In *Bacterial Membranes and Walls*, vol. 1, ed. L. Leive, Dekker Inc., New York, 1973, pp. 37-130.

Chapter 2

BIOSYNTHESIS OF PEPTIDOGLYCAN

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I. INTRODUCTION

Modern study of the main supporting structure of the bacterial cell started in the 1950's when the external surface structure, i.e., the cell wall, was first isolated from mechanically disrupted bacteria [1]. Walls isolated from cocci were found to be spherical, whereas walls isolated from bacilli were found to be cylindrical. It was soon discovered that the shape and tensile strength of the wall was imparted by a polymer, which, depending upon the bacterial species, represented 5% to perhaps 90% of the isolated walls. In marked contrast with other cell-supporting exostructures that consist of α-cellulose, hemicellulose, glucan, mannan, or chitin, the rigid matrix of the wall was characterized as an heteropolymer consistently composed of two different acetamido sugars and of from four to perhaps as many as eight different amino acid residues. This type of heteropolymer appears to be ubiquitous in the prokaryotic world except for Mycoplasma and some L-forms and halophiles. The two acetamido sugars were identified as 2-acetamido-2-deoxy-D-glucose (i.e., N-acetyl-D-glucosamine) and a previously unknown sugar 2-acetamido-2-deoxy-3-O-(D-1-carboxyethyl)-D-glucose (i.e., the 3-O-D-lactic acid ether of N-acetyl-D-glucosamine or N-acetylmuramic acid). In addition to these two acetamido sugars, L-alanine, D-alanine, and D-glutamic acid were always found to be present. There were also other amino acid residues, most often L-lysine or meso-diaminopimelic acid. Muramic acid, D-alanine, D-glutamic acid, and meso-diaminopimelic acid have been found only in prokaryotic cells. The occurrence of these compounds was thus a very striking feature that showed the uniqueness of bacterial walls. It was also observed that N-acetylglucosamine, N-acetylmuramic acid, L-alanine, D-alanine, D-glutamic acid, and either L-lysine or meso-diaminopimelic acid usually occurred in approximately equimolar amounts. This observation led to the conclusion that the assembly of the rigid part of the wall is probably brought about by polymerization of disaccharide-peptide units. The polymer was called peptidoglycan (glycopeptide, mucopeptide, glycosaminopeptide, or murein being synonymous to peptidoglycan).

The essential role played by the peptidoglycan in keeping the cell alive under ordinary hypotonic environmental conditions was established by several techniques. Both hen egg-white lysozyme, when added to resting bacteria, or in some cases to growing bacteria, and penicillin, when added to growing bacteria, were shown to result in dissolution of the cells. Lysis, however, could be prevented at least for a limited period of time,

if the external medium contained a solute to which the cell was impermeable, at a concentration that approximately balanced the high osmotic pressure of the cell [2]. Under these conditions, bacteria were seen to undergo transformation into spherical bodies. These physiologically active but osmotically fragile bodies were called protoplasts or spheroplasts [2a, 2b]. Essentially, they are either wall-less bacteria (i.e., protoplasts) or bacteria with some defect in their wall peptidoglycan component (i.e., spheroplasts). The spherical appearance and osmotic fragility of protoplasts and spheroplasts generally has been taken as evidence for the shape-maintaining and osmotically protective nature of the peptidoglycan. The targets of lysozyme and penicillin were thus seemingly the same or similar. In fact, although the overall target, the peptidoglycan, is the same, the mechanism of action of each is entirely different. Lysozyme enzymatically hydrolyzes insoluble peptidoglycans into soluble fragments. Penicillin acts as an inhibitor of the synthesis of insoluble and shape-maintaining peptidoglycan.

Studies of penicillin action on growing cultures of Staphylococcus aureus revealed that the intoxicated bacteria accumulate large amounts of a series of compounds containing uridine diphosphate sugars. The largest of these was later shown [3] to contain muramic acid, L-alanine, D-glutamic acid, L-lysine, and D-alanine in the molar ratio 1:1:1:2. Similar compounds were found in low concentration in many bacterial species. Because of their similarity in composition, these nucleotides appeared to be precursors of the peptidoglycan. Consequently, penicillin was thought to specifically inhibit the biosynthesis of the wall peptidoglycan.

An exhaustive survey of these early studies of the wall was presented by Salton in 1964 [1]. These studies prepared the way for future investigations dealing with the topology of the wall peptidoglycan in the cell envelope, its chemical structure, the reaction sequence and control sites involved in its biosynthesis and mode of growth, the functioning of the membrane in the process and the mechanism of action of several antibiotics. At present, many intriguing and fundamental questions are not yet satisfactorily answered. A number of monographs and recent reviews summarize much of this information [1, 4-14a].

II. STRUCTURE OF PEPTIDOGLYCAN

A. Primary Structure of Peptidoglycan

The wall peptidoglycan can be considered to be a single, enormous macromolecule that forms a more-or-less continuous network around the cellular permeability barrier and provides the cell with a supporting

structure of high tensile strength [1, 4, 5, 8, 11]. Basically, the network is composed of glycan strands that are interconnected through peptide chains (Figs. 1 and 2). Despite many variations, there is a remarkable consistency of structure of the wall peptidoglycans throughout the bacterial world.

1. The Glycan Strands

Basically, the glycan moiety consists of linear strands of alternating β-1,4-linked pyranoside-N-acetylglucosamine and N-acetylmuramic acid residues [15, 16] (Fig. 3). It is thus a chitin-like structure in which each alternate N-acetylglucosamine residue is ether-linked at C-3 to a lactyl group that has the D-configuration [17]. The carboxyl of the lactyl groups provides the point to which peptides are amide-linked to the glycan strands. Several variations of the glycan chains are known (Fig. 4). (1) In several Gram-positive bacteria [5] and in at least one Gram-negative species, Proteus vulgaris [18], some of the N-acetylmuramic acid residues are O-acetylated on C-6. (2) In many Gram-positive bacteria, the C-6 of some N-acetylmuramic acid residues is substituted by phosphodiester groups, which covalently link other wall polymers to the peptidoglycan matrix [19, 20, 21]. (3) Muramic acid can occur as N-glycolylmuramic acid, a structural feature, which thus far has been found to be unique to Nocardia and Mycobacterium sp. [22]. (4) In spores of Bacillus subtilis, a high proportion of the muramic acid residues occur in the form of a lactam derivative [23]. The same derivative has been found in trace amounts in Micrococcus lysodeikticus [24]. (5) A recent survey including species of Gram-positive and Gram-negative bacteria from a wide variety of taxonomic groups failed to detect the presence of galactosamine and galactomuramic acid in peptidoglycans [25, 26]. Only glucosamine and gluco-muramic acid were identified in the bacterial species examined. Small amounts of manno-muramic acid, however, were reported to occur along with gluco-muramic acid in Micrococcus lysodeikticus [24]. None of these variations would significantly affect the three-dimensional organization of the glycan chains [see Section II, D below].

2. The Tetrapeptide Units [5]

The D-lactyl groups of the muramic acid residues in the glycan strands, or at least some of them, are substituted by tetrapeptide units. These units have the general sequence L-alanyl-γ-D-glutamyl-L-R_3-D-alanine (Fig. 5). Except for the bond between the D-glutamyl residue and the L-R_3 residue, which is γ-linked, all peptide linkages are α. The L-R_3 residue varies. For example, it may be a neutral amino acid, such

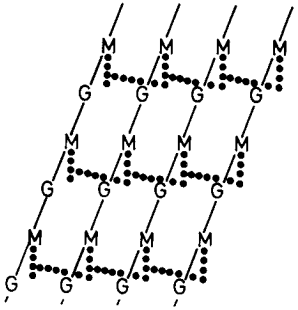


FIG. 1. Schematic representation of a wall peptidoglycan. Glycan chains are composed of N-acetylglucosamine (G) and N-acetylmuramic acid (M). Vertical dots from M represent the amino acids of the tetrapeptide subunits. Horizontal dots represent the peptide cross-linking bridges. Five bridging amino acids are shown, corresponding to the peptide bridges of Staphylococcus aureus presented in Fig. 2. [Reprinted from reference 9 by courtesy of American Elsevier Publishing Co., New York.]

as L-alanine or L-homoserine, or a dicarboxylic amino acid such as L-glutamic acid [26a], or a diamino acid such as L-2, 4-diaminobutyric acid, L-ornithine, L-lysine, L-hydroxylysine, LL-diaminopimelic acid, or meso-diaminopimelic acid. When meso-diaminopimelic acid is at the R_3 position, both the amino group, which is linked to D-glutamic acid, and the carboxyl group, which is linked to D-alanine, are located on the same L-carbon. Variations other than those occurring at the L-R_3 position are known. (1) The α-carboxyl group of D-glutamic acid can be either free, amidated, substituted by a C-terminal glycine or by a glycine amide [26b]. (2) The carboxyl group of diaminopimelic acid not engaged in a peptide bond may be substituted by an amide. (3) threo-3-Hydroxylglutamic acid [26a] can occur instead of glutamic acid. (4) L-Alanine at the N-terminus of the tetrapeptide can be replaced by L-serine or glycine. The aforementioned variations, however, fail to significantly alter the basic structure of the tetrapeptide backbone. Except for the occasional appearance of glycine at the N-terminal position, the backbone of all tetrapeptides exhibits an L-D-L-D sequence.

3. The Interpeptide Bridges

The peptide units belonging to adjacent glycan strands are, in turn, cross-linked through specialized bridges. The C-terminal D-alanine residue of one tetrapeptide unit is always involved in the bridging to a

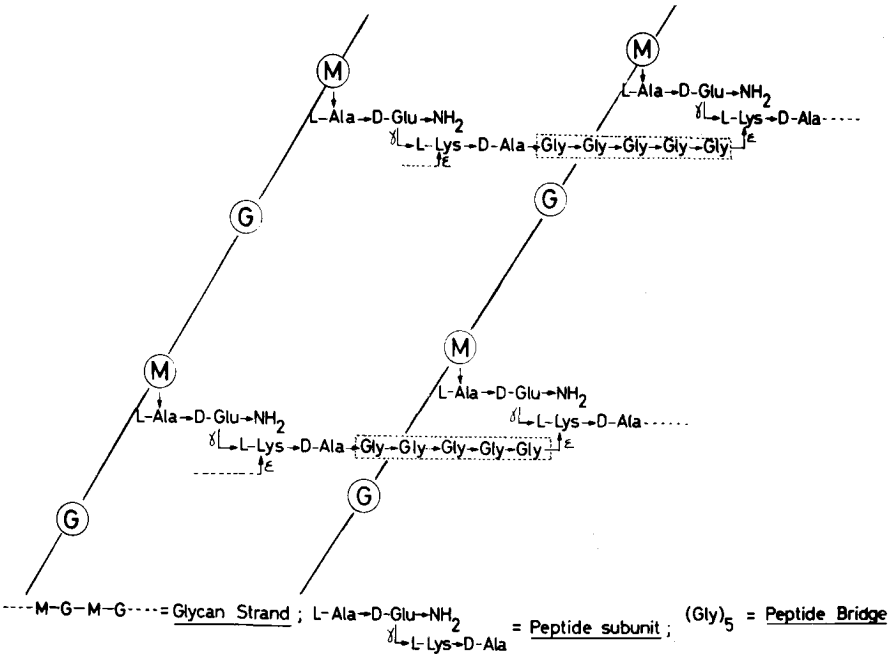


FIG. 2. Primary structure of the wall peptidoglycan in Staphylococcus aureus. In this and all subsequent figures, G = N-acetylglucosamine and M = N-acetylmuramic acid. Arrows indicate the CO→ NH direction of the linkages. Usual α-peptide bonds are represented by horizontal arrows; other peptide bonds (e.g., γ or ε) are also indicated. The pentaglycine bridges, which extend from the ε-amino group of L-lysine on one peptide subunit to the carboxyl group of D-alanine on another, are enclosed by a dashed rectangle.

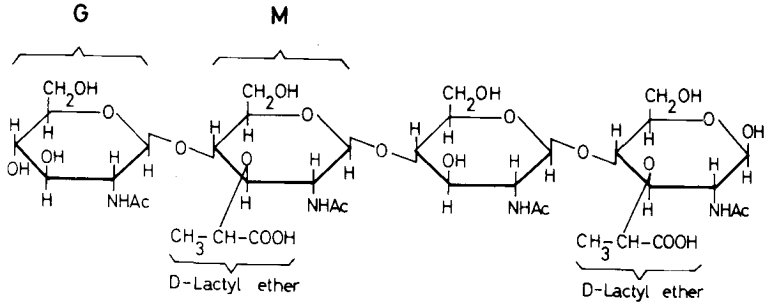


FIG. 3. A portion of a glycan strand. In the peptidoglycan network, the COOH of the D-lactyl groups is usually peptide substituted.

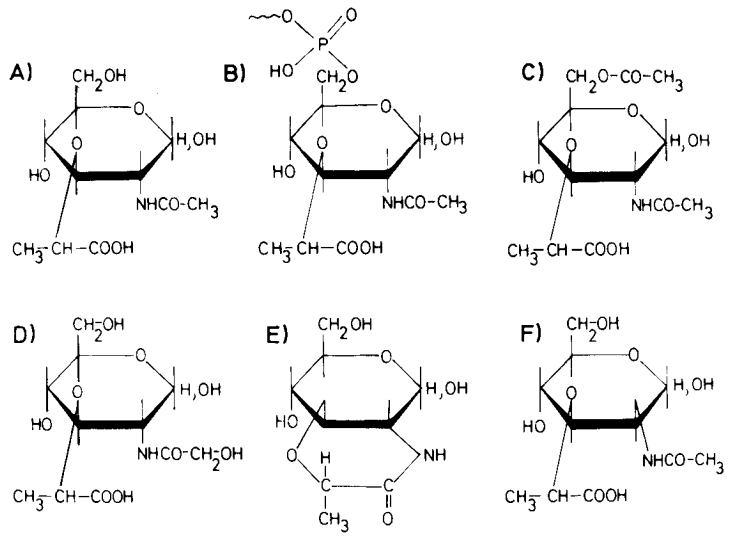


FIG. 4. Known structural variations of the N-acetylmuramic acid residue in glycan chains of various bacterial species. (A) N-acetylmuramic acid; (B) N-acetylmuramic acid with the C-6 substituted by a phosphodiester group; (C) N-acetylmuramic acid with the C-6 substituted by an acetyl group; (D) N-glycolylmuramic acid; (E) muramic lactam; (F) N-acetyl-manno-muramic acid.

second tetrapeptide unit. Considerable species variations in composition of the bridges have been observed. The amino acid composition and the location of the bridges have been used to divide bacterial species into chemotypes [5]. This has been considered to be a criterion of taxonomic importance [11, 27-29a].

In chemotypes I, II, and III, the bridges extend from the C-terminal D-alanine of one peptide to the ω-amino group of the diamino acid at the L-R_3 position of another peptide. In chemotype I, the bridging consists of direct N^{ω}-(D-alanyl)-L-R_3 peptide bonds. In many Bacillaceae and gram-negative bacteria, the interpeptide bond is a D-alanyl-(D)-meso-diaminopimelic acid linkage [5, 30] (Fig. 6). In Aerococcus sp. and Gaffkya homari, the interpeptide bond is a N^{ϵ}-(D-alanyl)-L-lysine linkage [31] (Fig. 7).

In chemotype II, the bridging is mediated via a single additional amino acid or an intervening short peptide. Variations appear to be endless, only a few examples of which are given in Figs. 8 and 9 [32-41].

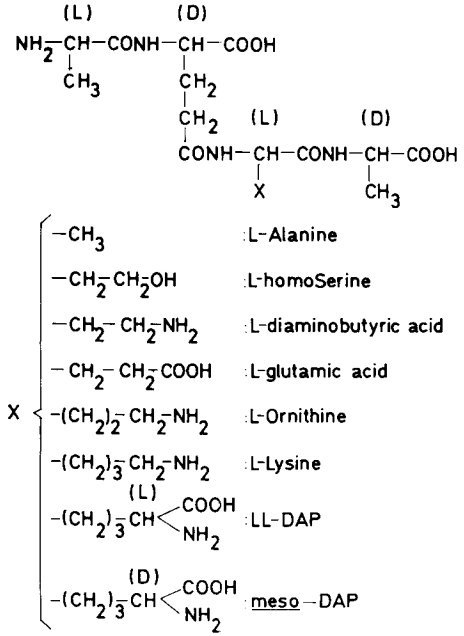


FIG. 5. General structure of tetrapeptide L-alanyl-γ-D-glutamyl-L-R_3-D-alanine subunits. Side chains of amino acids known to occur in the L-R_3 position are shown. DAP = diaminopimelic acid.

In chemotype III, the bridge is composed of one or several peptides, each having the same amino acid sequence as the peptide unit [Fig. 10]. This bridge occurs in M. lysodeikticus and related Micrococcaceae [42-44]. Seemingly, chemotype III is a variant of chemotype II. In fact, this bridge is quite unusual. In M. lysodeikticus many of the N-acetylmuramic acid residues in the glycan strands are not substituted by peptides, as if the peptide units had moved, at a certain stage of the biosynthesis, from these N-acetylmuramic acid residues into a bridging position [5, 42, 43]. The peptidoglycans of other Micrococci belonging to this chemotype III contain more peptide units than disaccharide units, but all of the N-acetylmuramic acid residues are substituted by peptides. It has been hypothesized that after translocation of some peptide units from the glycan to a bridging position, the unsubstituted segments of the glycans were, in turn, removed by excision [44]. Both D-alanyl-L-alanine and N^{ϵ}-(D-alanyl)-L-lysine linkages, sensitive to Myxobacter ALI enzyme and ML endopeptidase, respectively, are involved in the interpeptide bonding in all chemotype III peptidoglycans.

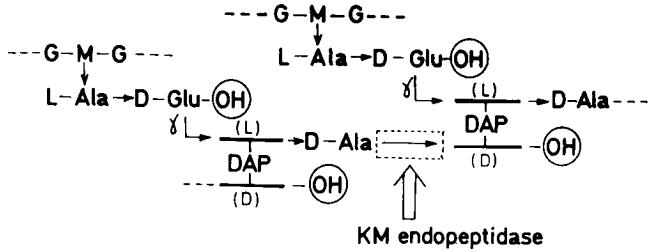


FIG. 6. Peptidoglycan of chemotype I with meso-DAP in the L-R_3 position. This structure occurs in the wall of E. coli, of probably all other gram-negative bacteria, and of Bacillus megaterium KM. Note that the interpeptide linkage, D-alanyl-(D)-meso-diaminopimelic acid is at a C-terminal position. The arrow indicates the site of action of the lytic KM endopeptidase (DD-carboxypeptidase) from Streptomyces strain albus G. In E. coli carboxyl groups are not amidated. Peptidoglycan of Streptomyces strain R39 has the same primary structure except that the α-carboxyl group of D-glutamic acid is amidated. In some Bacillaceae, the α-carboxyl group of D-glutamic acid and/or the carboxyl group of diaminopimelic acid that is not in a peptide bond are also amidated.

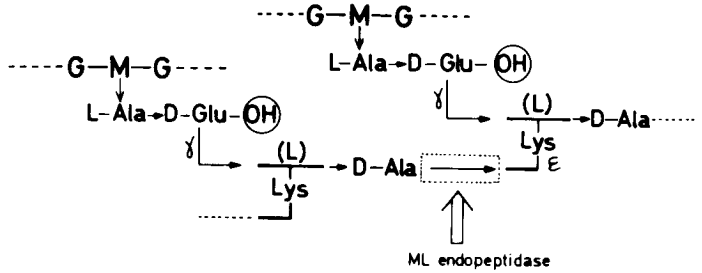


FIG. 7. Peptidoglycan of chemotype I with L-lysine in the L-R_3 position. This structure occurs in the wall of Aerococcus sp. and Gaffkya homari. Arrow indicates the site of action of the lytic ML endopeptidase from Streptomyces albus G.

In chemotype IV, the bridge extends from the C-terminal D-alanine residue of one peptide unit to the α-carboxyl group of D-glutamic acid of another peptide, i.e., between two carboxyl groups. Hence, it necessarily involves a diamino acid residue or a diamino acid-containing peptide. Examples are given in Figs. 11-13 [45, 46, 26a]. This type of bridging occurs between tetrapeptide units containing a neutral amino acid (Fig. 11), a diamino acid (Fig. 12), or a dicarboxylic amino acid (Fig. 13) at the L-R_3 position.

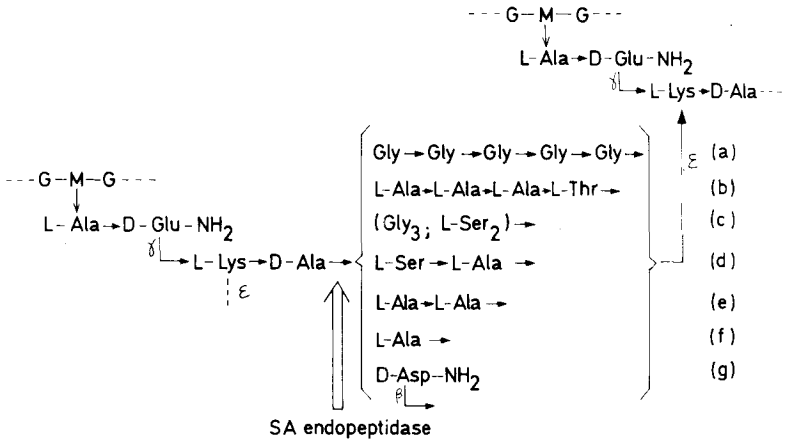


FIG. 8. Peptidoglycans of chemotype II, which occur in the walls of (a) Staphylococcus aureus Copenhagen [34, 39-41]; (b) Micrococcus roseus R27 [41]; (c) Staphylococcus epidermidis Texas 26 [33, 34]; (d) Lactobacillus viridescens [38]; (e) Streptococcus pyogenes Group A, type 14 [41]; (f) Arthrobacter crystallopoietes [32, 32a]; (g) Streptococcus faecalis (faecium) ATCC 9790 [35]; and Lactobacillus casei RO94 [36]. Arrow indicates the site of action of the lytic SA endopeptidase from Streptomyces albus G upon walls (a), (b), (e), and (g). This enzyme has not been tested upon walls (c), (d) and (f).

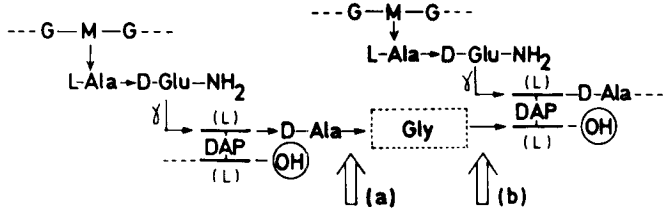


FIG. 9. Peptidoglycan of chemotype II, which occurs in the walls of Clostridium perfringens type A [27], and of Streptomyces strains albus G, R61, and K11. Arrows indicate the sites of action of Myxobacter AL1 enzyme. When acting on walls of C. perfringens, Myxobacter enzyme hydrolyzes both (a) and (b) linkages. When acting on walls of Streptomyces sp., Myxobacter enzyme hydrolyzes only (a) but not (b) linkages.

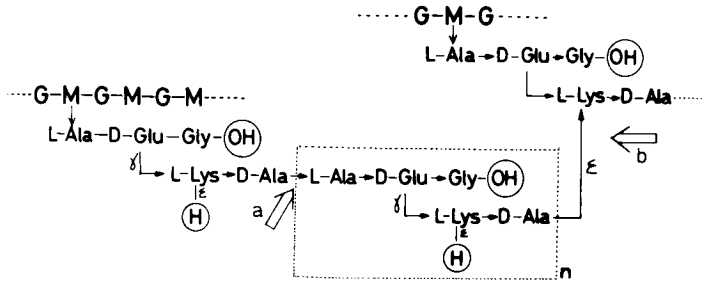


FIG. 10. Peptidoglycan of chemotype III, which occurs in the wall of Micrococcus lysodeikticus. The site of action of Myxobacter ALI enzyme is indicated by arrow a and that of the ML endopeptidase from Streptomyces albus G by arrow b. Note the similarity of action of the latter enzyme upon peptidoglycans of different chemotypes by comparing its action here with that on a peptidoglycan of chemotype I shown in Fig. 7. Also shown are the unsubstituted N-acetylmuramic acid residues that have been found in the wall of M. lysodeikticus, but not in some of the other peptidoglycans of chemotype III.

