Murein (peptidoglycan) structure, architecture and biosynthesis in *Escherichia coli*

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Abstract

The periplasmic murein (peptidoglycan) sacculus is a giant macromolecule made of glycan strands cross-linked by short peptides completely surrounding the cytoplasmic membrane to protect the cell from lysis due to its internal osmotic pressure. More than 50 different muropeptides are released from the sacculus by treatment with a muramidase. *Escherichia coli* has six murein synthases which enlarge the sacculus by transglycosylation and transpeptidation of lipid II precursor. A set of twelve periplasmic murein hydrolases (autolysins) release murein fragments during cell growth and division. Recent data on the in vitro murein synthesis activities of the murein synthases and on the interactions between murein synthases, hydrolases and cell cycle related proteins are being summarized. There are different models for the architecture of murein and for the incorporation of new precursor into the sacculus. We present a model in which morphogenesis of the rod-shaped *E. coli* is driven by cytoskeleton elements competing for the control over the murein synthesis multi-enzyme complexes.

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Keywords: Murein; Peptidoglycan; Sacculus; Penicillin-binding protein; Murein synthases and hydrolases; Bacterial cytoskeleton; Bacterial morphogenesis

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Abbreviations: GlcNAc, N-acetylglucosamine; m-A2pm, meso-diaminopimelic acid; MurNAc, N-acetylmuramic acid; nPB, non-Penicillin-binding domain; PBP, Penicillin-binding protein; TG, transglycosylase; TP, transpeptidase; UDP, uridine diphosphate

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1. Introduction

The essential murein (peptidoglycan) sacculus is located in the periplasm of Gram-negative bacteria and is a giant, bag-shaped macromolecule which encases the cytoplasmic membrane to protect the cell from rupture by its internal turgor (Fig. 1) [1]. Although the discovery of murein lies more than half a century back we still have to answer fundamental questions, as illustrated by three examples: (i) When the cell grows the sacculus is enlarged in such a way that the specific cell shape (sphere, rod, comma or other) is maintained. During cell division the newly synthesized septal murein will become the murein of the hemispherical caps of the daughter cells. The molecular mechanisms for bacterial cell shape maintenance, division site selection and septation are poorly understood. (ii) The chemistry of the sacculus is well known. Murein consists of glycan strands cross-linked by peptides forming a three-dimensional net-work structure. However, its peculiar physical properties made it impossible to determine the molecular architecture of a sacculus with the presently available techniques. For example, the orientation of the glycan strands and the peptides have to be modeled. (iii) The field also suffers from having only very few available structures of the key enzymes for murein biosynthesis, the Penicillin-binding proteins (PBPs). Surprisingly, there is no known crystal structure for any murein synthase from a Gram-negative bacterium. Having outlined the major tasks for future research at the beginning of this review, we will now summarize the present knowledge on the chemical structure, biophysics and biosynthesis of murein in Gram-negative bacteria, with an emphasis on the reactions and interactions of the murein synthetic enzymes. We will also present models for enlargement of the murein layer and for the spatial control of murein synthesis. The data described in this review were derived from studies in E. coli unless otherwise indicated.

2. Chemical composition and fine structure of murein

Murein is a hetero-polymer made of linear glycan strands of alternating, \( \beta1,4 \)-linked N-acetylglicosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues, that are cross-linked by short peptides [2] (Fig. 2). The terminal residues of the glycan strands are GlcNAc and 1,6-anhydroMurNAc, which is MurNAc with an intra-molecular ether-linkage from C-1 to C-6 [3]. Depending on the strain and the growth conditions, about \(3\%\)–\(6\%\) of the murein subunits have a 1,6-anhydroMurNAc residue. Hence, the average degree of oligomerization of the glycan strands is about 25 to 40 disaccharide units [4,5]. Isolated murein glycan strands with up to 30 disaccharide units can be separated by high-pressure liquid chromatography (HPLC) and these short glycan strands have an average length of 8.9 disaccharide units [6]. The long glycan strands with more than 30 disaccharide units that represent about 25–30\% of the total glycan material, have an average length of about 45 disaccharide units.

The peptides are attached by an amide linkage to the lactyl group of MurNAc and they are unusual because they contain rare \(\alpha\)-amino acids. The initial sequence of the newly synthesized pentapeptide is L-Ala–D-iGlu–m-A2pm–D-Ala–D-Ala, with the dibasic meso-diaminopimelic acid (m-A2pm) residing at position 3. The D-Glu residue is linked via its \(\gamma\)-carboxyl group to the L-center of m-A2pm. Depending on the species and growth conditions, a fraction of the peptides contains Gly instead of D-Ala at position 4 or 5. In murein sacculi isolated from E. coli, the fraction of pentapeptides is very low due to their rapid proteolytic degradation to tetrapeptides (L-Ala–D-iGlu–m-A2pm–D-Ala), tripeptides (L-Ala–D-iGlu–m-A2pm) and...
dipeptides (L-Ala–D-iGlu). Other species such as *Caulobacter crescentus* have a high percentage of pentapeptides in their murein, apparently due to the low activity of the murein-specific peptidases [7]. The chemical structure of the basic murein subunit, the disaccharide pentapeptide, is shared by nearly all Gram-negative bacteria, including a few rod-shaped Gram-positive species, for example *Bacillus subtilis* and *Listeria monocytogenes* [2]. In some species, the free carboxyl groups of D-Glu or m-A2pm (or both) are amidated. Several Gram-negative species, for example *Neisseria meningitidis* and *Helicobacter pylori*, contain a fraction of MurNAc residues carrying an additional acetyl group at C-6 [8,9] (Fig. 2B).

The murein lipoprotein (Braun’s lipoprotein, Lpp) is one of the most abundant proteins of *E. coli* and is anchored to the outer membrane by its N-terminal lipid residues. About one third of the Lpp molecules are covalently linked to the murein, firmly connecting the outer membrane to the murein layer and contributing to the stability of the cell envelope in Gram-negative bacteria. Lpp is attached to murein by a peptide bond between the ε-amino group of its C-terminal Lys residue and the L-carboxyl group of the m-A2pm residue in the murein peptide [10] (Fig. 2D).

