MINIREVIEW

The Architecture of the Murein (Peptidoglycan) in Gram-Negative Bacteria: Vertical Scaffold or Horizontal Layer(s)†‡

Waldemar Vollmer* and Joachim-Volker Höltje‡

Universität Tübingen, Fakultät für Biologie, Lehrbereich Mikrobielle Genetik, Tübingen, Germany

The murein (peptidoglycan) sacculus is the essential exoskeleton of all eubacteria (except Mycoplasma species and a few other species) that is needed to withstand the internal cytoplasmic turgor (osmotic) pressure (60, 78). Murein consists of oligo(GlcNAc-MurNAc) glycan strands that are cross-linked by short peptides and thus form a net-like polymeric structure that surrounds the cytoplasmic membrane (78). The sacculus of Escherichia coli is one giant macromolecule with a molecular mass of more than 3 \times 10^9 Da, which is in the same range as the molecular mass of the chromosome of this bacterium (2.32 \times 10^9 Da). Moreover, the sacculus is embedded in the cell envelope by virtue of its location in the periplasm of gram-negative bacteria. It carries approximately 10^5 molecules of covalently bound lipoprotein (Lpp, Braun’s lipoprotein) that links the outer membrane to the murein (6). It has been assumed that the murein glycans and peptides are arranged parallel to the membrane, forming a thin layer in gram-negative species and a thick multilayer structure in gram-positive species. This concept was challenged recently by Dmitriev et al. (20, 21), who proposed the scaffold model, in which the murein glycans extend perpendicular to the cytoplasmic membrane. In this communication we first review relevant data on gram-negative murein structure and biosynthesis that were obtained over the past few decades in many laboratories, most of which were obtained from studies of E. coli. Then we discuss these findings with respect to different structural models of the murein sacculus.

EXPERIMENTAL DATA

Size of the murein sacculus. The murein was isolated from gram-negative bacteria by boiling the cells in a sodium dodecyl sulfate (SDS) solution, followed by purification by enzymatic removal of glycogen and proteins (26, 56, 78). As visualized by electron microscopy, the purified murein sacculi are bag-shaped structures with the dimensions and form of the bacteria from which they were isolated (Table 1). Like the rod-shaped cells, the sacculi of E. coli consist of a cylindrical part that is closed by two polar hemispherical regions. Compared to the length (about 2 to 4 \mu m) and the diameter (about 0.5 to 1 \mu m) of the sacculi, the murein is very thin, which results in the observed appearance of an empty and sometimes crumpled envelope laying flat on a grid used for electron microscopy (18, 22, 78).

Thickness of murein. Three methods have been used to measure the thickness of the murein of E. coli: electron microscopy, neutron scattering, and atomic force microscopy. The results obtained by electron microscopy were different when different techniques were used (4). After successive fixation of cells with glutaraldehyde, osmium, and uranyl acetate, followed by dehydration with ethanol and embedding in araldite, De Petris observed a multilayer architecture for the cell envelope (19). One layer (the g2 layer) disappeared completely upon treatment with the murein hydrolase lysozyme and was therefore identified as the murein layer. The g2 layer appeared to be 1.5 to 3 nm thick, whereas isolated murein sacculi that were obtained by boiling cells in an SDS solution and were purified by treatment with amylase and protease were 1 to 1.2 nm thick. Another study revealed a similar thickness (2 to 3 nm) for the murein layer in the envelope of E. coli that disappeared after lysozyme-EDTA treatment (61). However, these findings were questioned by a study of Hobot et al. (31), in which different sample preparation techniques were used. For example, glutaraldehyde-fixed cells did not contain a typical murein layer after low-temperature dehydration, and the authors did not identify a central line that appeared after staining with uranyl acetate as the murein layer. Because of the high water content of isolated murein, Hobot et al. proposed the concept of a periplasmic gel, in which the murein is distributed throughout the periplasm and is more cross-linked near the outer membrane and less cross-linked near the cytoplasmic membrane. Phosphotungstic acid (PTA) staining was used, which is specific for carbohydrate compounds in a mutant lacking periplasmic membrane-derived oligosaccharides. PTA stained the whole space between the inner and outer membranes, which was about 15 nm thick. However, isolated murein sacculi that were obtained from exponentially growing cells and were stained with PTA were thinner (8.8 ± 1.8 nm), and if isolated sacculi were purified further by treatment with protease to release the bound lipoprotein, the thickness after staining with PTA decreased to 6.6 ± 1.4 nm (53). The murein of cells in the stationary phase was thicker, 10.7 ± 1.1 nm if the cells were grown in rich medium and 9.9 ± 1.3 nm if the cells were grown in minimal medium. Employing PTA staining dur-
### TABLE 1. Experimental data for the structure of murein and its constituents from E. coli

