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Layered murein revisited: a fundamentally new concept of bacterial cell wall structure, biogenesis and function

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Abstract The classical concept of the architecture of microbial murein assumes cross-linked glycan chains to be arranged in horizontal layers outside of the plasma membrane. It necessitates elaborate hypotheses to explain processes such as the biosynthesis, growth and division of the bacterial cell wall and provides no explanation for transenvelope macromolecular transport. Moreover, this model is difficult to reconcile with a number of basic chemical and electron microscopical data. According to a fundamentally distinct concept which is presented here, glycan strands in the microbial wall run perpendicular to the plasma membrane, each strand being cross-linked by peptide bridges with four other strands. This arrangement allows the formation of a structured matrix pierced with ordered ionophoric channels potentially harboring either lipoprotein or teichoic (lipoteichoic) acid molecules in Gram-negative and Gram-positive bacteria, respectively. New wall structures are synthesized in toto emerging from the cytoplasmic membrane as a condensed gel-like network below the old wall without being covalently attached to it, expanding due to inherent elasticity as the old wall is lyzed. This model reflects published genetic and biochemical data and offers a simple explanation for peptidoglycan biogenesis. As the biosynthesis is terminated by enzymic cleavage of all glycan strands, murein is irreversibly released from the membrane. The murein detachment prepares the membrane for de novo assembly of both the new wall synthesis machinery and the multicomponent factory for protein, DNA and phospholipid transfer. Being assembled in parallel, both new murein and the traffic complexes grow from the membrane together. This concept eliminates the necessity for the traffic complexes to penetrate intact murein. In the process of simultaneous assembly, the expanding murein functions as a lifting platform driven by the force of turgor pressure, transporting macromolecules through the perisplasmic space.

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Introduction

Unravelling the molecular principles of microbial cell wall structure has been a long and winding road, and it is perhaps fitting to first reassert certain milestone facts, such as Flemming's discovery of lysozyme [16] and penicillin [15]. Both compounds appeared to affect cell wall biogenesis in both Gram-positive and Gram-negative bacteria, causing either their disruption or, if the interaction took place in isotonic media, the formation of protoplasts and spheroplasts, respectively [7]. The bacterial cell wall was henceforth looked upon as a unique bag-shaped macromolecule surrounding the cell [59]. The term "envelope" was introduced [48] to emphasize that in Gram-negative bacteria the wall and the outer membrane are attached to one another, differentiating this structure from that of Gram-positive bacteria. The existence of the envelope was confirmed by electron microscopy establishing for the first time the concept of the periplasm with a thin and "rigid" murein layer sandwiched between two membranes [12]. The history of cell wall studies undoubtedly reached its first climax in the late 1960s when the chemical primary structure [17] and the pathway of murein biosynthesis [53] were elucidated, and when penicillin binding proteins were discovered [52].

Along the way, the only possible configuration of murein was envisioned as a stack of horizontally oriented two-dimensional layers running parallel to the cytoplasmic membrane [17]. Thus, the thin (2–2.5 nm) wall of *Escherichia coli* was considered to be monolayered whereas the thick (30–40 nm) wall of streptococci was described as multilayered [17], a concept which we would like to challenge in the present article. As discussed in detail below, this idea of layers has created a number of postulates [29, 32] and controversial models [35] that are difficult to harmonize with the basic chemical data.

The revision of the old concept has allowed us to take a fresh look at murein formation, its tertiary arrangement and its biological properties. This fundamentally new hypothesis of bacterial cell wall biogenesis, structure and function is described below.

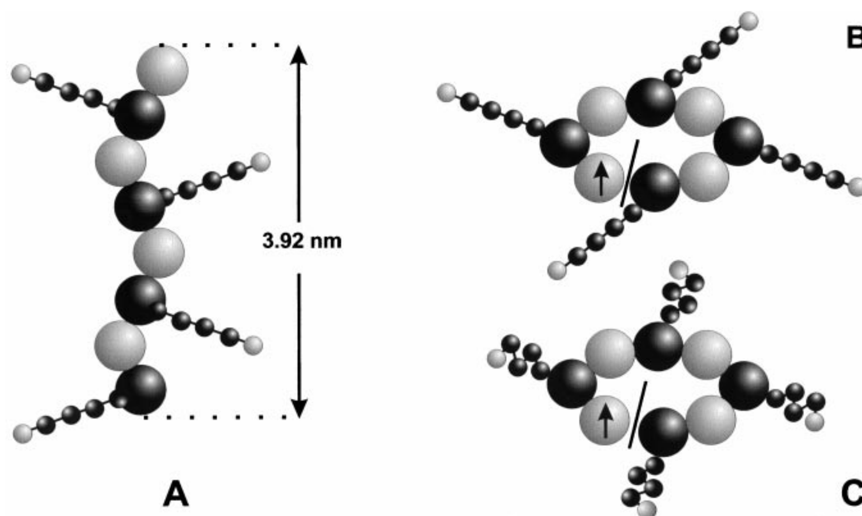

Fig. 1A–C Schematic representation of PG strand conformation. **A** Octasaccharide in extended form, forming one helix turn, with extended peptide side chains. **B** Octasaccharide in compressed form with extended peptide side chains. **C** Octasaccharide and peptide side chains both in compressed form (*PG* peptidoglycan, *black balls* MurNAc, *white balls* GlcNAc, *small balls* amino acids)