An average *E. coli* cell has a total of about $3 \times 10^7$ disaccharide peptide subunits in its murein sacculus [11]. Depending on the strain and growth conditions, between 40 and 60% of the peptides in the murein of *E. coli* [5] and other Gram-negative bacteria [12] are part of cross-links such that a polymeric, net-like murein structure is formed. Most cross-links are of the DD-type and formed between the carboxyl group of D-Ala (position 4) of one peptide and the amino group at the D-center of m-A2pm (position 3) of another peptide (Fig. 2C). A smaller number of LD-cross-links exists between the L-center of m-A2pm of one peptide and the D-center of m-A2pm of another peptide. Next to dimeric peptide structures, there are lower fractions of trimeric and tetrameric structures, that contain three or four cross-linked peptides [5].

From the analysis of the composition of the murein sacculus it became clear that it is not homogenous. Murein is composed of more than 50 different subunits termed muropeptides (disaccharide peptide units) that differ in the length of the peptide chain (di-, tri-, tetra-, pentapeptide), the presence of either D-Ala or Gly at positions 4 or 5, the state of cross-linkage (monomer, dimer, trimer, tetramer), the type of cross-linkage (DD or LD), the presence of 1,6-anhydroMurNAc residues (from the glycan strand termini), and the presence of a L-Lys–L-Arg dipeptide (at position 4), which remains after proteolytic digestion of Braun’s lipoprotein [4]. The major muropeptides present in the murein of *E. coli* are the disaccharide tetrapeptide monomer (>30% of the total material) and the DD-cross-linked bis-disaccharide tetra-tetrapeptide dimer (>20% of the total material). The typical muropeptide composition of *E. coli* is summarized in Table 1, although the muropeptide composition of isolated *E. coli* sacculi shows some variation depending on the strain, the growth medium and temperature, and the growth phase. For example, the fraction of tripeptides and L-Lys–L-Arg containing peptides as well as the total cross-linkage is increased in sacculi from stationary cells, as compared to sacculi from exponentially growing cells. On the other hand, stationary cells have, on average, shorter glycan strands (Table 1). The changes in murein structure during transition from exponential to stationary phase might be the consequence of down-regulation of the large number...
Table 1
The muropeptide composition of E. coli KN 126 grown in LB-medium [5]

<table>
<thead>
<tr>
<th>Muropeptide*</th>
<th>Relative molar percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exponential growth phase</td>
</tr>
<tr>
<td>Di</td>
<td>1.22</td>
</tr>
<tr>
<td>Tri</td>
<td>8.66</td>
</tr>
<tr>
<td>free</td>
<td>80.6</td>
</tr>
<tr>
<td>in DD-cross-links</td>
<td>16.5</td>
</tr>
<tr>
<td>in LD-cross-links</td>
<td>2.9</td>
</tr>
<tr>
<td>Trilysarg</td>
<td>4.98</td>
</tr>
<tr>
<td>free</td>
<td>68.1</td>
</tr>
<tr>
<td>in DD-cross-links</td>
<td>26.6</td>
</tr>
<tr>
<td>in LD-cross-links</td>
<td>5.1</td>
</tr>
<tr>
<td>Tetra</td>
<td>55.91</td>
</tr>
<tr>
<td>free</td>
<td>66.1</td>
</tr>
<tr>
<td>in DD-cross-links</td>
<td>29.8</td>
</tr>
<tr>
<td>in LD-cross-links</td>
<td>4.1</td>
</tr>
<tr>
<td>Penta</td>
<td>0.51</td>
</tr>
<tr>
<td>free</td>
<td>16</td>
</tr>
<tr>
<td>in DD-cross-links</td>
<td>84</td>
</tr>
<tr>
<td>in LD-cross-links</td>
<td>4.1</td>
</tr>
<tr>
<td>Tetra Gly(4)</td>
<td>2.35</td>
</tr>
<tr>
<td>free</td>
<td>73.2</td>
</tr>
<tr>
<td>in DD-cross-links</td>
<td>24.3</td>
</tr>
<tr>
<td>in LD-cross-links</td>
<td>2.6</td>
</tr>
<tr>
<td>Penta Gly(5)</td>
<td>0.17</td>
</tr>
<tr>
<td>free</td>
<td>53</td>
</tr>
<tr>
<td>in DD-cross-links</td>
<td>47</td>
</tr>
<tr>
<td>Gly(5)</td>
<td>3.88</td>
</tr>
<tr>
<td>uncross-linked</td>
<td>34.3</td>
</tr>
<tr>
<td>in DD-cross-links</td>
<td>42.5</td>
</tr>
<tr>
<td>in LD-cross-links</td>
<td>23.2</td>
</tr>
<tr>
<td>Monomers</td>
<td>51.52</td>
</tr>
<tr>
<td>Dimers</td>
<td>43.27</td>
</tr>
<tr>
<td>Trimmers</td>
<td>5.02</td>
</tr>
<tr>
<td>Tetramers</td>
<td>0.19</td>
</tr>
<tr>
<td>Average glycan strand length (disaccharide units)</td>
<td>25.8</td>
</tr>
<tr>
<td>Degree of cross-linkagea</td>
<td>25.12</td>
</tr>
<tr>
<td>DD-cross-links</td>
<td>22.36</td>
</tr>
<tr>
<td>LD-cross-links</td>
<td>2.76</td>
</tr>
</tbody>
</table>

* Di, GlcNacMurNAc(dipeptide); Tri, GlcNacMurNAc(tripeptide); Tetra, GlcNacMurNAc(tetrapeptide); Penta, GlcNacMurNAc(pentapeptide); Gly(4)/Gly(5), glycine at position 4 or 5 of peptide side chain; Lysarg, Lys–Arg residue from lipoprotein.

b For calculation see reference [4].

of murein synthesizing and hydrolyzing enzymes (see below) during cessation of growth. In addition, the cell’s preparation for stationary phase might involve strengthening of the cell envelope by increasing the number of cross-links in the murein and the number of contacts between the murein layer and the outer membrane via Braun’s lipoprotein. Among Gram-negative bacteria, there is also some variability in the fine structure of the murein, in particular with respect to the abundance of the LD-cross-linkage and the amount of bound lipoprotein [12].

3. Biophysical properties of murein sacculi

Murein sacculi have unique biophysical properties. They can be isolated intact from cells and visualized by electron microscopy as empty cell envelopes with the same length and diameter as a bacterial cell. The typical length of a sacculus from E. coli is about 2–4 μm and its diameter is about 0.5–1 μm. Compared to these dimensions, the sacculi have a very small thickness of approximately 6 nm, as determined by cryo-transmission electron microscopy of frozen-hydrated cell sections and by atomic force microscopy on isolated sacculi [13,14]. Isolated sacculi have also been analyzed by small-angle neutron scattering showing that approximately 75–80% of the surface of the sacculi have a thickness of 2.5 nm, and the remaining 20–25% of the surface is maximally 7 nm thick [15].

