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AN X-RAY STUDY OF THE CYTOPLASMIC MEMBRANES OF TWO GRAM-POSITIVE BACTERIA

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Summary

X-ray diffraction diagrams of partially disordered one-dimensional lattices of isolated bacterial cytoplasmic membranes are described and they provide a basis for suggesting possible molecular structures of bacterial membranes.

Biochemical and electron microscope evidence points towards a lipid bilayer with a high degree of fluidity. The protein molecules are in a disordered configuration in the membrane.

Introduction

We have studied isolated cytoplasmic membranes from two Gram-positive bacteria (*Micrococcus lysodeikticus* (strain NCTCC 2665) and *Bacillus licheniformis* (strain 6346)) by X-ray diffraction and electron microscopy. These are the first X-ray diffraction data to be reported for bacterial cytoplasmic membranes free of cell wall components.

With layered specimens we obtained preferred orientation in a direction perpendicular to the surface of the supporting slide. A comparison of relative intensities of lamellar X-ray reflections obtained in a series of hydration experiments shows that these reflections do not sample a unique curve, the squared Fourier transform of the electron density distribution through the membrane lamellae. This experimental fact is interpreted as a change in bacterial membrane structure with dehydration. Therefore, the conditions required by the

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method of phase determination introduced to membrane structure (myelin) by Finean and Burge [1] (also Ref. 2), are not fulfilled in the case of layered bacterial membrane specimens at different degrees of hydration.

A different approach has been taken by Wilkins et al. [3] and Engelman [4] who studied the X-ray scattering by membrane dispersions and proposed theoretical models to account for their observations. The Fourier transforms of centro-symmetric models of membrane profiles were compared with the square root of the corrected experimental X-ray scattering curve of membrane dispersions. The observed scattering curves showed either three or four low-angle maxima, and the corresponding calculated Fourier transforms presented the phase sequence (+, −, +, −) for the first four low-angle loops. The results were shown to be consistent with a lipid bilayer model. The experimental observation of temperature-induced transitions in the structure of *Mycoplasma laidlawii* membranes [4] is also consistent with this model. Lipid phase transitions have been also observed by Esfahani et al. [5] in membranes isolated from the Gram-negative bacterium *Escherichia coli* K12 (strain CR_{34}) and by Ashe and Steim [6] in *M. lysodeikticus* membranes. Therefore, it is conceivable that these membranes also include a lipid bilayer which might bear a resemblance to the bilayer proposed for *Myc. laidlawii* membranes. We have, therefore, investigated the possibility of phase transitions in the structure of bacterial cytoplasmic membranes by X-ray diffraction and our results are relevant to a lipid bilayer structure in this context. There are recent reviews [7,8] of the dependence of the lipid phase transition on the fatty acid composition of bacterial membrane phospholipids.

Materials and Methods

Preparation of bacterial cytoplasmic membranes. M. lysodeikticus (strain NCTCC 2665) cytoplasmic membranes were prepared by the 'standared' method of Salton and Freer [9] or by the modified version of Salton et al. [10] which includes a low concentration of glutaraldehyde in the bursting medium.

B. licheniformis (strain 6346) cytoplasmic membranes were prepared by the method by Rogers et al. [11]. The effects of different methods of preparation on the structure and biochemical activities of isolated bacterial membranes have been recently reviewed [12—14].

Preparation of electron microscope specimens. Thin sections of centrifuged membrane pellets were examined in a Philips EM 300 electron microscope after osmium tetroxide fixation followed by Epon embedding as in the currently used Ryter and Kellenberger method.

Preparation of X-ray specimens and X-ray equipment. Specimens for X-ray work consisted either of wet membrane pellets sealed inside capillary tubes 0.5 mm in diameter or of thin orientated films obtained by controlled dehydration on PTFE (Teflon) slides inside a temperature-controlled cell (temperature range −5—90°C, accuracy of control ±0.5°C). X-ray exposures were taken with an Elliott rotating anode (GX6) X-ray generator using an evacuated Searle camera. Intensity measurements above the background level were made using a Joyce-Loebl mark III C microdensitometer. The integrated intensities were corrected

by multiplying with the corresponding diffraction order of the lamellar X-ray reflection. This correction is similar to a Lorentz correction factor [15].

