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Biogenesis of the Wall in Bacterial Morphogenesis

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I. Introduction

For many years the insoluble material remaining after disruption of bacteria was discarded as "debris" by biochemists interested in preparing soluble enzymes from microbes. Material deposited during high- but not low-speed centrifugation, likewise unidentified, was used as the source of

particulate enzymes. New attitudes, towards the "debris" dawned about 20–30 years ago after Dawson (1949) and Cooper *et al.* (1949) had examined it under the electron microscope, and Salton (1953) had analysed it chemically. Likewise the origin and significance of the "particles" became clearer after Weibull's (1953, 1956) study of protoplasts and of the ghosts that could be obtained from them. Despite the sophistication of the subject that has since grown up around studies of bacterial cell envelopes, which comprise cell walls, the "debris" and cytoplasmic and other membranes—the "particles", large areas of ignorance still exist. The further the studies have progressed, the more intricate and numerous have become the structures and biosynthetic process necessary to make them. It has also become apparent that, far from being inert "hulls" or "cases" to the cells, they are dynamic, plastic and growing organs which control major cell functions ranging from cell growth and division through the export and uptake of molecules to the antigenic and adhesive properties of bacteria.

Numbers of reviews have been written describing the structure and biosynthesis of wall components and polymers or summarizing knowledge of bacterial membranes; some of these are quite recent (see Rogers *et al.*, 1978). There would therefore be little point in writing another one. Instead, the present article will be aimed at trying to understand more about the role of the envelope in growth, division and morphogenesis of cells. Growth of bacterial cells necessarily involves increase in the area and volume of wall material. It might be thought legitimate, therefore, to include a review of our knowledge of the growth of cells during the cell cycle and its relation to other major cell events such as DNA replication. Fortunately, these aspects have already been dealt with in a review by my colleague Sargent (1979); for information about these aspects of the problem, I shall lean heavily on his review. Greater attention will be given to ways in which cell division and the shape of the bacterial cell can be disturbed. This knowledge will be related to physiological and molecular knowledge about the envelope, but much will necessarily have to remain speculative.

II. Shape Maintenance

A. THE SHAPE OF BACTERIA

We are familiar enough with the appearance of bacteria at a magnification of about 1000-fold, and we think in terms of cocci, bacilli,