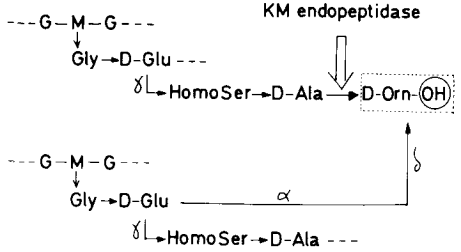


FIG. 11. Peptidoglycan of chemotype IV, which occurs in the walls of some Corynebacteria pathogenic for plants [45]. Arrow indicates the site of action of the KM endopeptidase from Streptomyces albus G (i.e., a lytic DD-carboxypeptidase; cf. Fig. 6).

B. Enzymatic Degradation of Peptidoglycan

The unraveling of the primary structure of the wall peptidoglycan by using nonspecific acidic or basic hydrolyses would have been an impossible task for two reasons. First, this enormous molecule, in contrast with other natural polymers that contain amino acids such as proteins, is built from a limited number of constituents. Second, other polymeric wall

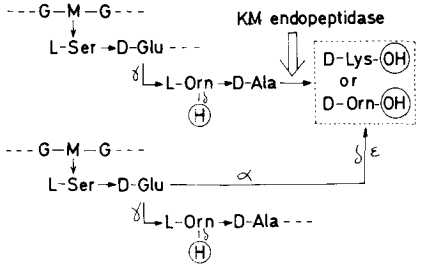


FIG. 12. Peptidoglycan of chemotype IV, which occurs in the wall of Butyribacterium rettgeri (46). Arrow indicates the site of action of the KM endopeptidase from Streptomyces albus G (i.e., a DD-carboxypeptidase that is lytic for walls; cf. Figs. 6 and 11).

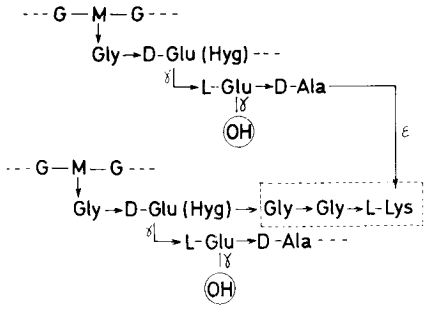


FIG. 13. Peptidoglycan of chemotype IV, which occurs in the wall of Arthrobacter J39 (26a). Hyg = threo-3-hydroxyglutamic acid.

components are often covalently linked to the peptidoglycan, and the resulting association exhibits an exceedingly high degree of complexity. The isolation of a collection of lytic agents selectively active upon different, well-defined linkages within the peptidoglycan was thus essential. Hen egg-white lysozyme, which had been discovered in the 1920's by Fleming, was such a tool. Lysozyme hydrolyzes β-1,4-linkages between N-acetylmuramic acid and N-acetylglucosamine in the glycan strands (Fig. 14, arrow a). The action of lysozyme on walls of M. lysodeikticus, which contain a peptidoglycan of chemotype III (Fig. 10), allowed the isolation and characterization of the disaccharide N-acetylglucosaminyl-N-acetylmuramic acid and of higher oligosaccharides. The choice of M. lysodeikticus for these early studies was rather fortunate. For several reasons, commercially available hen egg-white lysozyme proved to be of extremely limited usefulness for the determination of the chemical

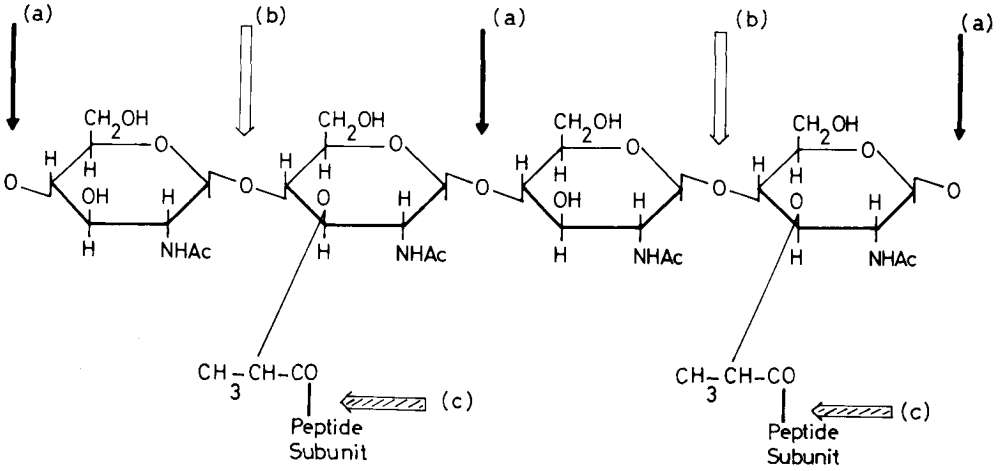


FIG. 14. Sites of action of endo-N-acetylmuramidases at arrows (a), of endo-N-acetylglucosaminidases at arrows (b), and of N-acetylmuramyl-L-alanine amidases at arrows (c). Note that the Streptomyces amidase hydrolyzes N-acetylmuramyl-L-alanine linkages (in many bacterial walls), N-acetylmuramyl-L-serine linkages (in B. rettgeri, cf. Fig. 12), but not N-acetylmuramylglycine linkages (in C. poinsettiae, cf. Fig. 11). Reprinted from reference 5 by courtesy of American Society for Microbiology.

structure of other peptidoglycans. In most cases the degradation products after lysozyme action were highly complex. This was due to a number of factors that include (1) the fact that lysozyme action fails to result in the complete hydrolysis of all sensitive bonds. This appears to be due to both inhibition of the reaction by products and to the transglycosidase activity of lysozyme [47-49]; (2) inhibition of hydrolysis by the presence of N, O-diacetylmuramic acid residues in the substrate; and (3) since most peptidoglycans contain peptide substituents on virtually all of their N-acetylmuramic acid residues, the action of a glycosidase alone cannot release unsubstituted di- or oligosaccharides.

Therefore a search for other lytic agents was undertaken [50, 51], which resulted in the discovery of three classes of lytic enzymes: glycosidases, N-acetylmuramyl-L-alanine amidases, and endopeptidases. The use of such enzymes in the determination of the structure of the wall peptidoglycans has been reviewed and discussed [5].

i. The glycosidases are either endo-N-acetylmuramidases or endo-N-acetylglucosaminidases (Fig. 14). Endo-N-acetylmuramidases, such as lysozyme, hydrolyze β-1,4-N-acetylmuramyl-N-acetylglucosamine

linkages, i.e., they produce fragments with N-acetylmuramic acid at the reducing end. Endo-N-acetylglucosaminidases hydrolyze β-1,4-N-acetylglucosaminyl-N-acetylmuramic acid linkages, i.e., they produce fragments with N-acetylglucosamine at the reducing end. Some of these newly discovered glycosidases exhibit lytic spectra broader than that of lysozyme, and they are not inhibited by the presence of O-acetyl groups on the glycan chains.

ii. N-acetylmuramyl-L-alanine amidases specifically hydrolyze the linkage between the carboxyl of the D-lactyl group of N-acetylmuramic acid and the amino group of the L-alanine residue at the amino terminus of the peptide unit (Fig. 14).

iii. Endopeptidases are of two types: Some hydrolyze peptide linkages in the interior of the peptide bridges of chemotype II peptidoglycans, whereas others specifically hydrolyze those peptide bonds that involve the C-terminal D-alanine residue of the peptide units. Figures 6-12 show the sites of action of some enzymes that hydrolyze D-alanine peptide bonds.

1. When acting on peptidoglycans of chemotype II (Fig. 8) the SA endopeptidase of Streptomyces albus G hydrolyzes D-alanyl-glycine, D-alanyl-L-alanine or D-alanyl-D-isoasparaginyl linkages, i.e., bonds located at the N-termini of the peptide bridges and at the C-termini of the peptide side-chain units.

2. The ML endopeptidase from the same strain of Streptomyces specifically acts on N^{ε}-(D-alanyl)-L-lysine linkages. This enzyme has been used for degrading peptidoglycans of chemotype I from Aerococcus sp. and G. homari (Fig. 7), as well as peptidoglycans of chemotype III from M. lysodeikticus and other Micrococcaceae (Fig. 10).

3. The Myxobacter ALI enzyme [52] hydrolyzes the D-alanyl-L-alanine linkages, which also occur as interpeptide bonds in peptidoglycans of chemotype III (Fig. 10) and the D-alanyl-glycyl-LL-diaminopimelyl sequence in walls of C. perfringens (Fig. 9).

4. The KM endopeptidase, also from Streptomyces albus G, hydrolyzes D-alanyl-(D)-meso-diaminopimelic acid linkages in peptidoglycans of chemotype I (Fig. 6), as well as other N^{α}-(D-alanyl)-D linkages in peptidoglycans of chemotype IV (Figs. 11 and 12). In such peptidoglycans these bonds occur simultaneously in both an internal and C-terminal position. The KM endopeptidase that hydrolyzes these bonds is in fact a D-alanyl-D-carboxypeptidase.

Figure 15 shows, as an example, how walls of S. aureus were enzymatically degraded in a controlled manner into small fragments. The process essentially involved four sequential steps [41].

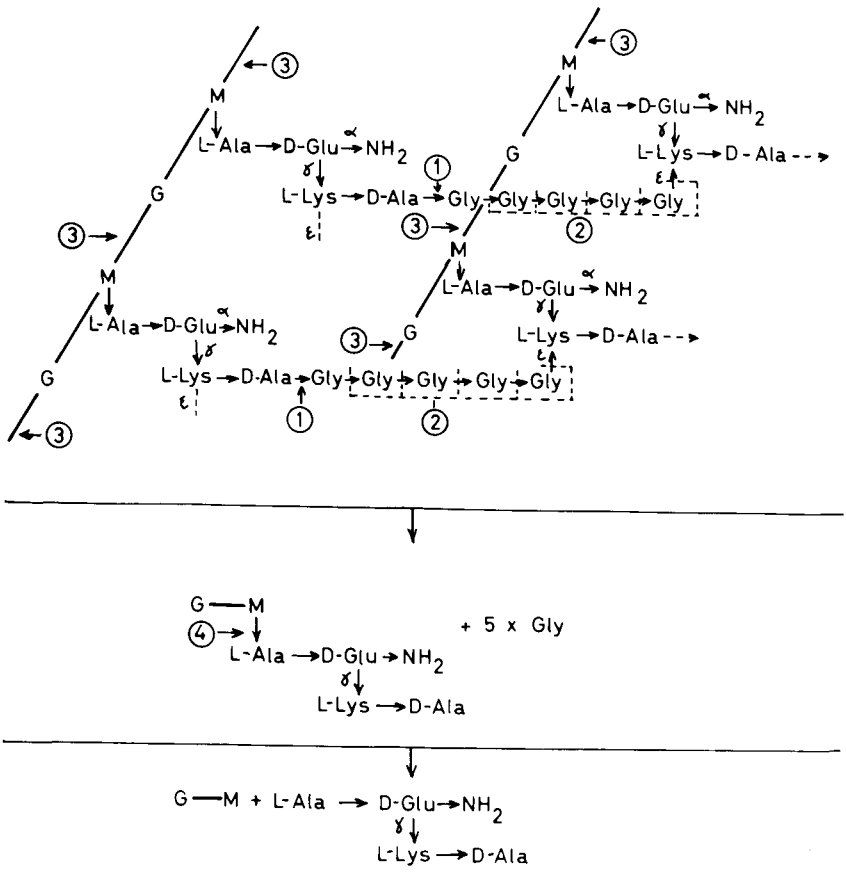


FIG. 15. Degradation sequence for the peptidoglycan of Staphylococcus aureus Copenhagen. Numbered arrows indicate the (1) site of action of SA endopeptidase; (2) degradation of the opened bridges with aminopeptidase; (3) site of action of endo-N-acetylmuramidase; (4) site of action of N-acetylmuramyl-L-alanine amidase.

1. Hydrolysis of the D-alanylglycine linkages by the SA endopeptidase, i.e., the opening of the peptide bridges at their N-termini. As a consequence of such hydrolysis, walls underwent dissolution.

2. Degradation of the opened bridges by aminopeptidase. The glycine residues were sequentially liberated and eventually, the ε-amino groups of L-lysine in the peptide units were exposed.

3. Degradation of the glycan strands into disaccharide units by means of a glycosidase (here an endo-N-acetylmuramidase). At this stage, the disaccharide peptide units were readily isolated and separated from the nonpeptidoglycan polymer of the walls. In S. aureus, this latter polymer is a teichoic acid. It was recovered undegraded, with small peptidoglycan fragments covalently attached to it through phosphodiester bridges [20].

4. Hydrolysis of the disaccharide peptide units into free disaccharides and free peptides, with the help of an N-acetylmuramyl-L-alanine amidase. Disaccharides and peptides were then isolated and characterized.

In the above procedure, an endo-N-acetylmuramidase was used at step 3, yielding β-1,4-N-acetylglucosaminyl-N-acetylmuramic acid disaccharide peptide units. (In the case of S. aureus, some of these disaccharide peptide units contained O-acetyl groups on C-6 of muramic acid). An alternate degradation procedure [53, 54] used an endo-N-acetylglucosaminidase, instead of an endo-N-acetylmuramidase, to degrade the glycan strands, yielding the isomeric β-1,4-N-acetylmuramyl-N-acetylglucosamine peptide disaccharides. Thus, a few enzymes of different specificities were sufficient to quantitatively dismantle the S. aureus peptidoglycan network into its building blocks: the isomeric disaccharides, the tetrapeptide units, and glycine residues originating from the interpeptide bridges. Similar enzymatic degradation techniques were applied to walls of all chemotypes [5]. More recently, partial acid hydrolysis techniques were developed by Kandler and his colleagues [27-29, 38, 42]. Based on the knowledge that all peptidoglycans are built according to the same basic framework, these techniques permitted an exhaustive survey of the peptidoglycan structure in many species, representing a wide variety of taxonomic groups.

C. Size of Peptidoglycans

Solubilization of the wall peptidoglycan can be brought about by the hydrolysis of any single type of linkage, but is achieved only if a sufficient number of bonds are actually hydrolyzed. This property is, of course, imparted by the netlike structure of the peptidoglycan. The organization of the peptidoglycan was initially thought to involve very long glycan strands that extended completely around the cell [8]. Accurate analyses, however, showed that, on the contrary, many terminal groups are present in both the glycan and the peptide moieties of the wall peptidoglycans. These terminal groups presumably reflect the dynamics of the bacterial growth and the active involvement of autolysins (see Sections IV, D and VI, F).

The average length of glycan chains have been estimated for walls of a few species [4, 32, 34, 55, 56]. They vary from 20 to 100 N-acetylhexosamine residues. More detailed analyses showed that the glycan chains are often, if not always, polydisperse. At present, there is no way of determining the actual distribution of chains of different lengths in the different parts of the wall. In several cases, N-acetylglucosamine has been identified at the reducing end of the glycan chains [57-59], suggesting the presence of endo-N-acetylglucosaminidase activity in the autolytic system. There does not appear to be a relationship between cell shape and average glycan chain length.

The average degree of polymerization of the peptide moiety has also been estimated. Escherichia coli has a "loose" peptidoglycan (Fig. 16) [8, 30]. About 50% of the peptide units occur as uncross-linked monomers and the other units as peptide dimers. Peptide oligomers were not detected. In walls from exponential-phase cultures of Lactobacillus acidophilus 63 AM Gasser [60], the average size of the peptide moiety was found to be 2.3 cross-linked peptides with 10% of the peptide units occurring as monomers, 37% as dimers, and 30% as trimers (Fig. 17). One of the most cross-linked peptidoglycans is that found in S. aureus (Figs. 1 and 2). Even in this case, the average size does not exceed 10 cross-linked peptide units [33]. Other examples can be found in reference 11. In S. aureus [39] and L. acidophilus [60], the peptides at the uncross-linked C-termini are pentapeptides (and not tetrapeptides) and end in a D-alanyl-D-alanine sequence (Fig. 17), a structural feature also found in nucleotide peptidoglycan precursors of all bacteria. In most bacteria, however, the uncross-linked C-termini of the peptides have not retained the D-alanine-D-alanine sequence. One of the D-alanine residues, or even both of them, are absent, presumably removed by a carboxypeptidase activity.

The tightness of the peptidoglycan network depends not only upon the frequency with which the peptide units are interlinked, but also upon the frequency with which the glycan chains are peptide substituted. The peptidoglycan of M. lysodeikticus is another example of a "loose" peptidoglycan (Fig. 10) [43]. It is unusual in that many N-acetylmuramic acid residues have unsubstituted D-lactyl groups (see Section II, A).

D. Three-Dimensional Organization of Peptidoglycan

The three-dimensional organization of peptidoglycan is unknown. Molecular models that suggest possible conformations have been constructed. The glycan chains of all of these models are based on that of chitin [61]. Chitin has a linear conformation that is stabilized by hydrogen bonding between the C-3-hydroxyl and the C-1 to C-5 ring oxygen

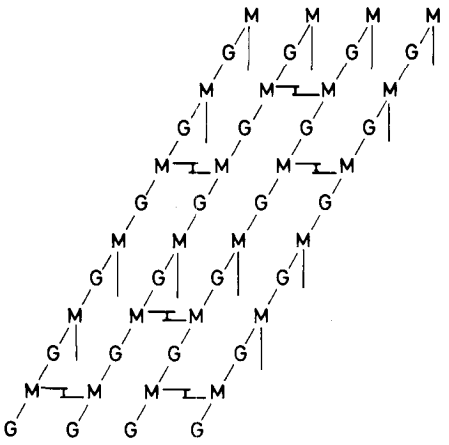


FIG. 16. Schematic representation of the wall peptidoglycan of E. coli. In this relatively loose network, all of the N-acetylmuramic acid residues are substituted, either by uncross-linked peptide monomers or by cross-linking peptide dimers (Fig. 6). Peptide oligomers larger than dimers have not been observed. Reprinted from reference 5 by courtesy of American Society for Microbiology.

of adjacent N-acetylglucosamine residues. As proposed by Tipper [11], a similar conformation is obtained for the glycan strands of the peptidoglycan (Fig. 18), if hydrogen bonding between one N-acetylglucosamine and its two adjacent N-acetylmuramic acids occur through the C-3-hydroxyl of N-acetylglucosamine and the C-1 to C-5 ring oxygen of one N-acetylmuramic acid (as in chitin), and through the C-6-hydroxyl of N-acetylglucosamine and the carbonyl of the lactyl group of the other N-acetylmuramic acid. As pointed out by Tipper [11], this latter hydrogen bond may only be possible with a lactyl group having the D-configuration. In this stabilized chitin-like conformation, the D-lactyl peptide side chains are aligned in parallel along a relatively rigid linear backbone. Moreover, the C-6 of the N-acetylmuramic acid residues are unhindered and hence are readily available for substitution either by acetyl groups or by phosphodiester bridges. It has been emphasized (see Section II, A) that galactosamine, rather than glucosamine, and galactomuramic acid, rather than glucomuramic acid, have not been found to occur naturally in the wall glycans, and that the only variations so far encountered do not alter the basic conformation of the glycan chains. This suggests that the conformation of the glycan is essential for the survival of bacteria, and that any mutation that would alter this conformation is probably lethal.

An α-helical conformation of the peptide units is not likely. Indeed, the glutamyl bond is always γ, and therefore all the carbon atoms of this

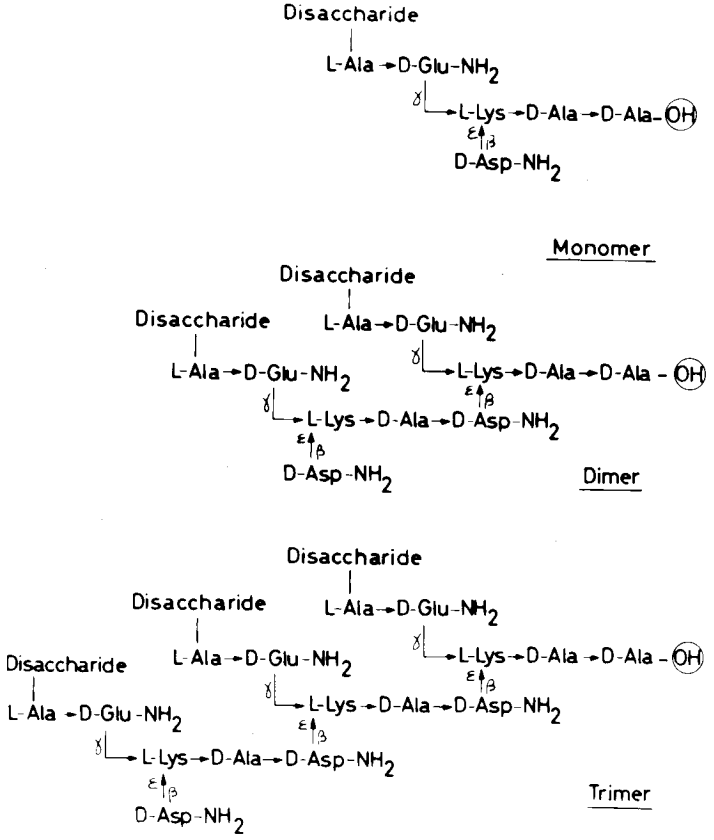


FIG. 17. Disaccharide peptide mono-, di-, and trimer from the wall peptidoglycan of Lactobacillus acidophilus 63 AM Gasser after degradation of the glycan strands into disaccharide units. All the uncross-linked C-termini of the peptides have a D-alanyl-D-alanine sequence. This latter linkage can be hydrolyzed by the DD-carboxypeptidase from Streptomyces albus G (i.e., the KM endopeptidase of Figs. 6, 11, and 12). The action of the SA endopeptidase on this peptidoglycan is the same as that shown in Fig. 8 for chemotype II g.

amino acid residue are in the chain. As shown by Kelemen and Rogers [62], extensive interpeptide hydrogen bonding is possible when the peptides are extended in the pleated sheet (β-like) conformation. A three-dimensional molecular model of the staphylococcal peptidoglycan indicates that the degree of hydrogen bonding between contiguous peptide chains can be as high as 80%.

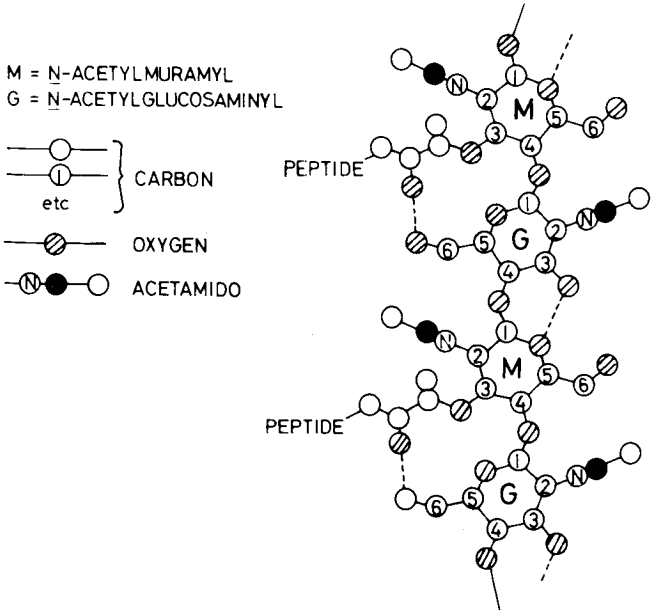


FIG. 18. A possible configuration for the glycan strands. The dashed lines represent hydrogen bonds. Reprinted from reference 11 by courtesy of Iowa State University Press.

