On the Plasma Membrane of Some Bacteria and Fungi

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With the assistance of John Marak

The contents of bacterial cells can be reversibly pulled away (plasmolyzed) from the wall by strong salt solutions, bacterial "protoplasts"—living cells divested of their walls—shrink or swell with changing concentrations of solutes in their environment and burst when this concentration falls below a critical level. These observations have long suggested that the cytoplasm of bacteria is covered by a selectively permeable plasma membrane.

In recent years electron microscopy has revealed the existence of a narrow profile at the surface of the cytoplasm of bacteria. In some bacteria, these profiles have the dimensions of sections of the "unit membrane" found in the interior and at the surface of plant and animal cells.

In the place of a unit membrane, single tracks of densely stained granules are found in many bacteria. New electron micrographs confirm the observation of Young and Fitz-James (1959) that the first spore membrane is derived from an infolding of the plasma membrane of the mother bacterium.

Illustrations are provided of the plasma membrane of B. mycoides and of the yeast Lipomyces lipofer.

The existence of an osmotic barrier at the surface of the retracting cytoplasm, which is suggested by these observations, is proved by the behavior of isolated bacterial protoplasts. These fragile forms are obtained when bacteria are divested of their walls by the action of lysozyme (or in some other manner) while they are suspended in a stabilizing medium of suitable tonicity. For protoplasts of B. megaterium, this is provided by 0.1-0.2 M sucrose. Staphylococci require 1.5 molar solutions of sucrose or NaCl. Granted the adaptability of bacteria, and in fact because of it, one would expect these values to vary with the osmotic pressure of the medium in which the bacteria were growing before they were transformed into protoplasts, but such variation has not been reported. Protoplasts shrink and swell with changes in the concentration of glucose (or other protective solutes) in the stabilizing medium. Quantitative experiments with many different solutes have shown that some, like glycerol, penetrate rapidly into protoplasts while others, like sucrose or NaCl, are unable to do so. It follows from all this that protoplasts are covered by a semipermeable membrane which is stable as long as the internal osmotic pressure of the protoplast is balanced by that of the cul-

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"The emphasis is illustrative rather than comprehensive." From a publisher’s advertisement, December, 1961.

IN THE PREPARATION of media for the cultivation of bacteria the matter of isotonicity is usually given little thought because, provided the needs of growth and multiplication have been met, bacteria can live in a wide range of concentrations of organic and inorganic solutes. Staphylococci, for example, grow well in nutrient media containing 0.1 M NaCl but also in media containing 20 times that amount of salt.1, 2 Many other examples of growth of one species proceeding at widely different osmotic pressures of the environment could be quoted. Adaptability notwithstanding, steep, abrupt increases in the concentration of external solutes may temporarily cause bacteria to shrink and the cytoplasm to retract in several places from the cell wall.3 Gram-negative bacteria are more easily plasmolyzed than Gram-positive ones, in which it may take 5 M NaCl to produce this effect.4, 5, 6 Spherical Gram-positive bacteria only shrink but do not plasmolyze in hypertonic solutions.
ture medium. Protoplasts will indeed burst and pour out their contents when the medium is greatly diluted. From preparations of dissolved protoplasts a membranous fraction has been retrieved which consists of lipid, protein, and carbohydrate and is regarded as consisting of the plasma membranes of the disrupted cells.

It is generally held that the tough cell wall of the intact bacterium protects the protoplast within from being destroyed by its own osmotic pressure (some 20 atm. in staphylococci) when the bacterium finds itself, as it often must, in a hypotonic medium. The reverse may also be true. The tenacity with which some protoplasts resist plasmolysis in the presence of high concentrations of salt may be due to stubborn linkages between plasma membrane and outer wall which prevent the membrane from shrinking in the presence of forces which it could not resist on its own at the surface of the naked protoplast. It needs to be remembered that the cell wall not only protects bacteria from bursting in water. Its presence allows them to survive in it for many hours or even days. Weibull's observation that protoplasts of B. megaterium, a strictly aerobic organism, are sensitive to aeration also emphasizes that the wall probably contributes more to the stability of the protoplast than mere mechanical restraint.

Current knowledge of the permeability of bacteria is largely derived from the work of Mitchell and Weibull. For detailed information on protoplasts, the reader is referred to recent review articles by these authors and by McQuillen.

On the basis of permeability studies made before protoplasts had become available, Mitchell suggested 100 Å as the probable thickness of the plasma membrane of staphylococci. A value of that order has also been derived from the light microscopy of Bacillus cells stained with Victoria blue 4R or B. These dyes have the ability to attach themselves selectively to the surface of the protoplast, staining (for a while) neither cell wall nor cytoplasm. The appearance of bacilli stained with Victoria blue can be imitated by depositing a thin film of gold on microscopic fibers of glass wool. Extrapolation from the heaviness of the metal film and the diameter of the fibers made Robinow and Murray suggest a thickness of 60 Å for the layer that accepts Victoria blue. Since then, electron microscopy of sections has revealed many instances of the existence of a membrane at the surface of the bacterial cytoplasm of the appearance and the dimensions (around 80 Å) which Robertson, Mercer, and many others have encountered in the interior and at the surfaces of the cells of higher organisms.