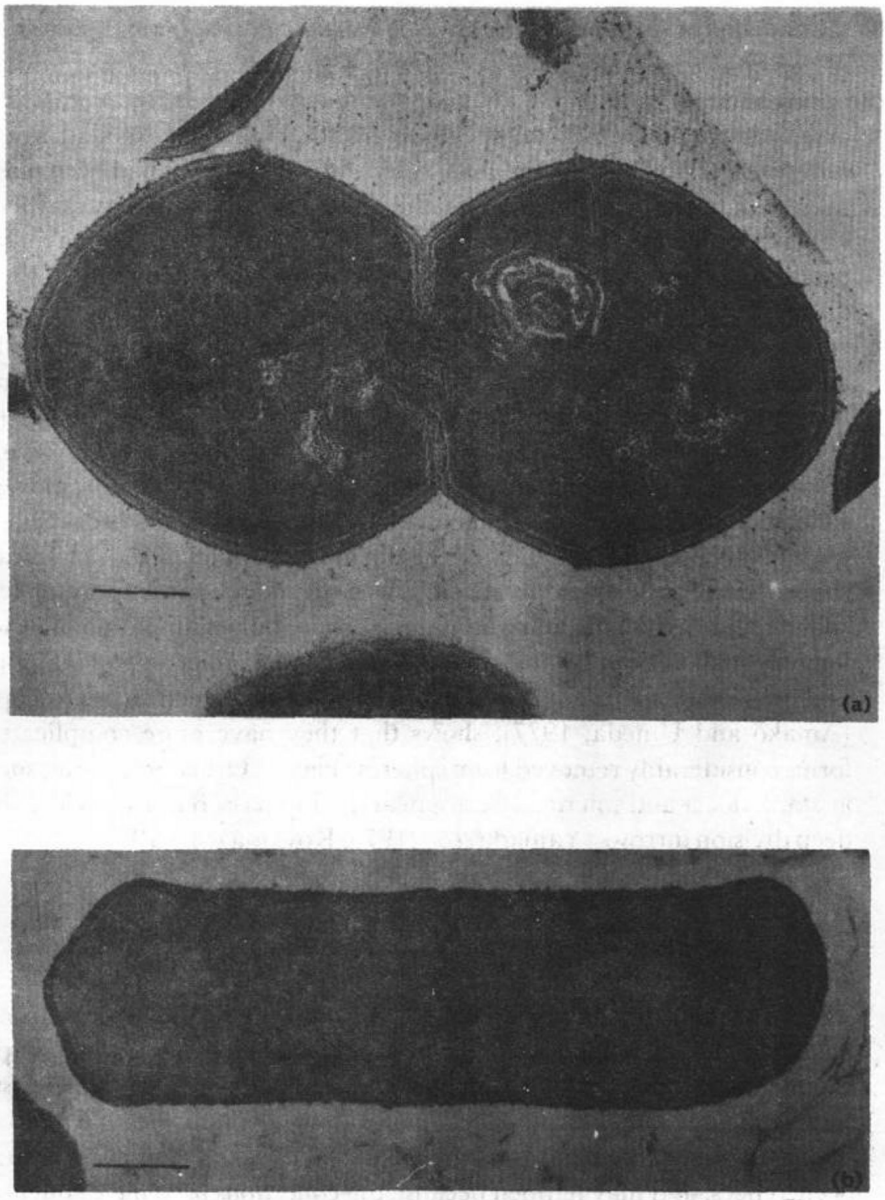

FIG. 1. (a) Section of *Streptococcus faecalis* obtained from a mid-exponential phase culture. The micrograph was kindly provided by Dr M. L. Higgins, Microbiology Department, Temple University, Philadelphia, U.S.A. (b) Section of *Bacillus subtilis* showing asymmetric modelling of cell poles. From Rogers *et al.* (1978). In both micrographs, the bar is equivalent to 0.25 μm.

salmonellae or sprilla knowing that some are roughly spherical, some rods of various lengths with differently shaped poles, and others approximately spiral in shape. We can also readily recall the arrangement of the individual cells as groups, regular packets, strings or independent units. However, until recently, little attempt had been made to designate precise cell-shapes or the forces required to maintain them, either as individuals or in specific arrangements. Still less have the evolutionary significance and the possible biological advantages of these different morphological forms yet been considered. The sphere is, of course, the form of minimum free energy and without constraints, is that adopted by fluids, or fluids contained within plastic envelopes such as membranes. One obvious function of coats of cells external to the cytoplasmic membrane is, therefore, to provide the constraints giving bacteria their characteristic shapes. When the walls are removed, spherical protoplasts or sphaeroplasts are usually formed. Shape maintenance applies not only to the obvious examples of rods or spiral-shaped bacteria but also to cocci. The latter may appear more or less spherical under the ordinary light microscope, but examination of exact longitudinal sections by the transmission electron microscope (Higgins and Shockman, 1976), or of whole cells by scanning electron microscopy (Amako and Umeda, 1977), shows that they have more complicated forms considerably removed from spheres (Fig. 1). Other organisms, such as staphylocci and micrococci, are nearer to spheres but are divided by deep division furrows (Yamada *et al.*, 1975; Koyama *et al.*, 1977.

B. THE ROLE OF THE WALL

The effects of removing or damaging the wall are in themselves strong evidence for functions as supporting structures and in shape maintenance, but a few experiments have been done in which protoplasts from bacilli adopted shapes other than spheres (Abram, 1965; Rogers *et al.*, 1967; van Iterson and Op den Kamp, 1969). These are unusual occurrences, and may happen because the conditions used have stiffened the membrane or even the cytoplasm itself. Protoplasts must be suspended in isomolar solutions to avoid lysis, and so the forces needed to maintain a rod shape, for example, are greatly decreased compared with those needed when the organisms are growing in nutrient media. The possible role of changes in lipid composition found when rod-shaped

protoplasts have been obtained from *Bacillus megaterium* and *B. subtilis*, grown or treated at low pH values, have not be examined further (Op den Kamp *et al.*, 1965, 1967, 1969). Despite these observations, acceptance or assumption of the shape-maintaining function of bacterial walls has been fairly general. The wall components supplying even the supportive strength, particularly in Gram-negative bacteria, have however been the subject of some discussion.