Basic facts and the classical concept

The terms currently emloyed to describe bacterial wall components may be used in different ways. In the present article, the following nomenclature is used: (i) "wall" signifies the organelle surrounding an intact microbial cell outside of its cytoplasmic membrane; (ii) "murein" refers to the structural carcass and the major component of the wall; (iii) "sacculus" is the purified murein isolated from bacteria by chemical and enzymatic methods; (iv) "peptidoglycan (PG)", the murein macromolecular structural unit, constitutes a glycan strand substituted by peptide side chains and produced by bacteria after blocking the transpeptidase activity of the murein synthases; (v) "glycan", being the polysaccharide core of PG, is prepared from the sacculus or PG by L-alanyl-peptidase treatment.

Chemically, murein is a superlarge macromolecule. In the case of Gram-negative bacteria, murein is relatively thin and moderately cross-linked. It is sandwiched between the inner and outer membranes, being attached to the latter via lipoprotein molecules to create a thick, elastic and firm envelope [6]. The murein content in the Gram-negative envelope does not usually exceed more than 10% [23]. In contrast, Gram-positive bacteria lack an outer membrane but possess a thick cell wall composed of long PG strands with a high degree of cross-linking [23]. The resulting matrix is strengthened by covalently attached teichoic acids which are present in equimolar ratios to PG [2]. The murein content in Gram-positive walls is about 40–45% [23].

The PG structural unit of murein is composed of repeating units of the disaccharide MurNAc(β1-4)GlcNAc carrying peptide side chains (L-Ala-D-Glu-Dap-D-Ala-D-Ala) [1] attached to the carboxyl group of the muramic acid residues [17, 35]. The subunits are interlinked via (β1-4)-glycosidic bonds and PG molecules are cross-linked via peptide bridges into a large unimolecular network. About three decades ago, it was assumed that PG strands in murein are arranged as horizontal macromolecular monolayers [17]. Although this assumption was hypothetical, it was widely adopted, presumably because it provided an easy explanation for the process of wall extension in the course of microbial growth. Indeed, "a mere cutting a few links between two or three adjacent spheres, squeezing in a new sphere, reconnecting the old links, and adding a few more to keep the newcomer in place" sounded simple enough [58].

As (β1-4)-glycosidic linkages are characteristic of chitin, two research groups proposed molecular models for both Gram-positive [31] and Gram-negative [5] bacteria, describing glycan strands as chitin analogs with peptide side chains oriented unidirectionally and proximally, i.e. sterically favorable for cross-linking. In both models, the major element of the wall architecture was a two-dimensional murein layer made of cross-linked PG strands arranged parallel to the membrane. Of note, the Gram-negative murein model was in agreement with early results of electron microscopy studies suggesting the wall in *E. coli* to be as thin as 2.5 nm [12]; however, these early data were later shown to be based on artifact formation and had to be revised [26].

Is murein arranged in layers?

The results of X-ray diffraction studies demonstrated that glycan strands are not straight and rigid like chitin, but mainly adopt a fourfold helical conformation with the symmetry order N = +4 [10]. Each turn of the helix is made of four disaccharide repeating units with four peptide side chains oriented outwards and perpendicular to each other, as shown in Fig. 1. It is important to keep in mind that macromolecular helices are flexible and can be either compressed or expanded like springs.

[1] Variations in peptide structure are not important for the discussion at this stage (*Dap* diaminopimelic acid)