An alternative possibility has been suggested by Tipper [11]. It rests upon the flexibility of the peptide chain because its γ-glutamyl linkage allows the chain to double back on itself, and on the similarity between the repeating peptide structure of the peptidoglycan and some peptide antibiotics such as enniatin and the gramicidins. These latter compounds are known for the high stability of their folded conformations and their chelating properties.

A third possible model has been constructed by Oldmixon and Higgins [63, 64]. Although the glycan strands are also based on that of chitin, this model differs from that of Kelemen and Rogers and Tipper in that it involves extensive hydrogen bonding between peptide side chains and the glycan chains. Extending from the lactyl group of N-acetylmuramic acid, the peptide side chains easily fold over one face of the glycan strands in a pattern that results in three hydrogen bonds between D-glutamic acid and D-alanine and the glycan, as well as an additional hydrogen bond between L-alanine and D-glutamic acid within the peptide chain. Because the tetrapeptide side chains consistently contain L-alanyl-γ-D-glutamyl-L-R_3-D-alanine (Section II, A) hydrogen bonding between tetrapeptide and

glycan chains is virtually unaffected by the known variations in tetrapeptide sequence. This model results in a somewhat more compact structure than the others.

The models proposed by Tipper, Oldmixon and Higgins, and Kelemen and Rogers, are not only consistent with, but take into account the constancy and variability of peptide structures discussed in Section II, A. It should be emphasized that there is little or no physical evidence to date in support of any of these or other molecular models of the three-dimensional structure of peptidoglycan.

Hydrogen bonding, both in the glycan and the peptide, probably plays a considerable role in defining the conformation and properties of the peptidoglycan. For example, hydrogen bonds may play a role in (1) binding the nascent peptidoglycan to active sites of enzymes involved in its biosynthesis (vide infra); (2) aligning this nascent peptidoglycan during wall synthesis and growth; (3) remodeling the shape of the wall during the cell division cycle; and (4) providing a three-dimensional organization compatible with rigidity and tensile strength together with the presence of gaps that are required for the insertion of other wall polymers, diffusion of nutrients and competence.

III. BIOSYNTHESIS OF PEPTIDOGLYCAN AT THE BIOCHEMICAL LEVEL

A. The Three Stages

Peptidoglycan biosynthesis can be divided into three stages, each of which occurs at a different site in the cell: in the cytoplasm, on the membrane, and within the wall itself. Peptidoglycan precursors made on a uridylic acid cytoplasmic carrier (stage 1) are transferred from uridylic acid to an undecaprenyl phosphate membrane carrier (stage 2), and then to a final acceptor in the expanding wall peptidoglycan (stage 3). At some point during the later stages of this process, the nascent peptidoglycan undergoes cross-linking, which is required to make it insoluble, and covalently linked accessory wall polymers are attached. Figure 19 shows the main reactions through which the synthesis of peptidoglycan of chemotype I is carried out. In this case, no additional amino acids are involved in peptide cross-linking. Incorporation of amino acids into interpeptide bridges and addition of specific groups such as amides, in other peptidoglycans, will be discussed in Sections III, F and G below. (For a more comprehensive list of references, the reader is referred to other reviews [9, 11, 65].)

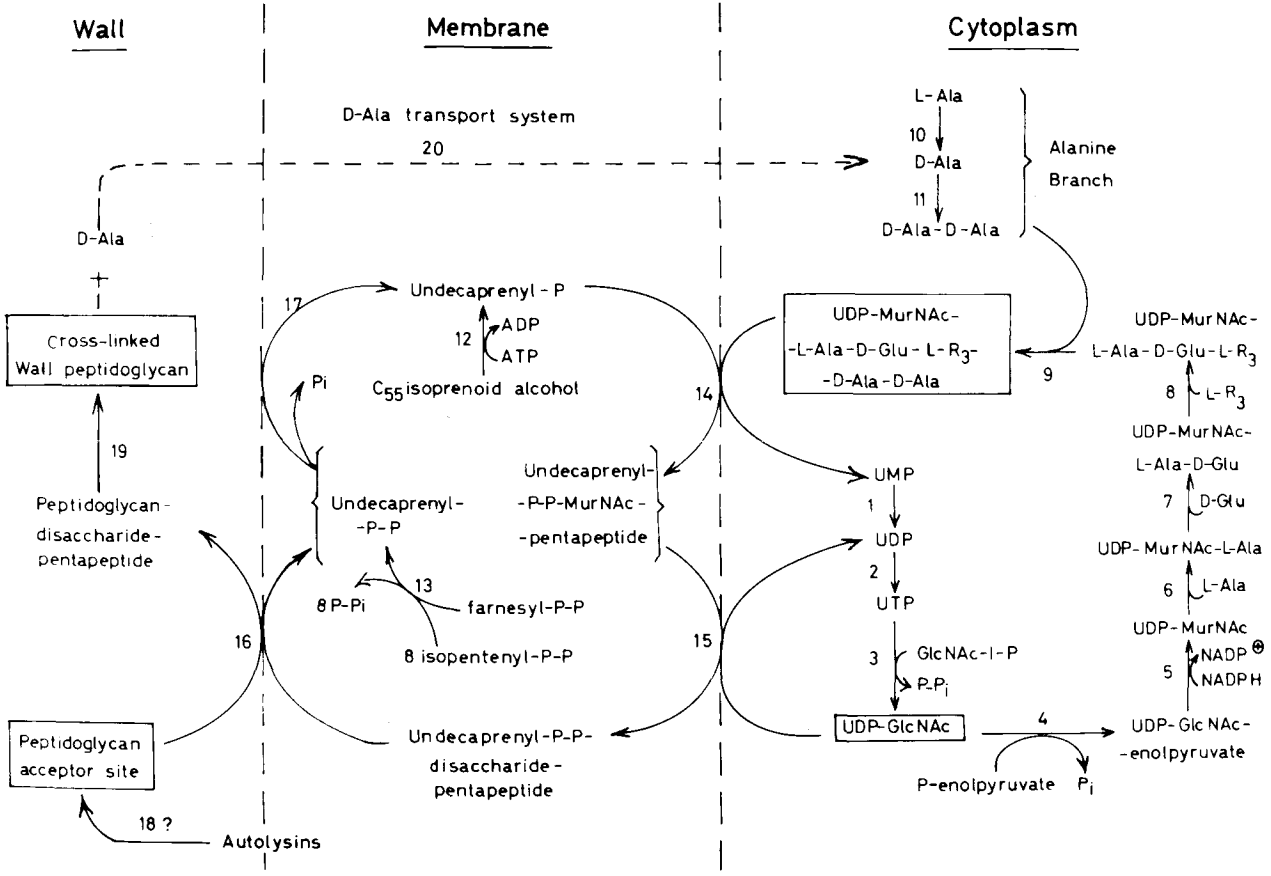


FIG. 19. Schematic representation of the biosynthesis of a peptidoglycan of chemotype I. The three stages — cytoplasmic, membrane-bound, and wall-bound — are separated by the dashed vertical lines. All the reactions are shown in the anabolic sense. GlcNAc = N-acetylglucosamine; MurNAc = N-acetylmuramic acid.

B. Stage 1: Synthesis of Nucleotide Precursors

The first two reactions are phosphorylations of UMP and UDP by ATP to yield UTP. UTP then reacts with α-D-N-acetylglucosamine 1-phosphate to yield UDP-N-acetylglucosamine and inorganic pyrophosphate (reaction 3, Fig. 19). This latter reaction is catalyzed by UDP-N-acetylglucosamine pyrophosphorylase and is analogous to reactions that lead to UDP-glucose and other compounds of this type.

Reaction 4 (Fig. 19) is, in fact, the first step in which the precursor is committed to becoming peptidoglycan. A three-carbon unit is transferred to UDP-N-acetylglucosamine from 2-phosphoenolpyruvate, the glycolytic intermediate. Inorganic phosphate and the pyruvate enol ether of UDP-N-acetylglucosamine are formed. The transferase that catalyzes the reaction in Enterobacter cloacae has been purified [66]. The reaction is reversible with a K_m for phosphoenolpyruvate of 3×10^{-5} M and a K_m for UDP-N-acetylglucosamine of 4.6×10^{-4} M. In this reaction, the enolpyruvate structure is preserved, in marked contrast with that observed in many other reactions involving phosphoenolpyruvate. The transferase is irreversibly inactivated by phosphonomycin. Phosphonomycin is believed to be a substrate analog of phosphoenolpyruvate and becomes covalently linked to the enzyme through a sulfhydryl group [67, 68]. The pyruvate UDP-N-acetylglucosamine transferase of E. cloacae has been separated from UDP-N-acetylglucosaminepyruvate reductase [66]. This latter enzyme catalyzes the next reaction (5, Fig. 19) in the sequence and yields UDP-N-acetylmuramic acid.

L-Alanine, D-glutamic acid, the L-R_3 residue, and finally, a preformed D-alanyl-D-alanine dipeptide are then added stepwise to UDP-N-acetylmuramic acid (reactions 6-9; Fig. 19). Each step is catalyzed by a separate enzyme requiring ATP and either Mg^{2+} or Mn^{2+}. Unlike the template-directed sequential peptide bond formation in protein synthesis, these reactions totally depend on the specificity of their respective enzymes for their substrates in order to result in a UDP-muramyl-pentapeptide of the correct sequence [9, 65]. The meso-diaminopimelic acid-adding enzyme of E. coli fails to add L-lysine, and the S. aureus L-lysine-adding enzyme fails to add meso-diaminopimelic acid to UDP-N-acetylmuramyl-L-alanyl-D-glutamate. A similar difference exists between the vegetative cell and "sporulation specific" L-lysine and meso-diaminopimelic acid-adding enzymes of Bacillus sphaericus 9602 (see Section IV, C) [69, 70]. In view of the absence of direction by a nucleic acid template, the relative constancy of peptidoglycan composition appears to be especially remarkable. The completed nucleotide is UDP-N-acetylmuramyl-L-alanyl-γ-D-glutamyl-L-R_3-D-alanyl-D-alanine (Fig. 20). Note that D-glutamic acid is γ-linked to the third amino acid, and that the peptide is not a tetrapeptide as is usually found in the completed wall peptidoglycans, but a pentapeptide ending in a D-alanyl-D-alanine sequence.

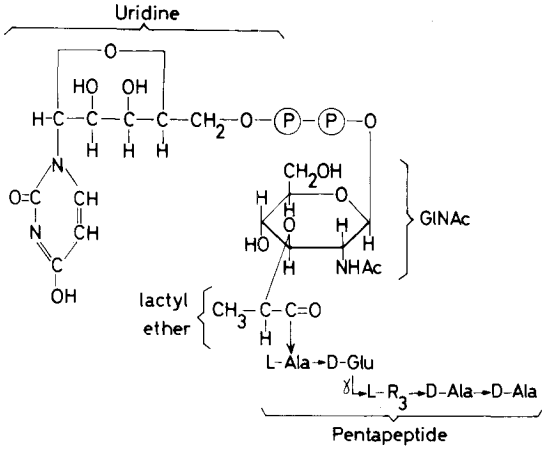


FIG. 20. The completed nucleotide precursor UDP-N-acetylmuramyl-pentapeptide.

C. The Alanine Branch [71, 72]

The synthesis of the dipeptide D-alanyl-D-alanine and its addition to the nucleotide tripeptide UDP-N-acetylmuramyl-L-alanyl-γ-D-glutamyl-L-R_3 have excited considerable interest. D-Alanine antagonists provided tools for studying what is called the alanine branch of peptidoglycan synthesis (Fig. 19). The details of this pathway have been recently reviewed by Neuhaus et al. [71, 72]. Three enzymes are involved in the alanine branch: (1) alanine racemase, (2) D-Ala:D-Ala ligase (ADP) or D-alanyl-D-alanine synthetase, and (3) UDP-MurNAC-L-Ala-γ-D-Glu-L-R_3: D-Ala-D-Ala ligase (ADP) or D-alanyl-D-alanine adding enzyme. Among the D-alanine antagonists, D-cycloserine is of prime importance. It is a competitive inhibitor of both the racemase and synthetase with K_i values considerably smaller than the K_m values for the substrates. D-Cycloserine does not inhibit the D-alanyl-D-alanine adding enzyme.

1. Alanine Racemase

In terms of the synthesis of D-alanyl-D-alanine, alanine racemase (reaction 10; Fig. 19) can function either in an anabolic (2L-Ala → 2D-Ala) or catabolic (2D-Ala → 2L-Ala) sense. The racemases from S. aureus [73], Streptococcus faecalis [74, 74a], B. subtilis [75], and E. coli [71] have been isolated and studied.

The S. aureus racemase is competitively inhibited by D-cycloserine when measured in either direction. However, even very high concentrations of L-cycloserine fail to inhibit the reaction in either direction. This observation was puzzling. Indeed, if D-cycloserine competes with D-alanine, L-cycloserine should also be a competitor for L-alanine. In an attempt to explain the lack of inhibition by L-cycloserine, Roze and Strominger [73] postulated the existence of a single site on the racemase on which L- and D-alanine would bind in the same conformation. The conformation specifically recognized by the enzyme would be that found in D- but not in L-cycloserine.

The E. coli racemase exhibits K_m values of 9.7×10^{-4} M for L-Ala and 4.6×10^{-4} M for D-Ala [71]. The V_{max} values (in μmole/h) are 2.22 for the L-Ala → D-Ala reaction and 0.95 for the D-Ala → L-Ala reaction. Hence, the anabolic velocity is larger than the catabolic one, but the apparent affinity for D-Ala is greater than that for L-Ala. Therefore, the Haldane relationship

$$K_{Eq} = \frac{(K_m \text{ D-Ala})\ (V_{max} \text{ L} \rightarrow \text{D})}{(K_m \text{ L-Ala})\ (V_{max} \text{ D} \rightarrow \text{L})}$$

is approximately equal to 1. D-Cycloserine competitively inhibits the E. coli racemase with either D- and L-alanine as substrate, and the K_i value is independent of the configuration of the substrate. In marked contrast with the S. aureus racemase, the E. coli enzyme is also competitively inhibited by L-cycloserine. The ratio K_m(L-Ala)/K_m(D-Ala) is approximately equal to the ratio K_i(L-cycloserine)/K_i(D-cycloserine). Neuhaus et al. [71] believe the above kinetics to be consistent with the existence of separate binding sites on the racemase, for L- and D-alanine. A pronounced substrate inhibition was observed in the D-alanine → L-alanine assay. This was interpreted to mean that, at high concentrations, D-alanine binds to both the L-alanine and D-alanine sites.

A unified model for alanine racemase cannot be proposed. It would appear that the alanine racemase from either S. aureus or S. faecalis is specifically inhibited by D- but not L-cycloserine, and that the alanine racemase from either E. coli or B. subtilis is inhibited by both D- and L-cycloserine. It may be that some racemases have a single site for both D- and L-alanine and that others have two binding sites. A third model has also been proposed [75a] according to which the racemase would have two forms, one which would bind L-alanine and the other D-alanine.

2. D-Alanyl-D-Alanine Synthetase

D-Alanyl-D-alanine synthetase catalyzes the reaction

$$2\text{D-alanine} + \text{ATP} \xrightarrow[K^+]{Mg^{2+}} \text{D-alanyl-D-alanine} + \text{ADP} + P_i$$

(reaction 11, Fig. 19). D-Cycloserine inhibits the reaction. It has been proposed by Neuhaus [71, 72, 76, 77] that (in the case of S. faecalis) the primary site of D-cycloserine action is at the donor site of the synthetase, and that the acceptor site of the enzyme is a secondary site of action. D-Alanyl-D-alanine and some analogs of D-alanyl-D-alanine are also inhibitors of the synthetase. The inhibition is specific for dipeptides. Additions to the N-terminal residue decrease the effectiveness of dipeptides as inhibitors. Additions to the C-terminal residue sometimes enhance their effectiveness. For example, D-norvalyl-D-alanine is not an inhibitor, and D-α-amino-n-butyryl-D-alanine is a very poor inhibitor. In contrast, D-alanyl-D-norvaline and D-alanyl-D-α-amino-n-butyric acid are better inhibitors than D-alanyl-D-alanine.

In the presence of D-cycloserine and of Mn^{2+}, the synthetase was found to bind ATP with high affinity. No complex formation was observed when D-cycloserine was replaced by D-alanine in the reaction mixture. D-Alanyl-D-alanine and analogs were tested for their ability to facilitate ATP binding to the enzyme. Two types of responses were observed, which were interpreted as indicating the presence of two types of sites [71]. One site, accessible to D-cycloserine and D-alanyl-D-alanine, facilitates ATP binding. The second site, accessible to D-alanyl-D-alanine, D-alanyl-D-valine and D-alanyl-D-norvaline, does not facilitate ATP binding. Although the inhibitor studies indicated product (or analog) multiple sites, it was not possible to distinguish them from substrate binding sites. It has been suggested that the function of multiple binding sites is to control the rate of dipeptide formation.

3. D-Alanyl-D-Alanine Adding Enzyme

This enzyme catalyzes the addition of D-alanyl-D-alanine dipeptide to UDP-N-acetylmuramyl-L-alanyl-γ-D-glutamyl-L-R_3 in the presence of Mg^{2+} and ATP (reaction 9 in Fig. 19). Its importance lies in its specificity pattern for the addition of dipeptide analogs of D-alanyl-D-alanine [78]. Neuhaus and Struve showed that in S. faecalis the substrate profile of this enzyme is complementary to that of the D-alanyl-D-alanine synthetase. For example, D-alanyl-D-alanine synthetase can incorporate D-norvaline (and D-serine or D-threonine) into the C-terminal end of a dipeptide, but

not into the N-terminal end. In contrast, D-norvalyl-D-alanine, but not D-alanyl-D-norvaline, can bind to and inhibit the adding enzyme. This combination of specificities results in the relative accuracy of synthesis of the complete nucleotide peptide and accounts for some of the growth-inhibitory effects of high concentrations of some D-amino acids.

D. Stage 2: The Undecaprenyl Phosphate Membrane Carrier

The membrane lipid carrier is one of the key compounds in peptidoglycan synthesis as it transports the N-acetylmuramyl-pentapeptide and N-acetylglucosamine precursors from the intracellular sites of synthesis through the membrane to the exocellular sites of polymerization. The peptidoglycan lipid carrier from S. aureus [79, 80] was isolated in a very high degree of purity, allowing its structure to be ascertained by mass spectrometry. It is a C_{55}-isoprenoid alcohol containing 11 isoprene units with the chain ending in an alcoholic function (Fig. 21). In S. lactis, a lipid carrier is used for the transport of teichoic acid precursors to the wall [81, 82]. The same peptidoglycan lipid carrier of S. aureus is also effective in the biosynthesis of the O-antigen of Salmonella [83] (i.e., the lipopolysaccharide of the outer membrane). Hence, in a single bacterium, the same lipid may be involved in the synthesis of more than one wall polymer and may be channeled into more than one synthetic route (peptidoglycan and O-antigen, for example). In agreement with this, competition for a limited amount of available lipid carrier was shown to occur in vitro when both precursors and enzymes of two biosynthetic pathways were present [84]. Nucleotide precursors for the synthesis of one polymer inhibit the synthesis of the other polymer. These effects can be enhanced or diminished by preincubation of the enzyme system with appropriate nucleotide precursors [84a]. However, recent evidence suggests that a polyisoprenoid carrier is not involved in teichoic acid synthesis [84b]. In addition, it should be mentioned that the lipid carrier involved in the synthesis of the capsular polysaccharide in Aerobacter [85] can function in mannan synthesis in M. lysodeikticus [86], and that a large number of long-chain isoprenoid alcohols of this type have also been isolated from plants and animals [87, 88]. All are probably relevant to problems of transport through membranes and perhaps to their orientation.

In order to be functional in peptidoglycan synthesis, the C_{55}-isoprenoid alcohol first must be phosphorylated. In S. aureus, phosphorylation is achieved in the presence of ATP by a membrane-bound isoprenyl alcohol phosphokinase [89] (reaction 12; Fig. 19). This enzyme is a lipoprotein and exhibits unusual properties. It is insoluble in water but is soluble and stable in several organic solvents. The enzyme clearly derives from the membrane where it must occur in a lipid phase. The active enzyme is composed of a protein fraction and a phospholipid (phosphatidylglycerol). The protein fraction is inactive and insoluble both in water and in organic

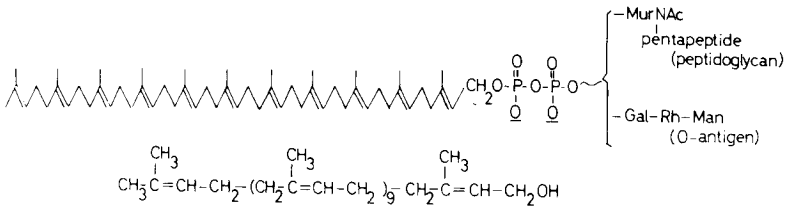


FIG. 21. The C_{55}-isoprenoid alcohol charged with either peptidoglycan or O-antigen precursors, via a pyrophosphate bridge.

solvents. Its activity and solubility in organic solvents are restored by addition of the phospholipid fraction [89, 90].

The presence of an ATP-dependent phosphokinase may be unique to S. aureus. In Salmonella newington, a particulate enzyme catalyzes the synthesis of C_{55}-lipid pyrophosphate (reaction 13; Fig. 19) from farnesyl pyrophosphate and isopentenyl pyrophosphate which, in turn, is probably derived from mevalonate [91, 92]. C_{55}-Lipid pyrophosphate is then dephosphorylated (reaction 17; Fig. 19; vide infra).

E. The Lipid Cycle

The lipid cycle achieves the assembly of the activated precursors into β-1,4-N-acetylglucosaminyl-N-acetylmuramyl-pentapeptide units and carries out the transport of the completed units to the exocellular sites of polymerization. Evidently, this mechanism is responsible for the alternating sequence of N-acetylglucosamine and N-acetylmuramic acid residues found in the glycan strands of the completed wall. The first two reactions (14 and 15; Fig. 19) consist of transferring the precursors from the hydrophilic environment of the cytoplasm to the hydrophobic environment of the membrane in "an interchange of carriers that are compatible with the particular microenvironments" [93]. Such processes are tranlocations, and the enzymes which catalyze them have been called translocases. First (reaction 14), phospho-N-acetylmuramyl pentapeptide is translocated from UDP-N-acetylmuramyl-pentapeptide to the membrane undecaprenyl carrier with formation of UMP and undecaprenyl-PP-N-acetylmuramyl-pentapeptide. N-Acetylglucosamine is then translocated from UDP-N-acetylglucosamine to undecaprenyl-PP-N-acetylmuramyl-pentapeptide with formation of UDP and undecaprenyl-PP-disaccharide-pentapeptide (reaction 15). Note that in this reaction, only the N-acetylglucosamine and not the phosphate is transferred. In order to complete the lipid cycle a third reaction (reaction 16; Fig. 19) transfers the disaccharide-pentapeptide unit from the membrane carrier into the expanding wall peptidoglycan.

1. Translocation of N-Acetylmuramyl-Pentapeptide [93, 94]

This translocation (reaction 14; Fig. 19) is a transphosphorylation reaction which proceeds without loss of energy: UDP-MurNAC (pentapeptide) + undecaprenyl-P $\rightleftharpoons$ undecaprenyl-PP-MurNAC (pentapeptide) + UMP. One phosphate group of the pyrophosphate is derived from UDP and the other from the phosphorylated lipid carrier. Forward and reverse reactions require Mg^{2+}. The transfer reaction is stimulated by K^+ and other monovalent cations. UMP is a very effective inhibitor of the forward transfer. The equilibrium may be reached by either route and the K_{Eq} value is about 0.25. The translocase has been solubilized from membrane fragments by a variety of diverse reagents into two major active fractions with apparent molecular weights of 2×10^6 and 100,000 to 200,000 [95]. Further purification has not yet been achieved.