Results and Analysis

Experimental results: X-ray diffraction by M. lysodeikticus membranes

Centrifuged membrane pellets containing between 40% and 80% water produced only a few broad low-angle reflections. As the layered speciments were slowly dehydrated at 5° C (92% relative humidity of air) to approximately 30% relative water content (of samples) a more detailed pattern gradually developed which comprised seven diffraction orders of a lamellar periodicity of 89 Å. This compares with the measured membrane thickness of approx. 70 Å in electron micrographs of edge-on thin sections of embedded membrane layers which contained 30% water before the fixation stage (Fig. 1), or in specimens resuspended after layering (Fig. 2a) and freshly prepared membranes (Fig. 2b).

Additional observations by freeze-fracturing of freshly prepared and trypsin-treated membranes suggest that membrane proteins penetrate the boundaries of the lipid bilayer and their density/unit of intact membrane area is considerably higher than in the freeze-fractured red cell membrane [14].

The lamellar reflections were observed only with the X-ray beam edge-on to the membrane layers which were stacked parallel to the smooth surface of the supporting PTFE slide. (The axis of the observed arcs will be called 'the equator' and the normal to the equator will be termed 'the meridian'.) The integrated intensities and the spacings of centroids of equatorial reflections from *M. lysodeikticus* for d = 89 Å, at 30% relative water content, are given in Table I.

At water contents slightly lower than 30%, the degree of distortion of the lattice increased and the first observed reflection at approx. 84 Å became diffuse, as shown in Fig. 3. The distribution of intensity in the equatorial pattern at this stage is given in Table II and Fig. 4.

These changes may be interpreted in terms of models of lattice distortions

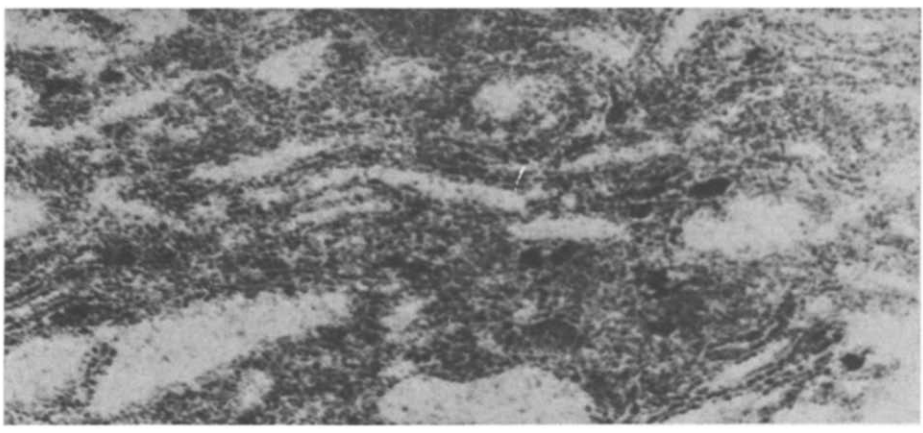

Fig. 1. Edge-on thin section of embedded *M. lysodeikticus* membrane layers (×168 000).

