Advances in

MICROBIAL PHYSIOLOGY

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VOLUME 7

1972

ACADEMIC PRESS · LONDON and NEW YORK

ACADEMIC PRESS INC. (LONDON) LTD.
24/28 Oval Road,
London NW1

United States Edition published by
ACADEMIC PRESS INC.
111 Fifth Avenue
New York, New York 10003

Library of Congress Catalog Card Number: 67-19850
ISBN: 0-12-027708-5

PRINTED IN GREAT BRITAIN BY
WILLIAM CLOWES AND SONS LIMITED
LONDON, COLCHESTER AND BECCLES

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Contents

Regulatory Phenomena in the Metabolism of Knallgasbacteria. H. G. SCHLEGEL and U. EBERHARDT

Energy Conversion and Generation of Reducing Power in Bacterial Photosynthesis. HOWARD GEST

Walls and Membranes in Bacteria

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I. Introduction

Almost one hundred years ago, Cohn (1872) suggested that the resistance of bacteria to attack by acids and alkalies was due to the presence of a rigid structure surrounding the cell. The phenomenon of plasmolysis, first observed twenty years later by Fischer (1891), produced the first visual evidence for a cell wall. During plasmolysis the cytoplasm loses water and retracts from an apparently rigid cell wall. The suggestion that the retracted cytoplasm is itself surrounded by a membrane responsive to osmotic changes followed naturally from Fischer's observations. Although some evidence for this membrane was obtained from observations with the light microscope, conclusive visual evidence came only with the advent of electron microscopy and the allied thin-section techniques (Kellenberger and Ryter, 1958; Glauert, 1962). This membrane has been variously termed a "cytoplasmic membrane", a "plasma membrane", a "protoplast membrane" and more recently a "plasmalemma". In this review it is proposed to use the term cytoplasmic membrane.

A correlation between reaction to the Gram stain and ease of plasmolysis was quickly established and it appears that in general only Gram-negative bacteria are plasmolysed. Mitchell and Moyle (1956) cited this observation as evidence for a stronger link between the cell wall and the cytoplasmic membrane in Gram-positive bacteria. Connections between specific regions of the cytoplasmic membrane and the inner layer of the cell wall can be seen in thin-sections of plasmolysed cells of *Escherichia coli* (Bayer, 1968). Photographs of replicas of freeze-etched bacilli (Holt and Leadbetter, 1969; Nanninga, 1968) indicate that strands or fibres connect the inner layer of the cell wall with the outer layer of the cytoplasmic membrane. The relationship between these fibres and the finger-like protrusions of material observed by negative staining to extend from the cytoplasmic membrane to the cell wall in many Gram-positive bacteria is not clear (Hurst and Stubbs, 1969). Bridges extending from the outer regions of the cytoplasmic membrane are clearly visible in thin sections of *Bacillus licheniformis* (Rogers, 1970)

and have been reported to be present in other Gram-positive bacteria (Leadbetter and Holt, 1968; Ghosh and Murray, 1967; Edwards and Stevens, 1963). An intimate connection between cell wall and cytoplasmic membrane is not unexpected in view of recent findings that the cytoplasmic membrane is involved in the biosynthesis of some cell-wall components (Ellar, 1970). Preparations of Gram-negative "cell walls" produced by mechanical disintegration often contain cytoplasmic membranes retained within the walls. Such preparations are better regarded as cell envelopes (Salton, 1967a).

Cell walls of Gram-positive bacteria consist principally of an insoluble polymer, the peptidoglycan (synonyms—murein, mucopeptide, glycosaminopeptide) which in some cases accounts for as much as 90% of the dry weight of the cell wall, together with one or more other macromolecular components which may be protein, polysaccharide or teichoic acid. The cell walls of Gram-negative bacteria are more complex containing only relatively small amounts (5–20%) of peptidoglycan and large amounts of protein, lipid and lipopolysaccharide (Salton, 1964; Rogers and Perkins, 1968). Isolated cell walls retain the shape of the cell from which they were obtained and, because of this retention of shape, they have often been described as rigid structures. Recent physico-chemical experiments (Ou and Marquis, 1970) on the cell walls of Gram-positive bacteria have demonstrated that the walls behave like polyelectrolyte gels expanding and contracting in response to changes in environmental pH value and ionic strength. Since the cell walls are also known (Gerhardt and Judge, 1964) to function as heteroporous molecular sieves, such a contraction or expansion may be important in controlling the size of molecules passing through the cell wall. Binding studies with monovalent and divalent cations show that cell walls also behave as weak ion-exchange resins (Cutinelli and Galdiero, 1967).