It has been proposed by Neuhaus [93-95] that an enzyme intermediate enzyme-P-N-acetylmuramyl (pentapeptide) is transitorily formed according to the reactions

Enzyme + UMPP-N-acetylmuramyl (pentapeptide) $\rightleftharpoons$ ...
... $\rightleftharpoons$ enzyme P-N-acetylmuramyl (pentapeptide) + UMP (14a)

Enzyme-P-N-acetylmuramyl (pentapeptide) + undecaprenyl-P $\rightleftharpoons$ ...
... $\rightleftharpoons$ enzyme + undecaprenyl-PP-N-acetylmuramyl (pentapeptide) (14b)

The enzyme intermediate has not been isolated, but several experimental evidences support the proposed mechanism. (1) The rate of exchange that occurs in reaction 14a largely exceeds the rate of transfer. (2) Kinetics of product formation showed that UMP continues to be formed after the lipid product undecaprenyl-PP-N-acetylmuramyl (pentapeptide) has attained a steady-state level. (3) After a lag period, phospho-N-acetylmuramyl (pentapeptide) is found in the reaction products. This product could arise through hydrolysis of the postulated enzyme-P-N-acetylmuramyl (pentapeptide) complex. (4) A small part (5-10%) of the maximal exchange activity was found to be sufficient to ensure a major part (about 50%) of the transfer activity. (5) The K_m values for K^+ ions are 1×10^{-2} M in the exchange assay and 2.4×10^{-3} M in the transfer assay. (6) At selected concentrations, certain detergents stimulate the transfer reaction as if they enhanced the ability of the enzyme to bind a recognized acceptor (i.e., the undecaprenyl-P), whereas they inhibit the exchange assay. Vancomycin stimulates the transfer reaction and inhibits the exchange reaction at low concentrations, but higher concentrations inhibit both reactions. These results have been interpreted to be an indication that vancomycin acts as a surfactant in the transfer assay and inhibits the exchange assay by forming an adduct with the terminal D-alanyl-D-alanine of the pentapeptide substrate [93].

The specificity profiles [93] of reactions 14a and b (Fig. 19) are also of interest with respect to their ability to complement those of the earlier reactions. The translocase exhibits a high specificity for the uracil moiety, suggesting a key role for pyrimidine in the reaction. While all of the biosynthetic enzymes will utilize the fluoro-substituted nucleotide, the translocase does not. Thus, fluorouracil causes great accumulations of FUDP-N-acetylmuramyl pentapeptide when various bacteria are grown in the presence of this analog [93, 93a]. Although complete studies of the use of analogs by the translocase have not yet been reported, it is known that a C-terminal D-alanyl-D-alanine sequence is an essential feature of the substrate. The utilization of UDP-N-acetylmuramyl pentapeptide with substituents on the ε-amino group of L-lysine (e.g., pentaglycine, L-alanine, or L-serine) in the transfer reaction by preparations from various microorganisms has been reported. An interesting example of the complementary specificities of the various reactions is the case of O-carbamyl-D-serine in S. faecalis [74]. The analog UDP-N-acetylmuramyl-L-alanyl-γ-D-glutamyl-L-lysyl-D-alanyl-O-carbamyl-D-serine is as effective as the normal UDP-N-acetylmuramyl pentapeptide in the exchange reaction (reaction 14a). O-Carbamyl-D-serine is used as a substrate by the D-alanyl-D-alanine synthetase to form D-alanyl-O-carbamyl-D-serine, which in turn can be incorporated into UDP-N-acetylmuramyl-pentapeptide. However, the analog is an effective inhibitor of alanine racemase (reaction 10) and so would prevent the initiation in vivo of a series of reactions resulting in an altered peptidoglycan.

2. Translocation of N-Acetylglucosamine [12, 96-98]

This translocation is a transglycosidation reaction (reaction 15; Fig. 19). N-Acetylglucosamine is transferred from UDP-N-acetylglucosamine to undecaprenyl-PP-N-acetylmuramyl (pentapeptide) carrier. The reaction results in the generation of UDP and of undecaprenyl-PP-disaccharide (pentapeptide). The disaccharide is β-1,4-N-acetylglucosaminyl-N-acetylmuramic acid and is linked to the pyrophosphate through the C-1 of N-acetylmuramic acid. This reaction differs from reaction 14 (Fig. 19) in that only N-acetylglucosamine, and not the terminal phosphate of UDP, is transferred to the lipid intermediate.

3. Translocation of Disaccharide-Pentapeptide into the Wall

This reaction (16; Fig. 19), which occurs at the interface of the membrane and wall, completes the lipid cycle. It is also the first of the series of reactions of stage 3 that occur in the wall itself, and is discussed in Section H.

F. Incorporation of Interpeptide Chains

In all peptidoglycans other than those of chemotype I, the bridges that extend between the peptide units consist of one or several additional amino acid residues. The incorporation of these bridging amino acid residues onto the pentapeptide units takes place before the transfer of disaccharide peptide units from the lipid carrier to the expanding wall. In the case of the peptidoglycans of chemotype II, the process of incorporation essentially consists of the extension of the length of the side chain of the L-R_3 residue through the substitution of the ω-amino group by one or several amino acid residues. Often, but not always, these amino acids are activated as aminoacyl-transfer RNA derivatives and are then transferred to the peptidoglycan unit on the lipid intermediate. Most of the reaction sequences investigated are catalyzed by particulate enzymes.

The synthesis of the N^{ϵ}-pentaglycyl-L-lysine sequence in S. aureus (Fig. 8a) has been studied in detail [102-104]. Five glycine residues from glycyl-tRNA are sequentially added to the ϵ-amino group of L-lysine on the undecaprenyl-PP-disaccharide-peptide intermediate. Four species of $tRNA^{Gly}$ exist in S. aureus [105]. All four support the incorporation of glycine into the peptidoglycan unit. Three of these $tRNAs^{Gly}$ participate in template-directed polypeptide synthesis. One of them, however, is inactive in polynucleotide-directed polypeptide synthesis and does not bind to ribosomes in the presence of any of the glycine codons. This apparently peptidoglycan-specific $tRNA^{Gly}$ might be a unique gene product. It has been extensively purified, shown to lack all of the minor bases except for a single thiouridine residue, but its sequence has not yet been published [12, 106]. Staphylococcus aureus contains only a single $tRNA^{Gly}$ synthetase which has been isolated and purified [107]. The synthetase functions in catalyzing the formation of glycyl-tRNA's used for both peptidoglycan and protein biosynthesis. In the presence of ATP and Mg^{2+}, it can also charge the tRNA's of other bacteria and yeast with glycine.

The synthesis of the N^{ϵ}-L-alanyl-L-lysine sequence in Arthrobacter crystallopoietes [108] (Fig. 8f) is carried out through a mechanism similar to that described for the synthesis of N^{ϵ}-pentaglycyl-L-lysine in S. aureus. L-Alanine is incorporated by particulate enzymes into the peptidoglycan unit at the level of the lipid intermediate from L-alanyl-tRNA. L-Alanyl-$tRNA^{Cyst}$ was prepared by reduction of L-cysteinyl-$tRNA^{Cyst}$ and was shown not to be utilized in peptidoglycan synthesis. This suggested that tRNA did not act simply as a carrier of activated L-alanine, but that some specific feature of this tRNA-Ala molecule was recognized by the peptidoglycan-synthesizing system.

In Staphylococcus epidermidis [109], four different pentapeptide chains have been found as substituents on the ϵ-amino group of L-lysine (Fig. 8c). All four contain three glycine residues and two L-serine

residues, but each has a different sequence. Particulate enzymes, glycyl-tRNA, and L-seryl-tRNA carry out the incorporation of glycine and L-serine at the level of the lipid intermediate. Maximum L-serine incorporation requires the simultaneous incorporation of glycine, but glycine incorporation is independent of L-serine incorporation. Four $tRNAs^{Ser}$ were isolated. All participate equally well in peptidoglycan synthesis. Two of them bind to E. coli ribosomes in the presence of either of two serine codons, UCA or UCG. A third binds in the presence of either of two other serine codons, AGU or AGC. The fourth does not bind in the presence of any of the known serine triplets. Hence, if tRNA is specifically recognized by the peptidoglycan-synthesizing system, the tRNA specificity is unlikely to be at the anticodon region. Whether or not each of the four $tRNAs^{Ser}$ is able to catalyze only one type of bridge is an interesting problem that has not yet been solved.

The incorporation of L-alanine to the ε-amino group of L-lysine in the peptidoglycan unit of Lactobacillus viridescens [110] (Fig. 8d) is unusual in that L-alanine is transferred from L-alanyl-tRNA to cytoplasmic UDP-N-acetylmuramyl-pentapeptide and not to the membrane-bound lipid intermediate. Moreover, the transfer is catalyzed by a soluble enzyme that exhibits a low specificity for the amino acid and a lack of specificity for tRNA. This transferase catalyzes the transfer not only of L-alanine from L-alanyl-tRNA but also, although with a lower efficiency, of L-serine, L-cysteine, and probably glycine, from their corresponding specific tRNA's, to UDP-N-acetylmuramyl-pentapeptide. Moreover, the transferase is able to utilize* L-alanyl-$tRNA^{Cyst}$ in peptidoglycan synthesis.

In all the aforementioned examples of incorporation of interpeptide chains, the amino acids that are added to the ω-amino group of the peptidoglycan unit are glycine or L-amino acid residues or both. The involvement of tRNA in the process seems to be general, although as yet a unified model cannot be proposed. The mechanism completely differs from mRNA-coded protein synthesis on ribosomes. However, there exist both in bacteria and in animal tissues, several soluble aminoacyl-tRNA transferases which catalyze the addition of certain amino acids to the N-terminus of proteins in the absence of ribosomes [111-113]. It should also be noted that a requirement for tRNA has also been demonstrated in the synthesis of aminoacylphosphatidylglycerols by bacterial enzymes [114].

In many Lactobacilli other than L. viridescens, the interpeptide bridge consists of a single isoasparaginyl residue that has the D-configuration (Fig. 8g). The syntheses of the N^{ε}-(D-isoasparaginyl)-L-lysine sequence in S. faecalis and L. casei have been studied. In these cases [115, 116] tRNAs do not participate. On the contrary, D-aspartic acid is activated in the form of a β-D-aspartylphosphate by an enzyme that can be released from the membrane by high salt concentrations. D-Aspartic acid is then transferred to the ε-amino group of L-lysine of the lipid intermediate, and finally its α-carboxyl group is amidated (in the presence of NH_3 and ATP).

By analogy with the foregoing, one can postulate that the synthesis of the peptide bridges in the peptidoglycans of chemotype IV (Figs. 11-13) also occur via the addition of one or several amino acid residues to the pentapeptide units. In this latter case, however, the extension of the pentapeptide must occur from the α-carboxyl group of D-glutamic acid. The interpeptide bridges of chemotype IV always contain a diaminoacid residue, which frequently has the D configuration. The mechanism by which these amino acids are incorporated is completely unknown. The peptide bridges in the peptidoglycans of chemotype III (Fig. 10), consist of one or several peptides, each having the same amino acid sequence as the peptide unit. It seems very unlikely that, in this latter case, bridge synthesis would occur through stepwise addition of amino acid residues. It has been hypothesized (Fig. 22) that some of the completed peptide units are translocated from N-acetylmuramic acid to a bridging position through the successive, alternating action of transpeptidase(s) and N-acetylmuramyl-L-alanine-amidase [43].

G. Further Alterations of Peptidoglycan Units

Variations occur both in the glycan and peptide portions of the peptidoglycans of many bacterial species (see Section II, A above). In terms of the general structure of the polymers, they are of minor importance. In most cases, the mechanisms involved are not understood. It is known, however, that in S. aureus [117], the conversion of D-glutamic acid of the pentapeptide unit into D-isoglutamine is carried out by amidation of the α-carboxyl group in the presence of ATP and ammonium ions at the level of the lipid intermediate. In M. lysodeikticus [118] the substitution of the same α-carboxyl group of D-glutamic acid by a glycine residue is also carried out in the presence of ATP at the lipid level. It should be noted that this glycine residue remains unsubstituted in the completed peptidoglycan and is not utilized as an interpeptide bridge. Another variation that has been described recently is the occurrence of small amounts of pentapeptides ending in either a glycyl-D-alanine or D-alanyl-glycine sequence, instead of the usual D-alanyl-D-alanine sequence, in the nucleotide precursors of some species of Arthrobacter and Corynebacteria [119, 120]. These alterations were observed only when inhibitory concentrations of glycine were present in the media. It is of interest to note here that the specificity profiles of the D-alanyl-D-alanine synthetase and the D-alanyl-D-alanine adding enzymes of S. faecalis would permit the substitution of glycine for D-alanine at the penultimate position of the UDP-N-acetylmuramyl pentapeptide [78].

The peptidoglycan of the cortex of bacterial spores is different from the wall peptidoglycan of the corresponding vegetative cells, although

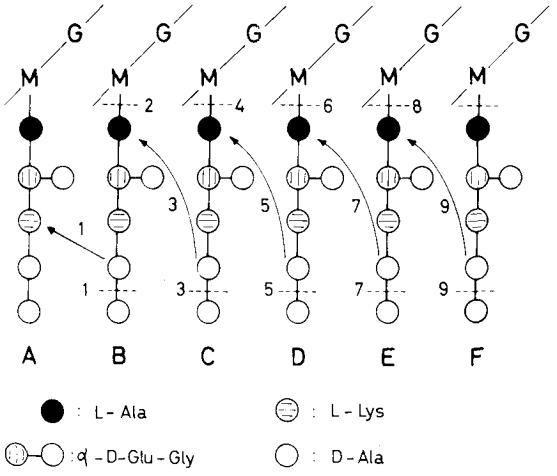


FIG. 22. Proposed biosynthetic sequence of the modification of the peptidoglycan in Micrococcus lysodeikticus. Reactions 2, 4, 6, and 8 are the result of hydrolysis by an N-acetylmuramyl-L-alanine amidase. Reactions 1, 3, 5, 7, and 9 are transpeptidations. Reprinted from reference 43 by courtesy of the American Chemical Society.

the two peptidoglycans are basically composed of the same constituents (see Section IV, C). It has been suggested that different segments of the genome are involved in the synthesis of the two peptidoglycans. Some of the enzymes that are responsible for the unique features of the cortex have been studied. Most of them, however, remain to be investigated. Similarly, the factors that must control the repression and expression of the various enzymes involved in the differentiation process are unknown.

H. Stage 3: Translocation of Disaccharide-Peptide Units into the Wall

The transfer of the disaccharide-peptide units from the membrane carrier into the expanding wall peptidoglycan should not be regarded as having been fully established. It is likely that each unit is transferred from the lipid intermediate to an endogenous wall acceptor, resulting in the addition of a disaccharide-peptide unit to the growing peptidoglycan (reaction 16; Fig. 19). In several cases the cell-free reaction product has been found to be solubilized by an endo-N-acetylmuramidase, suggesting the formation of glycosidic rather than peptide bonds. The formation of lipid-linked disaccharide-peptide oligomers, however, (such as those found in O-antigen synthesis in gram-negative bacteria [99]) has not been

eliminated as an intermediate step. Whatever the mechanism, the reaction generates undecaprenyl pyrophosphate, which in turn, is dephosphorylated (reaction 17; Fig. 19). This dephosphorylation reaction yields inorganic phosphate and the initial C_{55}-isoprenoid alcohol phosphate carrier, which then can begin a new cycle. Bacitracin [100, 101] was found to be an inhibitor of the phosphatase, which catalyzes the dephosphorylation of the undecaprenyl pyrophosphate.

The wall acceptors, which are assumed to be used for the insertion of the activated disaccharide-pentapeptide units, must be nonreducing termini of N-acetylglucosamine. Such termini can be generated by an endo-N-acetylmuramidase autolysin (reaction 18; Fig. 19; see also Section IV, D). There is no definite proof, however, that incorporation is actually carried out through such a mechanism. The suggestion has already been made (see Section II, D) that hydrogen bonding may also play an important role in aligning the nascent peptidoglycan in the expanding wall. It must also be remembered that somewhere in or after the peptidoglycan biosynthetic sequence, the addition of covalently linked accessory wall polymers, such as teichoic acids, must occur.

I. Transpeptidation Reaction

The insertion of newly synthesized, but as yet uncross-linked disaccharide peptide units into the growing wall peptidoglycan must be followed by closure of the bridges between the peptide units if the process is to yield an insoluble network. It may well be, however, that insertion and peptide cross-linking occur simultaneously. The mechanism for peptide cross-linking was first revealed by a study of the effects of penicillin on S. aureus [121-123]. Penicillin G, when added at sublethal doses to growing staphylococcal cells, was shown to reduce the extent of peptide cross-linking. Walls isolated from cells grown in the presence of low concentrations of the antibiotic contained higher amounts of uncross-linked peptide units than did walls isolated from cells grown in the absence of penicillin. Moreover, the peptide units that had not undergone transpeptidation because of the presence of penicillin, retained the C-terminal D-alanyl-D-alanine sequence of the peptidoglycan precursors and remained uncross-linked after subsequent growth in the absence of penicillin [123]. It was therefore proposed that the closure of bridges in S. aureus was achieved through a transpeptidation reaction, and that the transpeptidase, which catalyzes the reaction, was the target of the penicillin molecule. The mechanism of the transpeptidation reaction in S. aureus is shown in Fig. 23. The penultimate C-terminal D-alanine of a peptide donor is transferred to the N-terminal glycine of a peptide acceptor. Interpeptide bonds are formed, and equivalent amounts of D-alanine residues are liberated from the donor peptides.

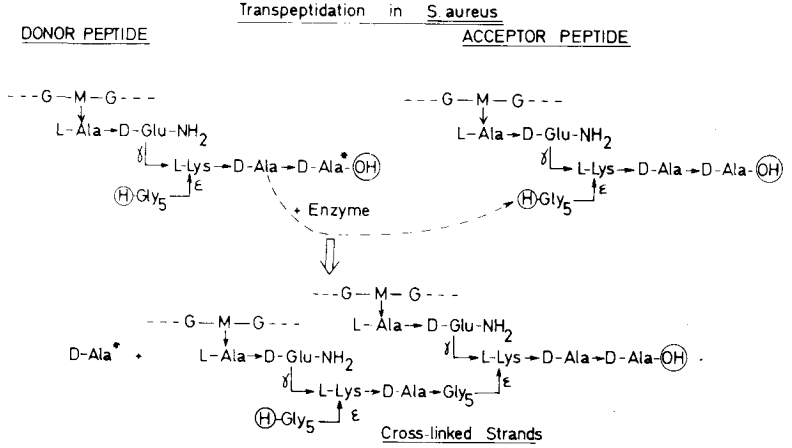


FIG. 23. Proposed transpeptidation reaction in Staphylococcus aureus Copenhagen. The completed, cross-linked peptidoglycan of S. aureus is shown in Fig. 2.

The use of the terminology for "donor" and "acceptor" here, and subsequently, is the same as that used in protein and polypeptide antibiotic biosynthesis [123a, 123b].

A cell-free system, which would carry out the reaction in vitro, has not yet been isolated from S. aureus. Fortunately, gram-negative bacteria allowed the study of the transpeptidation reaction in a more direct way. Particulate preparations [124-128] were obtained from E. coli and Salmonella, which performed the bridge-closure reaction (Fig. 24). In this case, the amino group involved in the reaction is located on the D-carbon of the meso-diaminopimelic acid residue of the peptide acceptor, and the peptide bond formed is D-alanyl-D-meso-diaminopimelic acid. The in vitro reaction was also shown to be inhibited by penicillin. Efforts of several laboratories to extend these studies to microorganisms other than E. coli and Salmonella have not yet achieved this goal. Transpeptidation, however, is thought to be ubiquitous among bacteria for several reasons. (1) It effectively explains why a single D-alanine residue is involved in cross-linking the peptide units in all bacterial peptidoglycans, whereas the largest peptides of the nucleotide precursors end in a C-terminal D-alanyl-D-alanine sequence. (2) Transpeptidation does not require an input of energy and therefore can result in peptide-bond formation at exocellular sites where ATP is not available. This is a particularly useful feature when one of the substrates is not only exocellular but may be insoluble. (3) Treatment with penicillin or related antibiotics results in lysis of bacteria from a wide variety of taxonomic groups.

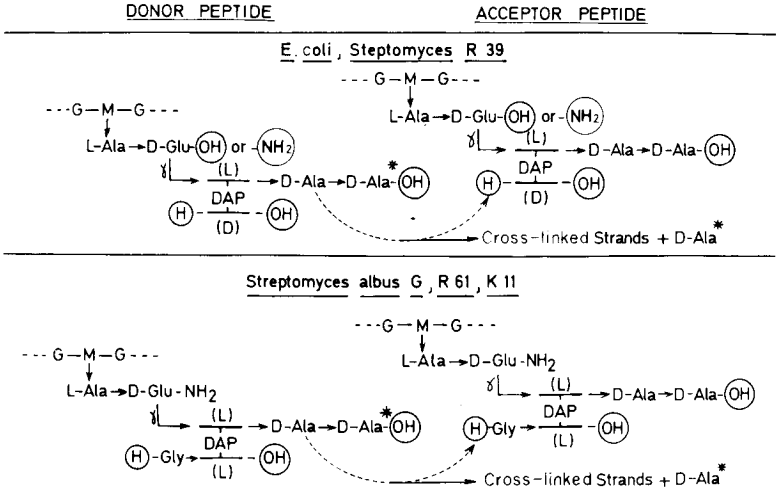


FIG. 24. Proposed transpeptidation reactions in E. coli and several Streptomyces sp. The structure of the cross-linked strands is shown in Fig. 6 for E. coli and Streptomyces R39, and in Fig. 9 for Streptomyces albus G, R61, and K11. Note that the proposed cross-linking reactions in Streptomyces sp. are based on the structure of the completed wall peptidoglycans.

The direction of growth of the peptide moiety is unknown, i.e., it is not known whether the D-alanyl-D-alanine donor group belongs to the newly transported disaccharide peptide unit or to the wall peptide oligomer. Whatever the mechanism, the D-alanine residue that is released can be reutilized by the cell either for peptidoglycan synthesis or for teichoic acid synthesis, by means of a specific transport system (reaction 20; Fig. 19). In E. coli the transport of D-alanine, L-alanine, and glycine has been partially resolved [71, 129]. It is mediated by at least two systems. The systems for D-alanine and glycine are related and are different from that for L-alanine. Study of some mutants resistant to D-cycloserine has shown that D-cycloserine is accumulated in sensitive cells through at least some part of the D-alanine/glycine transport system.

J. DD-Carboxypeptidase Transpeptidase

No bacterial membrane-bound transpeptidase has yet been released from the membrane, purified and characterized. Streptomyces sp., however, excrete into the culture medium enzymes that can function as carboxypeptidases (hydrolytic enzymes) or as transpeptidases (synthesizing enzymes), depending upon the availability of nucleophilic acceptor

(H_2O or NH_2-R). Such enzymes produced by several strains of Streptomyces were isolated and purified [130-135a]. They act on peptides ending in a C-terminal D-alanyl-D-alanine sequence and can differ in function since, after elimination of the C-terminal D-alanine residue of a peptide donor, the peptidyl-enzyme complex can be attacked by water (leading to simple hydrolysis; D-alanyl-D carboxypeptidase activity) or by a recognizable amino group (leading to peptide bond formation, transpeptidase activity) (Fig. 25). The two steps involved in the mechanism may occur either in a sequential manner, i.e., formation of a peptide-enzyme acyl intermediate and subsequent attack by a nucleophilic group with regeneration of the free enzyme, or in a concerted manner, i.e., via the formation of a donor-acceptor-enzyme tertiary complex. Structural studies of the wall peptidoglycan in several strains of Streptomyces suggest that the transpeptidation reaction in these organisms must be that shown in Fig. 24.

The substrate requirements for carboxypeptidase and transpeptidase activity of several purified enzymes isolated from different strains of Streptomyces were studied with the help of synthetic and natural peptides. The enzymes produced by strains R39, R61, K11, and albus G had high hydrolytic activity on the synthetic tripeptide N^{α}, N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine from which they release the C-terminal D-alanine residue. The discovery of these enzymes allowed the study of their carboxypeptidase action on peptides possessing the general structure acetyl-L-R_3-R_2-R_1 (OH) (Tables I-III). The series of enzymes differed in their K_m and V_{max} values with various peptides, but all of them exhibited the same general substrate profile [131-134], which was characterized by considerable specificity for the presences of (1) a D-amino acid residue, often preferentially D-alanine, at the C-terminal or R_1 position, (2) solely D-alanine at the penultimate R_2 position, and (3) a relatively long side chain on the L-R_3 residue (for further details, see reference 135a). This profile closely resembles the structure of the peptide units of the nascent Streptomyces peptidoglycans which undergo transpeptidation (Fig. 24). In fact, a C-terminal D-alanyl-D-alanine sequence occurs in all nascent peptidoglycans and an L-R_3 group with the same type of profile as above is ubiquitous in peptidoglycans of chemotypes I and II.