C. PEPTIDOGLYCANS

Isolated wall preparations from Gram-positive bacteria contain 40–90% of peptidoglycan, the remainder being composed of a variety of phosphate-containing teichoic acids, teichuronic acids and other polysaccharides. Preparations from Gram-negative organisms, on the other hand, may have as little as 1% of the polymer, the remainder mostly consisting of proteins and phospholipids. The fine structure of the peptidoglycans from a wide range of bacterial species is by now rather well understood (Schleifer and Kandler, 1972). They all consist of long glycan strands made from residues of *N*-acylglucosamine and *N*-

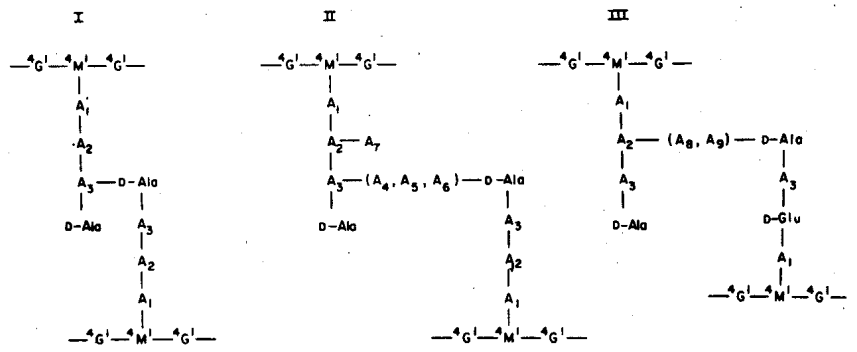

FIG. 2. General structures of peptidoglycans, G indicates *N*-acylglucosamine; M, *M*-acylmuramic acid. In most peptodoglycans, the acyl group is acetyl. A_1—A_3 are amino acids alternating in stereospecificity other than when glycine is involved. The amino acids A_4—A_6 form the so-called bridge between peptides directly attached to the glycan strands. A number of species, including all known Gram-negative organisms and bacilli, have type I peptidoglycan with A_1 indicating L-alanine; A_2, D-isoglutamine; and A_3, 2,6 mesodiaminopimelic acid. Many cocci have a type II peptidoglycan where A_1 indicates L-alanine; A_2, D-isoglutamine; and A_3, L-lysine. The bridge amino acids (A_4, A_5, A_6) may be all glycyl residues, glycyl and seryl or glycyl and threonyl. In *Micrococcus luteus* there are five instead of three bridge amino acids which repeat the main chain (i.e. -L-ala-D-glu-L-lys-D-Ala-). Fragments have been obtained from this peptidoglycan with up to four repeats of this peptide.

acylmuramic acid linked together by 1—4β bonds. The amino sugars are usually *N*-acetylated although, in some species, other acyl groups are involved. These polysaccharide chains are then joined together by short peptides each containing a very limited number of amino-acid residues with either the L- or D-configuration (Fig. 2). It is generally thought that these polymers form giant, cross-linked, mesh-like structures covering the whole surface of the cell. In Gram-negative organisms, the thin sacculus of peptidoglycan would have only 1–3 such meshwork layers (Braun *et al.*, 1973) whereas a Gram-positive organism, like *B. subtilis*, would have 30–40 (Kelemen and Rogers, 1971). Molecules of the other polymers, such as teichoic acids, are attached to the 6-hydroxl groups of muramyl residues in glycan strands of the peptidoglycan by single terminal bonds. Linker groups interposed between teichoic acid and peptidoglycan are now fully characterized in some species, and may be very common. The distribution of these substituents on glycan chains is as yet unclear. Likewise, the distribution of polymers through the thickness of the wall is uncertain (see Rogers *et al.*, 1978).