The murein net is not rigid but it can reversibly expand and shrink three-fold without rupture [16]. Isolated sacculi are two- to threefold more deformable in the direction of the long axis of the cell than in the direction perpendicular to the long axis [14]. In the same study the elastic modulus of hydrated sacculi was determined as 2.5×10⁷ N/m². In living bacteria, the surface area of the murein is 45% larger than that in the same bacteria upon relaxation of the murein by a sudden destruction of the cytoplasmic membrane [17]. These data indicate that the murein forms an elastic net which is expanded to some extend in the living cell due to the cells turgor pressure.

There are pores with a mean radius of 2.06 nm in the relaxed sacculi, and it is estimated that globular proteins up to a molecular weight of 24 kDa should be able to diffuse through the murein net [18]. However, in the living cell, the expanded murein might even be permeable for proteins of up to 100 kDa [19]. Many proteins are transported across the periplasm (and hence, through the murein net) to become inserted into the outer membrane. For example, the outer membrane lipoproteins are transported across the periplasm in a soluble complex with the chaperone LolA [20], that apparently can diffuse through the murein layer. On the other hand, the assembly of larger complexes through the periplasm, like flagella, fimbria or complexes secreting proteins or DNA, seems to require the activity of specialized murein hydrolases to locally open the meshes of the murein net [21,22].

4. Models for the architecture of murein

Murein has a heterogeneous, flexible structure and is not crystalline, making it impossible to directly determine its structure at high resolution with the presently available techniques. Molecular modeling suggested that the glycan strands are rather rigid structures, whereas the peptides are flexible. In the favorable conformation, a peptide bends back towards the glycan strand [23]. Under stretched conditions, the peptides adopt a more straight conformation, allowing the murein net to become expanded [24,25]. The flexibility of the peptides could be the reason for the observed elasticity of murein. According to a model of murein architecture [23,26,27], the peptides protrude in a helical pattern from the glycan strands, with about four disaccharide units being required for one complete turn. Recently, the smallest possible glycan fragment carrying two peptides has been analyzed by NMR [28]. It was suggested that the angle between the peptides is about 120 degrees, which would imply that three peptides are required for one turn. Monolayered, net-like structures can be modeled by cross-linking...
peptides from adjacent glycan strands. Peptides pointing away from the murein layer (up or down) could not be present in cross-links which is in accordance with the observation of a large fraction — about 40–60% — of monomeric peptides being present in isolated sacculi.

The classical model predicts the glycan strands to be arranged in parallel to the cytoplasmic membrane and being cross-linked by the peptides to form a planar murein layer. According to Koch, the glycan strands are not straight but follow a zigzag line to form a layer with hexagonal 'tessera' as the smallest pores (Fig. 1) [29,30]. The measured thickness of the sacculi (see above) led to the conclusion that 75–80% of the surface is single layered, whereas the rest is triple layered. The anisotropy in elasticity observed in isolated sacculi might indicate that the flexible peptides run predominantly in the direction of the long axis of the rod-shaped E. coli cell, whereas the glycan strands run predominantly perpendicular to the long axis.

The concept of a layered murein net has recently been challenged. In the 'scaffold' models of murein architecture the glycan strands protrude vertically from the cytoplasmic membrane [28,31]. As it has been discussed in a recent review [32], the scaffold models are not in accordance with the observed average length of more than 20 disaccharide units of the glycan strands isolated from E. coli. The glycan strands of average length are too long for a vertical arrangement in the periplasm.

5. Murein synthesis

Murein biosynthesis takes place in two different cell compartments: the precursor lipid II is synthesized in the cytoplasm and then flipped across the cytoplasmic membrane. The reactions for the enlargement of the murein sacculus occur at the periplasmic site of the cytoplasmic membrane. An excellent overview about the murein precursor synthesis is given by van Heijenoort [33]. Key intermediates of precursor synthesis are the nucleotide activated amino sugars uridindiphosphate-N-acteyl-glucosamine (UDP-GlcNAc) and uridindiphosphate-N-acteyl-muramic acid (UDP-MurNAc). The peptide side chain is formed at the lactyl group of UDP-MurNAc by the successive addition of L-Ala, D-Glu, m-A 2pm and a D-Ala–D-Ala dipeptide catalyzed by ATP-dependent ligases MurC, MurD, MurE and MurF, respectively. The D-amino acids are synthesized by racemases from the respective L-amino acids.

To facilitate transport of the hydrophilic precursors across the cytoplasmic membrane, a C 55-polysisoprenoid carrier is attached by MraY to form lipid I, the undecaprenyl pyrophosphoryl-MurNAc-pentapeptide. Next, GlcNAc (from UDP-GlcNAc) is added to lipid I by MurG forming the final murein precursor lipid II. The flipase(s) responsible for the transport of lipid II from the cytoplasmic to the periplasmic leaflet of the cytoplasmic membrane is (are) not yet known.

The following sections give an overview on the properties of the murein synthases and hydrolases from E. coli and on the recent data on the activities and interactions of the synthases. In the next sections we then present current models for the enlargement of the sacculus and for the spatial regulation of murein synthesis.

6. The E. coli murein synthases

Murein synthases catalyze the enlargement of the sacculus by incorporation of lipid II precursor. These proteins are present in 120 to 220 copies per cell [34] and are either monofunctional transglycosylases, bifunctional transglycosylases/transpeptidases or monofunctional transpeptidases (Table 2). All murein synthases are anchored to the cytoplasmic membrane by a single transmembrane region close to their N-terminals. They have a short N-terminal cytoplasmic part while the catalytic transglycosylation (TG) and transpeptidation (TP) domains are located in the periplasm (Fig. 3A). Murein synthases containing a TP activity are known as Penicillin-binding proteins (PBPs) because of their ability to covalently bind Penicillin and other β-lactams, which have structural similarity with the D-Ala–D-Ala termini of the pentapeptide side chains. Depending on the properties of the non-Penicillin-binding (nPB) motifs they are divided into two classes. Class A PBPs like PBP1A, PBP1B and PBP1C of E. coli are bifunctional enzymes harboring a TG domain as nPB module and a TP domain as Penicillin-binding domain.