A. Protoplasts and Sphaeroplasts

Treatment of many bacilli with lysozyme in hypertonic or isotonic media transforms the cells from rods into osmotically fragile spheres. These spheres, termed protoplasts by Weibull (1953), are free of cell-wall material and are surrounded simply by the cytoplasmic membrane. Comparable methods have been used to prepare protoplasts from other Gram-positive bacteria (Gooder, 1968; Weibull, 1968). The cytoplasmic membrane behaves as a porous differential dialysis membrane and the pore size can be increased by osmotic swelling of the protoplast (Corner and Marquis, 1969). The structure is highly extensible and enormous protoplasts may be prepared by slowly dialysing away the stabilizing solutes. The lysis of protoplasts appears to be caused by a rapid influx

of solutes when the pores reach a critical size leading to very rapid local extensions of the membrane and brittle fracture.

Historically, the absence of cell-wall material, spherical shape and osmotic sensitivity were taken as essential criteria for protoplasts (Brenner *et al.*, 1958). Rod-like forms, otherwise similar to protoplasts, are produced from *B. megaterium* (Op den Kamp *et al.*, 1967) and *B. subtilis* (Van Iterson and Op den Kamp, 1969) when cells previously subjected to acidic pH (5) values are treated with lysozyme. These forms which do not contain any residual cell-wall components are more resistant to osmotic lysis than spherical protoplasts produced from cells harvested at neutral pH values. The change in shape is correlated with the replacement of phosphatidylglycerol by glucosaminylphosphatidylglycerol in the cytoplasmic membrane of the former organism, and by the lysyl derivative in the latter. Bizarre shaped protoplasts of bacilli are produced when the cell walls are removed in the presence of high concentrations of magnesium ions (Rogers *et al.*, 1967).

The complete removal of the chemically more complicated Gram-negative cell wall to leave the organism surrounded simply by the cytoplasmic membrane is technically very difficult, and has only been achieved in one specialized case. A marine pseudomonad is converted into a wall-less form by lysozyme after prior removal of the outer layers of the cell wall by washing with 0·5 *M*-sodium chloride and resuspension in 0·5 *M*-sucrose. Similar forms may be produced by the action of lysozyme and EDTA in tris-HCl buffer made 0·5 *M* with respect to sucrose (Costerton *et al.*, 1967; De Voe *et al.*, 1970).

Spherical, osmotically sensitive forms of Gram-negative bacteria, termed sphaeroplasts, may be obtained by penicillin treatment or by the action of lysozyme (McQuillen, 1960). Gram-negative bacteria are more resistant to lysozyme than Gram-positive organisms and are only susceptible to the action of this enzyme if previously sensitized. Cells may be sensitized in several ways, which include heat treatment (Myerholtz and Hartsell, 1952), freezing and thawing (Kohn, 1960), plasmolysis (Birdsell and Cota-Robles, 1967) or by the action of EDTA in tris-HCl buffer (Repaske, 1956). Sphaeroplasts possess a weakened cell wall caused by the loss of the peptidoglycan (Salton, 1964).

B. Division of Protoplasts

Dumbell-shaped protoplasts reminiscent of division forms have been observed when *B. megaterium* is incubated in a stabilized medium for 8–9 hr at 28° (McQuillen, 1960) but, generally, true division of protoplasts in liquid media has yet to be observed (Weibull, 1968). Landman (1968) and Gooder (1968) have reported the propagation of protoplasts of *B. subtilis* and streptococci respectively on soft agar. The colonies

consist of wall-less forms of irregular size and shape which are morphologically indistinguishable from L forms. Such forms may be described as stable L forms since the organism will persist in the wall-less state. Lysozyme-produced protoplasts of *B. subtilis* still containing small amounts of cell wall revert to the bacillary state when plated on suitable media (Miller *et al.*, 1967). Apparently, for this organism, commitment to the L state is initiated by complete (or almost complete) loss of cell wall (Landman, 1968). However, cell wall damage rather than complete loss of cell wall is necessary for efficient propagation of lysozyme-treated cells of streptococci as L forms (King and Gooder, 1970).

C. Reversion of L Forms of *Bacillus subtilis* to Bacillary Forms

Stable L forms of *B. subtilis* propagated on hard agar (2·5%) or a medium containing 25% gelatin revert to the bacillary form (Landman *et al.*, 1968; Landman and Forman, 1969). In addition to hard agar and gelatin, reversion is stimulated by growth on certain membrane filters and by the addition of cell walls of whole organisms (Clive and Landman, 1970). The stimulation by whole cell walls is non-specific since similar stimulation is obtained by the addition of a wide range of autoclaved bacteria and yeasts. The factors involved in reversion have little in common chemically, and reversion must be dependent on the physical properties of the surface provided adjacent to the naked protoplast. How the barrier acts is not known. Synthesis of 2,6-diaminopimelic acid, a constituent of peptidoglycan is repressed in L forms and protoplasts (Bond and Landman, 1970), although cytoplasmic membranes of L forms retain the ability to synthesize peptidoglycan intermediates when supplied with appropriate substrates (Chatterjee *et al.*, 1967).