In most instances, enzymes that hydrolyse specific bonds in the peptidoglycans lead to lysis or to formation of protplasts or sphaeroplasts under suitable conditions. However certain organisms, such as pseudomonads, or even *Escherichia coli*, can be lysed by chelating agents, such as EDTA, which would not be expected to affect the peptidoglycan. The remaining "ghosts" are rod-shaped and not spherical. This work has been briefly discussed before (Rogers, 1974), and the suggestion made that damaged membranes may allow small activities of autolytic enzymes sufficient access to the thin layer of peptidoglycan present in Gram-negative bacteria for a hole or holes to be punched. This would either allow the contents of the cell to leak out or to a shattering of the "rigid" layer of peptidoglycan. The observations would not seem to provide a strong argument against a dominant role for peptidoglycan in providing strength. Nevertheless, that a variety of hydrolases specific for bonds in peptidoglycan can lead to loss of shape of non-spherical bacteria is not in itself sufficient evidence that peptidoglycan is solely responsible for shape maintenance. It is quite possible to argue that the characteristic shapes of bacterial cells can only be maintained by a contribution from all of the polymers in the walls, rather than by any one of them. The nature of the molecules attached to the peptidoglycan in the walls of Gram-positive species can be changed without apparently altering the function of the wall. For example, wall

teichoic acids can be almost wholly exchanged for teichuronic acids in many organisms by growth under phosphate limitation (Ellwood and Tempest, 1969, 1972; Forsberg *et al.*, 1973). The organisms do not change their shape as a result of this substitution. However, if certain mutant organisms are grown under phosphate limitation, their walls contain very little negatively charged polymer, and are made almost entirely of peptidoglycan; gross shape changes then occur. Instead of growing as rods, for example, bacilli grow as cocci. This change in morphology involves growth and probably is a problem of shape determination. Formal proof is lacking.

One argument commonly advanced for the dominant role of peptidoglycan in shape maintainance is not strictly valid. It is argued that, because molecules such as teichoic acids can be removed from peptidoglycan in isolated preparations without altering morphology of the walls, the latter is the only important shape-maintaining polymer. Walls in these preparations, however, are no longer subject to the osmotic pressure exerted upon them in the living cell. Therefore, even if teichoic acids had a role in maintaining the shape of the cell, little change in isolated wall preparations might be expected following their removal. Unfortunately, the conditions necessary for removal of polymers associated with peptidoglycan are too drastic to apply to living cells without damaging cytoplasmic membranes. Such damage would lead to a collapse of the osmotic forces due to leakage of internal solutes.

Among Gram-negative bacteria, mutants of *E. coli* entirely lacking detectable amounts of the lipoprotein attached to the peptidoglycan (Hirota *et al.*, 1977) provide some evidence for the latter's dominant role. These have abnormalities in the positioning of the outer membrane and in the process of cell division, consistent with a function for the lipoprotein in holding the peptidoglycan and the outer membrane together (Braun and Rehn, 1969). They, however, show no abnormality in their rod shapes despite reports of abnormal morphology in other mutants partially lacking lipoprotein (Wu and Lin, 1976). Another argument in favour of peptidoglycan as the sole shape-maintaining component of the wall is possibly to be found in a marine pseudomonad (Forsberg *et al.*, 1972). All the weight of the very thin insoluble layer of the wall that was isolated from "mureinoplasts" could be accounted for by peptidoglycan constituents, and it had the same shape as the original cell. However, the detection of leucine, isoleucine, glycine, serine and aspartic acid before treatment with proteolytic enzymes during isolation suggests the

presence of a covalently attached polypeptide in the living organism, similar to the lipoprotein of *E. coli*.

There can be no doubt about the importance of peptidoglycan in providing the strength of walls of bacteria necessary to resist the internal osmotic pressure of cells. The arguments for peptidoglycan as the sole shape-maintaining polymer in the wall are nevertheless rather weak.