### Table 2

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW [Da]</th>
<th>Gene</th>
<th>Domains (region and active site residue)</th>
<th>Intracellular localization</th>
<th>Copy number per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBP1A</td>
<td>94,519</td>
<td>pomA</td>
<td>TG: L65 to A266 (E94) TP: Q429 to G823 (S473)</td>
<td>Cytoplasmic membrane</td>
<td>221 ± 20</td>
</tr>
<tr>
<td>PBP1B α</td>
<td>94,292</td>
<td>(mrcA)</td>
<td>TG: L65 to A266 (E94) TP: Q429 to G823 (S473)</td>
<td>Cytoplasmic membrane</td>
<td>127 ± 13</td>
</tr>
<tr>
<td>PBP1B β</td>
<td>91,593</td>
<td>pomB</td>
<td>TG: D198 to 1385-S409 (E233) TP: Q477 to M780 (S510)</td>
<td>Lateral wall and septation site</td>
<td>120 ± 14</td>
</tr>
<tr>
<td>PBP1B γ</td>
<td>88,889</td>
<td>(mrcB)</td>
<td>TG: R64 to 1245-M263 (E94) TP: Q283 to G599 (S342)</td>
<td>Cytoplasmic membrane</td>
<td>132 ± 17</td>
</tr>
<tr>
<td>PBP1C</td>
<td>86,067</td>
<td>pBPc</td>
<td>TG: R64 to 1245-M263 (E94) TP: Q283 to G599 (S342)</td>
<td>Cytoplasmic membrane</td>
<td>ND</td>
</tr>
<tr>
<td>PBP2</td>
<td>70,857</td>
<td>pBPb</td>
<td>nPB: R68 to L254 TP: D255 to N627 (S330)</td>
<td>Cytoplasmic membrane</td>
<td>120 ± 14</td>
</tr>
<tr>
<td>PBP3</td>
<td>63,877</td>
<td>ftsI</td>
<td>nPB: R71 to I236 TP: D237 to V577 (S307)</td>
<td>Cytoplasmic membrane</td>
<td>132 ± 17</td>
</tr>
<tr>
<td>MtgA</td>
<td>27,342</td>
<td>mtgA</td>
<td>TG: V67 to D242 (E84)</td>
<td>Cytoplasmic membrane</td>
<td>ND</td>
</tr>
</tbody>
</table>

* See text for references.

b Active site residues: glutamate in TG domain and serine in TP domain.

* Not determined.
Both modules contain distinct motifs of highly conserved amino acids (Figure 3B). Class A PBPs contain 5 motifs (motif 1 to 5) within the nPB-module and are connected to the PB-module via a linker motif (motif A). The PB-module contains a further conserved motif B and the 3 distinct motifs of the acyltransferases: motif I \([S^*X_2K]\), harboring the active site serine (S*) residue, motif II \([(S/Y)X(N/C)]\) and motif III \([(K/H)(T/S)G]\) (X, any amino acid). Class B PBPs have only 3 conserved motifs within the nPB-module (motif 1′ to 3′), a linker-motif A′ between the two modules and a PB-module with motif B′ and the typical acyltransferase motifs I, II and III [35,36]. It has been proposed that the nPB domain plays a role in the assembly of class B PBPs in multi-protein complexes [37,38].

In *E. coli* the class A PBP1A and PBP1B are considered to be the enzymes with highest murein synthesis activity in the cell. Both enzymes are not required for cell growth, but a double deletion of the corresponding genes is lethal [39,40]. There is evidence that both proteins might play distinct roles during cell growth and division. Mutants lacking PBP1B are more sensitive to β-lactam antibiotics than mutants without PBP1A [41]. Furthermore, cell integrity is lost upon inactivation of PBP2, PBP3, or the cell division protein FtsQ, in mutants without PBP1B, but not in mutants without PBP1A [42]. PBP1A and PBP1B form homo- and heterodimers in vivo, further indicating that both proteins may have distinct cellular functions [43,44]. *E. coli* has a third class A protein, PBP1C, which cannot substitute for a double loss of function of PBP1A and PBP1B [45].

The monofunctional transpeptidases PBP2 and PBP3 belong to the class B PBPs and appear to have specific functions during the cell cycle. PBP2 is required for cell elongation while PBP3 localizes to mid-cell during septation and is required for cell division [46]. There is also a monofunctional transglycosylase

Fig. 3. Schematic representation (A) and sequence motifs (B) of the murein synthases from *E. coli*. 
(glycosyl transferase), MtgA, which shows sequence similarity to the nPB-module of the class A PBPs sharing the typical motifs 1 to 5.

So far only the C-terminal boundary of the TG-domain of PBP1B has been experimentally determined by activity assays using truncated forms of the protein [47]. The other boundaries of the TG and TP domains of the E. coli murein synthases were estimated by sequence comparisons (at http://genolist.pasteur.fr/Colibri/) with either PBP1B or PBP3, for which the boundaries had been suggested [48,49] (Table 2).

6.1. The Penicillin-binding protein 1A

The bifunctional transglycosylase/transpeptidase PBP1A [50] is encoded by the ponA (mrcA) gene [51]. Deletion of ponA has no significant effect on cell growth, morphology or on the sensitivity of the cell towards β-lactam antibiotics [41]. On the other hand, deletion of ponB (encoding for PBP1B) leads to a higher sensitivity of the cells to β-lactam antibiotics. This is consistent with the observations that PBP1A has a higher affinity to most β-lactams as compared to PBP1B, and that the inactivation of both, PBP1A and PBP1B is lethal for the cell [40,52]. PBP1A contains a transmembrane anchor, presumably from F15 to I36, and the typical TG- and TP-domains for class A PBPs [36]. Sequence comparison to PBP1B implies that the TG-domain reaches from about L65 to A266 followed by a typical linker region and the TP-domain from about Q429 to G823. The catalytically active residues in both domains have been shown to be E94 in motif 1 of the TG-domain and S473 in motif I of the TP-domain [53].

6.2. The Penicillin-binding protein 1B

The bifunctional PBP1B is encoded by the ponB (mrcB) gene and exists in three isoforms (α, β, and γ) which differ only in the length of their short cytoplasmic part and which can be separated by SDS-gel electrophoresis. The start codon of the γ-isoform is identical to the codon for methionine at position 46 of the α-isoform [51,54,55]. The β-isoform appears to be generated by an artificial cleavage of PBP1Bα after the N-terminal 25 amino acids by the outer membrane protease OmpT [56,57]. It is not clear whether PBP1Bα and PBP1Bγ have different functions although it was suggested that PBP1Bγ could be involved in murein synthesis during cell division while PBP1Bγ synthesizes new material for elongation of the murein sacculus [58].