Evidence that these Streptomyces enzymes are the transpeptidases that effect the closure of the bridges in the biosynthesis of the wall peptidoglycans includes the following.

1. In the presence of a suitable carboxyl donor, such as N^{α}, N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine, and a proper amino acceptor, the purified enzymes from Streptomyces R61, K11, and R39 were found to be able to catalyze transpeptidation with the concomitant release of the terminal D-alanine of the donor peptide [135]. The reactions occur in the absence of an exogenous input of energy. With either [^{14}C]-D-Ala, [^{14}C]-Gly, or [^{3}H]-meso-diaminopimelic acid as acceptor, these three

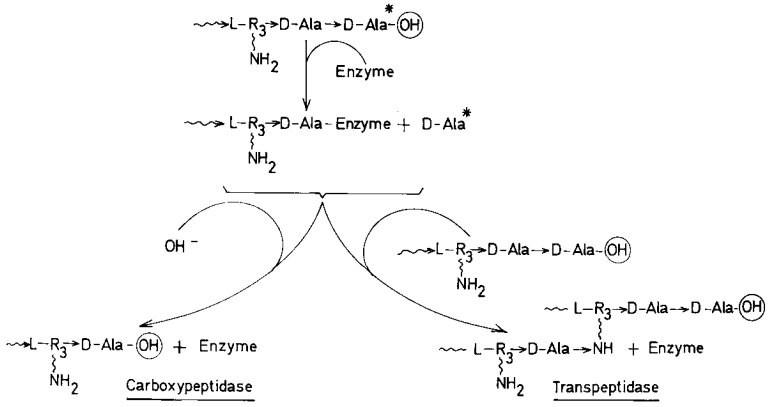


FIG. 25. Possible mechanism of hydrolysis and transpeptidation by "DD carboxypeptidase-transpeptidase" enzymes (see text).

enzymes catalyzed the formation of either diacetyl-L-lysyl-D-alanyl-[^{14}C]-D-alanine, diacetyl-L-lysyl-D-alanyl-[^{14}C]-glycine, or diacetyl-L-lysyl-D-alanyl-D-[^{3}H]-meso-diaminopimelic acid. In the presence of saturating acceptor concentrations, the time course of transpeptidation paralleled the time course of hydrolysis of the donor peptide when no acceptor other than water was present.

2. In some cases, transpeptidation was shown to occur at a molar ratio of amino acceptor:water as low as $1:1.8 \times 10^6$ (assuming that the concentration of water in the active site region of the enzyme is 55 M), demonstrating the exceedingly high efficiency of the enzymes as transpeptidases [135].

3. The Streptomyces enzymes exhibited differences with respect to their requirements for peptide acceptors and these differences reflected the differences in structure of the wall peptidoglycans of the corresponding strains.

As shown in Figs. 9 and 24, the interpeptide bond in strains R61 and K11 is a D-alanyl-glycyl linkage, which is in an endoposition, whereas the interpeptide bond in strain R39 is a D-alanyl-(D)-meso-diaminopimelic acid linkage, which is in an α-position to a free carboxyl group. As shown in Table IV, the R39 transpeptidase only catalyzes the synthesis of interpeptide bonds that are α to a free carboxyl group, whereas both R61 and K11 enzymes are able to catalyze the synthesis of peptide bonds either in an endo- or at a C-terminal position.

DD-Carboxypeptidase activity (as shown by the release of the C-terminal D-alanine residue from UDP-N-acetylmuramyl-pentapeptide precursors) also exists in gram-negative bacteria (E. coli; Salmonella) [125, 127], in gram-positive bacteria (particularly in several Bacilli) [136-139], and blue-green algae (Anaboena variabilis) [140]. Only cell-bound activity has been found so that carboxypeptidase-transpeptidase excretion may be a property unique to Streptomyces. In E. coli, the DD-carboxypeptidase is located in the inner plasma membrane of the cell envelope and has been solubilized by sonication of the cells [127] and by detergent treatment of the membrane [141]. The specificity of the soluble DD-carboxypeptidase from E. coli has not yet been studied, and so far an attempt to test it in a transpeptidase assay with defined peptide substrates has not been reported. In contrast, the crude "particulate" preparation obtained from E. coli exhibited transpeptidase activity as well as carboxypeptidase activity, in assays that involved concomitant peptidoglycan synthesis from UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-pentapeptide precursors [128]. It is possible that the E. coli enzyme requires a polymerized (at the glycan level) nascent peptidoglycan in order to function as a transpeptidase. It is also possible that E. coli, as well as other bacteria, contain a protein that exhibits only a carboxypeptidase activity, in addition to the carboxypeptidase-transpeptidase system such as that described for some Streptomyces enzymes.

K. Inhibition of the Transpeptidation Reaction by Penicillin

It is certain that an early and important step in the lethal action of penicillin G (and other penicillins and cephalosporins) upon bacteria is the abolition of, or reduction in efficiency of, the transpeptidase involved in peptide cross-linking. The purified Streptomyces R61, K11, and R39 enzymes were found to be inhibited by several penicillins and cephalosporins. With each antibiotic, inhibition of both carboxypeptidase and transpeptidase activity occurred at the same concentrations [135 and unpublished results]. This was additional evidence that at least in these Streptomyces sp. it is one and the same enzyme that is responsible for both activities.

The mechanism of inhibition by penicillin of hydrolysis of the peptide donor was studied in the absence of amino acceptor. Penicillin appeared to combine with the R61 enzyme at a site that was not identical with the substrate binding site. Kinetically, the inhibition of the R61 enzyme was competitive [133]. However, competitive kinetics do not necessarily exclude an inhibitory mechanism other than a direct competition between substrate and inhibitor for the same site on the

TABLE 1

Substrate Requirements of Streptomyces DD-Carboxypeptidases-Transpeptidases [a, b]

Ac → L-Lys → R_2 → R_1(OH) ↑ ε Ac		R61			K11			R39			albus G		
		K_m	V_{max}	Efficiency	K_m	V_{max}	Efficiency	K_m	V_{max}	Efficiency	K_m	V_{max}	Efficiency
Ac → L-Lys →	D-Ala → D-Ala	12	890	72	11	2,000	182	0.8	330	410	0.33	100	300
↑ ε	D-Ala → D-Lys	13	80	7							0.80	85	106
Ac	D-Ala → D-Leu	10	50	5	10	160	16	0.7	230	320	0.33	33	100
	D-Ala → Gly	36	200	6	12	220	18	2.5	100	40	2.50	60	24
	D-Ala → L-Ala	Virtually no hydrolysis			Virtually no hydrolysis			No hydrolysis			No hydrolysis		
	Gly → D-Ala	16	1.7	0.1	14	10	0.7	No hydrolysis			15.0	107	7
	D-Leu → D-Ala	10	10	1	13	38	2.9	No hydrolysis			No hydrolysis		
	L-Ala → D-Ala	Virtually no hydrolysis			Virtually no hydrolysis			No hydrolysis			No hydrolysis		

[a] Release of the C-terminal residue. Influence exerted by the C-terminal $R_2 \rightarrow R_1$ dipeptide sequence.

[b] K_m: mM; V_{max}: μ moles/mg/hour; Efficiency: V_{max}/K_m

TABLE 2

Substrate Requirements of Streptomyces DD-Carboxypeptidases-Transpeptidases[a]

~~→ L-R_3 → D-Ala ↓ D-Ala (OH)		Specific activity[b] R39	K11	R61	albus G
Ac → L-Ala →	D-Ala → D-Ala	200	1,100	650	300
R_1 → L-homoSer →		6,000	1,600	1,400	2,000
N^{α}, N^{γ}-bisAc → L-DAB →		14,000	6,500	4,000	22,000
N^{α}, N^{δ}-bisAc → L-Orn →		75,000	50,000	21,000	22,000
N^{α}, N^{ε}-bisAc → L-Lys →		90,000	90,000	47,000	40,000

[a] Release of the C-terminal residue. Influence of the length of the side-chain of the L-R_3 residue that precedes the C-terminal D-alanyl-D-alanine sequence.

[b] mμEq of linkage hydrolyzed/mg/h; R_1: UDP-MurNAC-Gly-γ-D-Glu.

TABLE 3

Substrate Requirements of Streptomyces DD-Carboxypeptidases-Transpeptidases[a,b]

(L) CH → D-Ala → D-Ala (OH); $(CH_2)_3$; R'-NH-CH-R''		R61			K11			R39			albus G		
		K_m	V_{max}	Efficiency	K_m	V_{max}	Efficiency	K_m	V_{max}	Efficiency	K_m	V_{max}	Efficiency
(1) Ac → ⊤ → $CH_3CONH-CH_2$	D-Ala → D-Ala	12	890	72	11	2,000	182	0.80	330	410	0.33	100	300
(2) Ac → ⊤ → NH_2-CH_2		15	4	0.3	30	9	0.3	0.20	600	3,000	6.0	20	3
(3) R_1 → ⊤ → $NH_2-CH-COOH$ (D)		11	8	0.3	17	18	1.06	0.25	400	1,600	0.4	10	25
(4) R_2 → ⊤ → $NH_2-Gly_5-NH-CH_2$		14	800	57	11	1,050	95	0.30	420	1,400	0.28	9	52

[a] Release of the C-terminal residue. Influence exerted by, the presence of charged groups at the end of the side chain of the L-R_3 residue that precedes the C-terminal D-alanyl-D-alanine sequence. The side chains are those of the following amino acids. (1) N^{ε}-acetyl-L-lysine; (2) L-lysine; (3) meso-diaminopimelic acid; (4) N^{ε}-pentaglycyl-L-lysine.

[b] K_m mM; V_{max}, μmole/mg/h; efficiency, V_{max}/k_m. R_1, UDP-MurNAC-L-Ala-D-Glu-γ-; R_2, [N^{α}-Disacch-L-Ala-D-Glu(NH_2)-γ].

free enzyme. Binding of penicillin G to the R61 enzyme was found to cause quenching of its fluorescence. The dissociation constant of the enzyme-penicillin complex as measured by fluorescence quenching (10^{-8} M) was in close agreement with the K_i value as determined by kinetic measurements. Both acceptor and donor peptide did not decrease the affinity of the R61 enzyme for penicillin [141a]. The penicillin molecule has a highly reactive CO-N bond in its lactam ring causing it to be a powerful acylating agent. The inhibition of the Streptomyces R61 carboxypeptidase-transpeptidase by penicillin G was found to be reversible and to occur in the absence of detectable acylation of the protein by the antibiotic.

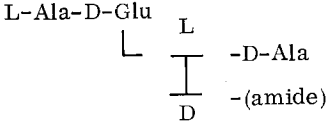
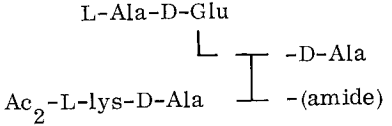
The transpeptidase activity of the particulate preparation from E. coli [128], the carboxypeptidase activity of the soluble preparation obtained by sonication of the same organism [127] as well as the carboxypeptidase activity of the particulate preparations from Bacilli [136, 137] and Anaboena [140] were also found to be inhibited by low doses of penicillin. The E. coli soluble DD-carboxypeptidase was competitively [127], and most probably, reversibly inhibited by penicillin. With some of the other enzyme preparations, irreversible fixation of penicillin was observed, but a direct demonstration that enzyme inactivation via penicilloylation occurred is lacking. It is possible, however, that different carboxypeptidase-transpeptidases from different organisms may be inhibited or inactivated in different ways.

Despite a number of hypotheses and experiments, even today little is known about the precise molecular basis of penicillin action [142]. The previously proposed ideas that penicillin could be a structural analog of the nascent peptidoglycan either at the level of N-acetylmuramic acid [143] or at the level of the D-alanyl-D-alanine donor site [122] were based on too few experiments and were probably premature. The resemblance between penicillin and D-alanyl-D-alanine is not close in two respects. The peptide bond between the two D-alanine residues is about 25% longer than the corresponding bond in the β-lactam ring of penicillin. Moreover, the angle around the D-alanyl-D-alanine peptide bond (normally 180 deg) is considerably different from the angle around the corresponding bond in the β-lactam ring (135.7 deg). It has been proposed [144] that cleavage and formation of peptide bond by the transpeptidase occurs via a transition state during which the D-alanyl-D-alanine peptide bond would be distorted to an angle of 135.7 deg. Such a distortion would be energetically unfavorable and the driving force required for it would be produced by the energy of binding to

TABLE 4

Substrate Requirements of Three Streptomyces DD-Carboxypeptidases-Transpeptidases Acting as Transpeptidases [a, c]

Acceptor used	Product of transpeptidation	Yield of product with enzymes [b] R61 or K11	R39
Gly-Gly	Ac_2-L-lys-D-Ala-Gly-Gly	+ + +	0
Gly-Gly-Gly	Ac_2-L-lys-D-Ala-Gly-Gly-Gly	+ +	0
Gly-L-Ala	Ac_2-L-lys-D-Ala-Gly-L-Ala	+ + +	0
Gly-(L) / Ac-(L)	Ac_2-L-lys-D-Ala-Gly-(L) / Ac-(L)	+ +	0
Gly-D-Ala	Ac_2-L-lys-D-Ala-Gly-D-Ala	+	0
D-Ala-Gly	Ac_2-L-lys-D-Ala-D-Ala-Gly	+	0
D-Ala-L-Ala	Ac_2-L-lys-D-Ala-D-Ala-L-Ala	+	0
L-Ala-L-Ala		0	0
L-Ala-D-Glu (L) -D-Ala / (D) - (OH)	Ac_2-L-lys-D-Ala (L-Ala-D-Glu (L) -D-Ala / (D) - (OH))	+ +	+ +

L-Ala-D-Glu 	-D-Ala -(amide)	+ +	0
L-Ala-D-Glu 	-D-Ala -(amide)		

[a] Transfer of diacetyl-L-lysyl-D-alanyl from diacetyl-L-lysyl-D-alanyl-D-alanine tripeptide donor to various peptides acceptors.

$\overset{L}{\underset{D}{\top}}$ = meso-diaminopimelic acid; $\overset{L}{\underset{L}{\top}}$ = LL-diaminopimelic acid.

[b] + + +, + +, +: high, good, and low yield of product of transpeptidation, respectively. 0 : no detectable product.

[c] Unpublished results from H. R. Perkins, M. Nieto, J. M. Ghuysen, J. M. Frère, M. Leyh-Bouille, R. Moreno, J. Dusart, and A. Marquet.

the protein. Since the penicillin molecule is already suitably distorted, its binding to the transpeptidase would be much more favorable. It has also been assumed that cleavage of the β-lactam amide bond would then follow with concomitant acylation and irreversible inactivation of the enzyme. Hence, according to this hypothesis, the molecular basis for penicillin action would rest upon a combination of two features: the resemblance to the transition state and the acylating capacity of the penicillin molecule. Experiments with the DD-carboxypeptidase-transpeptidase from Streptomyces R61 however, are in open conflict with this idea. Penicillin inhibition of this carboxypeptidase-transpeptidase does not rely on structural analogy as shown by fluorescent and peptide analog inhibition [141a, 144a] studies. In addition, penicillin inhibition was not accompanied by detectable acylation of the enzyme protein ([134] and unpublished results). The penicillin-treated enzyme could be recovered with full activity. Moreover, it has been recently found [144b] that the 6-methyl derivative of the methyl ester of penicillin which is a structurally closer analog of D-alanyl-D-alanine than the methyl ester of penicillin (since it has a methyl group in the position where penicillin has a hydrogen atom) is in fact completely inactive as an antibiotic. It is hoped that the Streptomyces transpeptidases and other soluble transpeptidases, which probably will be isolated from other bacteria in the near future, will provide accurate models for the study of penicillin-receptor interactions at the molecular level.

Much also remains to be done before the mechanism of action of penicillin at the cellular level is clearly understood. The problem is complicated by the fact that (1) on binding to living cells, irreversible fixation of the great majority of penicillin molecules takes place on sites that may not be concerned with peptidoglycan synthesis and that are probably irrelevant to the killing action of penicillin [145, 146], (2) more than one transpeptidase (for example, one for cell elongation and one for septum formation) may be present in the same bacterium, (3) transpeptidase may not be the only target of penicillin. It has been suggested that an autolytic glycosidase activity of E. coli was inhibited by very high doses of penicillin [147, 148]. Recent data on this topic can be found in reference 148a and 148b.

L. Mechanism of Action of Vancomycin [93, 149-154]

Vancomycin and ristocetin inhibit peptidoglycan synthesis. Vancomycin binds in vitro to wall peptidoglycan precursor nucleotides and other peptides terminating in R-D-alanyl-D-alanine or related structures in which both terminal residues are glycine or have the D-configuration. Thus, complex formation is thought to be the basis for the lethal action of this antibiotic. Experiments with radioactive iodovancomycin [155] showed that very little passed into the cytoplasm of M. lysodeikticus cells, although some was found in the membrane fraction. This suggests that vancomycin cannot

reach and bind to the nucleotide precursors in living cells. It may complex with the lipid intermediate that is about to be polymerized on the membrane surface (Section III, E). It may also interfere with later stages of maturation of the peptidoglycan involving transfer from the lipid intermediate into the wall and/or peptide cross-linking by transpeptidation. Reversal of vancomycin action has been obtained with the soluble peptide diacetyl-L-diaminobutyryl-D-alanyl-D-alanine [156]. This peptide binds vancomycin and counteracts both its inhibition of peptidoglycan synthesis and its enhancement of lipid intermediate accumulation. When the tripeptide was added to a culture of Bacillus megaterium at various intervals after vancomycin treatment, growth inhibition caused by the antibiotic was reversed.

IV. PEPTIDOGLYCAN AS PART OF THE CELL ENVELOPE

A. Location of the Peptidoglycan

In the bacterial cell, the peptidoglycan is located outside of the ultimate permeability barrier (protoplast membrane). In fact, the bacterial cell wall can be defined as that cellular organelle which is located outside of the protoplast membrane and contains peptidoglycan. Thus, the wall is an exocellular structure.

An important difference between the Gram-negative and Gram-positive species is that the envelope of Gram-negative bacteria contains an outer membrane very similar in appearance in thin sections to the inner plasma membrane. This outer membrane, sometimes called the L membrane [157], exhibits different permeability properties and contains lipopolysaccharides and lipoproteins. In a variety of Gram-negative species, a number of proteins and enzymes have been found to be released from the cells by various procedures involving osmotic shock, while other (intracellular) proteins are completely retained by the cells.

The complex layering in Gram-negative bacteria is clearly seen in thin sections [18, 157-158] (Figs. 26 and 27). An electron-dense layer G2 is separated from the outer L triple-layered membrane unit by a transparent layer G1, and from the inner triple-layered plasma membrane by another transparent layer M (the terminology is that proposed by De Petris [157]). The peptidoglycan has been located within the G2 layer, a dense line 1.5-2.0 nm thick, at the inner boundary of the wall part of the envelope. The peptidoglycan of E. coli was the first one to be isolated in the form of a rigid layer [8]. Granules of lipoprotein were shown to be covalently linked to it and they could be removed with proteolytic enzymes. In this organism a lysylarginine dipeptide extends from some of the meso-diaminopimelic acid residues of the peptidoglycan and covalently connects the structural lipoprotein to the peptidoglycan. On the average, one lipoprotein molecule

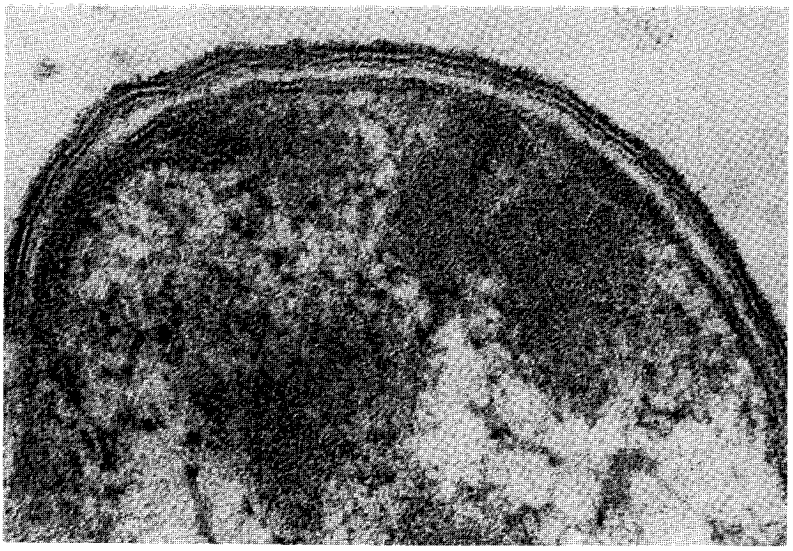


FIG. 26. Electron micrograph of a section of Proteus vulgaris P18 heated (5 minutes at 80°C) showing the complex multilayered structure of the cell envelope. Magnification x129,500. Reprinted from reference 18 by courtesy of Elsevier Publishing Co., Amsterdam.

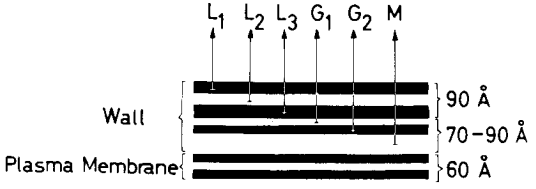


FIG. 27. Schematic representation of the structure of the cell envelope of Proteus vulgaris P18. The terminology is that used by De Petris [158] to describe the cell envelope of E. coli. The G_2 layer is thought to contain peptidoglycan. Reprinted from reference [18] by courtesy of Elsevier Publishing Co., Amsterdam.

would be linked to every tenth repeating peptide unit in the peptidoglycan [159]. In Gram-negative bacteria, this appears to be the only envelope polymer covalently linked to the peptidoglycan. Physical association between the plasma membrane and the peptidoglycan-containing G2 layer is not clear. It is thought that "adhesion sites" are present within the M

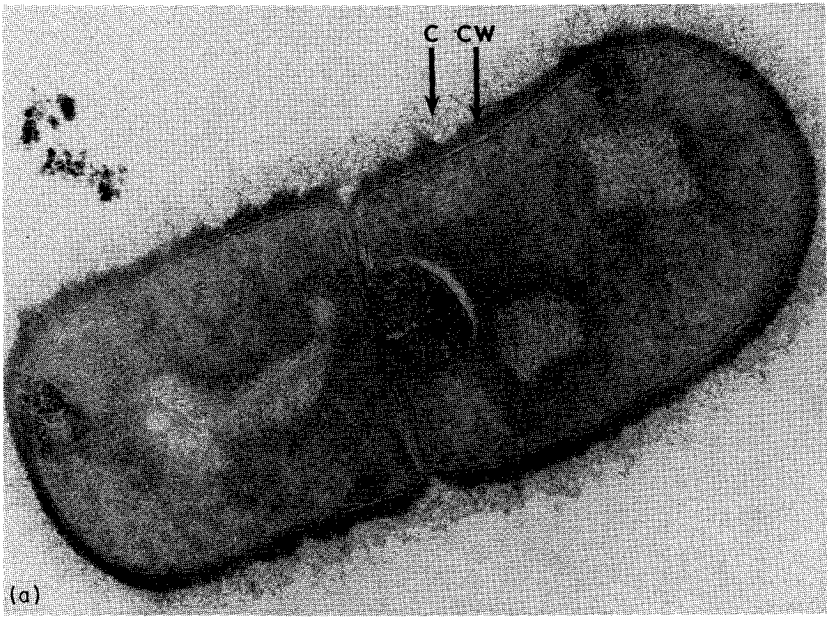
region [160]. Techniques are available which, after enzymatic degradation of the G2 layer, allow the separation of the inner membrane from the outer membrane and the purification of each of these organelles [161, 162].