The process of reversion has been monitored by electron microscopy (Landman *et al.*, 1968). In the initial stages of reversion, the chromosome of the protoplast becomes more condensed and then a thin layer of cell wall appears around the cytoplasmic membrane. It is not clear whether this cell wall originates at specific regions on the cytoplasmic membrane; during the process cell shape is extremely variable. Mesosomes of an unusual compartmented type not linked to the nucleus appear in about 25% of the cells but only after the cell wall has been laid down. Control experiments with bacilli suggest that these mesosomes are induced by gelatin and not by the reversion process.

Biochemically the process of reversion is divided into three stages (Landman and Forman, 1969). In the first stage, primed by casein hydrolysate, synthesis of both protein and RNA takes place. In the second stage, experiments with lysozyme and penicillin suggest that cell-wall synthesis is taking place. During this stage the cells must be continuously surrounded by the solid medium; heating the gelatin briefly

at 40° severely retards the reversion process. The third stage confers osmotic stability on the cells and is inhibited by actinomycin D and chloramphenicol.

II. Intracytoplasmic Membranes

A. Occurrence and Appearance

Classical intracellular organelles, such as mitochondria and chloroplasts, existing in the cytoplasm and carrying out specific cellular functions are not present in prokaryotic organisms. Such functions are performed in bacteria by the cytoplasmic membrane and intracytoplasmic membranes of varying complexity (Gel'man *et al.*, 1967). The intracytoplasmic membranes appear to be formed almost universally by invaginations of the cytoplasmic membrane and, as a result, they maintain contact with the extracytoplasmic space. A possible exception is the complex intracellular membrane system present in the nitrifying bacterium, *Nitrosocystis oceanus* (Remsen *et al.*, 1967); these membranes are apparently not formed by invagination of the cytoplasmic membrane and are distributed between daughter cells on division. Possibly this system represents an evolutionary intermediate in the formation of a true intracellular organelle. The photosynthetic organism, *Ectothiorhodospira mobilis*, contains an exceedingly complex internal membrane system comprising one to eight stacks of lamellar membranes (Remsen *et al.*, 1968). The stacks are penetrated by negative staining showing contact with the external environment and are formed initially by infolding of the cytoplasmic membrane. The membrane may infold again on itself at any point to produce disc-like structures which are reminiscent of chloroplasts in plants. Complicated internal membrane systems have also been demonstrated in the methane-utilizing bacteria (Davies and Whittenbury, 1970).

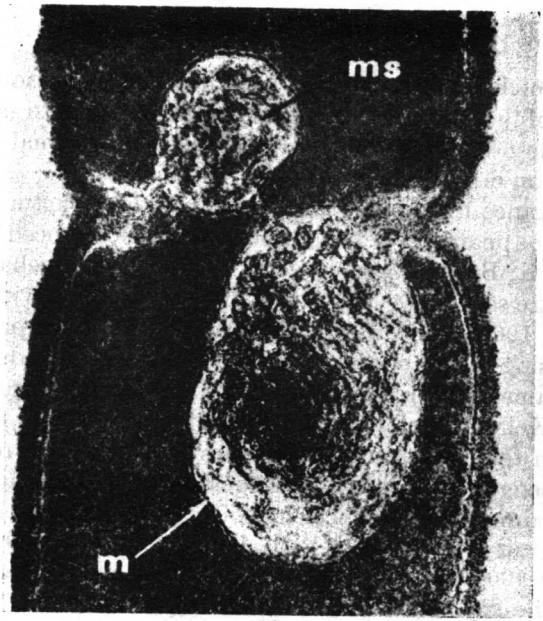
Simpler intracytoplasmic membranes systems are found in Gram-positive bacteria. Detailed studies of such systems, termed mesosomes (Fitz-James, 1960), have in the main been confined to bacilli, particularly *B. subtilis* (Ryter, 1968) and *B. licheniformis* (Rogers, 1970). The mesosome takes the form of a sac-like invagination of the cytoplasmic membrane into the cytoplasm. Examination of the cells, after fixation and thin-sectioning in the electron microscope, reveals that the sac may be filled with lamellar, tubular or vesicular structures (Salton, 1967a; Rogers and Perkins, 1968). In order to prevent confusion at later stages, it is proposed that the following terminology be used when discussing the mesosomes of Gram-positive bacteria. It is suggested that the entire invagination of the cytoplasmic membrane together with the contents of the invagination be called a "mesosome". The part of the cytoplasmic

membrane which invaginates will be called the "mesosomal sac" and the contents of the invagination (or sac) will be referred to as "mesosomal tubules", "mesosomal vesicles" or "lamellar mesosomal membranes" as the situation demands.