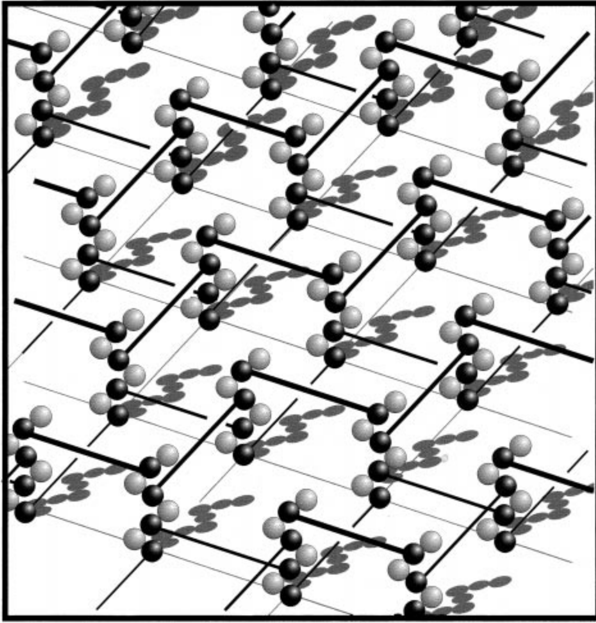

Fig. 2 Fragment of murein matrix constructed from peptide-cross-linked PG strands. This is a representation of the first turn of the PG helices to appear outside the cytoplasmic membrane (*black balls* MurNAc, *white balls* GlcN, *straight lines* peptide bridges in the upper turn of the helix). In Gram-positive bacteria, PG strands are considerably longer. Pores (channels), each made up by four cross-linked helices, are visible. To facilitate a spatial impression of the matrix, the outermost peptide bridges are drawn reinforced, and their decreasing width corresponds to their decreasing distance from the cytoplasmic membrane (drawn as a meshwork of thin gray lines). To further increase the three-dimensional aspect, shadows of the glycan strands are indicated on the surface of the membrane

After the conformation of the PG strands had been established, it became possible to assess the minimal chain length required to fit a two-dimensional layer. It is obvious that of the strands which make up a two-dimensional network one should be linked with four others, since less than quadruple connections would only generate a very loose network or none at all. To accomodate this requirement, the minimal strand length should correspond to a hexadecasaccharide because this molecule possesses four peptides in the horizontal plane, four others being arranged perpendicular to it. Thus, in agreement with geometry, the ratio of bridged to non-bridged peptides would be 50:50. However, numerous experimental data for Gram-negative bacteria unequivocally demonstrate this ratio to be approximately 25:75 [23, 53]. This shows that in bacterial glycan strands there must be at least four bridged and twelve non-bridged peptides. Therefore, the minimal glycan length, assuming a layer meshwork without gaps, would have to be as long as a 32-membered oligosaccharide.

There is, however, convincing experimental evidence demonstrating that the predominant lengths of the glycan chains in *E. coli* are five to ten repeating disaccharide units, i.e. a length much too short to fit the planar murein network [24]. Moreover, the original concept required peptide bridges to be distributed evenly along the glycan chains, a hypothesis which was never confirmed experimentally. Rather, it appeared that peptide bridges in *E. coli* are concentrated mainly in the last turns of the helices close to the *N*-acetylglucosamine (GlcNAc) termini [49]. These experimental data have been known for years but were never considered appropriately. Taken together, they cast serious doubts on the prevailing hypothesis that murein is arranged in layers, at the same time, however, providing a clue as to how PG strands may actually be arranged in the bacterial wall.

A new concept of bacterial cell wall structure

Our concept accomodates the available experimental data and assumes glycan chains to run *perpendicular* to the plasma membrane, each strand being cross-linked mainly with four other strands via peptide side chains. Peptide bridges are located mainly in the outermost turns of the helices, and are drawn as reinforced lines in Fig. 2, which shows only the first turns of the nascent PG helices to appear outside the cytoplasmic membrane. In this way, peptide bridges form three-dimensional, left-handed helices resembling pores, which may have the characteristics of ionophore channels because each peptide bridge carries one amino and five carboxyl groups. Evidently, both the charge and the pore size in channels may be influenced by local pH changes.

According to the new concept, the wall of Gram-negative bacteria is envisaged as a cross-linked matrix with the minimal height of 3.92 nm (Fig. 1) corresponding to the height of one turn of the glycan helix [10]. The remaining glycan moieties could be either less cross-linked or free. The upper cross-linked zone of the matrix is expected to possess the properties of a dense gel, whereas the inner membrane proximal zone with fewer cross-links should behave rather like a liquid gel. Presumably, a small fraction of peptide bridges is covalently linked to lipoprotein molecules, thus contributing to the tight connections between murein and the outer membrane, lipoprotein α-helices being buried in the channels.

Obviously, Gram-positive walls are much more solid as the glycan strands comprise 100–200 disaccharides and are cross-linked throughout the entire length [23], forming a thick matrix with helix-shaped channels that harbor either teichoic or lipoteichoic acids piercing outwards through the wall. A particularly attractive feature of the new concept is that the fundamental distinction between Gram-negative and Gram-positive cell wall architecture is reduced to mere quantitative differences in glycan chain lengths and peptide cross-bridging. Interestingly, an analogous conclusion was drawn by the authors who first proposed the model of the periplasm as a gel with a density gradient [26].