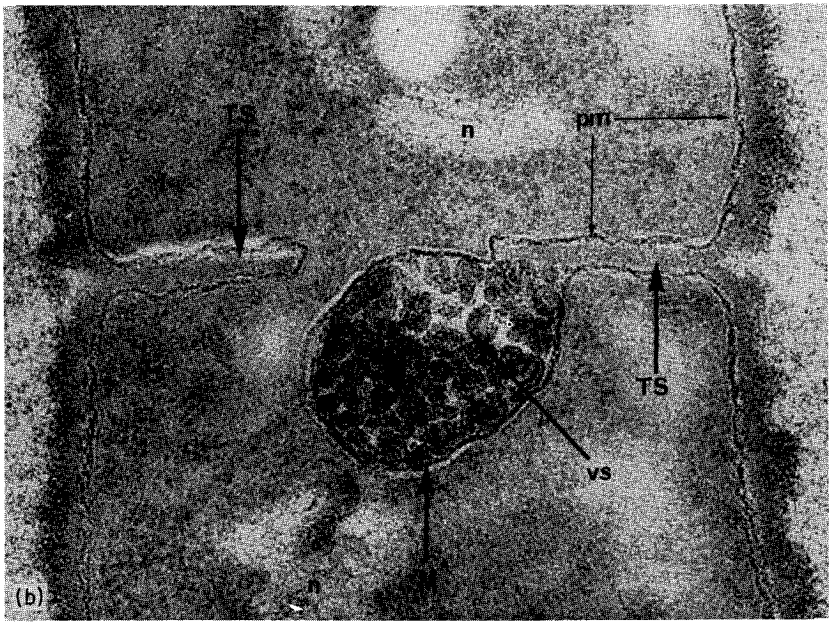
In general, the cell envelope of Gram-positive species lacks the outer L membrane [163] (Fig. 28). The wall, which surrounds the membrane, is considerably thicker (15-50 nm) than the equivalent G2 layer of the Gram-negative bacteria. It consists of 40-90% of peptidoglycan. An almost endless variety of polysaccharides that are frequently negatively charged and of polyolphosphate polymers that are collectively called teichoic acid [21] are covalently linked to the peptidoglycan and constitute the nonpeptidoglycan part of the wall. These latter polymers are highly antigenic. One, but probably not the only link between peptidoglycan and other wall polymers is via phosphodiester bridges from C-6 of N-acetylmuramic acid (see Section II, A above). By providing the chemical groups through which these polymers are anchored into the wall, the peptidoglycan contributes to the surface properties of the cell. In S. aureus [164, 165], for example, the N-acetylglucosamine residues of the teichoic acid are essential for phage fixation but, in order to be operative, these groups must possess a definite orientation that is imparted by the supporting insoluble peptidoglycan.

Truly layered wall profiles have been observed only infrequently in Gram-positive bacteria. One example is the layers seen after wall thickening had occurred during stationary-phase growth [166]. A second example is the highly ordered layer found at the surface of some species of Bacilli and Clostridia. For example, Nermut and Murray [167] suggest that (1) in Bacillus polymyxa the close-packed array (RS) of 7 nm units with a repeating frequency of 10 nm, which is visible on the outer surface, is a protein, (2) the intermediate layer is probably polysaccharide, and (3) the innermost layer is the site for teichoic acid and peptidoglycan.

B. Interrelationship between Wall and Plasma Membrane

The surface antigens of Gram-positive bacteria have two remarkable properties, i.e., their "mobility" and their functional importance in concentrating Mg^{2+} ions at the plasma membrane. Growth environment conditions exert deep influences on the composition of the wall anionic polymers in altering the relative proportion of polysaccharides and teichoic acids [168]. As noted by Rogers [14], "providing either one or another of the groups of negatively charged polymers is present on its surface, the microorganism seems content." This mobility occurs despite the fact that polysaccharides and teichoic acids are covalently linked to the peptidoglycan and thus implies the presence of an active system for turnover. The physiological importance of this phenomenon is revealed by the recent demonstration


C
CW
(a)


TS
n
pm
TS
vs
n
(b)

that teichoic acids seem to be essential for normal cellular activity and stability of the membrane [169]. In fact, two types of teichoic acids are involved. The wall teichoic acid is responsible for the capacity of isolated walls to bind Mg^{2+}. In addition to it, bacteria also contain a polyglycerol phosphate-teichoic acid, which is located in the periplasmic region of the cell, i.e., between the wall and the membrane. According to the model proposed by Baddiley and his colleagues, these two regions of anionic polymer would function as an integrated cation-exchange system between the exterior of the cell and the membrane, insuring to the latter the high concentration of Mg^{2+} required for many processes. One may assume that polymers other than teichoic acids have a similar function in Gram-negative bacteria.

C. Peptidoglycan in Resting and Germinating Spores

The peptidoglycan of the cortex in resting spores is extensively different from the wall peptidoglycan of vegetative cells. About 50% of the muramic acid residues in the spore peptidoglycan occur as nonacetylated internal amides [23]. The rest occurs as N-acetylmuramic acid, of which about half is substituted by only a C-terminal L-alanine residue, whereas the remainder is substituted by the usual type of peptide, which has a very low extent of cross-linking. Such profound alterations of the peptidoglycan suggest that the polymer fulfills an altered function in the spore cortex [69, 70]. The occurrence of muramic acid residues in the form of the δ-lactam prevents the lactyl groups from being involved in hydrogen bonding (see Section II, D). A likely consequence of this and of the low degree of peptide cross-linking, is a high flexibility of the polymer, which is, perhaps, better suited to its function in the spore cortex [11].

The envelope of resting spores has an exceedingly complex organization. Proceeding inward from the surface, thin sections of resting spores of B. polymyxa [170] reveal (1) the outermost sculptured surface of the sporangium, (2) the various laminae of the spore coats, (3) a zone of low

FIG. 28. Electron micrograph of a section of Bacillus megaterium ATCC 19213, taken from a synchronously dividing population at 3 hours. Note the absence of a distinctly layered appearance of the wall (CW) as compared with that usually seen in Gram-negative species (e.g., see Fig. 26). Also shown in the electron micrograph are the surrounding capsular material (C), the nascent transverse septum (TS), plasma membrane (pm), mesosomes (M), mesosomal vesicles (vs), and nuclear material (n). Note the close association of the nascent septum with a mesosome. Reprinted from reference 163 by courtesy of the American Society for Microbiology.

scattering corresponding to the cortex, (4) the wall primordium located at the inner boundary of the cortex in close vicinity to the plasma membrane, and (5) the spore body. The wall primordium presumably consists wholly or in part of peptidoglycan. The possession of a tripartite wall by the vegetative cells of B. polymyxa (see Section IV, A) implies that considerable change occurs to the wall primordium during spore germination. These changes involve elaboration of a new peptidoglycan structure and incorporation of other new wall components. They were followed by electron microscopy throughout germination [170] (Figs. 29 and 30). Rupture of the sporangial envelope and disintegration of the cortex were paralleled by a thickening of the wall primordium. This thickening was then reduced as cracks appeared in the laminae of the spore coats and the cell increased in size. At this stage of germination, patches of additional wall components composed of repeating elements, appeared under the cracks of the coat. The tripartite wall then enlarged from these centers and eventually covered the entire surface of the new cell. This remarkable study of the sequence of events in germination has made it possible to "see" a rearrangement and/or de novo synthesis of an apparent wall peptidoglycan and the delayed synthesis and assembly of an additional wall component, leading to a mature multilayered vegetative wall.

The presence of different peptidoglycan chemical structures in spores and vegetative cells offers interesting possibilities to study differentiation mechanisms at the biochemical and genetic levels. The peptidoglycan of vegetative cells of B. sphaericus 9602 contains L-lysine and D-isoasparagine (a peptidoglycan of chemotype II), whereas the spore peptidoglycan contains meso-diaminopimelic acid but not L-lysine or D-isoasparagine (peptidoglycan of chemotype I) [69, 70]. Both peptidoglycans, however, have a common precursor, uridine-diphospho-N-acetylmuramyl-L-alanyl-D-glutamic acid, which can accept either L-lysine or meso-diaminopimelic acid. Tipper and Pratt [70] found that as sporulation proceeds, the L-lysine-adding activity of the vegetative cells decays to a level that is maintained until meso-diaminopimelic acid-adding activity is detectable. The remaining L-lysine-adding activity then declines rapidly, whereas meso-diaminopimelic acid-adding activity increases rapidly. This process, as well as an increase in dipicolinate synthetase, was dependent on continued RNA and protein synthesis, and necessarily involves transcription and translation of at least one "sporulation-specific gene."

D. Autolytic Enzymes

Many bacterial species possess enzymes capable of hydrolyzing their own peptidoglycan. When such enzymes are permitted to act, the cells lose their osmotic protection, and autolyze. The presence and activity of such autolytic systems can be observed under a variety of conditions,

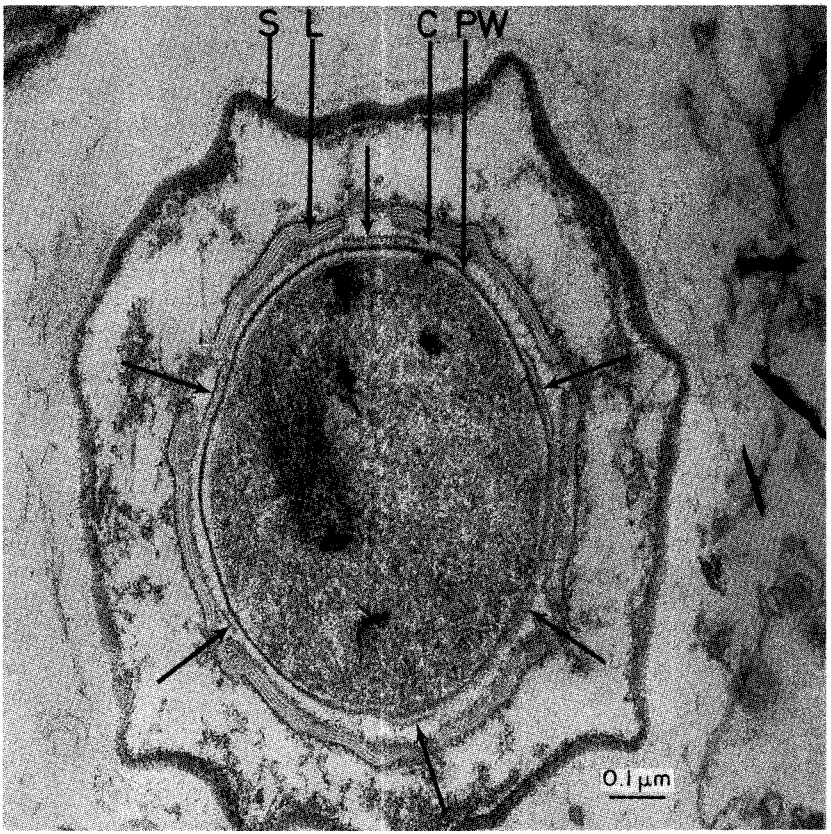


FIG. 29. Section of a germinating spore of Bacillus polymyxa. The exterior parts of the spore, i.e., the outermost surface of the sporangium (S) and the laminae of the spore coats (L), are disintegrating. The cortex (C) has all but disappeared. The primordial cell wall (PW), which is of slight density in the resting spore, now consists of a single highly scattering component. As shown by the six smaller arrows, the multilayered spore coats have cracked in several places and under the cracks, areas of the germ-cell wall show additional layers. The bar equals 0.1 μm. Reprinted from reference 170 by courtesy of the National Research Council of Canada.

many of which involve the inhibition of continued peptidoglycan synthesis. For example, the addition of specific inhibitors of peptidoglycan biosynthesis such as the penicillins or D-cycloserine, or the deprivation of nutrionally required peptidoglycan precursors such as glucose, glucosamine,

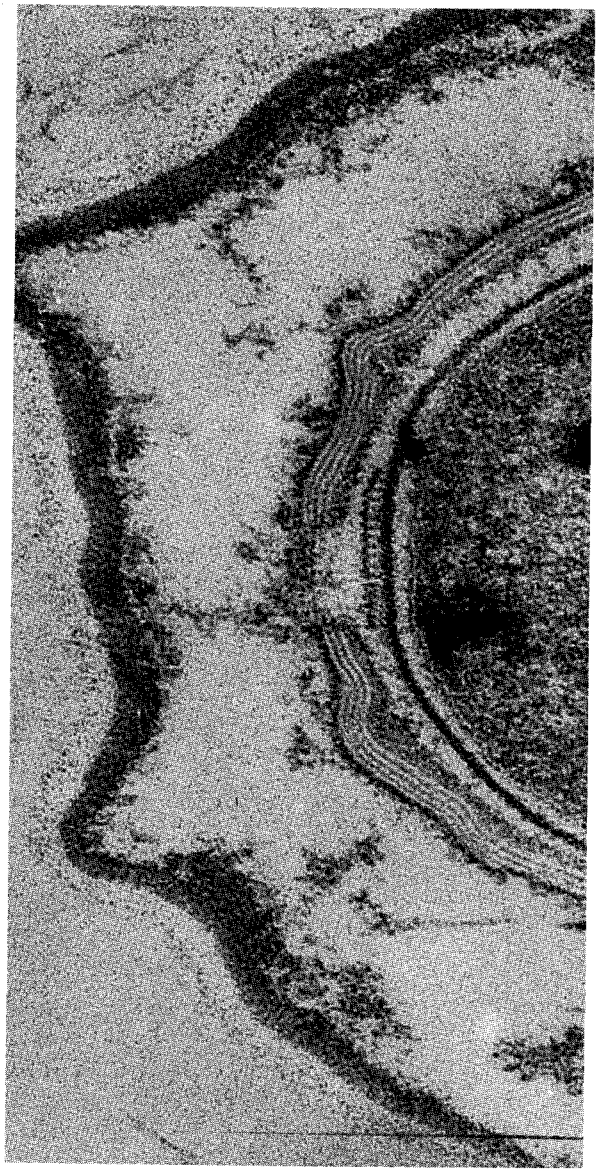


FIG. 30. High magnification of a portion of Fig. 29 to show detail of an early area of complete wall assembly during germination of spore of Bacillus polymyxa. Reprinted from reference 170 by courtesy of the National Research Council of Canada.

lysine, diaminopimelic acid, alanine, or glutamic acid, may result in cellular autolysis when the conditions of pH, salt concentration, etc., are suitable [171].

The relationship of inhibition of peptidoglycan synthesis to cellular autolysis is not due simply to the failure of wall growth to keep up with continued synthesis of protoplasmic constituents. This has been demonstrated in two ways. First, in S. faecalis ATCC 9790 (S. faecium) and other species, deprivation of lysine results in an inhibition of a net increase in both cellular protein and peptidoglycan, and, in cellular lysis. Second, both penicillin and D-cycloserine induce lysis of S. faecalis under conditions in which the cells are not significantly increasing their surface area but are engaged in wall thickening [172]. An increase in penicillin and cycloserine resistance occurs as the ability of the cells to autolyze decreases, and, as the walls thicken. An additional implication of these results is that inhibition of synthesis of the complete cross-linked peptidoglycan does not in itself result in cellular lysis, but that the active participation of one or more peptidoglycan hydrolases is required. A recent report of Tomasz et al. [173] showing that defects in the ability of Diplococcus pneumoniae to undergo cellular autolysis, induced nutritionally or via mutation, were accompanied by resistance to penicillin-induced lysis, serves to reinforce this concept.

The specificities of the autolytic enzymes found in various species correspond to the four described in Section II, B (N-acetylmuramidase, N-acetylglucosaminidase, N-acetylmuramyl-L-alanine amidase, and peptidases). Some have been found to be closely associated with the cell wall while others were found excreted in the growth medium, associated with the membrane or in the cytoplasm. In S. faecalis 9790 [174] and L. acidophilus [175] strain 63 AM Gasser, the only activity detectable in the wall fraction was an N-acetylmuramidase. In D. pneumoniae [176] various Bacillaceace [177] and Clostridia [177a,b] and in Listeria monocytogenes [177c], the major activity appears to be an N-acetylmuramyl-L-alanine amidase. Recently, however, the activity of a hexosaminidase of an as yet undetermined specificity, has been detected in a spore$^-$, proteinase$^-$ mutant of B. subtilis 168 [177]. It was postulated that the hexosaminidase activity may also be present in the parent Bacillus but was lost during preparative procedures, perhaps by proteolysis. Other species, such as E. coli [8] and S. aureus [57, 178-181], possess a variety of activities including N-acetylglucosaminidases, N-acetylmuramyl-L-alanine amidases, and cross-bridge-splitting peptidases. Evidence of an N-acetylmuramidase in S. aureus has not been obtained.

The presence of autolysins in rapidly growing and dividing cells led to the idea that such potentially dangerous enzymes may play an important role in bacterial growth [8, 14a, 182-185] as well as in other cellular

functions such as sporulation, the ability of cells to become competent for transformation and the excretion of toxins and exoenzymes. Peptidoglycan hydrolases of any specificity could effectively function for the latter processes.

Several potential roles for autolytic peptidoglycan hydrolases in cell wall growth and cell division have been proposed [5, 14, 14a, 64]. These include (1) hydrolytic action to provide new acceptor sites for the addition of peptidoglycan precursors, (2) the hydrolysis of bonds in selected areas of the wall so that changes in cell shape may occur ("remodeling" function), (3) in cell division, that is compartmentalization into two new cell units separated by wall or membrane or both, (4) cell separation, and (5) the potential biosynthetic capacity of a hydrolytic activity.

1. Provision of New Acceptor Sites

A number of years ago it was thought that the number of acceptor sites could be a limiting factor for the expansion of the cell surface [8]. As discussed in Section II, C, this does not seem to be the case for peptidoglycans of most species. In any event, from what is known of the final steps of peptidoglycan biosynthesis (Section III), the only peptidoglycan hydrolase that would provide suitable additional "ends" (nonreducing N-acetylglucosamine) would be an N-acetylmuramidase. Species thus far found to have such an activity include S. faecalis [174], L. acidophilus [175], Arthobacter crystallopoietes [186] and Bacillus thuringiensis [187]. It is possible that a bridge-splitting peptidase could also function in a similar way, if disaccharide-peptide monomers can be added to the growing peptidoglycan via transpeptidation before the glycosidic bonds are formed. It also remains possible that nascent peptidoglycan units are formed from small oligomers (e.g., on a lipid carrier as in O-antigen biosynthesis [99]) and that oligomers are added to the growing wall by transpeptidation after preparation of suitable sites via peptidase action [187a]. It seems certain that the frequently found N-acetylmuramyl-L-alanine amidase and N-acetylglucosaminidase activities cannot produce suitable acceptor sites. In fact, in some organisms that produce relative large amounts of amidase, such as S. aureus and Bacilli spp., evidence for the presence of substantial quantities of the products of amidase action, i.e., unsubstituted N-acetylmuramic acid and N-terminal L-alanine peptide residues in their peptidoglycan is lacking. For example, in S. aureus the number of N-terminal L-alanine residues is no more than 6% of the L-alanine in the wall [57].

2. Remodeling Function of Autolysins

To our knowledge, this idea was first proposed by Rogers [14]. There is no doubt that bacterial cells change their shape and surface area-to-

volume ratio throughout the cell division cycle. This is true not only for rod-shaped organisms but also for cocci. Hydrolysis of a few bonds at selected specific topological sites could permit a rearrangement of both the covalent and hydrogen bonding of the peptidoglycan network. Peptidoglycan hydrolase of any of the known specificities may serve this function.

Perhaps the most striking morphological change that has been observed to be accompanied by a change in level of autolytic enzyme activity, and a corresponding change in the structure of the resulting peptidoglycan, is the conversion of rods to spheres in A. crystallopoietes. A transient increase in N-acetylmuramidase activity was found to occur during rod-to-sphere conversion [186]. In addition, the average glycan chain length of the rods is 126, while that of the spheres is 34 hexosamine residues [32], which is consistent with the activity of a glycanase in morphogenesis. Similarly, the conversion of rod-shaped vegetative to spherical microcysts of Myxococcus xanthus was found to be accompanied by a transient increase in N-terminal meso-diaminopimelic acid residues [188], possibly the result of increased endopeptidase or decreased transpeptidase activity.

3. Cell Division

It is often hard to separate this function from cell separation (Fig. 31) since it is often technically difficult to determine the presence of a cross-wall, membranous septum, or some other type of physiological separation, especially in some rod-shaped species. The question then arises: does the division of one cell into two, or the fragmentation of a filamentous form, involve cross-wall growth, or merely the separation of preexisting cross-walls? To compound this problem, the time between initiation of nascent cross walls and their completion appears to be rather short, at least in some species. Also, the formation of a cross wall and its separation to become two new poles, results in subtle, sequential, and definable changes in cell shape and surface area-to-volume ratio, overlapping with the remodeling function discussed previously.

4. Cell Separation

Autolysins have been shown to play a role in cell separation in two Bacillus spp. [189-191], and D. pneumoniae [192], all of which possess potent amidase activities. For example, mutants of Bacillus licheniformis (lyt^-), deficient in ability to autolyze, grew at rates very similar to that of the wild type, but two of the least lytic mutants grew as very long chains of unseparated bacilli [189]. The wall chemistry of these two mutants also differed from that of the wild type [189].

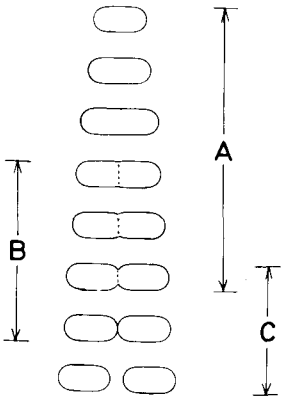


FIG. 31. Diagrammatic representation of the cell surface during the cell division cycle of a rod-shaped species. For simplicity the poles are drawn as hemispheres. Note the sequential changes in shape and surface area-to-volume ratio during the cell cycle. The overlapping elongation (A), division (B), and cell separation (C) sequence is also indicated.

5. Potential Biosynthetic Capacity of a Hydrolytic Activity

Some carboxypeptidases have been recently shown to be able to carry out transpeptidation reactions (Sections III, I and III, J. Endo-N-Acetylmuramidases, such as hen egg-white lysozyme, are known to be transglycosidases [47-49]. Transglycosidases could be useful for linking together short glycan chains, perhaps even at the level of the lipid intermediate, in a manner similar to that proposed for cross-bridge formation via transpeptidation (Section III, I).

6. Multiple Roles for Autolysins

As pointed out by Rogers [14], autolytic enzymes may have multiple functions. Mutants defective in ability to autolyze, as well as other types of mutation which affect wall chemistry, morphology, or function, are frequently pleiotropic. Without detailed genetic analysis, it is often difficult to differentiate between multiple mutagenic events and the multiple effects of a single mutation. However, in most cases, the defects in mutants investigated all seem to be related to surface function.