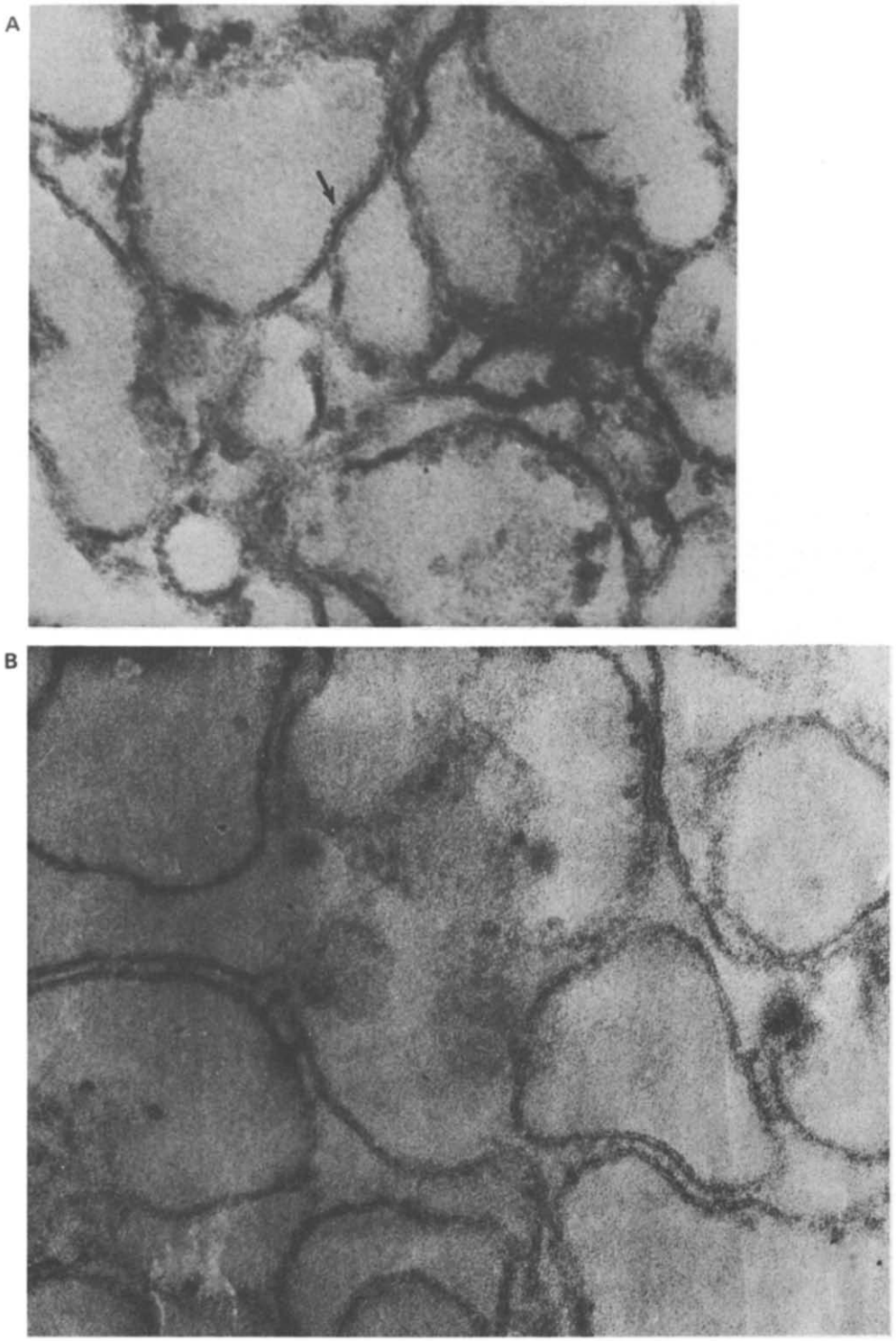

Fig. 2. (a) Thin section of *M. lysodeikticus* membrane specimens resuspended after layering (X168 000). (b) Thin section of freshly prepared *M. lysodeikticus* membranes (X145 000).

TABLE I

CORRECTED INTEGRATED INTENSITIES AND SPACINGS OF EQUATORIAL REFLECTIONS FROM *M. LYSODEIKTICUS* MEMBRANES AT 30% RELATIVE WATER CONTENT (5°C)

Diffraction order (k) ($d \approx 89$ Å)	Spacing (k/d) ($Å^{-1}$)	Corrected integrated intensity in relative units (±5% for strong reflections, ±20% for weak reflections)
1	1/(89)	60.6
2	1/(44.3)	27.8
3	1/(29.5)	0.5
4	1/(22.3)	2.4
5	1/(17.4)	0.5
6	1/(14.8)	7.2
7	1/(12.7)	2.1
8	1/(11.1)	0.5

TABLE II

SPACINGS AND INTENSITIES OF EQUATORIAL REFLECTIONS FROM *M. LYSODEIKTICUS* MEMBRANES AT HUMIDITIES LOWER THAN 30% RELATIVE WATER CONTENT (25°C)

Spacing ($Å^{-1}$)	Integrated intensities in relative units (±8% for strong reflections, ±20% for weak reflections)
1/(84.2)	24.2
1/(44.1)	70.6
1/(28.0)	0.5
1/(22.1)	2.5
— (absent reflection)	—
1/(14.2)	1.9
1/(12.1)	0.5
1/(10.4)	1.0