The recent discoveries that (i) the cell wall exterior in *Bacillus subtilis* is of fibrous appearance, and (ii) the long bundles composed of PG and teichoic acid strands run perpendicular to the cell surface [20] strongly support our pro-

posal. The new concept of murein architecture also implies that the wall thickness is proportional to the strand length and that the shape of microbial cells is determined by the ratio of glycan strands deposited along the corresponding cell axes.

The novel concept is in excellent agreement with results of recent electron microscopy studies which consider murein in Gram-negative bacteria to have gel-like properties. The height of the periplasmic space was determined to be up to 25 nm [20–22, 26, 36], which differs significantly from results of earlier studies. Surprisingly, these previous data coincided precisely with those of neutron small-angle scattering studies demonstrating that 75–80% of the *E. coli* sacculus is as thin as 2.5 nm [34]. A rational explanation for these facts is that 75–80% of the *E. coli* PG strands are rather short and consist, in average, of ten disaccharide repeating units [24]. In agreement with the molecular parameters of the PG chains [6], helices of this length, when present in a compressed conformation, would have a height of approximately 2.5 nm. Most likely, therefore, under denaturing conditions, murein is able to shrink considerably. Thus, PG helices appear to be ideal molecules to regulate the size of the outer surface and of the entire bacterium itself. This regulatory function of PG, however, appears only possible if glycan strands are arranged perpendicular to the cytoplasmic membrane and peptide bridges run parallel to it. This arrangement affords maximum elasticity to murein when it either shrinks or swells.

Implications of the new concept for murein biosynthesis and assembly

The genes and enzymes of murein biosynthesis have been extensively characterized [18, 40], except for an elusive “flippase” that would be required for translocation of precursor molecules accross the membrane. The biosynthetic steps may be briefly described as follows. The major precursor, an undecaprenyl pyrophosphoryl (UndPP) derivative of the PG repeating unit, is assembled in the cytoplasm [9] by at least two enzymes, transmembrane translocase I and membrane-associated translocase II. Precursors are somehow translocated across the membrane and repeating units are polymerized by successive transfer of one unit to the other followed by attachment to the preexisting PG via peptide bridges [53]. Both reactions, transglycosylation and transpeptidation, are performed by bifunctional transmembrane enzymes, known as penicillin-binding proteins (PBPs) [51].

Regardless of older [3, 53] or more recent [8, 18] considerations, the biosynthesis has been depicted in the form of a traditional biochemical cycle. After each elongation step, the released UndPP is dephosphorylated to yield UndP, which reinitiates another cycle [19]. Remarkably, it had been postulated [1] that multienzyme complexes are grouped around UndP molecules.

The novel concept is consistent with the aforementioned principles of murein biosynthesis [3, 18, 40, 53] and explains how transmembrane murein synthases may be organized into multienzyme complexes to ensure ordered cross-linking of PG strands. Indeed, UndP, which functions as a coenzyme, was shown to be present in minute concentrations in the cell, to be evenly distributed within the membrane hydrophobic interphase [11], and to be unable to translocate precursor molecules across the membrane spontaneously [60]. As glycan chains grow via the transfer mechanism and as the rate of the process is determined by the quantity of UndP molecules [3], the latter should be arranged in pairs, as otherwise they would just not be able to cooperate in elongation reactions because of their extremely low abundance. Also, transmembrane PBPs are very rare components [50, 62] with a short lifespan, and newly made enzyme complexes are permanently needed to ensure ongoing murein synthesis [57]. Therefore, the only way for these enzyme complexes to synthesize the large murein matrix is to cycle. This requires disassembly after each glycan strand synthesis and reassembly at a different location to initiate the synthesis of another strand. This conclusion is crucial because it implies that PG strands have to be detached from the membrane, and this is corroborated experimentally by the finding that glycan strands are terminated by 1,6-anhydromuramic acid residues in many bacteria investigated thus far, even in exotic species [47]. Although the exact disposition of PBPs within the membrane is not clear, it is known that the three major enzymes PBP 1A, PBP 1B and PBP 3 exist as dimers [43, 65] and that they functionally associate with PBP 2 [61], the latter also being assumed to be a dimer.

With these major principles in mind, it is easy to visualize the way in which glycan strands are formed and orderly cross-linked and how murein is assembled. Since peptides on the glycan helices are oriented in four directions perpendicular to each other, it is logical to assume that PBPs are arranged in quadruple complexes (dimer plus dimer), each enzyme cross-linking those two peptide chains which are oriented towards each other in one of four directions, as shown in Fig. 3. It follows that two molecules of UndP would occupy their position in the middle of each quadruple complex.