The change in wall chemistry accompanying a defect in cell separation and low autolytic activity in lyt$^-$ mutants of B. licheniformis is mentioned in the preceding text. In addition, a temperature-sensitive mutant of

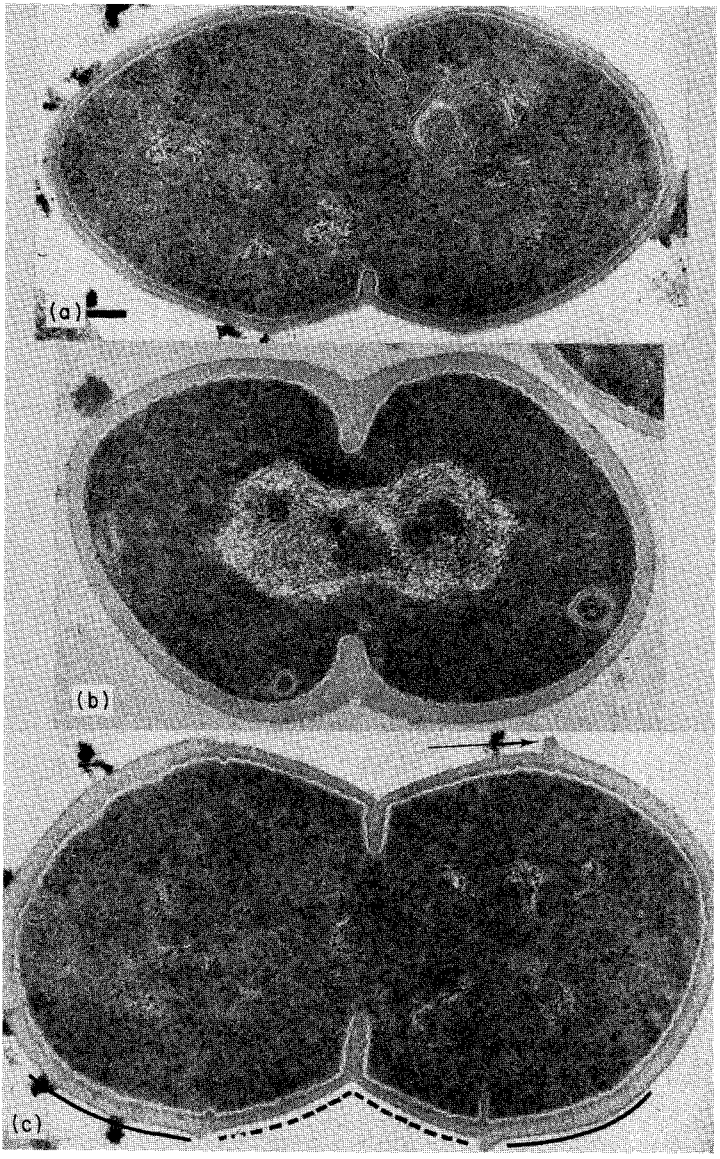
B. subtilis deficient in autolytic activity grew somewhat more slowly than the wild type, and had an altered morphology, at the nonpermissive temperature [191]. The cells were irregular in width and had many bends. Normal morphology and an increased growth rate occurred upon the addition of either B. subtilis autolysin or hen egg-white lysozyme.

Two mutants of S. faecalis with defects in their autolytic system were partially characterized [193]. Compared to the wild type, both mutants autolyzed more slowly, had walls of very similar chemical composition, and grew at nearly the same rate. One mutant, E81, grew in long chains, contained low levels of both the active and proteinase-activatable latent forms of the autolysin, had walls that were less cross-linked and more sensitive to added isolated autolysin. The other mutant, E71, contained low levels of active but high levels of latent autolysin, and had walls of a similar degree of cross-linking and sensitivity to added enzyme as the wild type. In addition, both mutants showed altered morphology, being longer and having thicker walls than the wild type.

In view of the problem of distinguishing multiple from single mutations, the pleiotropic effects of replacement of choline by ethanolamine in walls of D. pneumoniae are particularly significant [173, 176, 192]. Choline is required for growth and is a constituent of the wall teichoic acid of this organism. Replacement of choline by ethanolanine results in (1) loss of the ability of the cells to autolyze, (2) resistance of isolated walls to the action of the autolytic amidase, (3) physical association of daughter cells in long chains, (4) loss of the capacity to undergo transformation, and (5) resistance to dissolution by deoxycholate and to penicillin, cycloserine, and phosphonomycin-induced lysis. Since this change appears to be a single one that affects only the composition of a nonpeptidoglycan polymer of the wall, it would seem that similar pleiotropic effects could arise from a single mutation affecting surface growth or composition.

7. Relationship of Autolytic Activity to In Vivo Peptidoglycan Synthesis

There does not appear to be a requirement for autolytic activity for peptidoglycan synthesis per se. For example, inhibition of growth of S. faecalis via an inhibition of protein synthesis either nutritionally (valine or threonine deprivation), or by means of the addition of antibiotics (chloramphenicol or tetracycline), results in a rapid decrease in ability of cells to autolyze, but in the continued synthesis of peptidoglycan and the other wall polymers [64, 171, 184, 185, 194-196a]. However, instead of enlarging the surface, most of the newly made wall polymers go into a thickening of the wall [196]. The result is more wall and peptidoglycan per unit mass, and per cell. Wall thickening (Fig. 32), which is a normal part of wall growth [197], occurred over the entire


(a)
(b)
(c)

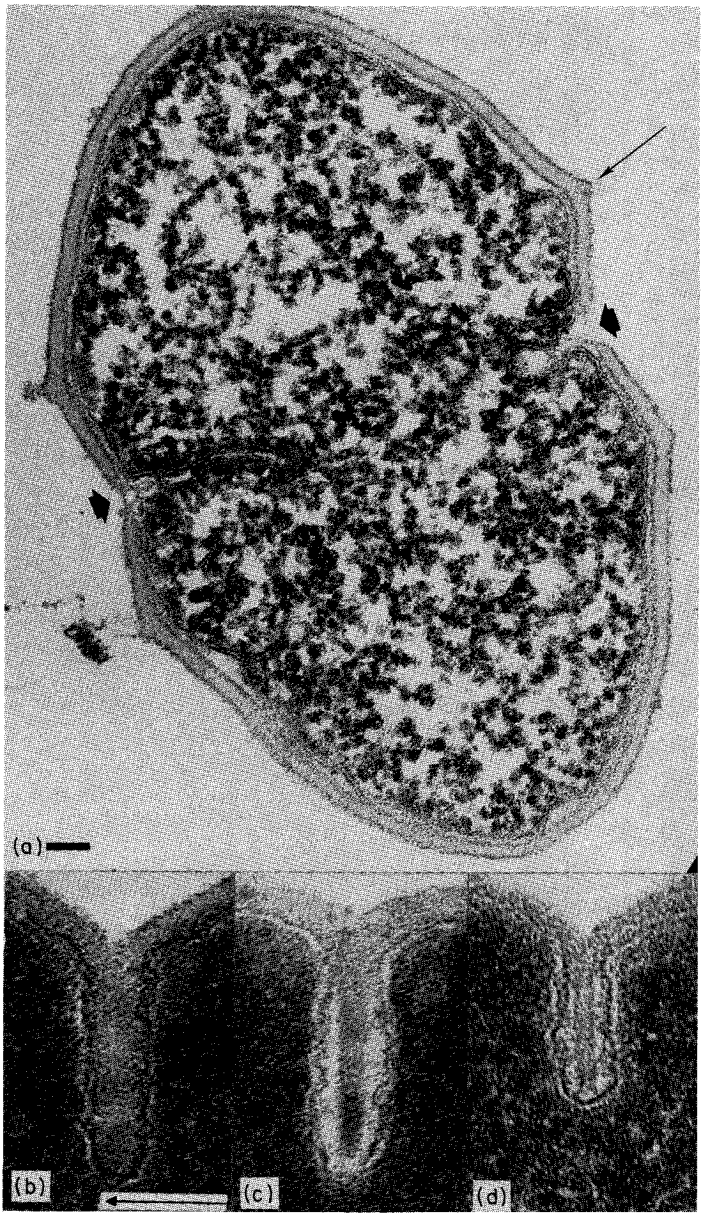
coccal surface [196], while it could be shown via electron microscopy (Fig. 33) that cellular autolysis was initiated at the tip and sides of nascent cross walls [198]. Using selective isotope-labeling techniques, it could be shown that the peptidoglycan made during chloramphenicol-induced wall thickening was not associated with autolytic enzyme activity [196a]. Thus, at least in this species it would appear that the autolytic N-acetylmuramidase can be associated only with peptidoglycan synthesis, which results in an increase in cellular surface area, a process which has also been shown to occur in the region of nascent cross walls [197]. A model for wall growth in this species has been presented recently [64, 197]. This model is consistent with multiple roles for the autolysin (remodeling, division, separation) and accounts for its continued action throughout most of the cell division cycle (see Section V, C).

This negative correlation between peptidoglycan synthesis per se and autolytic activity is not limited to S. faecalis. Similar though less complete information is available for other species including L. acidophilus [199], B. subtilis [166, 200] and B. megaterium [200]. In all cases thickening of the entire wall surface after an inhibition of protein synthesis was observed and correlated with a decrease in ability of cells to autolyze.

E. Heterolytic Enzymes

Some bacteria produce "heterolytic" enzymes, i.e., enzymes that are lytic only upon bacteria of other species. Both the ML endopeptidase, which acts on N^{ϵ}-(D-alanyl)-L-lysine linkages (Figs. 7 and 10), and the

FIG. 32. Electron micrographs of sections of Streptococcus faecalis. (A) Central longitudinal section of a cell from an exponentially growing culture. Arrow shows one of the wall bands that separate the equatorial wall made during the most recent generation from that made during a previous generation. Mesosomal membrane associated with the nascent cross wall can also be seen. (B) A central section of a cell from a culture deprived of threonine for 20 hr. Note the extensive thickening of the cell wall. (C) A central section of a cell from a culture recovering from 10 hr of threonine starvation. The culture had regrown for 80 min and had undergone 1.28 doubling in mass. Note the conservation of polar wall thickened during threonine starvation, which is now separated by thinner equatorial wall, synthesized during the 80-min period of regrowth. An increasing gradient of wall thickness from the nascent cross wall to the wall bands can be seen here as well as in part A of this figure. The bar equals 0.1 μm and applies to all three micrographs. Reprinted from references 196 and 227 by courtesy of the American Society for Microbiology.


(a)
(b)
(c)
(d)

KM endopeptidase, which acts on C-terminal D-alanyl-D linkages (Figs. 6, 11, and 12), are produced by Streptomyces albus G. Linkages sensitive to these enzymes do not occur in the Streptomyces peptidoglycan. It may be that heterolytic enzymes are weapons devised by bacteria in order to enhance their possibilities of survival in a natural niche. It may also be that the lytic activities of these enzymes are "accidental" and that their real function in the bacterial cell that produces them is to achieve a specific function unrelated to their ability to lyse other species. The KM endopeptidase from Streptomyces albus G is of particular interest since, in fact, it is D-alanyl-D-carboxypeptidase, and D-alanyl-D-carboxypeptidases are known to play an important role in peptidoglycan biosynthesis (see Sections III, I and III, J.).

F. Peptidoglycan Turnover

Not only do the nature and relative amounts of acidic accessory wall polymers change during shifts in nutritional conditions (Section IV, B), but turnover of the peptidoglycan portion of the wall has been observed in some species but not in others. Pitel and Gilvarg [201] saw no evidence of peptidoglycan turnover in a diaminopimelic acid-and lysine-requiring mutant of B. megaterium during exponential growth or the early part of the stationary phase. No loss of diaminopimelic acid from the peptidoglycan of E. coli during excretion of a lipopolysaccharide-lipoprotein complex was observed [201a]. Peptidoglycan turnover in S. faecalis appears to be below a detectable level during (1) exponential growth, (2) wall thickening during valine or threonine starvation, (3) growth reinitiation after amino acid starvation, (4) growth in the presence of a just subgrowth inhibitory concentration of penicillin, and (5) recovery from a period of wall damage inflicted by lysozyme treatment [202].

On the other hand, relatively rapid rates of turnover of peptidoglycan and other wall polymers have been observed in other bacterial species

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FIG. 33. Autolysis of Streptococcus faecalis. (A) Central, longitudinal section of a cell of Streptococcus faecalis 60 min after the beginning of autolysis (at 74% of the initial turbidity). The peripheral wall seems to be intact, but the entire cross wall is no longer visible (large arrows). The wall bands (small arrows) mark the separation of "new" equatorial, peripheral wall from "old" polar wall. Stages of cross wall dissolution. (B) Control, i.e., nonautolyzing cross wall. (C and D) Primary autolytic attack at the leading edges of the cross wall. The base of the cross wall remains intact and the septal membrane maintains its original invaginated position. Bar equals 0.1 μm. Reprinted from reference 198 by courtesy of the American Society for Microbiology.

[202, 203, 203a, 203b]. Peptidoglycan and wall teichoic acid were shown to turn over at the same rate in B. subtilis W23 [203]. The rate of peptidoglycan turnover was equivalent to a loss of almost 50% of wall material per generation in B. subtilis W23, and to about 30% per generation in B. megaterium KM. For B. subtilis the products of wall turnover were shown to result from the action of an N-acetylmuramyl-L-alanine amidase, and the turnover rate was decreased but not eliminated in the presence of 10 μg/ml of actinomycin D.

The peptidoglycan of L. acidophilus strain 63 AM Gasser was shown to turn over rapidly during exponential growth and upon recovery from amino acid starvation [199, 202]. The rate of turnover during exponential growth (generation times of 52-152 min) was approximately proportional to growth rate and 22-44% of the peptidoglycan was lost during each generation time. In this case, turnover does not appear to be related to autolytic activity since a slowly autolyzing mutant showed a turnover rate comparable to the controls. Peptidoglycan turnover slowed and stopped as the stationary growth phase was approached and was virtually completely prevented by chloramphenicol. A large number of anomalies of this turnover were noted of which some were as follows. (1) A lag of 0.8 to 2 generation times before turnover commenced even in random cultures that had been labeled for over six generations. (2) After pulse-labeling of less than 0.2 generation times, turnover was not observed while longer pulses resulted in turnover after the "normal" lag period. (3) Turnover was followed for many generations in a relatively undisturbed culture, maintained at a constant growth rate. After each doubling of mass, the culture was diluted (1:1) with fresh medium. After a lag of about one generation time, turnover occurred, with about a 40% loss of peptidoglycan per generation. Strikingly, however, peptidoglycan turnover stopped when about 10-20% of the initial labeled peptidoglycan was still present, suggesting that a significant fraction is immune to turnover. The observations do not appear to be either artifacts of the method of growth or of measurement of turnover, since, in L. acidophilus, significant turnover of protein was not observed, nor was detectable peptidoglycan (or protein) turnover seen in S. faecalis, grown and measured in the same way. A similar fraction of peptidoglycan immune to turnover was observed in experiments with B. megaterium, which turns over its peptidoglycan at a rate of about 15% per generation [203a].

At this time, it is difficult, if not impossible, to interpret the role of peptidoglycan turnover in the process of wall growth, in those species in which it occurs. Apparently, peptidoglycan turnover is somehow related to an increase in cell surface area and not to wall thickening. On the other hand, since the absence of detectable peptidoglycan turnover has

been observed during growth of E. coli, S. faecalis, and a mutant of B. megaterium, turnover does not seem to be an essential feature of wall growth. Its occurrence can, however, complicate the interpretation of several types of experimental approaches.

V. BIOSYNTHESIS OF PEPTIDOGLYCAN AT THE CELLULAR LEVEL

A. Localization of Peptidoglycan Synthesis

It seems clear that membrane-bound enzymes effect the final steps of peptidoglycan synthesis and that the final wall acceptor must be in very intimate association with the membrane sites. It remains to be determined whether or not there are specific topological sites at which this occurs and/or specific units of membrane that carry out an integrated process [204]. Some consideration has been given to the mesosome as the site of the membrane-bound peptidoglycan-synthesizing apparatus. Two types of evidence point to the presence of this system in the entire membrane surface. First, the undecaprenol lipid carrier has been found to occur in plasma as well as mesosomal membrane of L. casei [205]. Although this compound may be used by the cell for other purposes, we only know of its functions as a carrier in the synthesis, and perhaps transport, of precursors of exocellular substances (see Section III, D) such as peptidoglycan and O-antigens. Second, even in an organism such as S. faecalis, for which it is known that wall synthesis which results in an enlargement of the surface area, occurs at a rather restricted portion surface (in the vicinity of nascent cross walls), peptidoglycan biosynthesis, which results in wall thickening, not only can, but does occur over virtually the entire coccal surface (Fig. 32) even during rapid exponential growth [64, 196, 197].

Such findings are surely related to the regulation of peptidoglycan biosynthesis more in terms of exactly where on the cell surface and in what direction peptidoglycan (and wall) assembly occurs, rather than in terms of factors affecting the overall rate of peptidoglycan synthesis. Direction of peptidoglycan (and wall) assembly are not only important in the consideration of wall thickening versus enlargement of the surface but also for cross-wall versus peripheral-wall formation. These last factors are crucial in the determination of cell division, cell size, and morphogenetic changes during the cell division cycle, which, in turn, are related to the synthesis and regulation of synthesis of informational macromolecules (DNA, RNA, and protein). Only the peripheral wall elongation and cross-wall formation processes, and not the wall-thickening process, can be coupled to the synthesis of informational macromolecules. After the cessation of protein

synthesis, peptidoglycan synthesis proceeds at a virtually unchanged rate for a considerable period of time, thus emphasizing the complexity of the problem and the necessity to consider the topological location and direction of synthesis of the product [64, 196a, 206].

B. Relationship to Surface Enlargement

In cells of S. faecalis undergoing enlargement, newly synthesized peptidoglycan is closely associated with the active form of the autolysin [207], which has been in turn shown to be localized at nascent cross walls (Fig. 33) [198]. Electron microscopy (Fig. 32) showed that the wall elongation process also originates at nascent cross-walls [197]. Thus, in this case, and because of the covalent linkage of accessory wall polymers to the peptidoglycan, in probably all Gram-positive species, elongation of peptidoglycan is likely to be synonymous with elongation of wall. A limited number of discrete wall elongation sites have been seen in many cocci, which divide in one plane, by immunofluorescent or ferritin-labeled antibody or radioautographic techniques [208-213]. In all cases, antibodies to polymers other than peptidoglycan were used.

With rod-shaped organisms, results are conflicting. Hughes and Stokes [214] used an antipeptidoglycan-specific antiserum and were able to visualize a limited number of discrete sites of wall growth in a lyt^- strain of B. licheniformis, which exhibited very little peptidoglycan turnover. These investigators pointed out the complications of interpretation of wall-growth localization data in the presence of turnover. Chung et al. [210, 215] also observed a limited number of wall-growth sites in B. cereus, B. megaterium and E. coli using an immunofluorescence technique. Other data including the use of immunofluorescence, electron microscopic, and radioautographic techniques, are consistent with a large number of wall-growth sites in a variety of Bacillaceae and enteric bacteria [200, 209, 216-219a].

The use of penicillin to inhibit transpeptidation and thereby produce localized weakening in the protective wall has also produced conflicting results. In some cases, localized bulges in the wall were seen to develop [147, 220]. In others, multiple random weakened sites on the surface were seen [221].

For all of these techniques, there are a number of technical and other problems that are sometimes overlooked. The problem of wall turnover has been mentioned previously. In addition, there are the following problems. (1) Resolution and specificity of the techniques employed are limited. (2) Wall thickening has been shown to occur in a variety of bacterial species. In S. faecalis, it is a normal part of the wall elongation

process during rapid exponential growth. Thickening occurs over a significant portion of the wall surface, but mostly near the zone engaged in wall elongation. This could be a more significant problem in other bacterial species, especially with techniques that are unable to clearly differentiate wall enlargement from wall-thickening sites. (3) As pointed out by Donachie and Begg [222] for E. coli, the number, location, and direction of growth zones may depend on growth rate and cell size (which are related). (4) Lytic, or morphological, effects induced by penicillin may well depend on the conditions used.

Penicillin can undoubtedly inhibit the large number of sites of wall biosynthesis that are engaged in wall thickening as well as those that are engaged in wall elongation and/or cross-wall formation. The ability of penicillin to induce cellular lysis under conditions where wall thickening rather than surface expansion is occurring [171, 172, 185] clearly shows an effect of the antibiotic on wall thickening. The electron micrographs of Murray et al. [223] suggest that penicillin inhibits wall thickening as well as cross-wall formation in S. aureus. Penicillin also inhibits the incorporation of precursors into peptidoglycan in "resting cells" of S. aureus [224], and lysis also occurs.

The morphological consequences of penicillin treatment of S. faecalis appear to depend on the physiological state of the cells at the time of antibiotic addition [225]. Exposure of exponentially growing streptococci to 0.4 μg/ml of penicillin, a concentration just below that which affects the rate of growth, resulted in the appearance of mostly swollen, balloon-shaped, or lemon-shaped cells. The rate of peptidoglycan synthesis was the same as the control. It would appear that penicillin inhibition of peptidoglycan transpeptidation at sites engaged in wall thickening as well as at sites engaged in wall enlargement, resulted in a modified product over most of the coccal surface. In contrast, the addition of the same concentration of penicillin to approximately the same number of cells emerging from 3 hr of threonine starvation, at any time up to about 35 min after inoculation into fresh medium, resulted in the appearance of rod-shaped cells (about one-half of the population) at 100-130 min. In this latter case, it would seem that penicillin treatment was primarily modifying the peptidoglycan product at the midpoint of the cells. In the absence of penicillin, cells recovering from 3 hr of threonine starvation undergo two synchronized divisions, the first at 40-45 min, much the same as that reported for another strain of S. faecalis starved and regrown similarly [226].

A combination of factors may contribute to the appearance of a significant number of rod-shaped cells. The thickened walls of the threonine-starved cells are conserved to become the poles of the first generation of daughter cells [227]. Such thickened poles would tend to maintain the lateral dimensions of the cells. Also, since the polar wall has already

thickened during starvation, little peptidoglycan synthesis is presumed to occur at these sites. The inhibitory effect of penicillin would therefore be directed primarily toward peptidoglycan synthesis occurring at nascent cross walls and involved in wall enlargement. The localized effect of penicillin seen during regrowth of threonine-starved S. faecalis is similar to the localized effects observed during growth of an overnight culture of E. coli [147]. However, it should be noted that a similar degree of cross-linking in both the peptidoglycan of the control and penicillin-treated E. coli cultures was observed [147], consistent with similar observations on Proteus mirabilis and its peptidoglycan-containing, penicillin-induced L-form [228].

C. Relationship to Morphogenesis and Division

From the above and considerable additional information, it seems clear that factors that control the site, direction of assembly, and perhaps subsequent modifications of the peptidoglycan are those that govern surface enlargement, sites of division, shape, and changes in shape of the cell during the cell division cycle. At present, we know little about these factors except that they must be integrated with the other cellular processes. Elsewhere, a model for wall growth of cocci that divide in a single plane has been presented in some detail [64]. This model is based on considerable experimental evidence. The same article also presents a highly speculative model for the surface growth of rod-shaped bacteria, based on considerably less, and more conflicting information. These models were constructed on the principle that cocci are more primitive, in that they synthesize only cross walls (which become poles), while rods, in addition to making cross walls in order to septate the cytoplasm, form a cylindrical section of surface between cross walls. For increasing the surface area of rods, cylindrical extension is more economical in terms of surface area to volume ratio than is cross wall formation plus separation [64, 229]. Rods need to form and separate cross walls only to divide and increase in cell number. For several reasons, it has been assumed that rod-shaped species possess some additional genetic information, lacking in at least some cocci, for the production of a cylindrical wall section.

For both rods and cocci, cross-wall formation must be oriented not only topologically but also in direction of assembly. In virtually all cases examined, cross-wall formation proceeds centripetally from the cell surface, forming a continuously closing annular ring. Thus, even in rod-shaped species that appear to have a large number of peptidoglycan growth sites, those sites involved in cross-wall formation and cell division must be localized. The location of these cross-wall sites relative to the poles, and the relative rates of elongation of cross-wall and peripheral wall, govern the ultimate length (shape) of the cells as well as the shape changes that occur during the division cycle. When the size and shape of rod-shaped

bacteria, such as E. coli or Salmonella, grown at different growth rates were compared, it was found that the faster-growing population had a greater mean cell volume [229]. The larger cell volume is due not only to an elongation of the cylinders but also to an enlargement of cell diameters. Thus, when cells are shifted up from a poor to a rich medium they increase not only in length but also in width, by a mechanism that is not yet understood.

The interrelationship of the processes of cross-wall closure and peripheral-wall enlargement is perhaps most simply illustrated by the streptococcal wall-growth model [64], where both processes are known to occur at the same sites (Fig. 34). In this model, peptidoglycan, which is synthesized and assembled at, or near nascent cross-walls, can find its way into peripheral wall, cross wall, or thickened wall. The fraction of peptidoglycan precursors that finds its way into each of the various products is influenced by growth conditions, and appears to depend upon the relative rates of three processes (diagramed in Fig. 34): (1) the rate at which precursors go into the wall-elongation process at the leading edges of the cross wall (R_1 in Fig. 34); (2) the rate at which precursors go into the wall thickening process which, even in rapidly growing cells, takes place at a much larger number of sites covering a larger portion of the surface and; (3) the rate at which newly elongated wall peels apart at the base of the cross wall into two layers of peripheral walls (R_2 in Fig. 34).

