THE BACTERIAL CELL SURFACE

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PREFACE

It is a common statement that because of its simplicity the bacterial cell makes an ideal model for the study of a wide variety of biological systems and phenomena. While no-one would dispute that much of our understanding of biological function derives from the study of the humble bacterium, the concept of a simple life-form would be hotly disputed by any scientist engaged in the determination of the relationship between structure and function within the bacterial cell. Bacteria are particularly amenable to intensive study; their physiology can be probed with powerful biochemical, genetical and immunological techniques. Each piece of information obtained inevitably raises as many questions as answers, and can lead to a highly confused picture being presented to the lay reader. Nowhere is this more evident than in the study of the surface layers of the bacterial cell. Examination of the early electron micrographs suggested that the bacterial cytoplasm was surrounded by some sort of semi-rigid layer, possessing sufficient intrinsic strength to protect the organism from osmotic lysis. The belief that the surface layers were rather passive led to their neglect, while researchers concentrated on the superficially more exciting cytoplasmic components. Over the last twenty years our view of the bacterial envelope has undergone extensive revision, revealing a structure of enormous complexity. In this short monograph we have attempted to explain the known properties of the bacterial envelope and hope to convince the reader that the surface represents a highly integrated series of components that serve as an interface between the metabolic activity of the organism and its environment. In the early chapters we describe the structure and biosynthesis of those layers lying exterior to the cytoplasmic membrane. In the later sections we have attempted to relate the importance of these surface structures to the life of the organism in its natural habitat. At the end of each chapter we suggest some review-type articles which extend and complement the given text.

We are grateful to those people in our respective institutions who have contributed to the production of this book. During the writing of the book we have relied extensively upon the published work of scientists of many disciplines. In attempting to produce a concise, and we hope readable, account we have been unable to acknowledge more than a handful of the research workers involved by name; to these unnamed scientists we dedicate this book.

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CONTENTS

1 THE PEPTIDOGLYCAN LAYER

The bacterial cytoplasm is enclosed by a rigid, highly structured layer of great mechanical strength, termed the cell wall. The wall is responsible for the maintenance of the shape and integrity of the cell. The cytoplasmic membrane which surrounds the bacterial protoplast has little intrinsic strength and if not overlaid by the wall would be ruptured by the high internal osmotic pressure, if the organism were placed in hypertonic solution. The wall's great strength derives from the unique highly cross-linked aminosugar polymer, called peptidoglycan, of which the wall is built. The importance of the bacterial cell wall to the organism can be demonstrated by treatment with enzymes or certain antibiotics. Many body fluids (e.g. serum, tears) and leucocytes, contain the enzyme lysozyme, which is an important component in the host defences against invading bacteria. Addition of the enzyme to a suspension of a Gram-positive bacterium, e.g. *Bacillus subtilis*, degrades the peptidoglycan, the cells rapidly lyse and the suspension becomes clear and viscous due to the release of the bacterial chromosomal material (DNA) as the cells burst. Lysozyme appears to have unrestricted access to the peptidoglycan of Gram-positive cells. In Gram-negative bacteria the peptidoglycan is masked by a protective layer, the outer membrane (Chapter 3), which prevents lysozyme from penetrating through to the peptidoglycan. Lysis by lysozyme of Gram-negative bacteria can only be achieved by first increasing the permeability of the outer membrane by treatment with a chelating agent, such as ethylenediamine-tetra-acetic acid (EDTA). EDTA exhibits strong binding affinity for metal ions, especially magnesium. In the presence of the organic buffer, tris-hydroxymethylaminomethane, treatment with EDTA withdraws magnesium ions from the outer membrane, leading to the release of specific outer membrane components and greatly increasing the permeability of the membrane. Only under these conditions can lysozyme gain access to the underlying peptidoglycan and bring about cell lysis. Many antibiotics, particularly those possessing a β-lactam ring structure, e.g. penicillins and cephalosporins, bring about their lethal action by inhibiting a number of enzymes involved in peptidoglycan assembly. This leads to a progressive weakening of the cell wall until the structure possesses insufficient strength to resist the internal turgor pressure. Often bulges develop at the site of cell division, which may enlarge until the cells ultimately burst.

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Structure of Peptidoglycan

Determination of the composition of bacterial peptidoglycan must be preceded by the preparation of bacterial cell walls free from contamination by other cell constituents. Gram-positive bacteria are susceptible to mechanical breakage, usually achieved by vigorous shaking in the presence of small glass beads in an apparatus such as the Braun Disintegrator. Walls can be readily separated from such a suspension by differential centrifugation and freed from contamination by exhaustive washing.