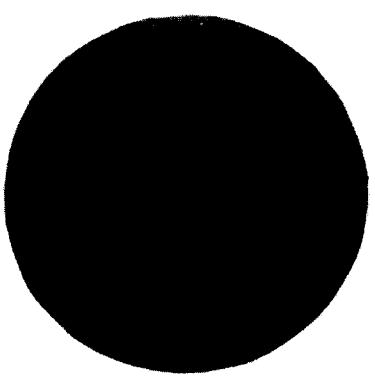

Fig. 3. Low-angle X-ray pattern of orientated *M. lysodeikticus* membranes ($d \approx 84$ Å, 'Bragg' orders k = 1 and 2).

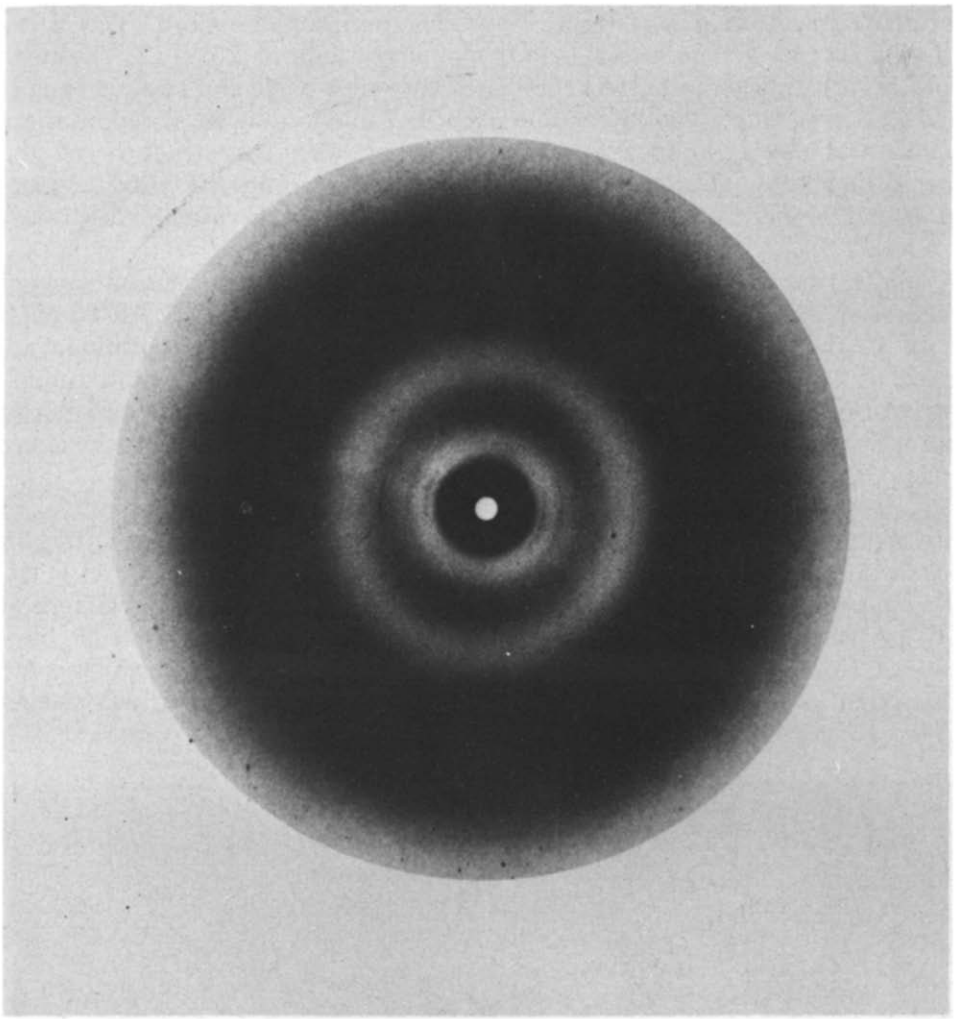

Fig. 4. High-angle X-ray pattern of orientated *M. lysodeikticus* membranes ($d \approx 84$ Å, 'Bragg' orders k = 4, 6, and 8 are visible on this over-exposed film).

