grown cells appear morphologically similar, exhibiting the typical cell envelope structure of Gram-negative bacteria in electron micrographs of thin sections or freeze-etched cells (Figures 1 and 5).[5]

A. Hydrocarbon Inclusions

The hydrocarbon inclusions appear in thin sections of hexadecane-grown *Acinetobacter* as electron-transparent, spherical cytoplasmic bodies averaging 0.2 μm in diameter, situated at the periphery of the cell or in contact with the intracytoplasmic membranes (Figure 2).[3,5] These inclusions contain the unmodified growth hydrocarbon, as determined by X-ray diffraction analyses.[3] Hydrocarbon inclusions isolated from hexadecane-grown *Acinetobacter* contain 71.5% hexadecane, 15.8% protein, 9.5% phospholipid, and 3.2% neutral lipid (Table 1).

The limiting membrane of the hydrocarbon inclusion is most easily visualized in thin sections of partially lysed cells containing little cytoplasmic material (Figure 3). The hydrocarbon inclusions are limited by a unique monolayer membrane which does not exhibit the typical bilayer structure of a unit membrane.[5] Freeze-etch studies of isolated hydrocarbon inclusions and of hexadecane-grown cells provide further evidence for the presence of a smooth-surfaced, monolayer inclusion membrane (Figure 4).[5] Freeze-etch studies of nutrient broth-grown cells illustrate the absence of inclusions characteristic of alkane-grown cells (Figure 5).

Since the discovery of these unique cytoplasmic inclusion bodies in *Acinetobacter*, similar structures have been described in other hydrocarbon-grown bacteria and yeasts.[6] Scott and Finnerty[6] documented the presence of hydrocarbon inclusions in hexadecane-grown *Arthrobacter* sp. 80 (Figure 6), *Corynebacterium* sp., *Mycobacterium album* 7E4, *Nocardia rubra*, *Nocardia* sp. 72 (Figure 7), *Mycobacterium vaccae*, *Candida lipolytica*, tetradecane-grown *C. tropicalis*, and naphthalene-grown *Pseudo-*

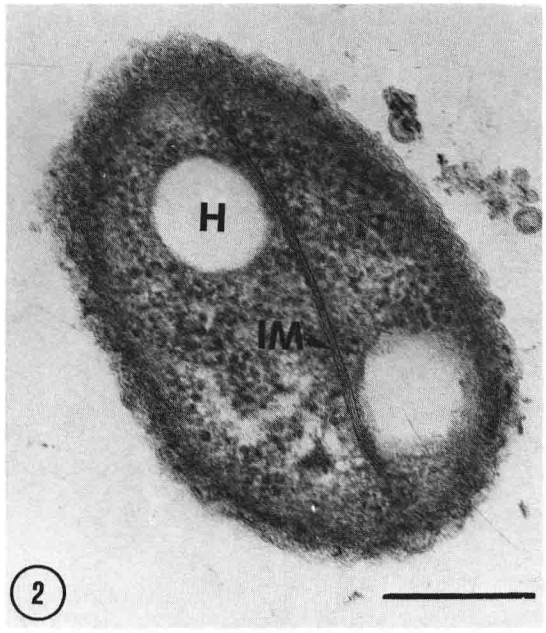

FIGURE 2. Thin section of *Acinetobacter* grown on hexadecane. H, hexadecane inclusion; IM, intracytoplasmic membrane. Bar represents 0.2 μm. (From Scott, C. C. L. and Finnerty, W. R., *J. Bacteriol.*, 127, 481, 1976. With permission.)

Table 1
BIOCHEMICAL COMPOSITION
OF HYDROCARBON
INCLUSIONS

Component	Total μg	Percentage
Wax ester	140	2.2
Triglyceride	15	0.2
Free fatty acid	8	0.1
Fatty alcohol	22	0.3
Diglyceride	14	0.2
Monoglyceride	2	0.03
Hexadecane	4520	71.5
Phospholipid	600	9.5
Protein	1000	15.8

monas sp. With the exception of *N. rubra,* these microorganisms do not contain electron-transparent inclusions when grown on nonhydrocarbon-containing media. *N. rubra,* however, contains significant amounts of poly-β-hydroxybutyrate when cultured with nutrient broth, but only trace amounts when grown on hexadecane. Both of the *Nocardia* sp. also contain electron-dense bodies as a result of growth on hydrocarbon and nonhydrocarbon substrates. These inclusions most likely represent an accumulation of lipids other than hexadecane. Alkane-grown *Nocardia* characteristically accumulate large amounts of waxes and glycerides.[7] Scott and Finnerty[6] demonstrated that those microorganisms with hydrocarbon inclusions contain intracellularly localized hydrocarbon.