Approximately 50 per cent of the weight of the Gram-positive cell wall is peptidoglycan, the remainder made up of a variety of polymers depending upon the organism. These accessory polymers, teichoic acids, teichuronic acids and proteins will be discussed in Chapter 2. In order to study the chemical composition of the peptidoglycan, enzymic or chemical treatments are used to cleave the covalent bonds linking the accessory polymers to the peptidoglycan, producing pure peptidoglycan. In Gram-negative organisms, peptidoglycan makes up only 10–20 per cent of the weight of the wall. Although this percentage is much lower than in Gram-positive organisms the peptidoglycan layer is of no less importance to the life of the cell. Purification of Gram-negative peptidoglycan also begins with the mechanical rupture of the cells, usually by extruding the cell suspension through a narrow constriction in a pressure cell, e.g. as in the French Press. The Gram-negative layer is covalently linked by lipoprotein molecules to an outer membrane consisting of phospholipid, protein, lipopolysaccharide and polysaccharide. The peptidoglycan can be readily freed from the majority of the outer membrane components by heating the wall fraction with an anionic detergent such as sodium dodecyl sulphate (SDS), followed by extensive washing to remove the detergent. However, simple detergent-treatment cannot free the lipoprotein which remains covalently attached to the peptidoglycan. Its removal can be effected by treatment with proteolytic enzymes. When stripped of all its associated polymers peptidoglycan is an insoluble macromolecule, which retains the characteristic shape of the original cell from which it was prepared (Figure 1.1).

The chemical composition and structure of the peptidoglycan has been determined for many bacterial species. Although there is considerable variation in detailed composition amongst different organisms, the basic structure is essentially the same. Peptidoglycan is made up of a network of linear polysaccharide chains (glycan strands), up to 200 disaccharide units in length, cross-linked by short peptide chains. The structure of the peptidoglycan found in *Escherichia coli* (Figure 1.2) is typical of most other Gram-negative bacteria and many bacilli (i.e. rod-shaped bacteria). The glycan strands are composed of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid joined by a 1,4-β glycosidic linkage