III. The Physical Properties of Walls

A. WALLS OF THE LIVING ORGANISM

Knowledge of the chemistry of the components of wall preparations is well advanced and now presents no major difficulties of principle. However, when we come to try to decide their arrangement and distribution in the growing organism, major problems arise. Even our knowledge of the volumes in which they are contained in the walls of the living cell is uncertain. As has so frequently been stated, walls of Gram-positive bacteria appear, in sectioned material, to vary from about 15–30 nm in thickness, according to the species of organism and growth conditions. In Gram-negative organisms an outer wavy membrane is seen, separated by a less dense region from a very thin layer identified as peptidoglycan. How far does the appearance of sectioned material reflect the situation in the living cell? Frozen-etched preparations of whole cells provide some basis for comparison since it is likely that a very high proportion of the population of an organism, such as *E. coli*, survives the freezing procedure; in one test 85% survived (Bayer and Remsen, 1970). Freeze-fractured preparations of the Gram-positive *Strep. faecalis* have been compared (Higgins, 1976) with bacteria first fixed in glutaraldehyde followed by osmium tetroxide and then embedded and dehydrated. The walls of these latter organisms, when sectioned, appeared about 30% thinner than in the freeze-fractured material but the other dimensions of the cells did not seem to differ. *Escherichia coli*, on the other hand, shrank 30% in overall dimensions when fixed with osmium tetroxide, prepared and sectioned (Bayer and Remsen, 1970). More subtle changes also occurred. For example, the outer membrane became wavy in the sectioned material but, after freeze-etching, presented an almost smooth surface with a little residual waviness which was said to be influenced in degree by the growth medium (Nanninga, 1970).

These effects are not negligible but may only be indicative of greater

difficulties if the results obtained by application of physico-chemical methods to bacteria are correct. For example, a value for the wall thickness of living *M. luteus* cells of 80 nm was recorded from measuring the difference between the cell volumes impermeable to a dextran of molecular weight 150,000 and to sucrose or phosphate (Carstensen and Marquis, 1968). Such a value is two or three times greater than that seen in sections of the micro-organism. Likewise, a value of 86 nm was deduced for the wall thickness in *Staph. aureus* from light scattering and refractive index measurements (Wyatt, 1970). In this instance, the measured thickness in sectioned material was 18 nm. Again, calculations of wall density from the chemically measured content of wall substances and the approximate volumes of walls measured from electron-microscope pictures, seem to differ very much more from directly determined values than would be expected (Kelemen and Rogers, 1971; Ou and Marquis, 1972). Moreover, densities of isolated walls determined by various more direct methods differ more among themselves than seems easy to explain, except by very considerable expansion and contraction of the preparations according to their treatment.

B. EXPANSION AND CONTRACTION OF THE WALL

The physical properties of the peptidoglycan meshwork are likely to be greatly modified by attachment to the glycan chain of highly negatively-charged teichoic or teichuronic acid molecules. The effect of the removal of teichoic acid from wall preparations on their physical properties has been studied. For example, the dextran-impermeable volume of walls of *Staph. aureus* is regulated not only by the presence or absence of teichoic acid but markedly by the amount of D-alanine that is ester linked to teichoic acid (Ou and Marquis, 1970). Removal of esterified amino acid, leaving the *N*-acetylglucosaminyl substituted polyribitol phosphate still in place, doubled the wall volume impermeable to large dextrans; it changed from 5.1 to 10.1 ml per g. Subsequent removal of the substituted polyribitol phosphate lowered this value to 6.2 ml per g. Changes in surface location of teichoic acids, or even more of the D-alanine substituent on it, within the wall of growing organisms could possibly have profound effects upon the morphology of the wall and hence upon cell shape.

Extensibility of the envelope has been demonstrated by other approaches. Examples from two groups of workers (Knowles, 1971;