The major part of PBP1B is located in the periplasm. Directly after the transmembrane region (G64 to D88) there is a region of about 100 amino acids not present in PBP1A and responsible for the correct folding of the protein [49]. The TG domain starts at D198 and contains the five conserved motifs characteristic for class A PBPs [35]. Homology searching has identified conserved motifs, which suggest the catalytic residues E233, D234, and E290 are involved in the catalysis. The exchange of E233 by Q resulted in the complete loss of enzymatic activity. Replacement of D234 by N and E290 by Q lead to a reduction in activity. A protein, in which Y310 was substituted by F was degraded immediately and could therefore not be purified [49].

The C-terminal border of the TG domain has been located between I385 and S409 [47]. The TP domain lies in the C-terminal part of the protein (Q447 to M780) and shows three typical motifs of the penicillinyl-serine-transferase superfamily. S510 is part of the catalytical triade and directly involved in the enzymatic reaction. The C-terminal part from R781 to N844 is dispensable for the activity of PBP1B [54,59,60].

6.3. The Penicillin-binding protein 1C

The non-essential PBP1C is encoded by the pBP1C gene [45]. PBP1C cannot support cell growth in the absence of PBP1A and PBP1B and could have a specialized cellular function. The calculated mass of PBP1C of 85,067 kDa differs significantly from the apparent molecular weight of about 70 kDa observed by SDS-gel electrophoresis. PBP1C contains a transmembrane region (presumably G9 to D29). We predict that the transglycosylase domain of PBP1C ranges from R64 to the region between I245 and M263. Despite the absence of three highly conserved amino acid residues (R136 and E140 of motif 3, and R218 of motif 5) in the TG domain, PBP1C is capable of binding the inhibitor moenomycin and shows transglycosylation activity. We predict that the transpeptidase domain starts with Q281 and ends with G598, and that it contains the three typical conserved motifs of class A PBPs, with S342 as the active site serine. Nevertheless, as compared to other PBPs, PBP1C has significantly different affinities to β-lactam antibiotics and binds virtually only the oxacephalosporin latamoxef and a Bolton/Hunter derivative of ampicillin [61,62].

6.4. The monofunctional glycosyltransferase MtgA (Mgt)

MtgA is capable of glycan strand polymerization but not of cross-linking the peptides [63]. The enzyme has a molecular weight of 27,342 kDa and is similar in sequence to the TG domain of the class A HMW PBPs. The mtgA gene could be deleted in several species like E. coli [45], Haemophilus influenzae [64], and Brucella abortus [65] without severe growth defects under laboratory conditions. On the other hand, the mtgA-deletion strain of B. abortus was less virulent in a mouse infection model [65]. MtgA contains a predicted transmembrane region from L19 to V39. It was suggested that the TG-domain reaches from V67 to the end of the protein (D242) [66]. Experimental data obtained with Staphylococcus aureus MtgA showed that the strictly conserved residue E100 (corresponding to E84 in MtgA from E. coli) is essential for catalytic activity.

6.5. The Penicillin-binding protein 2

The monofunctional transpeptidase PBP2, encoded by the pBP2 gene, is essential for cell elongation and maintenance of the rod shape of E. coli [46,67,68]. From sequence comparisons of PBP2 with PBP3 we predict that the nPB-domain covers the region from R68 to L254 and the transpeptidase domain begins with D255 and ends with N617. Both domains contain the
typical motifs of class B PBPs. The \textit{pbpA} gene is located in the same operon as the \textit{rodA} gene encoding for an integral membrane protein which is (like PBP2) required for the enlargement of the side wall of the sacculus [69,70]. Inhibition of PBP2 with mecillinam [67] results in spherical cells with constitutive septal physiology of the maturation has remained unclear.

7. Studies on the murein synthesis reactions

7.1. Transglycosylation and transpeptidation reactions

The transglycosylation step in murein synthesis produces the glycan strands from lipid II substrate. It has been suggested that the growing glycan strand acts as donor during TG reaction such that the glycosidic bond is formed between C1 of MurNAc of the growing chain and C4 of the GlcNAc residue of the next lipid II [33,88]. This is supported by two recent publications on the first known crystal structures of murein transglycosylases, PBP2 from \textit{S. aureus} [89] and PBP1A from \textit{Aquifex aeolicus} [90]. Interestingly, both structures do not resemble structures of other known glycosyl transferases but have striking similarities with bacteriophage \(\lambda\)-lysozyme. Because transglycosylases utilize a membrane-bound substrate (lipid II) it is not unexpected that the structures show hydrophobic surface sites for the interaction with the membrane. PBP2 from \textit{S. aureus} was crystallized with and without the TG-specific inhibitor moenomycin. Lipid II and the growing glycan chain were modeled into the structure of PBP2. The lipid II acceptor fits between the glycan chain (donor) and the catalytic residue E114. A glycan chain of more than two disaccharide units (not counting the disaccharide of lipid II) can protrude out of the TG domain without any steric hindrance [89]. The TG-domain of PBP1A of \textit{A. aeolicus} contains an active site cleft, which can be covered by a flap. It was possible to model a growing glycan chain into the cleft with the reacting end anchored behind the flap and the lipid chain extending past the hydrophobic patch and into the membrane. The acceptor lipid II is suggested to bind in the more open site of the cleft with the GlcNAc moiety directed to the MurNAc of the donor. The flap prevents the glycan product from dissociation and the elongated glycan strand shifts within the cleft so that the new reducing end is positioned in the active site again [90]. \textit{E. coli} PBP1B strongly prefers acceptor molecules with a lipid moiety while the donor substrate requires both GlcNAc and MurNAc but no lipid residue. This indicates that the mechanism of glycan chain elongation by \textit{E. coli} PBP1B is the same as for \textit{S. aureus} PBP2 and \textit{A. aeolicus} PBP1A [91]. An overview about the transglycosylation step and its inhibition by antibiotics is given in a recent review by Welzel [92].

Cross-linking of the peptides occurs by transpeptidation between the D-Ala at position 4 of the donor peptide and the m-A2pm residue at position 3 of the acceptor peptide. The energy for this reaction is gained from the cleavage of the D-Ala–D-Ala bond of the pentapeptide of the donor peptide. The active site serine of motif I attacks the peptide bond between the two terminal D-alanines of the donor to form an intermediary acyl-enzyme complex which is cleaved by the \(\epsilon\)-NH2-group of the m-A2pm residue of the acceptor peptide to release the enzyme and form the cross-link [49]. The crystal structure of the TP domain of the class A PBP1B of \textit{S. pneumoniae} has been solved
together with the linker region and a short peptide of the TG domain [93]. Interestingly, in the absence of an antibiotic the enzyme assumes a closed conformation with a blocked active site. The presence of substrate results in an opening of the catalytic gorge with an elongated binding cleft.