To complete the multi-enzyme complex, additional enzymes should be added from the cytoplasmic side, i.e. two translocases (I and II) and UndPP-phosphatase, three for each UndP molecule. Presumably, to ensure the transfer reactions, two UndP molecules are located in a manner that enables them to be shared by all enzymes, as was predicted earlier [1], i.e., in the middle of the multi-enzyme complex as shown in Fig. 3. Evidently, each elongation step of the growing chain by one disaccharide-pentapeptide unit is followed by cross-linking with the adjacent chain emerging from the membrane. Both the synthesis and the translocation of the glycan chain across the membrane occur concomitantly as the polymer grows out of the enzyme complex. In fact, this could be a more general principle for the biosynthesis of all those bacterial heteropolysaccharides which are elongated by growing from their reducing ends proximal to the UndPP moiety of the precursor, as tentatively discussed previously [2]. Remarkably, the same

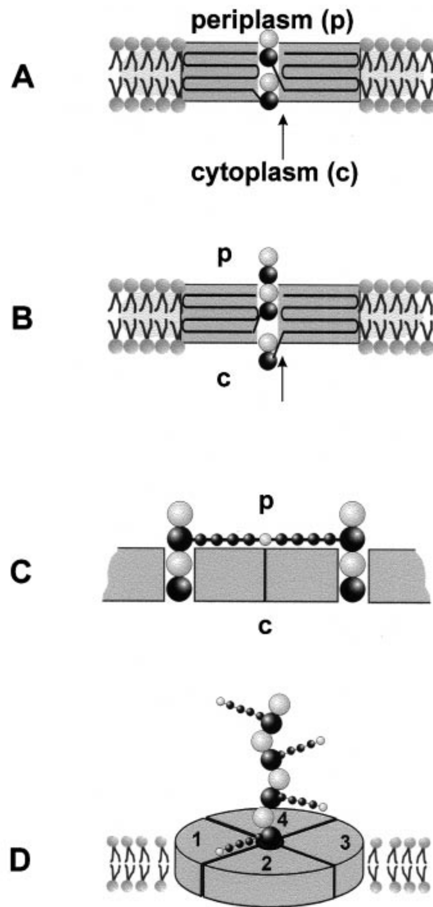

Fig. 3A–D Schematic presentation of peptide-cross-linked PG strand assembly. **A, B** The transglycosylase domain of the murein-synthase complex transfers the growing PG chains from one undecaprenyl pyrophosphate molecule to another assuring translocation of the polymer through the cytoplasmic membrane to the periplasm. **C** Two proximal peptides are cross-linked by the transpeptidase domain of the murein synthase complex forming a peptide bridge between two adjacent strands. **D** Tentative model of the organization of the murein synthase as a quadruple transmembrane complex allowing PG chain elongation, translocation and cross linking, and thus, formation of the murein matrix. Six additional enzymes cooperate with the quadruple complex from beneath the membrane (see text)