The following pages will be mainly concerned with the plasma membrane of Bacillus cells. The plasma membrane of two fungi will also be illustrated.

Methods and Materials

Several organisms have been examined: three bacilli (B. mycoides, B. cereus, and B. subtilis), a yeast (Lipomyces lipofer), and a mycelial fungus (Basidiobolus ranarum). The bacteria were grown on Difco heart infusion agar or in heart infusion broth and were prepared for electron microscopy by the methods of fixation and embedding of Ryter and Kellenberger. The yeast was fixed with potassium permanganate and uranyl nitrate in the manner of Vitols et al., and the fungus was fixed in 2 per cent potassium permanganate followed by 1 per cent osmium tetroxide, a technique recommended to the writer by Dr. Royall T. Moore. Some cultures of bacteria were treated with ether to produce retraction of the cytoplasm from the cell wall. This was done either by holding a culture on agar over a dish of ether for 7 minutes and fixing it 10 to 20 minutes later or by shaking 3–4 ml. of ether with 25 ml. of a culture of bacilli in heart infusion broth in a separatory funnel. After 7 to 10 minutes the broth was run off, incubated for another 10 to 20 minutes, and fixed in the usual manner. To demonstrate the role of the plasma membrane in spore formation, B. cereus was grown on the surface of potato extract agar at 18° C. and for another six hours at 37° C. The confluent growth of bacilli was then washed off and fixed according to Ryter and Kellenberger. All specimens were embedded in Vestopal epoxy resin, sectioned on a Porter-Blum microtome, and examined in a Phillips 100 electron microscope.
Results

Many electron micrographs of bacterial plasma membranes have been published in the last few years.\textsuperscript{19–30} They are of two kinds. In some micrographs the plasma membrane appears as a pair of dense lines separated by a light space. In others only a single dense track is found. The former kind of membrane, being more akin to that found commonly in cells of higher organisms, will be dealt with first.

We have consistently found a double-contoured plasma membrane in vegetative forms growing out of germinating spores of \textit{B. mycoides}. It appears to be approximately 80 Å thick. Typical examples are illustrated in figures 1–5. The best view of the membrane is obtained when the protoplast is pulled away from the cell wall by some means or other. Phage infection has served this purpose in \textit{E. coli},\textsuperscript{31} contact with ether achieves it in \textit{Bacillus} cells. A pretreatment with ether so weakens the adhesion of the protoplast to the cell wall that fixation with chemicals or dehydration, neither of which normally greatly alters the appearance of \textit{Bacillus} cells,
Figures 1–5

B. mycoides (1–3). Inside the cell wall (cw) may be seen the double-contoured plasma membrane (pm). In figure 3 the plasma membrane sends a convoluted intrusion into the cytoplasm. × 200,000. B. cereus (4–5). Fixed during the early stages of lysis induced by ether. The plasma membrane has pulled away from the wall.

now causes a visible separation of cell wall and contents. If some time is allowed to elapse between exposure to ether and fixation then many cells will undergo autolysis (figs. 4, 5) initiated, presumably, by injury to their plasma membranes.

Since the widespread adoption of the fixation technique of Ryter and Kellenberger, many students of bacteria have learned that the plasma membrane of Gram-positive bacteria sends convoluted intrusions (the "mesosomes" of Fitz-James) into the cytoplasm, which may be tubular, vesicular, or take the form of concentric layers of evenly spaced lamellae of remarkably uniform thickness. Neat intrusions of the latter type are regularly found in suitably fixed cells of B. mycoides (figs. 6–8). They show the same proximity to the nuclear material and the initials of transverse partitions that has already been noted in other bacilli. Ether treatment increases the likeness (already pronounced in normal cells), between the intrusions of the plasma membrane and myelin figures, more precisely their likeness to myelin figures that had been soaked in distilled water.

The inrush of water which presumably attends the ether induced lysis of bacteria is first reflected in a widening of the spaces between the lamellae of the intrusions (figs. 9–12). The thickness of the lamellae themselves is only slightly increased or not at all. This behavior is compatible with the view that, like myelin figures, the lamellae of the mesosomes may be composed of bimolecular leaflets of phospholipid bearing hydrophilic groups on their outside (scheme of fig. 3 of Stoeckenius et al.). Before these analogies are carried too far, it ought to be remembered that the analyses carried out by Weibull and Bergstroem and by Gilby et al. have shown that bacterial plasma membranes contain a considerable amount of carbohydrate, a feature they do not share with myelin figures. Also unlike the latter, the convoluted intrusions could not, in our hands, be preserved with...
Figures 6–8

Convoluted intrusions of the plasma membrane ("mesosomes") in B. mycoides. (cw) cell wall, (pm) plasma membrane. × 200,000.
Swollen, waterlogged, membranous intrusions in B. mycoides fixed after brief exposure to ether. The interlamellar spaces are wider than normal. The continuity of the lamellae appears disturbed. All figures × 200,000.
B. subtilis, × 200,000 (13 and 14); B. subtilis, × 150,000 (15); Streptococcus, × 150,000 (16). Short lengths of a double-tract plasma membrane (pm) can be made out in figures 13 and 14. There are also traces of the connections between the plasma membrane and the cell wall which have been described by Glauert et al.24 Figure 15 shows part of a germinating spore of B. subtilis. The plasma membrane is represented by a single line. This is also true of the plasma membrane of the streptococci in figure 16.