7.2. In vitro murein synthesis studies

To study murein synthesis in vitro turned out to be a challenging task for several reasons. Firstly, the enzymes are membrane-bound, of low cellular abundances and some of them are intrinsically unstable. Secondly, the substrate lipid II has long been a limiting factor until recently a new method was developed to produce larger amounts [94]. And thirdly, the analysis of the synthesized murein requires particular analytical techniques. Murein synthesis studies have been performed in vitro since the late 1960s, either with isolated cell membranes [52,69,95–98], ether-permeabilized cells [99], or proteins which had been purified under denaturing conditions followed by renaturation [100–102]. Later the proteins were specifically produced with a tag and purified by affinity chromatography [49]. As substrate, either UDP-MurNAc-pentapeptide and UDP-GlcNAc were added to the membrane preparations [52,95–99,102–104], or lipid II was added to the purified proteins [49,100,101]. Reaction products were analyzed by paper chromatography.

We have recently developed a simple in vitro murein synthesis assay based on the detection and analysis of the reaction products formed from radioactively labeled lipid II by high-pressure liquid chromatography (HPLC) [105]. Interestingly, the activities of PBP1B, and in particular of the TP reaction, are highest at conditions favoring dimerisation of the enzyme (see below). Further, time-course experiments showed that in the presence of lipid II both TP and TG reactions occur simultaneously, suggesting that a dimer of PBP1B may produce two glycan strands and simultaneously cross-link the peptides between them (Fig. 4). Also, when the available lipid II was consumed, peptide cross-linking occurred at a rate 12-fold lower than the initial cross-linking rate. At optimum reaction conditions, PBP1B is capable of converting lipid II in vitro to murein with an average length of the glycan strands of about 25 disaccharide units, with almost 50% of the peptides being part of cross-links. PBP1B not only produces dimeric, but to a lower extent trimeric peptide structures, which result from a cross-linking reaction between a dimeric and a monomeric peptide. Furthermore, PBP1B is able to use artificial tri-, tetra-, and pentapeptide compounds (present as UDP-linked precursors) as acceptors for the TP reaction. Such reactions were observed before in ether-treated cells and cell membrane preparations of E. coli [99,104]. Kinetic studies of the TG-activity of the full-length PBP1B protein [49] as well as of two truncated forms L433b and S409 have shown that the kinetic efficiency ($k_{cat}/K_m$) of the full-length protein is 4 to 5 fold higher than the one of the truncated forms indicating that the TP domain may influence the turnover rate of the TG-domain [47].

PBP1A with lipid II as a substrate produces in vitro murein with an average length of 20 disaccharide units with about 20% of peptides being part of cross-links [53]. PBP1A has poor TP activity in the absence of transglycosylation (i.e. after lipid II substrate is consumed). The TP activity of PBP1A with lipid II is also greatly reduced in a variant with inactive TG domain. Furthermore, TP reactions are not detected when two PBP1A variants, one with inactive TP domain and the other with inactive TG domain were mixed. Thus PBP1A requires ongoing transglycosylation reactions in the same molecule for the

Fig. 4. Murein synthesis reactions catalyzed by PBP1B in vitro. PBP1B elongates the glycan chains by transglycosylation (TG) and forms cross-links by transpeptidation (TP). Grey bars, MurNAc; white bars, GlcNAc; zigzag line, undecaprenyl residue; black circle, phosphate group.
expressing of its full cross-linking activity. Such an effect has been observed before for other class A PBPs and points to a probable intra-molecular activation mechanism involving inter-actions between the TG and TP domains [49,53,101,105]. Time-course experiments revealed that PBP1A-catalyzed transpeptidation requires the presence of already polymerized glycan strands (transglycosylation product), indicating that PBP1A might prefer high-molecular weight murein as acceptor for cross-linking reactions (Fig. 5). Interestingly, in vitro PBP1A is able to attach a fraction of the new murein synthesized from radioactive lipid II to isolated murein sacculi by transpeptidation reactions. Attachment of new material to the existing sacculus occurs in the growing cell but had not been demonstrated in vitro before.

Two other synthases from *E. coli* have been reported to have transglycosylase activity in vitro. The isolated MtgA produced from lipid II glycan strands carrying un-cross-linked pentapeptides in vitro [63]. Early reports identified both TG and TP activities of PBP3 from *E. coli* [79], this was later shown to be a monofunctional transpeptidase [83]. It is possible that the PBP3 preparation used in the early study contained contaminating PBP1B, because both proteins were shown to interact with each other (see below). The in vitro activities of PBP2, PBP3 and PBP1C need to be characterized in future.

8. The *E. coli* murein hydrolases

Murein hydrolases are enzymes that cleave covalent bonds in murein or murein fragments [106]. Autolysins are, by virtue of their enzymatic specificities and their periplasmic localization, capable to digest the murein sacculus to small, soluble fragments (Fig. 6). Muramidases, e.g. lysozyme, hydrolyze the β1,4-bond between MurNAc and GlcNAc in the murein glycan strands. Unlike lysozyme, lytic transglycosylases are muramidases which cleave the glycosidic linkage with the concomitant formation of a 1,6-anhydro bond at the MurNAc residue [3] (Fig. 6B). *E. coli* has six known lytic transglycosylases, with five being lipoproteins residing in the outer membrane (MltA, MltB, MltC, MltD, and EmtA) and facing into the periplasm, and one being soluble (Slt70) (Table 3). EmtA is an endo-specific lytic transglycosylase, whereas Slt70, MltA and MltB are exo-enzymes that remove disaccharide units presumably from the 1,6-anhydroMurNAc glycan strand end. Other autolysins cleave either the bond between the glycan strands and the peptides (the N-acetylmuramyl-L-alanine amidases AmiA, AmiB and AmiC) or hydrolyze peptide bridges (the DD- and/or LD-endopeptidases MepA, PBP4 and PBP7). Obviously, the activities of these autolysins can destroy the integrity of the sacculus, and they must be strictly controlled in the cell to avoid autolysis. Table 3

![Fig. 5. Murein synthesis reactions catalyzed by PBP1A in vitro. (A) PBP1A initially forms an uncross-linked product with lipid II substrate. Cross-links are formed only later, presumably with glycan strands carrying monomeric peptides as acceptors (scheme II). (B) PBP1A is capable of attaching newly formed oligomeric murein to isolated sacculi in vitro by transpeptidation reactions. Grey bars, MurNAc; white bars, GlcNAc; zigzag line, undecaprenyl residue; black circle, phosphate group.](image-url)
lists the known murein hydrolases of *E. coli* and refers to the available structures [107–114].