<table>
<thead>
<tr>
<th>Property of murein or its constituents</th>
<th>Method</th>
<th>Experimental data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of sacculi</td>
<td>EM</td>
<td>Same size as cell, 2 to 4 μm long, 0.5 to 1 μm in diameter</td>
</tr>
<tr>
<td>Thickness of sacculi</td>
<td>EM</td>
<td>1 to 3 nm</td>
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<tr>
<td></td>
<td>EM</td>
<td>6.6 to 15 nm</td>
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<tr>
<td></td>
<td>Cryotransmission EM of frozen-hydrated sections</td>
<td>6.35 nm</td>
</tr>
<tr>
<td></td>
<td>Small-angle neutron scattering</td>
<td>2.5 nm (75 to 80% of surface), 7 nm (20 to 25% of surface)</td>
</tr>
<tr>
<td></td>
<td>Atomic force microscopy</td>
<td>6 nm (hydrated), 3 nm (nonhydrated)</td>
</tr>
<tr>
<td>Elasticity of murein</td>
<td>Low-angle laser light scattering</td>
<td>Approximately threefold reversible increase in surface is possible</td>
</tr>
<tr>
<td></td>
<td>Atomic force microscopy</td>
<td>Two- to threefold-higher deformability in the direction of the long axis of the sacculi compared to the short axis</td>
</tr>
<tr>
<td>Porosity of murein</td>
<td>Penetration of fluorescently labeled dextrans</td>
<td>2.06-nm pore radius (relaxed murein)</td>
</tr>
<tr>
<td>Size of proteins released by osmotic shock</td>
<td>Up to 100 kDa (pores with a radius of up to 3.1 nm may exist in the stretched murein)</td>
<td></td>
</tr>
<tr>
<td>Amount of murein per cell</td>
<td>Incorporation of radioactive Dap and chemical determination of radioactive composition</td>
<td>2.7 to 3.5 × 10^8 subunits per cell</td>
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<tr>
<td></td>
<td>HPLC analysis of isolated glycan strands</td>
<td>21 disaccharide units</td>
</tr>
<tr>
<td></td>
<td>HPLC quantification of 1,6-anhydro-MurNAC-containing muropeptides</td>
<td>25 to 40 disaccharide units</td>
</tr>
<tr>
<td>Murein glycan strands</td>
<td>X-ray analysis (β-chitin)</td>
<td>1.03 nm</td>
</tr>
<tr>
<td></td>
<td>Average length of glycan strands</td>
<td>21 disaccharide units</td>
</tr>
<tr>
<td></td>
<td>HPLC analysis of isolated glycan strands</td>
<td>21 disaccharide units</td>
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<td>HPLC quantification of 1,6-anhydro-MurNAC-containing muropeptides</td>
<td>25 to 40 disaccharide units</td>
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<tr>
<td>Murein peptides</td>
<td>Theoretical value</td>
<td>4.1 nm</td>
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<td></td>
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<td>21 disaccharide units</td>
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<td></td>
<td>Cross-links</td>
<td>34 to 48% of peptides are present in cross-links</td>
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<td></td>
<td>High-performance liquid chromatography (HPLC)</td>
<td>34 to 48% of cross-links are present in cross-links</td>
</tr>
</tbody>
</table>

**Notes:**
- EM, electron microscopy; HPLC, high-performance liquid chromatography.
ing autolysis of *E. coli* led to similar results. However, the fact that a thickness for regularly structured murein of about 15 nm (the thickness of the PTA-stained layer) contradicts a number of other experimental data, including the measured amount of murein per cell, was discussed by Leduc et al. (54). It was pointed out in two publications that thickness measurements resulting from electron microscopy images should be viewed with caution. First, the thickness of stainable material in the periplasm is not equivalent to the thickness of murein (79), and second, the measurement indicates the thickness of the contrasting metal and not of the murein itself (19). Recently, cryotransmission electron microscopy was used to visualize the envelope structure of gram-negative bacteria (58). This technique involves neither chemical fixation nor staining procedures and is therefore believed to produce fewer artifacts. Murein was visualized by cryotransmission electron microscopy in the periplasm of *E. coli* K-12 as a thin line below the outer membrane with a thickness of $6.35 \pm 0.53$ nm (58).

The thickness of isolated murein sacculi was also directly measured by small-angle neutron scattering (52). The advantages of this method were that the sacculi were fully hydrated, they contained no bound lipoprotein, and no staining procedure was used. The purified sacculi of exponentially growing *E. coli* W7 were not uniform in thickness. It was found that 75 to 80% of the surface was 2.5 nm thick and the remaining 20 to 25% was about 7 nm thick. An important finding was that the maximum thickness of the sacculi did not exceed 7 nm. Atomic force microscopy allowed determination of the thickness of nonhydrated murein and hydrated murein (81). As determined by this method, nonhydrated murein of *E. coli* was 3 nm thick, whereas hydrated murein was 6 nm thick.