During rapid exponential growth, most peptidoglycan synthesis appears to occur at the more limited number of sites at the nascent cross wall that are engaged in wall elongation (R_1 in Fig. 34). However, a significant portion of synthesis results in a continuously decreasing gradient of the rate of the wall thickening, extending from the tip of the nascent septum (most rapid) to the poles (slowest). The size of the resulting daughter cells as well as their exact shape and the shape changes that occur during the division cycle, are, according to this model, governed by the relative rate at which peeling apart of the two new wall layers occurs at the base of the nascent cross wall (R_2 in Fig. 34). Relatively rapid peeling apart results in longer peripheral walls and shorter cross walls, i.e., longer cells. Relatively slow peeling apart (R_2 in Fig. 34) results in shorter peripheral wall and more cross wall, i.e., shorter and smaller cells. The timing and degree of the peeling apart process are also essential for cell separation. At present, little is known about the factors that govern the relative rates of these processes or if and when they vary in a cell division cycle. It is thought that the activity of the autolytic N-acetylmuramidase of S. faecalis plays a role, especially with regard to the rate of R_2 in Fig. 34. Factors thought to be involved in the regulation of surface enlargement are discussed in Section VI, E.

The streptococcal model is intended to suggest the basis for a generalized mechanism for cross-wall growth in bacteria of a variety of shapes

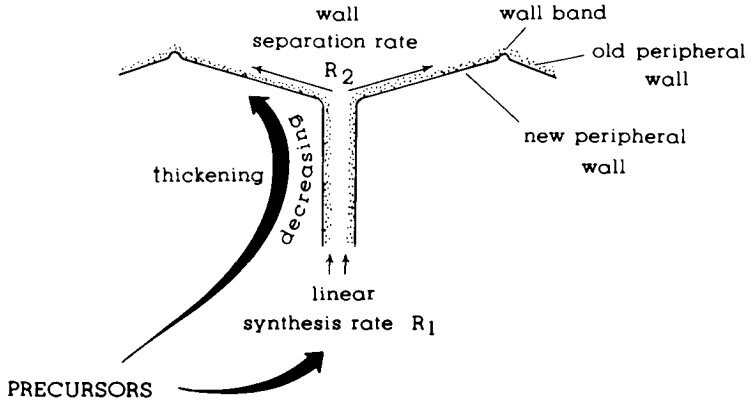

FIG. 34. Model of wall growth for Streptococcus faecalis. During exponential growth most of the wall precursors are fed into the leading edges of the growing cross wall. This forms two layers of cross-wall that separate into two layers of peripheral wall. By controlling the rate (R_2) at which the two layers of cross wall peel apart in relation to the rate of linear extension (R_1), the shape of the cell can be controlled. When the rate of separation (R_2) is approximately equivalent to the rate of linear extension (R_1), peripheral wall synthesis is favored and the cell increases in length experiencing a large increase in surface area. By inhibiting the rate of separation (R_2) relative to the rate of wall extension (R_1), the cross wall migrates centripetally into the cytoplasm to septate the cell. On completion of the septation process, the two layers in the cross wall complete separation and the two daughter cells then split apart. Besides linear wall extension, wall precursors are also used to thicken the cross wall to about twice the thickness of nearby peripheral wall and to continuously thicken peripheral wall. Most of the thickening occurs at the cross-wall tip, and it appears that the rate of wall thickening decays with wall age (as the wall is found further away from the cross-wall tip). The wall bands appear to mark the boundary between equatorial wall produced in the current generation and polar wall produced in a previous generation. Entrance of a culture into the stationary phase of growth interrupts the process of linear extension. During the early stages of this period of growth (1) precursors are preponderantly diverted into an overall thickening of the wall surface at the expense of linear extension; and (2) the rate of wall separation (R_2) is greatly inhibited. Reprinted from reference 64 by courtesy of the Chemical Rubber Co., Cleveland, Ohio.

and planes of division. Superimposed on the model, would be other factors such as cylinder formation by rod-shaped species. On the other hand, rod-shaped bacteria may use entirely different mechanisms. It also seems possible, both for this model and others, that changes occur in the primary,

secondary or tertiary structure of the peptidoglycan subsequent to its initial incorporation into the wall. Such changes could be important in shape determination and morphogenesis and may be governed by other factors such as those discussed by Dr. Henning and Dr. Schwarz in Chapter 9.

VI. REGULATION OF PEPTIDOGLYCAN SYNTHESIS

A. General Considerations

The peptidoglycan is the most rigid part and one of the largest molecules of the bacterial cell. At the same time, it is a dynamic structure. This enormous molecule completely surrounds the cell, exhibits a high degree of mechanical and tensile strength, maintains shape, and yet undergoes constant remodeling during cell expansion and division. The peptidoglycan and the other wall polymers sometimes share a common constituent. D-Alanine and N-acetylglucosamine, for example, are constituents of the peptidoglycan and of some teichoic acids [21]. A given intermediate can participate in the synthesis of more than one wall polymer. The C_{55}-polyisoprenoid alcohol, for example, is utilized as a membrane carrier in peptidoglycan, and O-antigen syntheses [169]. Many factors including changes in cell size and shape with growth rate suggest that the peptidoglycan covering not only expands in order to keep up with an increase in cell volume but that the quantity, topological localization, and direction of peptidoglycan synthesis is, in a largely unknown way, regulated and integrated with the synthesis of other macromolecules and structures. These considerations emphasize the importance of the regulation of enzyme activity, formation and localization for peptidoglycan synthesis itself and its interrelationship with the synthesis of the rest of the cell.

B. At the Level of Nucleotide Precursors

Osmotically fragile temperature-sensitive mutants of *E. coli* K12 impaired at the levels of UDP-N-acetylglucosamine enolpyruvate reductase (proposed genetic symbol: *MurB*), of L-alanine-adding enzyme (*MurC*), of diaminopimelic acid-adding enzyme (*MurE*) and of D-alanyl-D-alanine-adding enzyme (*MurF*), respectively, were isolated and studied [230-235]. Mutants which would be impaired at the level of UDP-N-acetylglucosamine-2 phosphoenolpyruvate transferase (proposed genetic symbol: *MurA* [231]) and at the level of D-glutamic acid-adding enzyme (*MurD*) were not obtained. The *MurC*, *E*, and *F* genes were located extremely close to each other (between *Leu* and *azi*: 1 - 1,5 min.) on the *E. coli* chromosome

so that they may form or be part of an operon. In contrast, the MurB gene was located at 77 min. Mutants with impaired L-alanine racemase (alr, at 3 min) and D-alanyl-D-alanine ligase (ddl, at 17 min) activities, respectively, were also obtained.

A number of mutants probably altered in one of the membrane-bound enzymes were also isolated. The exact lesions or genetic loci have not yet been determined. Strikingly, accumulation of the completed UDP-N-acetylmuramyl-pentapeptide precursor could not be detected in any of these mutants [231]. Similarly, the amount of UDP-N-acetylmuramyl-pentapeptide in the K12 parental strain was not increased significantly by penicillin or vancomycin treatment, whereas in contrast, the amount of UDP-N-acetylmuramyl-tripeptide could be enormously increased by D-cycloserine. As suggested by Lugtenberg, the apparent difficulty in accumulation of large amounts of UDP-N-acetylmuramyl-pentapeptide in K12 strains, either under the action of antibiotics or by mutation, strongly suggests that UDP-N-acetylmuramyl-pentapeptide regulates its own biosynthesis by feedback inhibition [231]. It would appear that the choice of S. aureus in the early studies of penicillin action was rather fortunate since the accumulation of UDP-N-acetylmuramyl-pentapeptide may be limited to perhaps relatively few species that lack regulation at this level.

C. At the Level of the Alanine Branch

Regulation of the amount of D-alanyl-D-alanine available for condensation with the UDP-N-acetylmuramyl-tripeptide has been studied. The mechanism is complex and involves (1) regulation of the size of the intracellular pools of D- and L-alanine: Two unrelated transport systems, one for each isomer, have been identified [129]; (2) competition for available D-alanine by the several reactions that utilize this compound: A D-alanine membrane acceptor (ADP), which participates in the incorporation of D-alanine into membrane teichoic acid has been purified from L. casei [236]. A specific transaminase, which utilizes D-alanine as an amino donor for D-glutamic acid synthesis, has been purified from B. subtilis [237]; (3) D-alanine regulates its own biosynthesis. By acting as a specific inducer of L-alanine dehydrogenase in B. subtilis, D-alanine limits the amount of L-alanine available to the alanine racemase [238]. In Pseudomonas aeruginosa, D-alanine (as well as other D-amino acids) induces a D-amino acid dehydrogenase which directly limits the level of D-alanine [239]; (4) a high intracellular concentration of alanine apparently represses the synthesis of alanine racemase in E. coli [71]; (5) the intracellular ratio of L- to D-alanine influences the anabolic activity (L → D) of the racemase: This is an intrinsic property of the racemases of both E. coli and S. faecalis. The anabolic (L → D) velocity is higher than the catabolic (D → L) velocity and the K_m value for L-alanine is higher than for D-alanine (see Section

III, C). The Haldane relationship K_{Eq} is one. Thus, the intracellular L-alanine pool must be larger than the D-alanine pool in order to insure the necessary anabolic velocity; (6) The D-alanyl-D-alanine synthetase contains several product-binding sites, some of which probably function in the control of enzyme activity, which results in D-alanyl-D-alanine formation (see Section III, C). Since K_{Eq} of the racemase is one, it follows that inhibition of the D-alanyl-D-alanine synthetase by its product would, in fact, control utilization of L-alanine by the racemase.

D. Of Peptide Cross-linking

Whether it occurs in single or multiple enzyme forms, the DD-carboxypeptidase-transpeptidase system is likely to play a role of prime importance in controlling the degree of peptide cross-linking of wall peptidoglycans. Studies of several Streptomyces enzymes which perform both carboxypeptidase and transpeptidase activities, indicated the presence of several sites or subsites in the active region of the protein involved in substrate binding, or inducing a correct alignment of the catalytic groups, or both. These multiple sites may control the actual functioning of the system either in an anabolic (transpeptidase activity) or catabolic (carboxypeptidase activity) sense. The direction of the reaction could also depend upon the exact location of the enzyme within the plasma membrane, the availability of a nucleophilic group (water or a recognizable amino group), and upon the structure of the peptides.

The efficiency of the transpeptidation reaction is obviously determined by the intrinsic properties of the enzyme that catalyzes it. Hence, efficiency is likely to vary with bacterial species (and possibly, in the course of the cell division, or more complex life cycle of a given cell). In S. aureus and in L. acidophilus 63 AM Gasser, for example, the residual noncross-linked C-termini of the wall peptide moieties have retained the D-alanyl-D-alanine sequence of the nucleotide precursors (Fig. 17). Hence, in these cases, peptide hydrolases and particularly carboxypeptidase activity cannot be involved in the control of the size of the peptide moieties. Estimations of the extent of peptide cross-linking in the isolated walls indicate that the transpeptidation reaction is very efficient in S. aureus (80%) but poorly efficient in L. acidophilus (30%).

In most bacteria, the residual noncross-linked C-termini of the wall peptides have not retained the D-alanyl-D-alanine sequence of the nucleotide precursor. Elimination of the donor site involved in transpeptidation necessarily limits the size of the peptide moiety of the wall peptidoglycan. This can be carried out by carboxypeptidase activity upon the nascent disaccharide peptide units (during or after wall insertion), or at the level of cross-linked peptide oligomers within the wall itself. Note that

carboxypeptidase action at the UDP-N-acetylmuramyl-pentapeptide level would prevent transfer of this unit to the lipid intermediate. Indeed, the translocase has a considerable specificity for C-terminal D-alanyl-D-alanine (see Section III, E). Consequently, removal of the C-terminal D-alanine residue from the precursor would prevent, or at least interrupt, peptidoglycan synthesis.

Limitation of the size of the peptide moiety could also result from the hydrolysis of D-alanyl interpeptide bonds in the completed peptide moiety of the walls through the action of endopeptidase autolysins. The C-termini appearing as a consequence of endopeptidase action would contain a single D-alanine residue. Such a reaction also could be carried out by carboxypeptidase activity at least when the interpeptide bonds are mediated through C-terminal D-alanyl-D linkages. When these interpeptide bonds are in a different position, other endopeptidases (see Section II, B) would be required to fulfill this function. The endopeptidase activity of the DD-carboxypeptidases is explained by the fact that these enzymes do not require a specific side chain of the C-terminal residue, providing that it is located on a D-amino acid center. When acting on peptide oligomers in which the interpeptide bonds are C-terminal D-alanyl-D-linkages (Figs. 6, 11, and 13; for a more complete list, see Chart I in reference 130), the carboxypeptidases hydrolyze the interpeptide bonds and degrade these oligomers into monomeric peptides.

E. At the Cellular Level

As mentioned in Sections V, B and V, C, not only is it necessary to regulate the activity and biosynthesis of the many enzymes involved in peptidoglycan biosynthesis, but it is also essential to control the selection of sites at which the final assembly takes place, the direction of synthesis, and the integration of the process with the biosynthesis of other macromolecules and cell division. It should be pointed out again that peptidoglycan synthesis per se can be relatively easily uncoupled from the synthesis of other macromolecules. Continued RNA, DNA, and protein synthesis may occur in the absence of peptidoglycan synthesis. For example, growth in the presence of inhibitors of peptidoglycan synthesis results in the formation of spheroplasts or L-forms in osmotically protective environments. Conversely, there are many examples of continued cellular synthesis of peptidoglycan in the absence of synthesis of informational macromolecules. In this latter case, the overall rate of peptidoglycan synthesis is virtually unchanged, but the topology of the process is altered. One of these changes is the wall-thickening phenomenon mentioned in Section V, A. Recent studies revealed specific changes in the ultrastructure of S. faecalis, well within the first hour, following the relatively selective inhibition of DNA, RNA, and protein synthesis [206]. Selected concentrations of various

antibiotics were added to balanced exponentially growing cultures. The antibiotics and their concentrations were selected on the basis of their relative specificity as inhibitors of the synthesis of one type of macromolecule. This was essential since high concentrations of most of the agents used were not as selective in their target as one might judge from the literature. Even at the antibiotic concentrations selected, specificity was only relative in degree of inhibition or in time. All of the inhibitors used permitted the continuation of peptidoglycan synthesis at or near the preexisting rate for periods of 10 min or more. After this time, incorporation of lysine into peptidoglycan still continued but at reduced rates. This occurred upon inhibition of protein synthesis with chloramphenicol (50 μg/ml) or 5-azacytidine (5 μg/ml); RNA synthesis with actinomycin D (0.25 μg/ml) or rifampicin (0.1 μg/ml); or DNA synthesis with mitomycin C (0.5 μg/ml). As expected from previous work on amino acid starvation [196], chloramphenicol treatment was accompanied by a rapid thickening of the wall over the entire surface of the cocci. In contrast, mitomycin-C treatment was not accompanied by wall thickening during the first half-hour of treatment. When this was analyzed in more detail, it was found that, in contrast to the chloramphenicol-inhibited cells, the mitomycin-treated cells almost doubled in cell number. Also, the mitomycin-C-treated cells differed in shape. In general they were longer and had shorter nascent cross walls, as if the rate of splitting (R_2 in Fig. 34) remained high. Quantitative measurements of electron micrographs of central longitudinal sections demonstrated that the ratio of length of peripheral wall to cross wall increased after mitomycin treatment.

Results obtained with these inhibitors have been interpreted in terms of the model for cell-wall growth discussed in Section V, C and shown in Fig. 34. While selective inhibition of either protein or DNA syntheses is accompanied by continued peptidoglycan synthesis, the ultrastructure, size, and shape of the resultant cell population is strikingly different. On the one hand, inhibition of protein synthesis appears to be accompanied by inhibition of both wall elongation (R1 in Fig. 34) and cross-wall separation (R2 in Fig. 34). Thus, the additional peptidoglycan (and other wall polymers) made result in about the same number of cells with thicker walls. On the other hand, inhibition of DNA synthesis appears to be accompanied by both continued wall elongation and, more strikingly, cross-wall separation (R2). Also a large fraction of the cell population continues to divide. Thus, wall thickening is not an early consequence of inhibition of DNA synthesis. Only later, when other cellular processes slow and/or stop, and further cell division ceases, is wall thickening seen. As yet we know little about the timing or regulation of these processes during a cell division cycle. However, it does seem likely that, at the correct time in a cell division cycle, centripetal closure of a cross-wall would be favored by an inhibition of cross-wall separation, so that more of the wall elongation product would end up as cross-wall and less as peripheral wall [206, 239a].

F. Of Autolytic Enzymes

Potential functions of autolytic enzymes are discussed in Section IV, D, some of which are related to the occurrence and direction of cellular sites of peptidoglycan synthesis. At the very least, the hydrolytic action of such enzymes must be limited so that in the growing cell the protective nature of the wall is maintained. This is particularly true for those species that show extensive peptidoglycan turnover, and those species that excrete autolysins into the growth medium. In the latter case, there must be some mechanism that protects the wall not only from extracellular enzymes but during release from the cells.

Mechanisms of control of autolytic enzymes may differ. Two examples of physiologically related, but morphologically different species S. faecalis and L. acidophilus will serve to illustrate this point. In S. faecalis (Fig. 35) it appears that the N-acetylmuramidase is made as a proenzyme, which is transported to the wall sites engaged in wall enlargement, where it is activated by a proteinase [240]. Both active and latent enzymes have an exceedingly strong affinity for the wall so that once wall-bound, they cannot easily be released [207, 241-243]. This affinity for the wall plus the occurrence of the cytoplasmic form in a latent state and the absence of peptidoglycan turnover, are all properties which made it possible to experimentally find an association of the enzyme with newly made wall at nascent cross walls. Lactobacillus acidophilus also has an N-acetylmuramidase [175]. Many properties of the autolysins of the two species differ [199]. Important for consideration of the L. acidophilus system is (1) the apparent absence of a latent form and the presence of non-wall-bound soluble active autolysin in the cell, (2) the occurrence of peptidoglycan turnover, and (3) an affinity of autolysin for the wall that appears to be not as strong as that of S. faecalis enzyme. Thus, the techniques that have been used to localize autolytic activity in S. faecalis have not been successful with L. acidophilus.

All of the steps in the S. faecalis system from synthesis of the latent form to the activity of the active form could be subject to regulation. Evidence has been obtained that suggests that the conversion of latent to active form is carried out by a process requiring protein synthesis [196a]. More interesting is the probability that cellular autolysis is controlled at the level of autolytic enzyme activity in S. faecalis and perhaps also in L. acidophilus. In both organisms the ability of cells to autolyze drops sharply when a culture enters the stationary phase. Walls from stationary-phase L. acidophilus cultures apparently still contain substantial amounts of autolysin [175]. A more detailed study has been carried out with S. faecalis. In this organism, inhibition of protein synthesis with chloramphenicol or threonine starvation results in a very rapid drop in the ability of cells to autolyze while the isolated walls from these cells retain

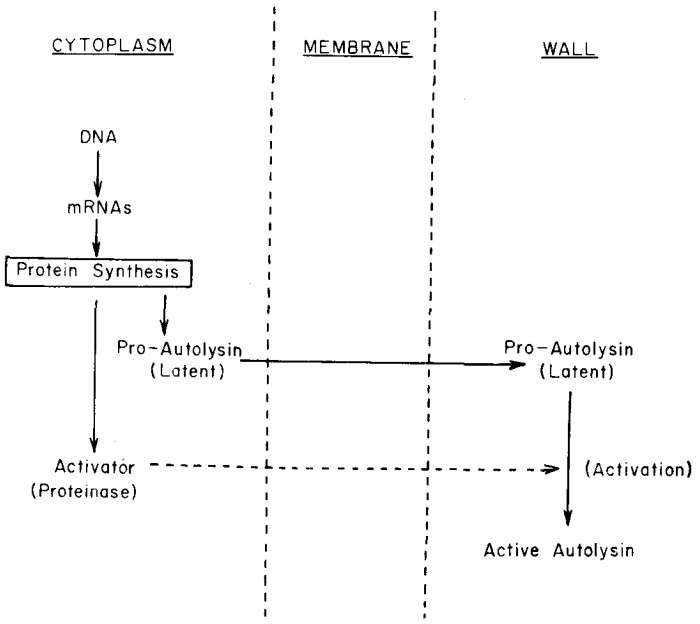

FIG. 35. Possible relationship between the latent and active forms of autolytic enzyme of Streptococcus faecalis 9790. The latent form is shown as a precursor (proenzyme) of the active form. Activation of the latent enzyme, by means of a proteinase, takes place after the enzyme has passed through the membrane and become bound to the cell wall. Reprinted from reference 240 by courtesy of American Society for Microbiology.

nearly the same amount of active and latent autolysin activity [196a]. The retention of autolysin activity long after inhibition of further protein synthesis suggests that the enzyme has a relatively long half-life, and that some other mechanism rapidly prevents its ability to lyse cells. Further studies, using selective inhibition of DNA, RNA, and protein synthesis showed that the rapid decrease in ability of cells to autolyze was much more closely correlated with inhibition of protein synthesis than with inhibition of RNA or DNA synthesis [244]. An as yet unidentified low molecular weight substance that inhibits autolytic activity can be extracted from cells of S. faecalis with hot water. Larger amounts of it can be extracted from chloramphenicol-inhibited than exponential-phase cells. Thus, availability of inhibitor could be inversely related to protein synthesis.

These observations on the effects of DNA, RNA and protein synthesis are consistent with a role for the autolysin in peeling apart the two layers

of wall at nascent cross walls, as discussed in Section V, C. Inhibition of DNA synthesis is accompanied by a great deal of peeling apart so that short cross walls and long peripheral walls were seen. This correlates with the absence of an inhibition of autolytic activity. On the other hand, little peeling apart was observed after inhibition of protein synthesis, this correlating with the rapid drop in cellular autolysis seen under these conditions.

In order for an autolysin to carry out a morphogenetic function coupled to other cellular biosynthetic systems during the cell cycle, it would be essential to have a mechanism that would control autolytic activity in a rapid and sensitive way. A small molecule that can bind to an exocellular protein to modify its activity would be such a tool.

There is little evidence of cycles of autolytic activity during the cell division cycle. Groves and Clark have obtained evidence for an increase in autolytic activity before division in synchronized cultures of E. coli B/r [245]. A cyclic decrease and increase in ability of L. acidophilus to autolyze during recovery from valine starvation has been noted [199]. Such cyclic autolytic behavior has not been observed with S. faecalis. It is not yet known if the L. acidophilus cultures were dividing with some degree of synchrony, but recovery of growth after a period of amino acid starvation resulted in synchronized division in S. faecalis and other species.

ACKNOWLEDGMENTS

The work in the authors' laboratories was supported by research grants 515 and 1000 from the Fonds de la Recherche Fondamentale Collective, Brussels, and l'Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture, Brussels (1699) to J. M. Ghuysen, and by research grants from the National Science Foundation (GB 20813) and U. S. Public Health Service (AI 05044) from the National Institute of Allergy and Infectious Diseases) to G. D. Shockman. We wish to thank the following for providing some of the illustrations used in this chapter: Dr. D. J. Tipper (Fig. 18), Dr. J. Fleck (Fig. 26), Dr. D. J. Ellar (Fig. 28), Dr. R. G. E. Murray (Figs. 29 and 30), and Dr. M. L. Higgins (Figs. 32 and 33); and for their comments on this chapter, Dr. O. Kandler, Dr. H. R. Perkins, Dr. F. C. Neuhaus, Dr. H. Martin, Dr. P. Reynolds, Dr. R. Tinelli, Dr. M. Guinand, Dr. J. F. Petit, Dr. M. L. Higgins, and Dr. L. Daneo-Moore.

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