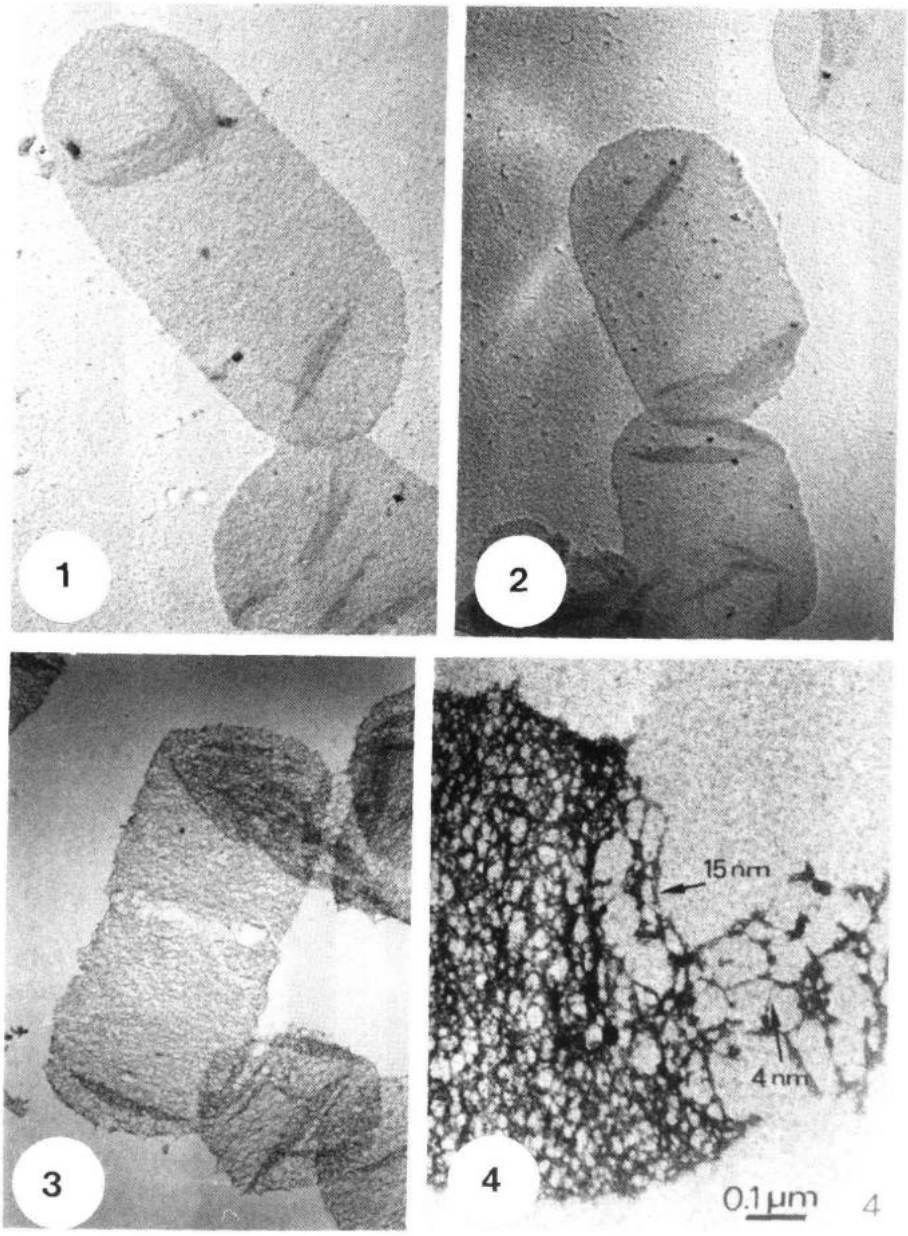

Figure 1.1: Electron Micrograph Showing Isolated Purified Sacculi from *Escherichia coli*. 1 and 2 show the purified sacculi which is in effect one enormous peptidoglycan molecule, 3 and 4 show the sacculi after treatment with specific endopeptidase. It can be seen that the glycan chains appear to run perpendicular to the long axis of the cell. Reproduced by permission, Verwer, R.W., Nanniga, N., Keck, W. and Schwartz, U. (1978) *J. Bacteriol., 136*, 723.

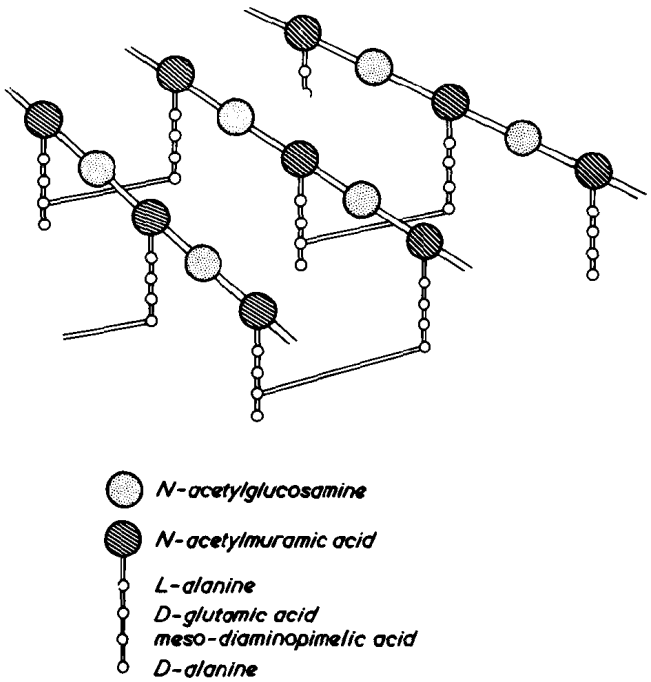

Figure 1.2: Generalised Representation of the Structure of Bacterial Peptidoglycan

(Figure 1.3). *N*-acetylmuramic acid is unique to peptidoglycan and is a derivative of *N*-acetylglucosamine, bearing an ether-linked D-lactyl on carbon 3. To the carboxyl group of each muramic acid residue is attached a chain of four amino acids, joined by a peptide bond. The amino acids have alternating L and D centres, the sequence being L-alanine, D-glutamic acid, meso-diaminopimelic acid and D-alanine. D-amino acids are rare in nature, their occurrence being confined chiefly to peptidoglycan. The third amino acid of the sequence, meso-diaminopimelic acid is also unique to peptidoglycan, and is a symmetrical molecule with two centres of optical activity, one D and one L (hence the name meso). The tetrapeptide chains on adjacent glycan strains are linked together by various means to give a cross-linked polymer. In the case of most Gram-negative bacteria and many bacilli the cross-linking is formed by a direct peptide linkage between the carboxyl group of the fourth amino acid on one glycan chain, D-alanine, and the free amino group of the third amino acid on an adjacent chain, i.e. meso-diaminopimelic acid (Figure 1.4). Similar linkages at other points along the glycan strands build up the cross-linked structure of peptidoglycan. It is thought that the peptidoglycan component of the bacterium is