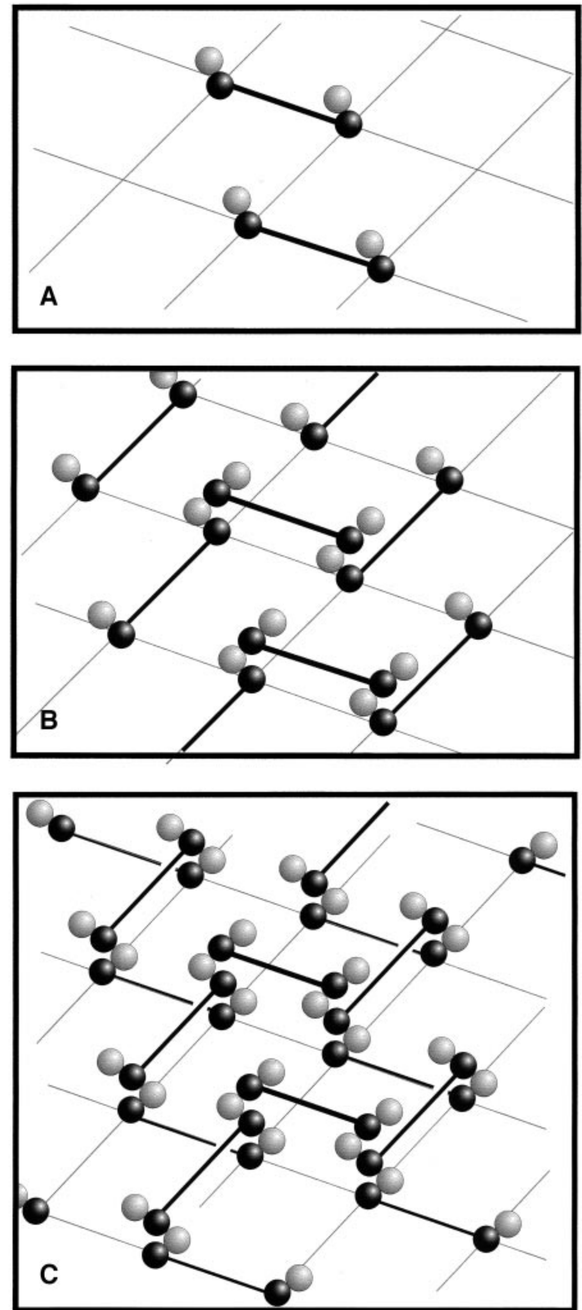

Fig. 4A–C Schematic representation of murein biosynthesis proceeding in toto. The stepwise-growing PG strands emerge from the cytoplasmic membrane to form small patches which become confluent to form a unimolecular murein blanket. **A** Growing chains start to emerge from the membrane and the first disaccharide-pentapeptide ends get cross-linked. **B, C** The subsequent elongation steps result in the emergence of additional strands that can be cross-linked. The following fourth elongation step completes the synthesis of one turn of the peptidoglycan helices and reveals pore formation within the progressively growing murein patches, as detailed in Fig. 2

mechanism for PG strand translocation was proposed for the first time in 1972 by Baddiley's group who rejected the idea of a "flippase" from the very beginning [1].

Thus, according to the new concept, the murein biosynthesis occurs in toto, resembling a conveyer belt starting from the assembly of precursors in the cytoplasm followed by their polymerization within the membrane and concomitant transportation through it and, finally, cross-linking at the periplasmic exterior of the membrane, the intensive septal murein synthesis being the final stage of de novo cell wall creation. Thus, glycan strands grow like grass and become more and more cross-linked with each other on the membrane exterior (Fig. 4). The proposed mode of murein assembly is in agreement with the observation that nascent material appears evenly on the membrane exterior as small discrete patches which gradually connect to each other to make up the entire network [42].

The most striking feature of the new concept for murein assembly is that it proceeds independently of the preexisting murein. Naturally, the old murein still exists, func-

tioning as a stress-bearing surface to withstand the turgor pressure. Notably, however, there are no connections to the nascent murein. To emphasize the molecular basis of this phenomenon, it should be remembered that glycan strands in murein of different bacteria are terminated either by free muramic acid or by 1,6-anhydromuramic acid residues. The enzymatic cleavage of the growing chains has two important biological implications. First, it signals that synthesis is terminated and, second, it implies that synthesized chains are detached from the membrane. In fact, the microbial wall is detached in toto from the cytoplasmic membrane. As soon as detachment occurs, the nascent wall starts to assemble, creating a situation in which two walls are present in the bacterial cell, a mature and a nascent one. Of note, two walls were clearly identified on electron micrographs some time ago but were interpreted in terms of “old and new wall material” [42] or “innermost and stretched wall” [20], the authors trying to accommodate their findings within the dogma of the classical concept [32]. More recently, two types of walls were unequivocally distinguished on micrographs of dividing staphylococci to reveal two daughter cells each being covered by common and individual walls [55].

The old wall is gradually destroyed by lytic enzymes, as the cell continues to grow, and is substituted by the newly synthesized wall. The latter is in “condensed form of high electron density” [20] and sprouts up from beneath as the old wall is worn out. It is conceivable that, for a short time, the new wall exists in a compressed form (masking it from hydrolytic enzymes working on the old, extended murein) and is associated with the membrane to allow for attachments to be introduced in the form of either lipoproteins in Gram-negative or teichoic acids in Gram-positive bacteria.

Cell wall growth: elasticity versus insertion

According to the classical postulate [32], the wall grows by virtue of continual insertion and covalent attachment of the nascent strands into the preexisting murein, and numerous models have been developed to visualize the mechanisms involved, as reviewed in [29, 35]. Concerning the covalent attachment deduced from pulse-chase experiments with [^{3}H]diaminopimelic acid, it is pertinent to remember that murein is a bag-shaped molecule (sacculus) and will retain any polymer. With similar success, labeled nucleotides and amino acids could be used in pulse-chase experiments to suggest that DNA, RNA, and proteins are covalently attached to the wall. These experiments conducted more than a quarter century ago did, of course, not prove the covalent attachment of these molecules, but rather demonstrated that nascent murein was trapped inside the old sacculus.

In considering the question of how the wall is actually growing, it may be assumed that it simply expands in response to the turgor pressure making use of its high inherent elasticity [56]. The largest increase in area would represent the elastic limit of the wall and the latter is 300% above the area of isoionic sacculi [33]. This degree of expansion is consistent with flexible conformations of the peptide bridges (transition from relaxed to stressed conditions) without causing rupture of the wall fabric.