The periplasmic murein hydrolases are quite active during growth and division of the cell. In one generation, an *E. coli* cell loses 40 to 50% of its total murein by the activity of hydrolases [115,116]. This is a surprisingly high turnover rate, considering the mainly single-layered architecture of murein and the need to constantly maintain the integrity of the sacculus. The chemical structures of the turnover products indicate that all autolytic activities – lytic transglycosylases, *N*-acetylmuramyl-L-alanine amidases and endopeptidases – participate in murein turnover. This is particularly true for the cleavage of the division septum where the amidases and also the lytic transglycosylases and the endopeptidase PBP4 are required to allow cell separation [117–119]. For example, a mutant lacking three amidases and the soluble lytic transglycosylase Slt70 grows in chains of up to 40 non-separated cells. Also it has been shown that the amidase AmiC localizes at the site of septation, whereas AmiA is dispersed throughout the periplasm [120]. Analysis of these chaining mutants lacking murein hydrolases has also shown that the permeability of the outer membrane is increased for unknown reasons [118,121].

Only a minor fraction of the murein fragments released by the hydrolases is lost into the growth medium because *E. coli* has an efficient uptake and recycling system for murein turnover products. Uptake from the periplasm into the cytoplasm occurs via the unspecific oligopeptide permease Opp and the murein peptide permease Mpp. Once taken up, different cytoplasmic enzymes hydrolyze the murein fragments to compounds that can re-enter the biosynthetic pathway of murein precursors [122]. Interestingly, some Gram-negative bacteria, e.g. *Citrobacter freundii* and *Enterobacter cloacae*, elegantly couple murein turnover with signaling for the production of chromosomally encoded β-lactamase, an enzyme that is secreted to degrade exogenous β-lactam antibiotics [123]. The inhibition of murein synthesis by β-lactams causes an uncontrolled activity of autolytic enzymes which is characterized by a sudden increase in murein turnover products that are taken up into the cytoplasm. A transcriptional activator, AmpR, is inactivated by the binding of murein precursor UDP-MurNAc pentapeptide,
but becomes activated by binding a turnover product 1,6-anhydroMurNAc-tripeptide, leading to the expression of AmpC β-lactamase. Mutants without lytic transglycosylases (that produce the 1,6-anhydroMurNAc compounds) or without the permeases required for the uptake of turnover products, fail to induce β-lactamase and lyse in the presence of exogenous β-lactam antibiotic [121].

Carboxypeptidases are capable of removing the terminal amino acid residues from the peptides in the murein. The function of these enzymes is not yet clear since there are species, e.g. C. crescentus, which appear to lack these activities [7]. On the other hand, the DD-carboxypeptidase PBP5 has been implicated in a regulatory function in murein synthesis in E. coli because mutants lacking PBP5 show remarkable morphological defects, including kinks, bends and even branches [124]. These effects are exacerbated if additional PBPs are deleted, but the normal rod-shaped cell morphology can always be restored by the production of PBP5 from a plasmid-born dacA gene in these multiple mutants [125]. The branches can arise either from de novo generation of poles on the side wall or from splitting of an existing pole by areas of active murein synthesis [126]. The tip of the deformed regions behave like misplaced, fully functional cell poles [127]. The combination of dacA deletion with certain fisZ (ts) mutations results – at restrictive temperature – in E. coli cells with a helical cell shape [128]. The molecular mechanism(s) by which PBP5 contributes to maintenance of rod-shape and

![Diagram](image_url)
the reasons why the other DD-carboxypeptidases cannot restore rod-shape in the absence of PBP5 are not known.

9. Interactions between murein synthases and hydrolases

Because of its network-like structure the enlargement of the sacculus requires not only synthetic reactions, but also hydrolysis of covalent bonds within the sacculus to allow incorporation of subunits into the stress-bearing layer. It has been proposed by Höltje that synthetic and hydrolytic reactions need to be coordinated temporarily and spatially for a safe enlargement of the sacculus. He suggested that this is achieved by the formation of multi-enzyme complexes combining different murein synthases and hydrolases thus ensuring that the hydrolases are active only at sites of new synthesis

Table 3
Murein hydrolases of *E. coli*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specificity a</th>
<th>MW b [Da]</th>
<th>Gene</th>
<th>Intracellular localization</th>
<th>Function/remarks</th>
<th>Reference for structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slt70</td>
<td>LT</td>
<td>73,353</td>
<td>sltY</td>
<td>Periplasm</td>
<td></td>
<td>[112]</td>
</tr>
<tr>
<td>MltA</td>
<td>LT</td>
<td>40,411</td>
<td>mltA</td>
<td>Outer membrane</td>
<td>Lipoprotein</td>
<td>[114]</td>
</tr>
<tr>
<td>MltB</td>
<td>LT</td>
<td>40,256</td>
<td>mltB</td>
<td>Outer membrane</td>
<td>Lipoprotein, degradation to Slt35</td>
<td>[113]</td>
</tr>
<tr>
<td>MltC</td>
<td>LT</td>
<td>40,113</td>
<td>mltC</td>
<td>Outer membrane</td>
<td>Lipoprotein</td>
<td>[107]</td>
</tr>
<tr>
<td>MltD</td>
<td>LT</td>
<td>49,417</td>
<td>mltD</td>
<td>Outer membrane</td>
<td>Lipoprotein</td>
<td></td>
</tr>
<tr>
<td>EmIA</td>
<td>LT</td>
<td>26,575</td>
<td>emIA (mltE)</td>
<td>Outer membrane</td>
<td>Lipoprotein</td>
<td>[107]</td>
</tr>
<tr>
<td>AmiA</td>
<td>Ami</td>
<td>31,412</td>
<td>amiA</td>
<td>Periplasm</td>
<td>Cell separation</td>
<td></td>
</tr>
<tr>
<td>AmiB</td>
<td>Ami</td>
<td>47,985</td>
<td>amiB</td>
<td>Periplasm</td>
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<tr>
<td>AmiC</td>
<td>Ami</td>
<td>45,634</td>
<td>amiC</td>
<td>Periplasm, septation site</td>
<td>Cell separation</td>
<td></td>
</tr>
<tr>
<td>AmpD</td>
<td>1,6Ami</td>
<td>20,536</td>
<td>ampD</td>
<td>Cytoplasm</td>
<td>Recycling</td>
<td>[109]</td>
</tr>
<tr>
<td>PBP4</td>
<td>DD-EP/CP</td>
<td>51,798</td>
<td>ddcB</td>
<td>Periplasm, membrane d</td>
<td></td>
<td>[106]</td>
</tr>
<tr>
<td>PBP7</td>
<td>DD-EP</td>
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<td>pbgC</td>
<td>Periplasm, membrane e</td>
<td>Proteolytic cleavage to PBP8</td>
<td>[110]</td>
</tr>
<tr>
<td>MepA</td>
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<td>mepA</td>
<td>Periplasm</td>
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<td></td>
</tr>
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<td>NagZ</td>
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<td>nagZ (ycfO)</td>
<td>Cytoplasm</td>
<td>Recycling</td>
<td>[110]</td>
</tr>
<tr>
<td>PBP5</td>
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<td>ddcA</td>
<td>Inner membrane</td>
<td>Cell shape maintenance</td>
<td>[111]</td>
</tr>
<tr>
<td>PBP6</td>
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<td>43,609</td>
<td>ddcC</td>
<td>Inner membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBP6B</td>
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<td>43,346</td>
<td>ddcD</td>
<td>Inner membrane</td>
<td></td>
<td></td>
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<tr>
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<td>ldcA (ycgQ)</td>
<td>Cytoplasm</td>
<td>Recycling, essential in stationary phase</td>
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</tr>
<tr>
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<td>envC (yibP)</td>
<td>Septation site</td>
<td>Cell separation</td>
<td></td>
</tr>
<tr>
<td>FlgJ</td>
<td>Unknown f</td>
<td>34,475</td>
<td>flgJ</td>
<td>Flagellum</td>
<td>Flagellum synthesis</td>
<td></td>
</tr>
</tbody>
</table>
PBP1A was also shown to form dimers, but there are no heterodimers formed by PBP1A and PBP1B [43].