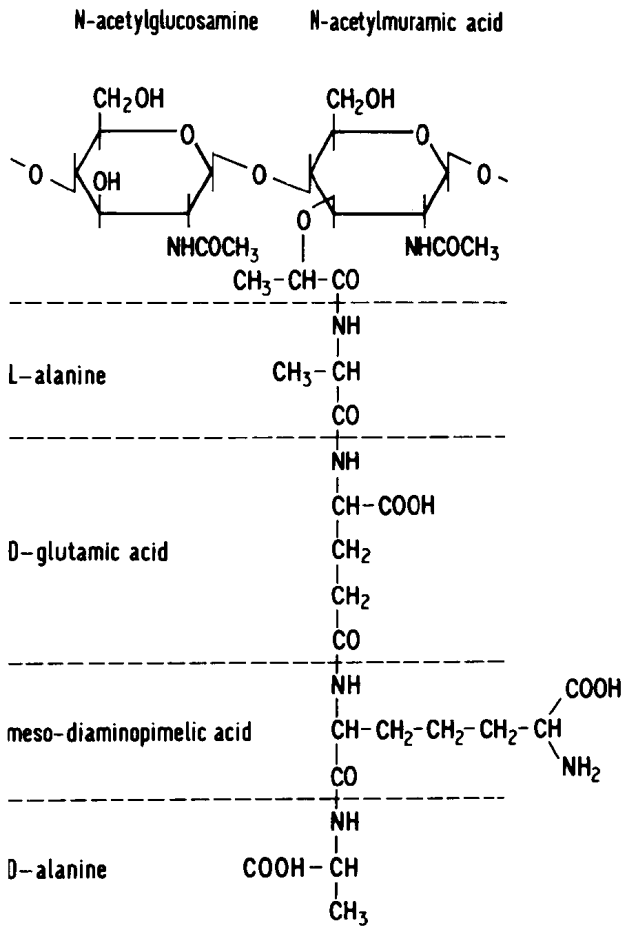

Figure 1.3: Linkage of *N*-acetylmuramic Acid and *N*-acetylglucosamine in the Glycan Strands and Attachment of Amino Acids to Muramic Acid

a single macromolecule covering the entire cell surface. The number of tetrapeptide chains which participate in cross-linking varies considerably from organism to organism. It can be as low as 20 per cent in Gram-negative organisms, such as *E. coli*, with the majority of tetrapeptides remaining unlinked. In contrast, in the Gram-positive organism *Staphylococcus aureus*, the cross-linking may be greater than 90 per cent. In this organism a bridge of five glycine residues links the terminal carboxyl group of D-alanine in one chain to the free amino group of the third amino acid on an adjacent chain, which in this case is L-lysine (Figure 1.5). The

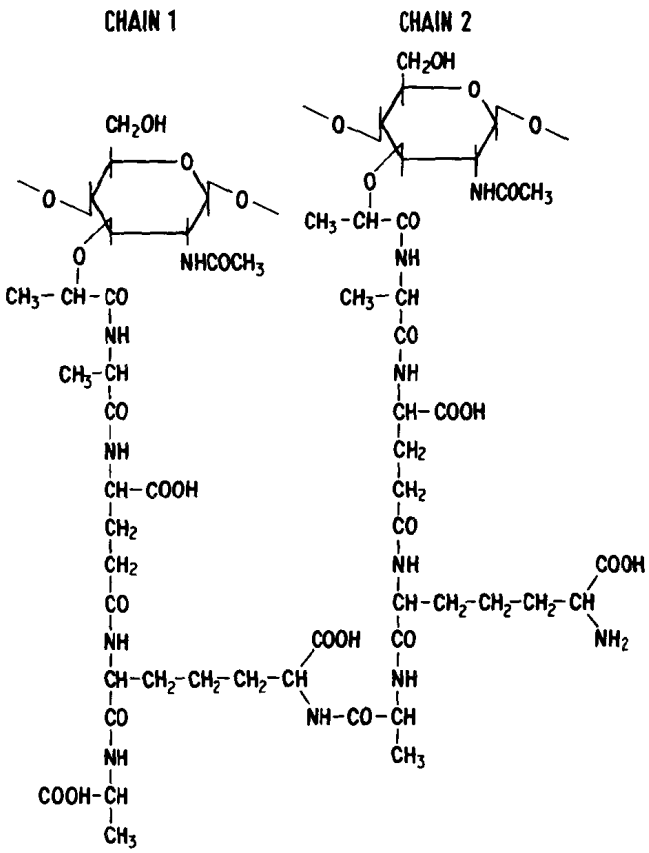

Figure 1.4: Formation of the Peptide Cross-links Between Adjacent Glycan Strands. The linkage is formed by a peptide bond between the ε-NH_2 group of diaminopimelic acid on chain 1 and the carboxyl group of the terminal D-alanine on chain 2

peptidoglycan of *S. aureus* has relatively short glycan chains, which necessitates the high degree of cross-linking to produce a structure of equivalent strength to that of other bacteria.