**Elasticity of the sacculus.** The murein net is quite elastic and can reversibly expand and shrink, and this property is mainly due to the flexibility of the peptide part (2), whereas the glycan strands are rather rigid (48). This flexibility was demonstrated in filamentous cells of strain TOE13, which contain a temperature-sensitive *ftsA* allele. If the osmotic pressure of filaments of this strain was suddenly removed by destruction of the cytoplasmic membrane, the length of the filaments decreased by 17%, and it was calculated that the surface area of the murein in vivo was 45% greater than that in the relaxed state (46). The elasticity of isolated murein sacculi was also estimated by measuring the surface area by low-angle laser light scattering under different conditions (47). It was concluded that the surface area of the sacculi could reversibly increase threefold without rupture. Furthermore, atomic force microscopy revealed that sacculi are two- to threefold more deformable in the direction of the long axis (elastic modulus, $1.5 \times 10^7$ to $3 \times 10^7$ N/m$^2$; average, $2.5 \times 10^7$ N/m$^2$) than in the direction perpendicular to the long axis (elastic modulus, $3.5 \times 10^7$ to $6 \times 10^7$ N/m$^2$; average, $4.5 \times 10^7$ N/m$^2$ (81)). The elastic modulus is lower for material with greater elasticity. It was suggested that the observed anisotropy in elasticity was the consequence of the alignment of the murein glycan strands mainly perpendicular to the long axis of the cell.

**Porosity of the murein meshwork.** Demchik and Koch determined the size of fluorescently labeled dextrans that could penetrate isolated murein sacculi of *E. coli* ATCC 11775. They estimated that the pores in the murein had a mean radius of 2.06 nm, which would theoretically allow the penetration of a globular protein with a molecular mass of 22 to 24 kDa (17). Because in the living cell the murein is under tension, it was roughly estimated that in vivo the stretched murein may allow free diffusion of globular proteins with a maximum molecular mass of 50 kDa. In another study it was demonstrated that EDTA treatment of *E. coli* cells in combination with a hyperosmotic shock released a subset of cytoplasmic proteins that were almost identical to the proteins that are able to pass through a 100-kDa-cutoff filter (74). It was speculated that this limitation was caused by the molecular sieving property of the murein sacculus that was impermeable for proteins with molecular masses of more than 100 kDa under the osmotic shock conditions.

**Amount of murein per cell.** The amount of murein per cell was estimated by determining the amount of *m*-diaminopimelic acid (*m*–Dap), an amino acid that is present exclusively in murein. In early work two methods were used, chemical determination and determination via incorporation of radioactive *m*–Dap, and these methods gave essentially the same results ($2.7 \times 10^6$-m–Dap molecules per cell for strains HrH and W945/3282) (7). Using both methods, workers in a later study determined that the amount was $3.5 \times 10^6$ molecules of *m*–Dap per cell for strain MC4100 *h*sd (79). The surface area of the same cells was measured by using electron micrographs and was found to be 8.3 or 8.9 $\mu$m$^2$, indicating that the average surface area per disaccharide unit was 2.5 nm$^2$ (79). In a third study with *E. coli* B/r H266 grown in a variety of different media, an average surface area of 1.6 to 1.8 $\mu$m$^2$ per disaccharide unit was determined, and this value was found to be nearly constant at different growth rates ranging from 0.40 to 2.93 doublings/h (82). In the *m*–Dap auxotrophic strain MC6RF3, the amount of murein as measured by incorporation of radioactive *m*–Dap into SDS-insoluble material could be reduced by 50% without any growth defect if the external concentration of *m*–Dap was decreased from 40 to 1.4 $\mu$g/ml (68).

**Chemical composition of murein.** Murein is composed of glycan strands of alternating *β*-1,4-linked GlcNAc–*β*-1,4-MurNAc disaccharides that are cross-linked by short peptides (72). In *E. coli* and other gram-negative bacteria, the MurNAc residue at the end of the strand has a 1,6-anhydro modification (34, 69). The stem peptides that are linked to the lactyl group of MurNAc consist of two to five amino acids, and the sequence of the pentapeptide is as follows: d-Ala–d-1-Glu–(γ)-m-Dap–d-Ala–d-Ala. Most cross-links are formed between the d-Ala at position 4 of one stem peptide and m-Dap at position 3 of a second stem peptide of a neighboring glycan strand (1D cross-links), but there is also a small percentage of cross-links between two m-Dap residues (1D cross-links). Besides such dimeric cross-links, there is a smaller fraction of trimeric and tetrameric cross-linked structures (26).