The fine structure of hydrocarbon-grown *C. lipolytica* has been described previ-

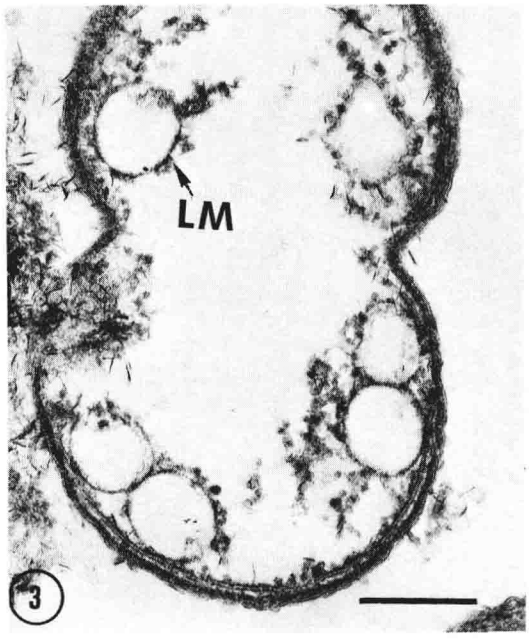

FIGURE 3. Thin section of a partially lysed cell of *Acinetobacter* grown on hexadecane. H, hexadecane inclusion; LM, limiting membrane of hexadecane inclusion. Bar represents 0.2 μm. (From Scott, C. C. L. and Finnerty, W. R., *J. Bacteriol.*, 127, 481, 1976. With permission.)

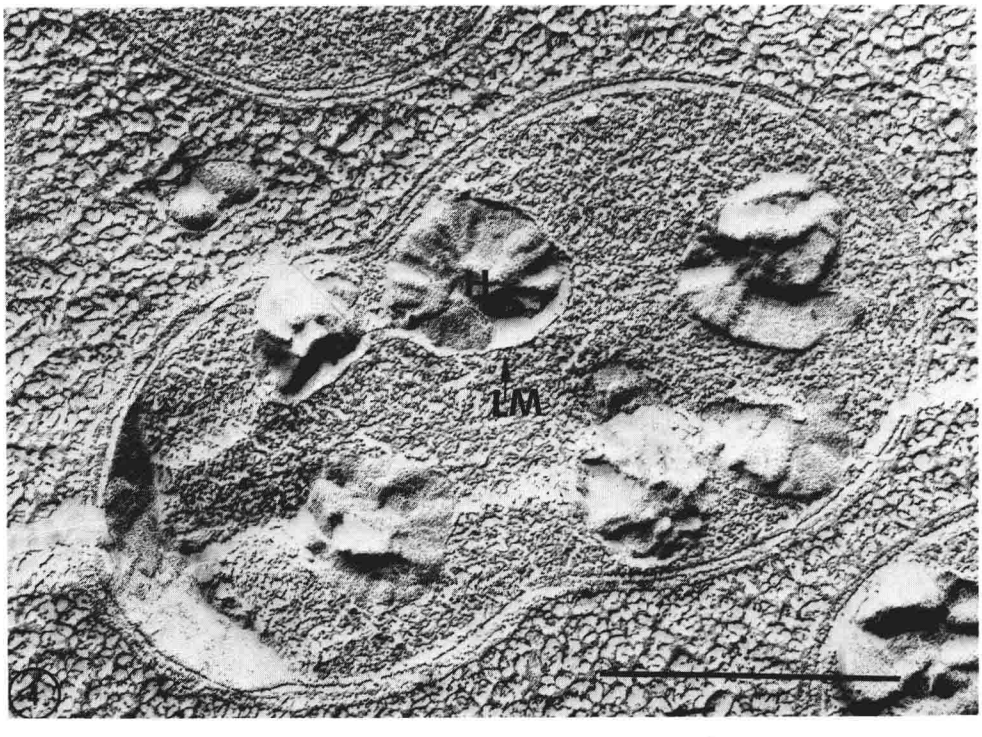

FIGURE 4. Freeze-etch of *Acinetobacter* grown on hexadecane. H, hexadecane inclusion; LM, limiting membrane of hexadecane inclusion. Bar represents 0.5 μm. (From Scott, C. C. L. and Finnerty, W. R., *J. Bacteriol.*, 127, 481, 1976. With permission.)