Matts and Knowles, 1971; Marquis, 1968; Ou and Marquis, 1970) show that the dimensions of living bacteria can be changed by altering the balance of forces exerted on the envelope. This can be done by increasing the concentration of solutes on the outside, using compounds such as sucrose. Volumes of the cells can be deduced from changes in the extinction value of the suspension (Koch, 1961). This method must be applied with circumspection, and possibly a better one is to use measurements of the volume of the suspension that is impenetrable to high molecular-weight substances such as dextrans. Applied both to isolated wall preparations and to whole cells of *Micrococcus luteus*, *Staphylococcus aureus* (Ou and Marquis, 1970) and *B. megaterium* (Marquis, 1968), this method showed a small decrease of 10–33% in cell volume for the whole bacteria when transferred to concentrated sucrose solutions. This change was deduced to be associated with a degree of plasmolysis. Definite plasmolysis could not be seen under the microscope, but could be estimated from the decrease in the sucrose-impermeable volumes of cells. Leakage of cytoplasmic contents was not detected, so that gross damage to the cytoplasmic membrane had presumably not occurred. The volumes of cell-wall preparations, at least of *B. megaterium* (Marquis, 1968), were not affected by concentrated solutions of sucrose. Greater alterations in the volumes of bacteria could be obtained by varying the ionic strength of the medium. Decreases of the order of 25–30% were found with *B. megaterium* and *M. luteus* in progressing from an external ionic strength of 0.001 to 1.0. The changes obtained with staphylococci were much smaller, of the order of 9% (Ou and Marquis, 1970). Similar changes in the volumes of isolated cell-wall preparations from the former two organisms were obtained with altered ionic strength. The patterns of change with wall preparations and increases in ionic strength differed considerably from those obtained with whole cells. They started at lower ionic strengths and decreased more rapidly. Similar, rather large, effects on the volumes of isolated wall preparations from these two organisms could also be obtained by varying the pH value of the suspending media at constant ionic strength. Significant changes could be obtained by varying the pH value but not the ionic strength of suspending media for wall preparations from *Staph. aureus*. These latter results were complicated, and are possibly explained by removal of esterified D-alanine from teichoic acid at the higher pH values. Such losses incur their own increase in wall volume, as has been noted, and cause a hysteresis in the volume/pH value curves. It seems reasonable to conclude from these

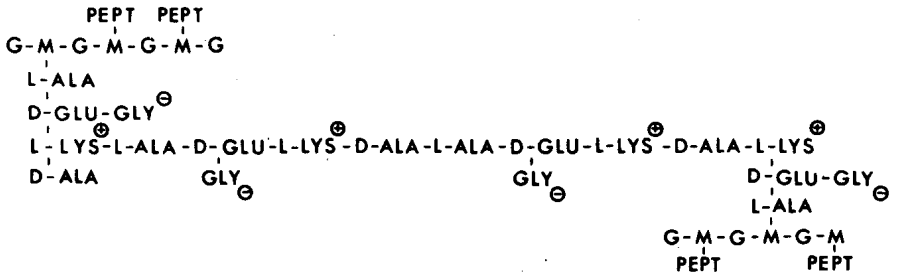

FIG. 3. One part of the peptidoglycan of *Micrococcus luteus* showing potential fixed charges in the structures. PEPT indicates peptidoglycan.

results that small volume changes in the whole cells of *M. luteus*, *B. megaterium* or *Staph. aureus* can occur due to gross alterations in the osmotic forces acting on the envelope. Larger changes in the two former organisms can be obtained by varying the ionic strength or pH value of the medium. The effects on the walls are consistent with alterations in ionization of groups fixed within the wall polymers.

As can be seen from Fig. 3, a number of charged groups exist within the peptidoglycan structure, apart from those due to polymers attached to it. When charged groups of like sign are relatively close together, they will repel each other, tending to expand the wall. Such expansion presumably occurs at the expense of the conformation of the peptide chains in the peptidoglycan. The $1\rightarrow4\beta$ linkages of the sugar residues in the glycan chains are such as to make impossible a tightly coiled configuration which would allow expansion. The differences between the behaviour of walls and cells at different ionic strengths probably reside in the absence of osmotic pressure on wall preparations which would oppose wall contraction in whole cells. Thus, the volume changes in cells are the result of a balance between osmotic effects and electrostatic forces in the walls.

The mechanisms underlying volume changes of Gram-negative bacteria have been the subject of some disagreement between the protagonists of purely osmotic forces and purely electrostatic ones. In general, when Gram-negative bacteria are moved to fluids containing higher concentrations of low molecular-weight solutes, the optical density of the suspensions increases rapidly (e.g. Mager *et al.*, 1956). This should indicate a decrease in the volume occupied by the micro-organisms, if due precautions have been observed (Koch, 1961). Careful study has been made of the relationship of such increases in optical