Variations in Composition

Detailed examination of the peptidoglycan from a range of bacteria has revealed many variations in chemical composition, for example the nature of the amino acids joined to muramic acids and forming the cross-links between glycan strands. Attempts have been made to classify peptidoglycans according to the way in which the cross-links are formed. The

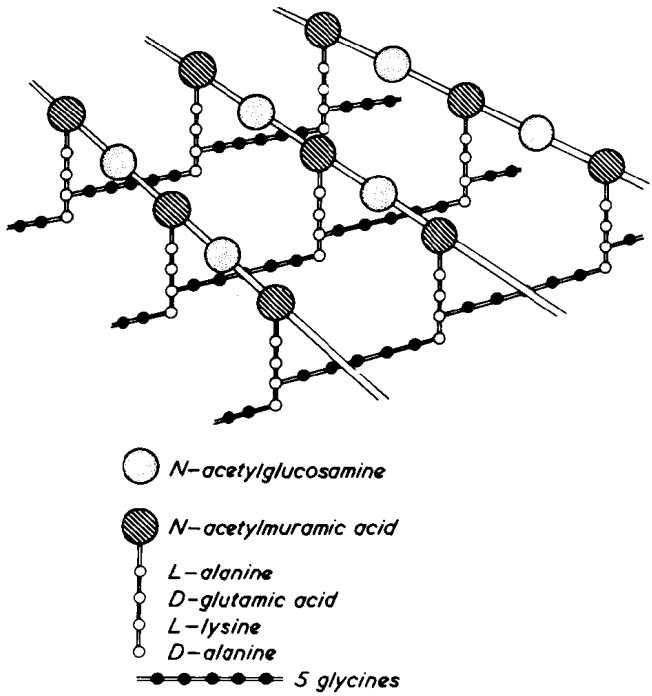

Figure 1.5: Structure of Peptidoglycan in *Staphylococcus aureus*

major variation concerns the amino acid at position 3 in the peptide chain on muramic acid. Although meso-diaminopimelic acid is by far the most common occupant of the 3-position, L-lysine is found in many cocci (e.g. *Staphylococcus aureus, Micrococcus luteus, Sarcina flava, Streptococcus faecium, Leuconostoc mesenteroides* and *Gaffkya homari*) and some other species contain L-ornithine, L-diaminobutyric acid or L-homoserine. The cross-linkage in most Gram-negative and Gram-positive bacilli is directly formed between the free amino group of the amino acid in position 3 and the terminal carboxyl group of the D-alanine at position 4 on an adjacent glycan strand. In some other organisms an intervening chain of amino acids is present between the glycan chains; for example in *S. aureus* five glycine residues link the two peptide chains on adjacent glycan polymers. In *M. luteus* this bridging polypeptide has the same sequence as the peptide bound to the muramic acid (i.e. L-Ala-D-Glu-L-Lys-D-Ala). In this species a number of muramic acid residues are unsubstituted, suggesting that the bridging polypeptide is derived from tetrapeptides that have been detached from muramic acids.

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The D-glutamate residue at position 2 in the muramic acid tetrapeptide is also subject to modification. It should be noted that this moiety is usually linked to the first residue, L-alanine, by its α-amino group (i.e. a normal peptide linkage), but to the third residue through a peptide linkage involving its γ-carboxyl group, leaving the α-carboxyl free. In some species, including *S. aureus, Corynebacterium diphtheriae, Clostridium perfringens* and *Lactobacillus plantarum* the α-carboxyl group of D-glutamate is present as an amide; in *M. luteus* it is modified by addition of a glycine residue and in other species it remains unsubstituted. Diaminopimelic acid also contains a free carboxyl group (at the D centre), which is not involved in any linkage. This group can be unsubstituted (e.g. *E. coli* and *Bacillus megaterium*) or amidated (e.g. *C. diphtheriae, B. subtilis* and *L. plantarum*). In species where lysine occupies position 3 no free carboxyl is present.

Variations also occur in the muramic acid itself. In *S. aureus* approximately half of the muramic acid residues bear an acetyl group on the 6 position. This modification renders the peptidoglycan insensitive to degradation by lysozyme. Similar modifications have been reported in some strains of *Proteus, Neisseria* and *Pseudomonas.* Loss of some of the *N*-acetyl residues from the *N*-acetylglucosamine of some *Bacillus cereus* strains also has the effect of rendering the peptidoglycan resistant to lysozyme.

Despite the numerous variations encountered in the peptidoglycan backbone, the peptide side chains and the bridge linking the chains, the overall structure of the peptidoglycan is essentially constant. It forms a cross-linked lattice structure which is responsible for the shape and integrity of the bacterial cell wall (Figure 1.1).

The enzyme lysozyme breaks the linkages between the *N*-acetylmuramic acid and *N*-acetylglucosamine units. The structure of lysozyme has been determined by X-ray diffraction and found to contain a cleft, or groove, into which the glycan strand fits perfectly. Once attached to the groove, or active site, the glycosidic bond is broken, significantly weakening the peptidoglycan lattice. The acetylation of muramic acid residues in *S. aureus,* prevents effective binding of lysozyme to the peptidoglycan. However, peptidoglycan of *S. aureus* is sensitive to the enzyme lysostaphin. Lysozyme and lysostaphin are both *N*-acetylmuramidases, capable of hydrolysis of the *N*-acetylmuramyl-1,4-β-*N*-acetylglucosamine bonds in the glycan chains.