**Length of the murein glycan strands.** One GlcNAc–MurNAc disaccharide in the murein is the same length as a GlcNAc–GlcNAc disaccharide in crystalline α-chitin, namely, 1.03 nm (7, 12, 13, 48). The average degree of oligomerization of the murein glycan strands of *E. coli* can be determined in two different ways: first, by analyzing the length distribution of isolated glycans and second, by quantification of the 1,6-anhydro-MurNAc-containing muropeptides that are a hallmark of one of the ends of the glycans. Murein glycan strands can be
released from isolated murein by treatment with an amidase from human serum. The glycans can be purified by cation-exchange chromatography, and the length distribution can be analyzed by C18 reversed-phase chromatography (30). However, the standard method allows only separation of glycans that are not longer than 30 disaccharide units. The average length of the glycans containing from 1 to 30 disaccharide units was 8.9 disaccharide units in strain W7. Glycans that are longer than 30 disaccharide units eluted together in one peak. These long glycans represented about 25 to 30% of the total material, and their average length was 45.1 disaccharide units. The average length of all glycans was estimated to be 21 disaccharide units by this method (30). If the chromatography was prolonged, a regular pattern of peaks corresponding to glycans with up to 80 disaccharide units could be resolved (Y. Chen and J.-V. Höljlje, unpublished).

The glycan strands in the E. coli murein do not contain a reducing end but do contain a 1,6-anhydro-MurNAc moiety. Therefore, digestion of murein with a muramidase, such as lysozyme or Cellosyl, yields a fraction of mucopeptides with 1,6-anhydro-MurNAc representing mucopeptides from the end of the glycan strands. Mucopeptides can be separated by reversed-phase high-performance liquid chromatography (26), and the proportion of mucopeptides containing 1,6-anhydro-mucopeptides was shown to depend on the strain and on the growth conditions and ranged from 3 to 6% of the total mucopeptides. For example, strain KN126 from an exponential-phase culture in Luria-Bertani medium contained 3.88% 1,6-anhydromucopeptides, indicating that the average length of the murein glycan strands was 25.8 disaccharide units. In murein of cells from a stationary-phase culture the average length was greater, 33.3 disaccharide units at 42°C. In contrast, digestion with muramidases (Sl70 or lysozyme) did not result in gaps with a preferential orientation. Assuming a layered murein architecture, Verwer et al. concluded that the material resistant to endopeptidase digestion (namely, the glycan strands) is oriented predominantly perpendicular to the long axis of the cell (76). However, the results for the appearance of muramidase-treated sacculi are conflicting. It was found recently by de Pedro et al. that the digestion of murein sacculi with the muramidase Cellosyl produced oriented oblong gaps (18) similar to those seen after endopeptidase treatment (76). Therefore, it might not be possible to determine the orientation of the murein glycan strands by analyzing electron microscopic images of partially digested murein (42).

**Cross-linkage of murein.** A cross-linking (transpeptidation) reaction occurs between the carboxyl group of a donor peptide and a free amino group of an acceptor peptide such that the energy for the formation of the new peptide bond is generated by the release of the D-alanine at position 5 of the donor peptide. About 50% of the peptides in the murein of E. coli are part of cross-linked (dimeric, trimeric, or tetrameric) mucopeptides. For example, the murein of exponentially grown strain KN126 contains 51.51% free (non-cross-linked or monomeric) peptides, 43.27% peptides in dimeric structures, 5.02% peptides in trimeric structures, and 0.19% peptides in tetrameric (cross-linked) structures (26). Glauner et al. defined the degree of cross-linkage as follows: degree of cross-linkage = 100 x (1/2 dimers + 2/3 trimers + 3/4 tetramers)/all mucopeptides. By this definition, the degree of cross-linkage is equal to the molar percentage of peptides that functioned as donors in cross-linking reactions and is not the same parameter as (and is much smaller than) the molar percentage of cross-linked peptides, a number that is given frequently in publications. Thus, KN126 has a degree of cross-linkage of 25.12%, and 48.48% of all peptide side chains are part of cross-linked structures. The degrees of cross-linkage are similar for different strains and mutants of E. coli, and analysis of the mureins of nine other gram-negative species revealed that in all cases more than 40% of the peptides were part of cross-links (69). Only in E. coli cells grown in the presence of D-amino acids was the cross-linkage dramatically reduced to a value of 34% of cross-linked peptides (11). Pulse experiments showed that newly incorporated material had a lower degree of cross-linkage (16% after a 20-s pulse), but this value increased to the normal value, 25.1%, after a 150-s pulse (25). The degree of cross-linkage decreased to 23.9% during a 90-min chase. The murein glycan strands have two ends, and it was shown that not only the 1,6-anhydro-MurNAc end of the glycan (26) but also the GlcNAc ends (71) are predominantly part of cross-linked structures.