The appearance in thin section (Fig. 1) of the structures within the mesosomal sac apparently depends upon the fixation procedure (Highton, 1969, 1970a, b; Burdett and Rogers, 1970); thus lamellar mesosomal membranes are seen only very rarely after prefixation with glutaraldehyde (Ryter, 1968; Burdett and Rogers, 1970) but are present in cells fixed at 0° using the standard Ryter-Kellenberger procedure (Highton, 1969; Fitz-James, 1968). When fixed at room temperature by the standard Ryter-Kellenberger procedure, the mesosomal sac of *B. licheniformis* is seen to contain inflated tubules constricted at regular points along their length (Burdett and Rogers, 1970). After using a fixing solution containing a lower concentration of Ca^{2+} or a lower ionic strength the sacs appear to be filled with lamellar mesosomal membranes. Vesicles, 300–400 Å in diameter, are observed in the sacs if the ionic strength of the fixative is increased or alternatively the cells are fixed with a solution of unbuffered osmium tetroxide containing sucrose (0·05–0·3 *M*).

Better understanding of the nature of mesosomes under physiological conditions is obtained from freeze-etch studies. This procedure does not permanently damage living cells and the rapid freezing used might be expected to preserve cells in an *in vivo* condition. The mesosomes of *B. subtilis* appeared as collections of vesicles (size 300–1000 Å) implanted into or attached to the cytoplasmic membrane (Remsen, 1968); in contrast to the cytoplasmic membrane both inner and outer surfaces of the vesicles were smooth. Comparable pictures have been obtained by Nanninga (1968). A lamellar-type of mesosome covered with particles similar to those present on the inner side of the cytoplasmic membrane appeared prior to spore formation. Remsen suggests that this mesosome is functionally different from the type containing vesicles.

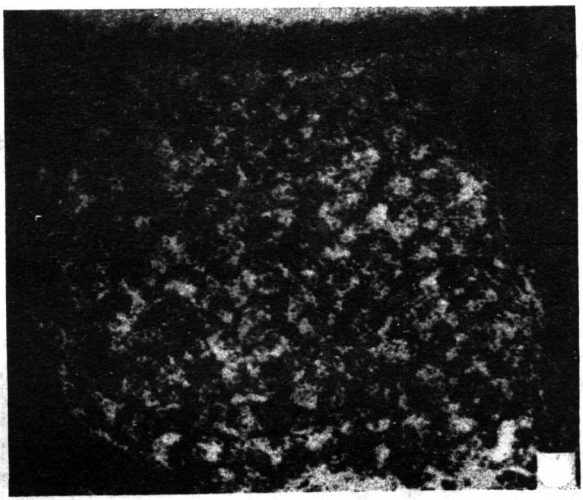
A disorganization of mesosomes occurs when cells are chilled to 15° (Fitz-James, 1965; Neale and Chapman, 1970). The extent of the disorganization is probably dependent on the nature, function and position of the mesosome in the cell. The mesosomes associated with growing septa are severely disorganized whereas those associated with the nucleus appear intact. Changes in mesosomal structure have frequently been observed when cells are subjected to low doses of antibiotics such as streptomycin, cycloserine (Fitz-James, 1967) or bacitracin (Rieber *et al.*, 1969). A rapid increase in the areas of mesosomes, as observed in thin section, occurred when cells of *Streptococcus faecalis* were starved of valine or threonine for 1–2 hr. (Higgins and Shockman, 1970b).



(a)

(b)

Fig. 1



(c)

FIG. 1. Mesosomes of *Bacillus licheniformis*. Photographs are reproduced from Burdett and Rogers (1970). (a) shows a thin section through a cell fixed by the standard Ryter-Kellenberger procedure. Mesosomes (ms) are associated with a forming septum and are filled with tubular structures. The membrane (m) is continuous with the cytoplasmic membrane, and is termed the mesosomal sac. Magnification × 104,800. (b) shows the appearance of a mesosome when the ionic strength of the Ryter-Kellenberger fixative was decreased. Lamellar membranes (l) are present in the mesosome. The smaller arrows point to a fine densely stained line present in the centre of the sheets of membrane and which may be composed of globular units. Magnification × 372,000. (c) shows a thin section through a mesosome when the ionic strength of the fixative is increased by addition of sodium chloride (0.1 *M*). The mesosome appears to be filled with vesicles. Magnification ×184,000.