[16] similar to those introduced for dehydrated layers of bacterial cell walls. The shift of the first odd-order reflection from the corresponding 'Bragg' position and its diffuseness for a relative water content less than 30% suggest an asymmetric membrane profile distributed with the denser side at random in a distorted lattice. That is, different membrane pieces are orientated with their dense site either to the left or to the right, at random [16].

Long exposures of specimens maintained in saturated water vapour at 5°C allowed the observation of six diffraction rings showing no preferred orientation. The spacings of these rings are integral orders of a fundamental repeat of 130 Å. It is assumed that this periodicity corresponds to the bacterial membrane thickness in the fully hydrated state plus the intermembrane fluid layer. The estimation of the intensities of these rings is rendered difficult due to the

high diffuse scattering on which these rings are superimposed. The X-ray scattering by *M. lysodeikticus* membrane dispersions at 25° C (1—5% membranes by dry weight, in buffer) comprised three low-angle and two high-angle, broad-scattering rings. The intensities given in Table I could be scaled on these maxima after background correction indicating that the structure of the bacterial membrane at approx. 30% water content is consistent with the structure of membranes in dispersion. The remaining high-angle rings are centred at 8.7 Å and 4.6 Å, respectively.

A phase transition was observed by means of X-ray diagrams of layered specimens of *M. lysodeikticus* membranes recorded at 5° C, 10° C, 15° C, 25° C and 40° C. The phase transition takes place at 5° C when a sharp meridional arc centred at 4.2—4.3 Å is observed together with a weaker unorientated maximum centred at 4.6 Å. Above 5° C there is only a broad ring in this region of reciprocal space centred at approximately 1/4.6 Å and this ring becomes broader above 25° C.

X-ray diffraction by B. licheniformis membranes: comparison of results with those for M. lysodeikticus membranes

The X-ray diagrams obtained with *B. licheniformis* membrane specimens at 25° C are generally similar to those obtained with specimens from *M. lysodeikticus*. However, the number of equatorial diffraction maxima which could be recorded in the case of *B. licheniformis* membrane specimens was smaller

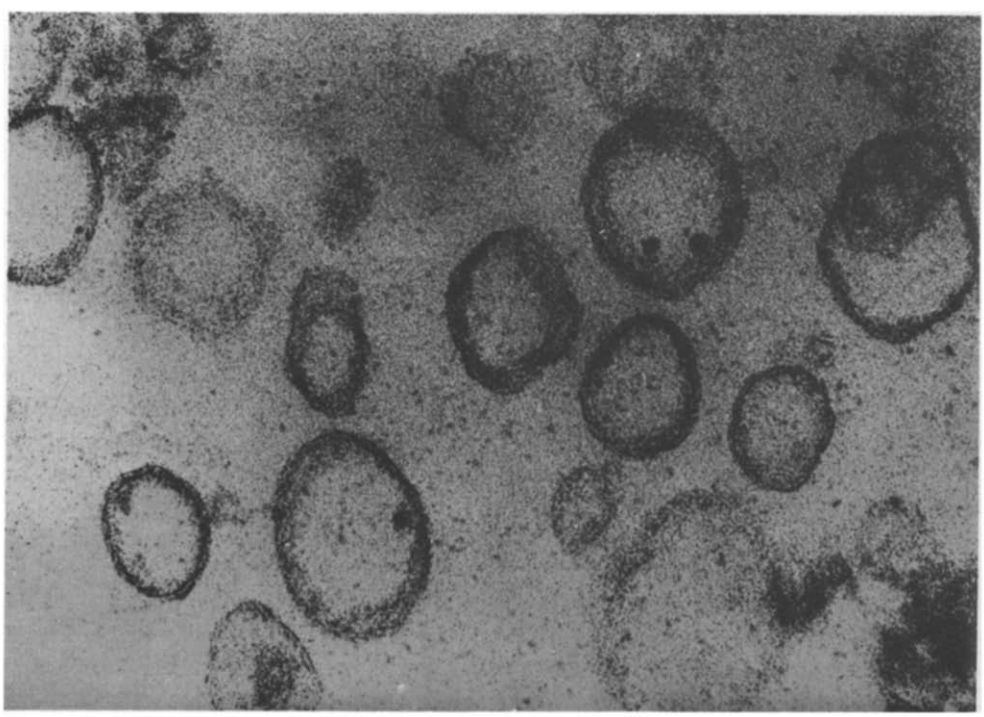

Fig. 5. Electron micrograph of a thin section of isolated *B. licheniformis* membranes in suspension showing membrane profiles of approximately 70 Å in width. (×176 000).