**Growth of the sacculus and murein turnover.** Growth of the sacculus occurs by incorporation of new material by two reactions, transglycosylation and transpeptidation. The transpeptidation reaction results in a new cross-link in which the donor peptide is characterized by a free ε-amino group at m-Dap, whereas on the acceptor peptide the ε-amino group at m-Dap is linked to D-Ala at position 4 of the donor peptide (see
above). By determining the radioactive label distribution among donor and acceptor sites of the cross-bridges in pulse-chase experiments, several conclusions could be drawn. First, new (labeled) material is cross-linked to the existing (old) material (10, 14, 16, 25), and this is proposed to occur during elongation of the cell. During cell division, there is also a cross-linking reaction between new donor and new acceptor peptides (16, 25). It appears that free oligomeric murein intermediates are not formed prior to incorporation into the sacculus. Rather, the lipid-linked precursors are directly linked to the sacculus without passing through an oligomeric stage (28).

Furthermore, during growth of E. coli a dramatic release of murein material from the sacculus (murein turnover) takes place, which is subject to an effective recycling process (27, 29). It was estimated that in one generation 40 to 45% of the murein of the sacculus is released by the actions of lytic transglycosylases, endopeptidases, and amidases (27).

**Growth pattern of the murein glycan strands.** By using the m-Dap auxotrophic strain W7 the average length of newly synthesized glycans was determined by quantification of the 1,6-anhydromuropetides in pulse experiments to be 50 to 60 disaccharide units (25). Pulse-chase experiments showed that the average length of the new glycans decreased within 5 min to 35 to 40 disaccharide units. Even a longer chase for 60 min resulted only in a minor reduction to 31.2 disaccharide units, which is the characteristic value for uniformly labeled murein material from the sacculus (murein turnover) takes place, which is subject to an effective recycling process (27, 29). It was estimated that in one generation 40 to 45% of the murein of the sacculus is released by the actions of lytic transglycosylases, endopeptidases, and amidases (27).

**Structural Models**

Because the three-dimensional architecture of murein cannot be determined with high resolution by the techniques available at this time, the structure was modeled based on the basis of the relevant experimental data for the physical properties and the chemical composition. As in other scientific fields, the models were changed or refined as new techniques were developed and more data became available. Below, we describe and discuss the different structural models for murein from gram-negative bacteria. For historical reasons, we divide this section into three parts: (i) the first models of the glycan strands and the peptides and early models of a layered murein, (ii) the more recent (new) model of a layered murein, and (iii) the scaffold model. The relevant experimental data for the structure of murein and its constituents are summarized in Table 1.

**First models of the structures of the glycan strands and peptides and early models of a layered murein.** The first models included mainly predictions for the conformation of the basic murein constituents, the glycan strands, and the peptide side chains, as well as predictions for the three-dimensional architecture of the sacculus. The glycans are similar in terms of their primary structure to the strands in chitin in that they are β-1,4-linked GlcNAc oligomers. In the murein every second sugar residue carries a lactyl group (the MurNAc residues) with the peptide side chains at position 3. In a chitin chain, the glycans are twisted such that successive GlcNAc residues are rotated 180° relative to each other, and in the most common form, α-chitin, adjacent chains run antiparallel (59). A chitin-like tertiary structure was assumed for the murein glycan strands in the early models (7, 23, 37, 63, 77) (Fig. 1A). The glycans were modeled as straight rods that run parallel, almost touching each other, with the peptides protruding in the same direction above or below the glycan plane. Some peptides form cross-links to peptides of neighboring strands. With this arrangement only a horizontally layered murein model can be envisioned, in which the glycan strands run parallel to the cytoplasmic membranes. The horizontal models are in accordance with the conclusion that the murein glycan strands must lie predominantly in the plane of the surface because they are too long for a vertical arrangement (70). Furthermore, it was speculated based only on considerations of possible growth mechanisms and without any experimental proof that the glycan strands might be arranged perpendicular to the long axis of the cell (66). Later, indirect evidence for such an arrangement was obtained (81).

Conformational calculations of the non-cross-linked pen-tapeptides revealed that there are several energy-minimized states. However, the favorable conformation is likely to be the one in which the pentapeptide is not straight but bends back to the glycan strand (2). In two additional studies the authors concluded that the cross-linked peptide might adopt either a compact or a more extended configuration (63, 77) and that this flexibility of the peptides might explain the observed elasticity of murein.

**More recent (new) model of a layered murein.** The interpretation of new data obtained by X-ray diffraction studies, together with stereochemical considerations and quantum chemical studies, led to the conclusion that the glycan strands in the murein cannot adopt a chitin-like structure with two sugar residues per turn (9, 48, 80). Instead, the presence of the rather bulky lactyl group at MurNAc allows less rotation. About four disaccharide units (eight sugar residues) are required for one turn, and consequently, the peptides protrude from the glycans in a helical pattern (3, 50, 55) (Fig. 1B). If the glycans are arranged parallel to the membrane, then every second peptide lies in the same plane, and a monolayer of murein can be formed by cross-linking such peptides of neighboring strands (Fig. 2, right side). One quarter of the peptides would point up,
and another quarter would point down, and these peptides could not take part in cross-links. Thus, theoretically, a perfect monolayer with this structure would contain 50% of the peptides as part of cross-links, and 50% of peptides would not be cross-linked. This is similar to the experimentally observed fraction (40 to 50%) of peptides that are part of cross-links (24, 26).