This new simple and rational principle of cell growth is in contrast to the assumption that concerted and precisely coordinated action of murein synthases and hydrolases is needed to assure growth [29]. The hypothetical molecular interplay [28] of enzymes with opposing activities would constantly place the cell on the verge of suicide and, therefore, is unlikely to exist. The new model proposes that each group of enzymes just does safely and independently what it was designed for: to synthesize the new wall and to hydrolyze the old one. The hypothetical idea of a holoenzyme [27] composed of eight to nine enzymes (synthases and hydrolases) aggregated in one giant hovercraft shuttling along the strands to ensure concomitant cutting-and-sewing appears intellectually appealing, but oversophisticated, not least because the different enzymes are normally anchored to different membranes.

Apart from DNA replication and chromosome segregation, the bacterial cell cycle includes a division step which leads to cytokinesis and cell separation. During the division cycle, the cell identifies the mid-cell site, differentiates this site in preparation for cytokinesis and finally forms the division septum. The latter is made via coordinate ingrowth of the cytoplasmic membrane accompanied by intensive murein synthesis controlled by enzymes (PBP 3 being the major component) within the invaginated membrane. The latter more precisely represents the juxtaposition of two membranes, each of which assures continued murein biosynthesis until two new walls are formed. In fact, the cell wall is not divided in the course of cell division because this would represent an enormously difficult technical problem under the strong stress caused by turgor pressure. Instead, two new walls are synthesized beneath the old one which is destroyed by lytic enzymes in due course. The postulate of surface stress inducing cell wall division (“PUSH” model) does, therefore, not seem to apply [32].

Concerning the outer membrane in Gram-negative bacteria such as *E. coli*, it will become passively invaginated, as it is still connected with old murein. The process of *E. coli* cell division starts from the polymerization of the membrane-associated FtsZ protein to form the self-constricting Z-ring resulting in an intensive membrane invagination (septum formation) and is accompanied by recruitment of other Fts proteins [39]. Our concept is in complete accordance with this “PULL” model of cytokinesis and considers the septal murein synthesis as the final stage in the de novo process of creating the new bacterial wall.

Recycling and murein turnover

The phenomenon of peptide recycling and murein turnover has been known for years. However, since all cell wall growth has been postulated to proceed by cutting and in-

sertion, there is no need to degrade the entire peptidoglycan layer and the reason why bacteria destroy their murein and reuse the destruction products has remained obscure. In contrast, our new concept implies that the old sacculus *must* be destroyed to offer an opportunity to two newly born daughter cell walls to appear on the surface and allow the new cells to be separated. Murein turnover is almost undetectable (though existant) during the growth phase, because it is compensated for by intensive reuse (recycling) of the degradation products. Yet it proceeds exponentially during the stage of division [44], when new walls are assembled and lytic enzymes destroy the remnants of the old wall to facilitate the separation of the daughter cells.

In Gram-negative bacteria PG strands would begin to be cleaved by lytic exo-glycosidases from the loose (i.e. cytoplasmic membrane-proximal) ends carrying 1,6-anhydromuramic acid residues, i.e. from inside, whereas peptide bridges remain intact to retain the stress-bearing zone. The released anhydrodisaccharide-peptide units may be reused by the cell for new wall synthesis. This "inside-to-outside" mode of the wall lytic degradation is well known in *E. coli* [37]. In contrast, in Gram-positive bacteria which lack the outer membrane, the wall destruction proceeds from outside [20] to make the old wall worn and fibrous. The entire old sacculus in both types of bacteria would be destroyed in parallel with the cell division and this process would be accompanied by extensive murein turnover. We believe that it may be the murein tertiary structure (more accurately, the degree of its extension) that triggers lytic enzymes. Thus, the lysis of murein may be both initiated and controlled by the substrate itself.

According to the considerations presented here, bacteria seem to shed their walls very much like insects, crustacea and snakes when they substitute their integuments, with the only difference that old material is partially reused and, thus, turned over.

Macromolecular transenvelope transport: new murein as a lifting platform

In recent years, there has been an explosion of information identifying bacterial factors that are needed to transport macromolecules to the cell surface and into the environment. The term transenvelope transport is used here in its broadest sense, covering physiological processes as diverse as secretion and uptake of polymers regardless of their chemical nature and the assembly of different surface-associated complexes including fimbriae and flagellae. The common link unifying these biologically different processes is the assumed penetration across two structurally different barriers: the cytoplasmic membrane and the wall, the outer membrane to be added in case of Gram-negative bacteria. The transportation systems are usually known to be composed of multiprotein complexes arranged in the form of channels protruding across the wall. However, the crucial question of how they penetrate the firm cross-linked murein matrix is as yet unanswered. Current studies aimed at solving this problem are governed by the general idea that appropriate holes in the murein could be tailor-made via topologically controlled and elaborate murein rearrangements with the use of either lytic enzymes present in the periplasm or their homologs positioned in the trafficking complexes [13].