TABLE III

SPACINGS AND CORRECTED INTEGRATED INTENSITIES OF EQUATORIAL REFLECTIONS FROM *B. LICHENIFORMIS* MEMBRANES AT 30% RELATIVE WATER CONTENT (5°C)

Diffraction order (k) (d = 170 Å)	Spacing (k/d)	Corrected integrated intensity relative units (±5% for strong reflection, ±20% for weak reflections)
1	1/(170)	7.0
2	1/(85.1)	32.0
3	1/(54.6)	0.2
4	1/(42.4)	56.0
5	1/(34.5)	0.4
6	1/(28.4)	4.8

than in the case of *M. lysodeikticus* membranes in the same conditions. The equatorial reflections observed with wet *B. licheniformis* membrane specimens at 5° C (approx. 30% relative water content) were integral orders of a spacing of 170 Å, which is interpreted as a double membrane repeat by comparison with the thickness of approx. 70 Å measured in electron micrographs of thin sections of a single membrane profile (Fig. 5). The corrected intensities of these reflections are given in Table III.

The double repeat is correlated with a higher RNA and protein content of *B. licheniformis* membranes as compared with *M. lysodeikticus* membranes, which is presumably due to differences in the preparation procedures and is probably related to disordered material adhering to the membrane surface. These differences in biochemical composition seem to result in a higher adherence of the denser sides of *B. licheniformis* membranes, in comparison with *M. lysodeikticus* membranes. Electron micrographs of embedded *B. licheniformis* membrane layers show pairs of membrane layers with a 20—30 Å dense layer of disordered material (presumably RNA and protein) between the membrane profiles; if *M. lysodeikticus* membranes are subjected to low glutaraldehyde concentrations at the stage of hypotonic treatment, or at the next stage of preparation, similar double repeats are observed both by electron microscope and X-ray diffraction [14].

The X-ray scattering curves of *B. licheniformis* membrane dispersions showed four to five broad maxima which could be in principle related to the squared Fourier transform of the electron density profile [5], if the appropriate intensity corrections can be made. The calculation of incoherent and coherent scattering terms of partially ordered membrane lattices on the basis of stochastic membrane models is essential for the interpretation and correction of the experimental data obtained with polydisperse membrane suspensions. This problem is presently under investigation (see Ref. 17).

Discussion

Recent structural studies of a specialised part of *Halobacterium halobium* cell membrane (e.g. Refs. 18 and 19) showed considerable detail due to the high degree of ordering of structural proteins in the purple membrane. The

electron density profile through the thickness of the purple membrane is thought to be asymmetric [19].

Our present investigations of typical bacterial membranes also show that the electron density profile of *M. lysodeikticus* and *B. licheniformis* membranes is asymmetric and that the protein arrangement in the membrane is a highly disordered one. The asymmetry of the electron density profile of typical cytoplasmic membranes is most probably due both the asymmetrical distribution of lipids [20] and the asymmetric positioning of membrane proteins [12,21]. In the studies on the intact protoplasts of *M. lysodeikticus*, electron microscope observations revealed an asymmetric distribution of ATPase molecules in the cytoplasmic membrane. These are located preferentially on the inside surface of the protoplast membrane [12]. However, the isolated *M. lysodeikticus* membrane suspensions contain a mixture of vesicles ordered with both inside-out and right-side-out (that is, with the same orientation as in the intact protoplasts). This limitation of the preparation procedure and the highly disordered arrangement of protein, as well as the membrane sensitivity to hydration changes, are major obstacles in the derivation of electron density profiles for bacterial cytoplasmic membranes.

Preliminary data on the cytoplasmic membrane of a Gram-negative bacterium, a Marine pseudomonad, suggest that similar problems are encountered with this bacterial membrane.

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