In the layered arrangement, one subunit can cover approximately 5.2 nm² in the maximally stretched conformation (21). Considering the number of subunits (3.5 × 10⁶ subunits), a maximum total area of 18 μm² can be covered, which is about twice the measured surface area of the cells (79). In vivo, murein that is not maximally stretched could cover less surface; therefore, it is likely that the number of subunits allows only one to two complete layers. The horizontally layered model is in excellent agreement with the thickness of isolated murein (not more than 7 nm) determined by small-angle neutron scattering and atomic force microscopy. The data obtained by small-angle neutron scattering led to the interpretation that 75 to 80% of the surface of the sacculus is single layered and is 2.5 nm thick, whereas the rest is triple layered and has a maximum thickness of 7 nm (52).

FIG. 2. Possible arrangement of the murein glycan strands with respect to the cytoplasmic membrane. Part of the murein structure with glycans consisting of 12 disaccharide units that are maximally cross-linked is shown. In the scaffold arrangement (left side) the glycans are arranged perpendicular to the cytoplasmic membrane (xy plane). In a horizontally layered murein (right side), both the glycans and cross-linked peptides are oriented parallel to the cytoplasmic membrane, whereas the non-cross-linked peptides point out of the layer. Every dark gray bar and light gray bar represents one GlcNAc-MurNAc disaccharide unit; solid lines represent peptides.

A modification of this model was introduced by Koch, who pointed out that in the stress-bearing murein the glycans would not be straight but would follow a zigzag line (38, 42, 45) and termed the smallest pore that is formed by two glycan strands and two peptide cross-links a tessera. A tessera would have the form of a hexagon (Fig. 3), and it would be more deformable in the directions of the peptides. It was shown experimentally that sacculi are two- to threefold more deformable in the direction of the long axis (81). The theoretical elastic constant of a perfect single-layer murein network with the dimensions of the cell consisting of hexagonal tesseras was calculated to be ~10⁷ N/m² (5), which is in good agreement with the experimental value, 2.5 × 10⁷ N/m² (in the direction of the long axis of the cell). Thus, Boulbitch et al. (5) concluded that potential defects in the (imperfect) cylindrical network of the real murein (see below) might play only a minor role in determining the elastic properties.

FIG. 3. Tessera is the smallest structural unit of the layered murein. The glycan strands are arranged in a zigzag pattern. The peptides in the plane of the glycans form cross-links to peptides of neighboring glycans. The peptides pointing out of the plane that are present on every second disaccharide are not shown. Dark gray bars, MurNAc; light gray bars, GlcNAc; solid lines, peptide cross-links.

Given the experimental data, the layered murein net cannot be perfect for two reasons: (i) compared to the dimensions of the cell, the glycan strands are rather short (average length, 25 to 35 nm), and (ii) the percentage of cross-linked peptides is not the theoretical value (50%) but is slightly less (40 to 50%). Imperfections are holes or slits that are larger than the hole of a single tessera or, in other words, consist of fused hexagonal tesseras. The distribution of such holes in a layered murein with glycans of the observed length was modeled by Pink et al. (67). Representing fused tesseras, the larger holes were found to have the form of slits that run predominantly perpendicular to the long axis of the glycans. If these slits were distributed over the surface of the sacculus and did not accumulate at distinct sites, such an imperfect murein would be a stable network (67), and it was concluded that such a murein would have a permeability that is consistent with the observed data (17). A structurally stable murein network can be modeled with short glycans consisting of seven disaccharide units and 50% cross-linked peptides (Fig. 4). The structure of the murein shown in Fig. 4 is far from the structure of the real murein. The latter molecule would have a reduced number of connected tesseras because the slightly lower level of cross-linkage is more than compensated for by the three- to fivefold longer
glycan strands. It is not known what number of larger pores and what maximum pore size in the layered murein can be tolerated without destroying the integrity of the cell wall. Experimental data indicate that isolated (relaxed) murein of E. coli has pores with a mean radius of 2.0 nm (17). In vivo, the stretched murein allows penetration of proteins with molecular masses of up to 100 kDa (17, 74), indicating that larger pores with a radius of about 3 nm may exist (according to the formula given in reference 17, a globular protein with a molecular mass of 100 kDa has an estimated radius of 3.1 nm). The possible existence of larger holes indicates that the murein net is not perfect and is consistent with the data on the glycan length distribution and on the degree of cross-linkage.