A number of enzymes capable of degrading peptidoglycan at other points has been identified. For example, the glycan chains can also be broken by *N*-acetylglucosaminidase; the linkage between *N*-acetylmuramic acid and the peptide units is broken by *N*-acetylmuramyl-L-alanine amidase and the peptide bridges between glycan chains can be hydrolysed by endopeptidase (Figure 1.6). Nearly all bacteria produce enzymes capable of degrading their own peptidoglycan. These autolysins are found

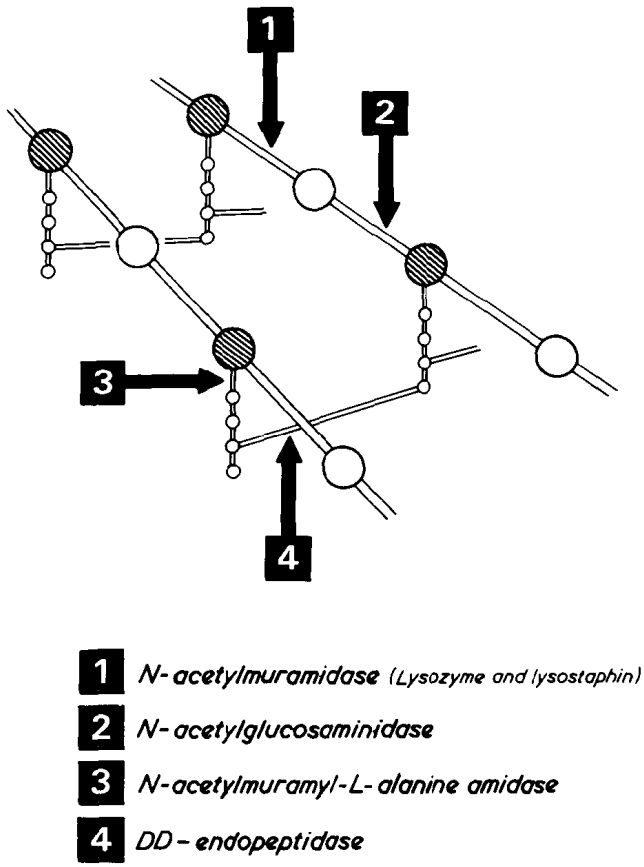

Figure 1.6: Sites at which Enzymes Attack Peptidoglycan (Symbols for peptidoglycan structure as in Figure 1.2)

in the cell walls of the organism and their function seems to be to modify existing peptidoglycan permitting extension of the wall during cell growth and separation of the cells during division. Clearly their activity must be carefully regulated or these enzymes would rapidly degrade the bulk peptidoglycan ultimately causing cell autolysis. The enzymes are actually bound to, and inhibited by, the polymers in the cell wall. Understanding of the mechanism by which tailoring of existing peptidoglycan and insertion of new material is controlled in the growing bacterial cell in such a way that the daughter cells retain the characteristic shape presents an enormous challenge to microbial biochemists.

Arrangment of Peptidoglycan in the Wall

Surprisingly little firm evidence is available to indicate the way in which peptidoglycan is arranged in the cell wall. The glycan chain length of the peptidoglycan of Gram-positive bacilli is about 100 disaccharide units. This extended polymer represents a length of approximately 0.1 μm. A rod-shaped organism 5 μm long and 0.5 μm wide would require 50 glycan chains to extend the length of the cell, or about 15 to wrap around the circumference of the short axis of the cell. There is some evidence to suggest that in some bacteria, e.g. *B. subtilis* and *E. coli*, the glycan chains are orientated so they wrap around the short axis of the cell, perpendicular to the long axis. Direct observation of *B. subtilis* walls under the electron microscope reveals striations aligned around the short axis circumference of the organism, which persist even after removal of accessory polymers. A similar pattern can be seen in *E. coli* after careful controlled digestion of some of the peptidoglycan cross-links with endopeptidases, thereby loosening the polymer network (Figure 1.1).

Attempts have been made to use X-ray diffraction to determine the exact molecular arrangement of peptidoglycan. Unfortunately the X-ray diffraction patterns produced to date do not give a clear idea of the structure. Suggestions have been made that the glycan chains are arranged in a helix, similar to that of cellulose or chitin, which are linear polymers of poly-β1→4-glucose and poly-β1→4-*N*-acetylglucosamine respectively, but other data fail to support this view. The difficulties encountered in obtaining sharp X-ray diffraction patterns indicate that the walls do not contain large areas in which the peptidoglycan has ordered repeat structure. This presumably reflects the way in which the polymer is synthesised and inserted into the existing wall during cell growth.