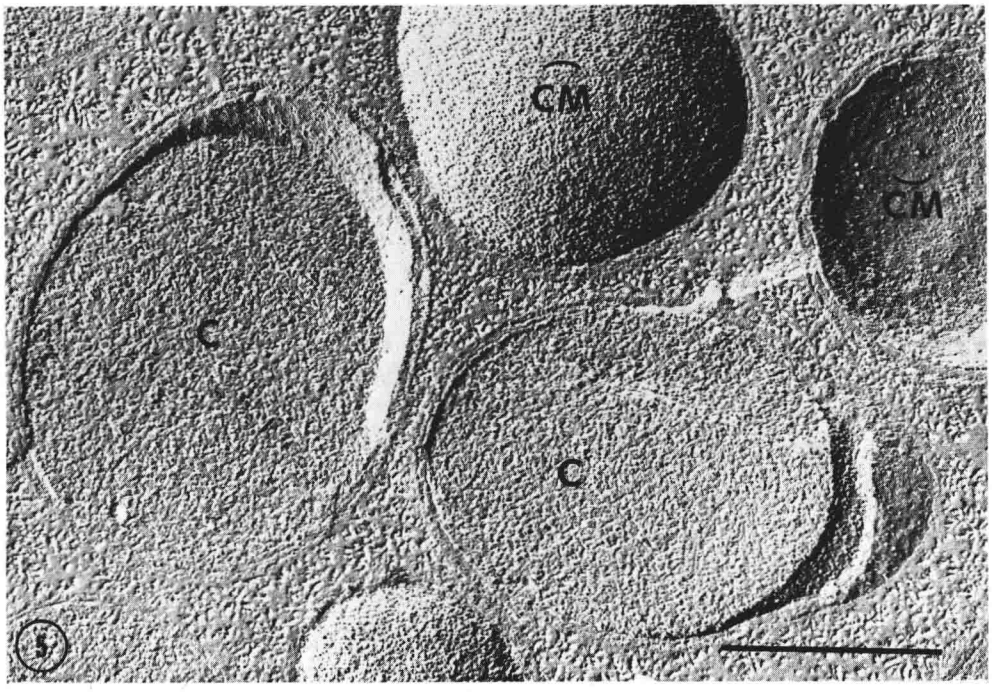

FIGURE 5. Freeze-etch of *Acinetobacter* grown on nutrient broth. $\widehat{CM}$, convex face of cytoplasmic membrane; $\widetilde{CM}$, concave face of cytoplasmic membrane; C, cytoplasm. Bar represents 0.5 μm. (From Scott, C. C. L. and Finnerty, W. R., *J. Bacteriol.*, 127, 481, 1976. With permission.)

ously.[8,9] Hexadecane-grown cells were reported to contain a large number of fat vacuoles which were not positively identified. Hexadecane accumulation at the external surface of the cytoplasmic membrane was suggested but not proven. Scott and Finnerty,[6] however, showed no accumulation of hexadecane at the cytoplasmic membrane of *C. lipolytica*, but rather the occurrence of typical cytoplasmic hydrocarbon inclusions (Figure 8). *C. tropicalis* appears to accumulate tetradecane at the exterior surface of the cytoplasmic membrane in small vesicles projecting into the cytoplasm (Figure 9). Occasionally, the vesicles appear within the cytoplasm at the cell periphery, suggesting an endocytotic type of mechanism for hydrocarbon transport. This mechanism was suggested by Ludvik et al.,[8] who observed pinocytotic vesicles at the ends of deep invaginations of the cytoplasmic membrane in hexadecane-grown *C. lipolytica*.

Several other reports document the occurrence of intracytoplasmic structures resembling hydrocarbon inclusions. Bertrand et al.[10] described disc-shaped, electron-transparent cytoplasmic "vesicles" in thin sections and in negatively stained preparations of a hexadecane-grown marine bacterium. These vesicles are not observed in acetate-grown cells. In freeze-etched preparations with fractures through the intracytoplasmic vesicles, the vesicles appear to be membrane limited. The internal structure of the vesicle appears to have a smooth to finely granular texture, indicating the possible hydrocarbon or lipoidal nature of the enclosed material. Although the nature of the material sequestered in these inclusions was not determined, hexadecane-grown cells were shown to contain 20-fold more nonsaponifiable lipid than acetate-grown cells. Results were inconclusive as to whether these cytoplasmic vesicles contain hexadecane, since no distinction was made between externally absorbed and internally accumulated hexadecane. *Thermomicrobium fosteri*, an obligate thermophile capable of growth on hydrocarbons, contains electron-transparent intracytoplasmic inclusions resulting from