Because the glycan strands with average lengths are longer than the peptide cross-bridges, the arrangement of the glycan strands is a major structural determinant in a layered murein. One could envision the following possibilities for arrangement of the glycan strands in one layer: (i) the glycan strands run parallel and mainly in the direction of the long axis of the cell, (ii) the glycan strands run parallel and mainly perpendicular to the long axis, (iii) the glycan strands run parallel and along helices around the cell surface, (iv) there are patches or areas on the surface with regularly arranged parallel glycan strands, together with areas of random glycan orientation, and (v) the glycan strands are arranged in a random orientation without any order. Koch favored an irregular or random structure for the arrangement of the horizontally layered glycan strands to form a “carded, non-woven fabric” structure (39, 43), and he discussed other models (42).

At this time, no technique allows direct determination of the orientation of the glycan strands in the sacculus. Limited fragmentation of sacculi by sonication and different murein hydrolases gave contradicting results regarding the direction of the slits generated on the sacculi (18, 42, 75, 76). The anisotropy in elasticity of the sacculi (81) mentioned above would be in accordance with a layered murein in which the flexible peptides are arranged predominantly in the direction of the long axis of the cell and the glycan strands are predominantly perpendicular to the long axis. If the murein is the main stress-bearing layer, it was estimated on the basis of the elasticity measurements that the length of the bacterium would increase by 12% and the diameter would increase by 8% for every 1 atm of turgor pressure (81).

The murein sacculus is not a static structure. It is enlarged and divided into two sacculi during the cell cycle. Weidel and Pelzer pointed out that not only the incorporation of new subunits but also the hydrolysis of covalent bonds is required to increase the surface area of the bag-shaped sacculus (78). Two major aspects need to be understood with respect to enlargement of murein: (i) how is the site of insertion of new material selected, and (ii) what is the mechanism of insertion of precursors into the sacculus during growth. The selection of the insertion sites for new material might be controlled merely by surface tension, as proposed by the surface stress theory of Koch (40, 41, 44). Briefly, it has been proposed that elongation of the rod-shaped stress-bearing sacculus is possible with maintenance of a constant diameter by random insertion of precursors into the cylindrical part. This model requires inerrness of the polar regions, a property that has been proven experimentally (18). On the other hand, murein synthesis might be controlled directly or indirectly by the recently discovered MreB/Mbl proteins that form spirals at the inner site of the cytoplasmic membrane and that are required for the rod shape in Bacillus subtilis and in other rod-shaped bacteria (15, 36).

There are different models for the mechanism of insertion of new material into the murein of a layered structure. Burman and Park have proposed that local hydrolysis within the murein net precedes the insertion of two newly synthesized and cross-linked glycan strands (10, 65). On the other hand, the 3-for-1 model follows the make-before-break strategy that demands that synthetic reactions precede hydrolysis of bonds in the stress-bearing sacculus (38). Accordingly, three new glycan strands are synthesized, cross-linked to each other, and linked to both sides of an existing glycan strand in the sacculus. Upon removal of the so-called docking strand, the new triplet of glycan strands is inserted into the sacculus (32, 33). Further experimental work is required to determine both the mechanism for selection of the insertion sites and the mode of insertion of the new material.

**Vertical scaffold model.** Recently, a novel scaffold model for murein structure was proposed (20, 21), in which the glycan strands extend perpendicular to the cytoplasmic membrane and are cross-linked by peptides that are parallel to the surface of the membrane (Fig. 2, left side). The 1,6-anhydro-MurNAc ends of the glycan were assumed to be located near the cytoplasmic membrane in a region of high cross-linkage, whereas the GlcNAc ends were located close to the outer membrane in a poorly cross-linked region. According to the authors who proposed this model, the murein almost completely fills the
periplasmic space. However, several experimental findings do not fit the scaffold model, as discussed below.

E. coli contains about 3.5 \times 10^6 molecules of m-Dap per cell, which are present in murein consisting of glycan strands that are, on average, 25 to 35 disaccharides long. If an average of 30 disaccharides is used, this yields a total number of 3.5 \times 10^6/30 or 1.17 \times 10^6 glycan strands. According to the authors who proposed the scaffold model, one unit (one glycan strand oriented perpendicular to the membrane) covers an area of about 27 nm^2 (21). We realize that this is the maximum possible surface area of a unit that was calculated from the length of a maximally stretched peptide cross-link (4.1 nm), the length of one disaccharide (1 nm), and the thickness of one disaccharide (1.1 nm). With 1.17 \times 10^6 glycan strands a maximum total surface of 3.15 \mu \text{m}^2 could be covered in the maximally stretched scaffold arrangement, which is less than 40% of the surface area of the cell. The discrepancy becomes even greater if one considers that strain MC6RP3 could grow with 50% less murein at a low m-Dap concentration. The average glycan strand length was 24.4 disaccharide units under these conditions (68). It follows that a maximum area of 1.9 \mu \text{m}^2 can be covered with scaffold-like murein, which is only 23% of the cell surface area (assuming that the amount of m-Dap per cell and the average size of the cells are not much different from the values for other strains). We concluded that E. coli does not contain enough murein for the proposed scaffold murein structure with glycan having the measured length distribution.