Plain osmium or permanganate. Very probably what Finnean has stressed in another context is also true of these membranes: "... that in view of the considerable shrinkage that takes place during sectioning and viewing the exact dimensions observed in electron micrographs are not meaningful in terms of the normal dimensions of lipid and protein molecules."35

The similarity to unit membranes and myelin figures ceases when only a single, dense, wavy, dotted line is seen where one ex-
Figure 17
An early stage in the development of the spore membrane (sm) in B. cereus. The membrane is seen growing inward from the plasma membrane (pm) at a point near the junction of sister bacilli whose walls are still in contact. X 150,000.

In their spores, bacteria are completely at rest, very dry, and chemically different from the cells of the growing phase. The morphology of the early phases of the process that produces the highly specialized spore cell (nuclear events apart) is very simple. The region of the future spore is carved out of the mother cell by influences whose first visible agent is the plasma membrane. In bacilli

The Role of the Plasma Membrane in the Formation of Spores
The anatomical details of spore formation were first understood and clearly described by Young and Fitz-James. With their findings the new micrographs in this article are in full accord.

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Figure 18
Advanced stage in the development of a spore of B. cereus. In two places on the right the spore membrane is still connected with the plasma membrane (pm) of the mother bacterium. X 150,000.
in the appropriate state (which can be precisely established, \textit{vide} Young and Fitz-James\textsuperscript{36}) the plasma membrane grows inward into the cytoplasm a little way back from one of the poles of the rod-shaped mother cell. Along a wavy, rambling course it traces out and eventually completely encloses an area in which the synthesis of the spore’s protoplasm and its dehydration will presently begin (figs. 17, 18). The folded, ample contours of the spore membrane, or spore plasma membrane (as it must now be called), are later

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figures19-22}
\caption{Figures 19-22}
\textit{Further illustrations of the relationship between plasma membrane and spore membrane. Figure 21 is an enlarged part of figure 18. For abbreviations, see legends to earlier figures. All figures $\times$ 150,000.}
\end{figure}
smoothed by the growing spore which is round or oval in its finished state. The outer envelopes of the spore, of which there may be several, are assembled in the cytoplasm of the mother bacterium and do not arise from pre-existing membranes. It is important to realize that the original spore membrane does so arise. In this way continuity is preserved between the plasma membrane of the sporangium and that of the next vegetative generation.

In our material, the plasma membrane of the mother cell presented the appearance of a single scalloped track of closely spaced dark dots and dashes. By contrast the spore membrane is bounded by two such lines. The relationship of these two contours to the single-track plasma membrane from which they originate is not always entirely clear (figs. 19–22). It seems simplest to regard the outer contours of the spore membrane as direct continuations of the inner (cytoplasmic) surface of the plasma membrane of the mother bacterium (fig. 23). This explanation is in agreement with the idea of Fitz-James that the spore membrane arises through an infolding of the plasma membrane followed by fusion, back to back, of the walls of the fold.

The activities of the plasma membrane at the start of spore formation display one of its most remarkable capabilities and bring the observer face to face with fundamental problems of cellular differentiation.

**Observations on Fungi**

Yeast enzymes in common with bacteria that they are sensitive to abrupt changes in the concentration of solutes in their environment and respond to them with plasmolysis or swelling or contraction. But, again like bacteria, they are capable of adapting themselves rapidly to growth in media of a much wider range of osmotic pressures than would be tolerated by most cells of plants and animals. For details of permeability studies on yeasts see Orskov and Eddy.

The plasma membrane of Lipomyces (figs. 24, 25) is much indented and scalloped, a feature that has also been noted in Saccharomyces and in Neurospora.

Little more can be said about the plasma membrane of Basidiobolus in figure 26 than that it appears as a single dense line. Of interest are its many points of contact with the endoplasmic reticulum.

**Acknowledgment**

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**References**

Figures 24–26
Lipomyces lipofer (24, 24a, 25). Figures 24 and 24a are from adjoining regions of the same cell and partly overlap. A long stretch of the plasma membrane is visible as a double-contoured, clear, wavy track just inside the cell wall (cw). The latter, as is common in fungi, lacks scattering power except for a region close to the plasma membrane, v = vacuole. Figures 24, 24a × 100,000, figure 25 × 60,000. Figure 26 shows the edge of a hypha of Basidiobolus ranarum. × 60,000.

14. Mitchell, P. D.: The osmotic barrier in bac-
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