We propose that the new concept provides a simple solution of this problem. As soon as the synthesis of murein is terminated followed by detachment from the membrane, the latter becomes free for the assembly of new complexes, including, for example, trafficking systems. This implies that nascent murein and nascent transporting systems are generated in parallel. In this case, the problem of protrusion through murein does not arise because there is nothing to penetrate. In fact, transportation would occur in parallel with the synthesis and, actually, as a result of the latter. Moreover, as it was discussed above, the newly synthesized murein is temporally retained in the condensed form to help assembly of the trafficking system. The more the old wall is lyzed, the more the new wall grows up functioning as a lifting platform for the trafficking complexes. In agreement with the proposed mechanism is the recent discovery that trans-envelope proteins in Gram-negative bacteria have coiled-coil domains with properties of springs able to decompress [45].

To illustrate the advantage of the proposed mechanism for the assembly of various bacterial cell surface-associated complexes "protruding" the wall fabrics, a simple example is considered, i.e. the trafficking of the lipoprotein (LP) molecule which, being the major component of the outer membrane in Gram-negative bacteria, is linked covalently to murein [4]. Irrespective of their fine chemical structure, all LP are synthesized as precursors with signal peptides at the N termini and then translocated across the cytoplasmic membrane via the *Sec*-dependent route [54]. A specific signal peptidase cleaves the signal peptide, after the Cys residue has been modified in the lipid moiety and anchored in the membrane. At the same time, the C terminus is released from the cytoplasm and attached covalently to the peptide bridge in murein. Afterwards, the sorting protein recognizing the Ser residue proximal to the lipid anchor detaches LP from the cytoplasmic membrane and targets it to the outer membrane [41]. Thus, in the course of biogenesis, there is a moment when LP is linked to both the inner membrane and murein, and this situation, if preserved, is irreversibly lethal [63]. Here, the question arises as to how LP molecules traverse the periplasm after the N termini are released and the C termini are attached to murein. The assumption [41] that a sorting protein shuttles through the periplasm is unlikely for many reasons, the energy dependence being a major problem. We rather propose that it is the growing murein that lifts up the LP molecules from the inner to the outer membrane. The polysaccharide portions of lipopolysaccharides, teichoic and lipoteichoic acids are assumed to traverse the periplasm in an analogous fashion.

The same mechanism may apply to the transportation of more complex systems: the assembly of either com-

mon type 1 [30] or type 4 pili [25] with accompanying secretory complexes will be considered here more closely. Common and related pili of Gram-negative bacteria are heteropolymeric fibers with a diameter of about 6.5 nm composed of several different types of subunits in a distinct order. To be assembled into a pilus, subunits must be transported through the periplasm and through both membranes. The trafficking and assembly processes require two specialized proteins: a chaperone that caps tightly interactive domains in subunits to prevent their spontaneous aggregation, and an usher that serves as an assembly platform [30]. Chaperones are coenzyme-like proteins that are involved in the folding of other proteins but are not part of the ultimately assembled structure [14]. Ushers interact specifically with the chaperone-subunit complexes to dissociate them and release the chaperone which recycles to interact with another subunit. The newly formed usher-subunit complex functions as a receptor for another subunit which is donated by a chaperone. This assembly principle is supported by the observation that every pilus grows from the base [38]. Type 4 pili are assembled without chaperone and usher participation [25], using mainly the components of the general secretory pathway [46].

It is obvious that a coordinated trajectory of both chaperone-associated complexes and multicomponents of the *Sec*-dependent system through the cross-linked murein by protrusion is absolutely incompatible with the generally assumed physical structure of the latter. The situation would be different if there was no murein (as is often suggested by omission of murein in schematic representations of the functional cell envelope). Indeed, if the murein was absent there would be no problem with the pilus assembly, and we propose that this is, in fact, the case. Thus, at the moment of pilus assembly, the nascent murein is also being assembled, and the old wall is detached from the membrane and no longer represents a barrier for the macromolecular transenvelope trafficking.

Perspective

The life of bacteria in natural environments depends largely on the orderly functioning of the outer cell surface components, the wall being of utmost importance. Because of its significance, the major wall component, murein, has been intensively studied during recent decades. Although its primary structure and biosynthesis are well known, plausible models explaining its tertiary organization and function are still lacking. In the present paper we have proposed a novel concept which is based on previously reported data of other investigators. While it appears to integrate our current knowledge of murein biogenesis, arrangement and activity, it is obviously not based on new experimental evidence of our own. We thus present the model as an intellectual challenge and hope that it may stimulate the design and execution of experiments proving or disproving it. We are confident that the new concept will have a major impact on our understanding of the mechanisms of antibiotic agents affecting cell wall biogenesis.

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