Biosynthesis of Peptidoglycan

Bacteria are faced with the problem of expanding their cell wall in order to grow and divide. In particular, the peptidoglycan matrix must be extended in a controlled way so that the shape and integrity of the cells are preserved. At first sight it is difficult to imagine how the cells achieve this: peptidoglycan forms an enormous cross-linked network which is outside the cytoplasmic membrane and therefore outside the direct metabolic control of the cell. Synthesis of the precursors occurs inside the cell with the utilisation of considerable metabolic energy. How are they assembled, transferred across the cytoplasmic membrane, and finally inserted into the wall?

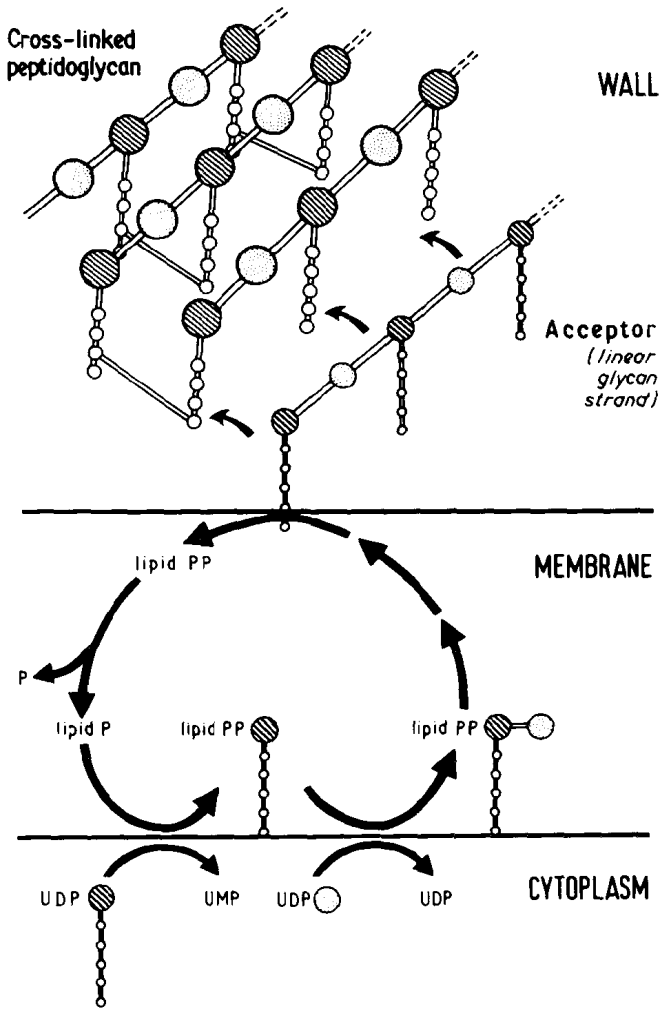

Figure 1.7: Sequence of Peptidoglycan Biosynthesis in *Escherichia coli*. Sugar nucleotides transfer *N*-acetylglucosamine (with its attached pentapeptide) and *N*-acetylmuramic acid to the C_{55}-isoprenoid lipid carrier which serves to translocate the precursor across the cytoplasmic membrane. At the outer face of the membrane the wall precursor is transferred to the growing linear glycan strand, releasing the isoprenoid for another round of synthesis. The acceptor glycan is subsequently cross-linked with existing wall peptidoglycan. (The symbols used for peptidoglycan structure are as in Figure 1.2)

Synthesis of Precursors

The biosynthesis sequence can be divided into three stages: synthesis of the precursors in the cytoplasm; transfer of the precursors to a lipid carrier molecule which transports them across the membrane; insertion into the