According to small-angle neutron scattering and atomic force microscopy experiments, isolated sacculi are rather thin. About 75 to 80% of the surface is 2.5 nm thick, and the remaining 20 to 25% is at most 7 nm thick (52). These measurements were obtained with purified murein sacculi that were fully hydrated and not subjected to staining procedures, which could influence thickness measurements determined by electron microscopy. Taking these facts in account, the scaffold-like murein would consist mostly of glycan strands consisting of 2.5 disaccharide units and would contain glycans that have a maximum length of only 7 disaccharide units. However, the measured average length of the murein glycan strands is 25 to 35 disaccharide units. Given the determined average length of the murein glycans, the thickness of the proposed scaffold-like murein would be 25 to 35 nm, which is about 10-fold greater than the measured thickness of murein. Furthermore, a high proportion (25 to 30%) of all glycan material consists of glycan strands that are longer than 30 disaccharide units. Thus, many of the murein glycan strands are longer than the distance from the cytoplasmic membrane to the outer membrane, which is about 13 to 25 nm (58, 64). In the attempts to model both the planar murein and the scaffold-like murein, the long murein glycan strands were not included (21). We concluded that a scaffold-like arrangement with glycan strands arising perpendicularly from the membrane is not in accordance with the observed length distribution of the murein glycan strands and the measured thickness of the sacculus.

The authors who proposed the scaffold model stressed the point that a layered murein with a low percentage cross-linked peptides (33%) and short glycan strands (on average, 12 disaccharide units) would have large holes (21). However, both assumptions are far from reality. The glycan strands have an average length of about 30 disaccharide units. High-perfor-

mance liquid chromatography analysis of muropeptides revealed that about 40 to 50% of the peptides in the murein of E. coli and other gram-negative bacteria are part of cross-links (26, 69). E. coli grown in the presence of \(d\)-amino acids showed strongly reduced cross-linking; only 34% of the peptides were part of cross-links. However, these cells contained longer glycan strands (average length, 39 disaccharide units), as determined from the proportion of anhydro-MurNAc-containing muropeptides (2.6%) (11). It is possible that the cells can (at least partially) compensate for a low degree of cross-linkage by increasing the length of the glycan strands to stabilize the murein net.

The murein sacculus is elastic both in vivo and in vitro. As mentioned above, atomic force measurements revealed that the elasticity is greater in the direction of the long axis of the cell and less perpendicular to this direction (81). This finding does not seem to fit into the scaffold model, in which the elastic peptide bridges point in both directions.

Both ends of the murein glycan strands are subject to greater cross-linkage than internal fragments (26, 71). This may reflect either the mechanism of insertion of new glycans or the fact that non-cross-linked glycan ends that do not contribute to the stability of the net are trimmed down by enzymatic degradation to cross-linked structures. This finding is inconsistent with the scaffold model, in which only the 1,6-anhydro-MurNAc ends are located near the cytoplasmic membrane in a zone of high cross-linkage and the GlcNAc ends are localized in a less cross-linked zone near the outer membrane (loose ends) (21).

The authors who proposed the scaffold model present a model in which in one cell cycle “two new walls are synthesized beneath the old one which is destroyed by lytic enzymes in due course” (20). However, biochemical studies performed in different laboratories have clearly shown that during growth of the sacculus cross-links are formed between new material and the old murein of the existing sacculus (10, 14, 16, 25), excluding the proposed mechanism of synthesis of two new walls beneath the old wall.

**CONCLUSION**

In summary, we believe that many experimental results, including the amount of murein per cell, the thickness of the sacculus, the analytical data on the length distribution of the glycan strands, the growth pattern of the glycan strands, the degree of cross-linkage, and the fact that cross-links are formed between the existing murein and the newly synthesized murein, are in accordance with a model in which very few layers of glycan strands are cross-linked by peptides and are arranged parallel to the cytoplasmic membrane (horizontally). Given the experimental data, a scaffold-like murein structure with glycans that extend perpendicularly from the cytoplasmic membrane is highly unlikely. However, our interpretation does not exclude the possibility that occasionally or even in particular areas on the sacculus glycan strands may bend out of the horizontal layer to adopt another orientation, but the major stress-bearing part of the murein is likely to be formed by a planar layer(s). We hope that in the future high-resolution techniques will become available that allow direct visualization of the orientation of the murein glycan strands in vivo.