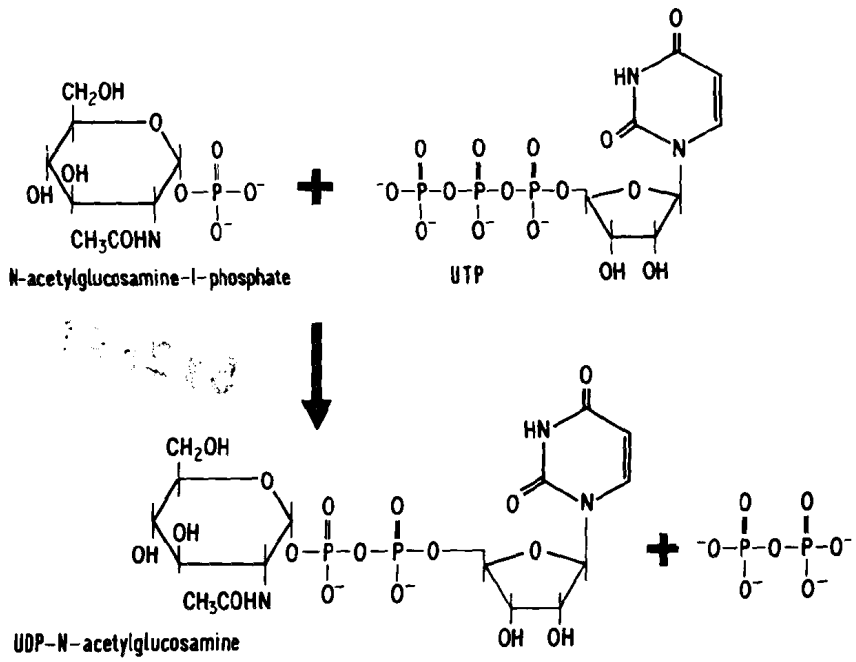

Figure 1.8: Structure of UDP-*N*-acetylglucosamine and its Synthesis from UTP and *N*-acetylglucosamine-1-phosphate

wall and coupling to existing peptidoglycan (Figure 1.7). The precursors synthesised in the cytoplasm are the sugar nucleotides: UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramylpentapeptide. In nature most polysaccharides are synthesised from sugar nucleotides: the energy for formation of the polymers being provided by hydrolysis of the sugar-nucleotide linkage. UDP-*N*-acetylglucosamine is the precursor of *N*-acetylglucosamine in many polymers and is found in a wide variety of cells. It is synthesised from UTP and *N*-acetylglucosamine-1-phosphate (Figure 1.8) by the enzyme UDP-*N*-acetylglucosamine pyrophosphorylase. By contrast, *N*-acetylmuramic acid is unique to peptidoglycan and its precursor, UDP-*N*-acetylmuramylpentapeptide is used exclusively by bacteria. It is synthesised from UDP-*N*-acetylglucosamine by addition of a 3-carbon fragment from phosphoenolpyruvate to the 3-position of *N*-acetylglucosamine (Figure 1.9). The pyruvyl group is reduced by an NADPH-reductase enzyme giving UDP-*N*-acetylmuramic acid, the 3-*O*-D-lactyl ether of UDP-*N*-acetylglucosamine.

To function as the precursor of peptidoglycan UDP-*N*-acetylmuramic acid is converted into the pentapeptide. A chain of five amino acids is

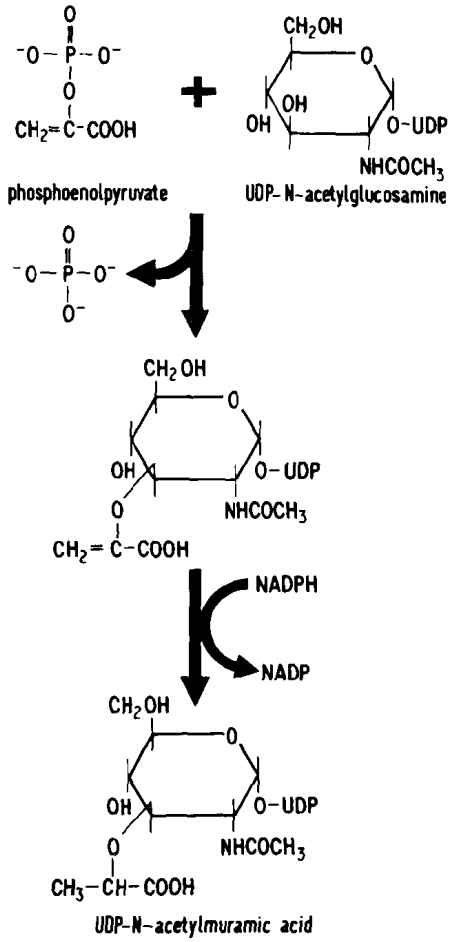

Figure 1.9: Structure of UDP-*N*-acetylmuramic Acid and its Synthesis from UDP-*N*-acetylglucosamine and Phosphoenolpyruvate

linked by an amide bond to the free carboxyl of the lactyl ether substituent, giving the sequence: *N*-acetylmuramic acid-L-alanine-D-glutamic acid-meso-diaminopimelic acid-D-alanine-D-alanine. Note that at this stage the precursor has an extra D-alanine residue which is removed during incorporation into peptidoglycan leaving the familiar tetrapeptide. The first three amino acids are added sequentially to UDP-*N*-acetylmuramic acid by specific enzymes which use ATP as an energy source. The remaining two D-alanine residues are added together as a pre-formed dipeptide, D-alanyl-D-alanine (Figure 1.10). The dipeptide is synthesised from L-alanine by alanine racemase and D-alanyl-D-alanine ligase. The first enzyme converts two molecules of L-alanine into the D forms and the

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