A number of affinity chromatography experiments with immobilized murein enzymes led to the identification of several direct and/or indirect interactions. Immobilized Slt70 retains PBP1B, PBP1C, PBP2, PBP3 as well as the hydrolase PBP7/8 from crude membrane extract, whereas immobilized MltB specifically binds PBP1B, PBP1C and PBP3 [135,136]. Immobilized MltA retains the murein synthases PBP1B, PBP1C, PBP2, and PBP3 as well as non-PBP proteins [137]. One of these non-PBP-proteins was identified as a structural protein mediating the interaction between MltA and PBP1B, and was therefore named MipA (MltA interacting protein A). A trimeric protein complex containing PBP1B, MipA and MltA could be reconstituted in vitro on a sensor chip for surface plasmon resonance (BIACore). If the membrane fraction was supplemented with periplasmic proteins, PBP1A is retained on the MltA-column, indicating that the binding of PBP1A to MltA is mediated by a so far unknown periplasmic factor. In other work it was shown that immobilized PBP1C specifically retains MltA as well as the murein synthases PBP1B, PBP2 and PBP3 [45].

The interaction between PBP1B and PBP3 was demonstrated by different in vivo and in vitro methods [138]. Firstly, immobilized PBP3 retains PBP1B from crude membrane extract in an affinity chromatography experiment. A direct interaction between the purified enzymes was then shown by surface plasmon resonance using a sensor chip with immobilized PBP3. This interaction was shown to exist in vivo by the bacterial two-hybrid system, which, in addition, revealed, that the first 56 amino acids of PBP3 are sufficient for the interaction with PBP1B. PBP1B and PBP3 can be cross-linked in vivo and co-immunoprecipitated with either an anti-PBP1B or an anti-PBP3 antibody. The interaction between PBP1B and PBP3 is independent of the activity or the correct localization of PBP3. The latter suggests that both proteins interact with each other before PBP3 gets recruited to the cell division site. Consistent with these results is the finding that only a proportion of PBP1B localizes at mid-cell and that this is dependent on the presence but not the activity of PBP3. The dissociation constants of the interactions between murein synthases and hydrolases determined by surface plasmon resonance are given in Table 4.

Interactions between class A and class B PBPs were reported for other bacteria. For example, β-lactam resistance of S. aureus is mediated by a functional interaction between the TG activity of PBP2 (class A) and the TP activity of PBP2A (class B) [139]. Only in the presence of PBP2A a β-lactam-inactivated PBP2 is able to localize correctly at the cell division site [140]. In B. subtilis, the septal localization of PBP1 (class A) depends on the presence of PBP2b (class B) [141]. In Enterococcus faecalis, β-lactam resistance is mediated by PBP5 (class B) only if PBP6 (class A) is active [142]. Cross-linking of proteins in H. influenzae cells revealed the existence of two different high-molecular-weight complexes containing class A and class B PBPs [143]. And finally, in C. crescentus several PBPs, probably PBP1a, PBP2a, PBP2b and PBP3a, co-immunoprecipitated with PBP2 [144].

### 10. Interactions of murein synthases with cell cycle proteins

PBP3 had a different affinity to cephalaxin when labeled either in cells with or without ongoing cell division, and it was concluded that the transpeptidase function of PBP3 was activated in dividing cells by allosteric interactions or substrate binding [145]. PBP3 and PBP1B are likely part of a murein synthesis complex active at the cell division site and interact with other proteins of the divisome [146]. FtsZ is the bacterial homolog of tubulin and forms at mid-cell a ring-like, contractile structure to which more than 12 other cell division proteins assemble. The mature divisome is assembled in two steps [147], with the cell division proteins FtsZ, FtsA, ZipA, FtsE, FtsX, and FtsK localizing early in the cell cycle at mid-cell. Later, FtsQ, FtsL, FtsB, FtsW, PBP3 (presumably together with PBP1B) and FtsN are recruited to form the mature divisome. The integral membrane protein FtsW is required for septal localization of PBP3 [148], and a periplasmic loop between transmembrane helices 9 and 10 in FtsW was found to be essential for recruitment of PBP3 [149]. PBP3 itself was found to be required for proper localization of FtsN [150], an essential cell division protein which harbors a periplasmic murein-binding domain of unknown function [151,152]. Indeed, the use of the bacterial two hybrid system indicated interactions of PBP3 with FtsW and FtsN, in addition to further interactions with itself (dimerization), FtsA, FtsK, FtsL, FtsQ and YmgF [153,154]. Bacterial two-hybrid experiments with truncated forms of PBP3 showed different interaction sites for various proteins. The interaction between PBP3 and FtsQ is mediated by amino acids 19 to 51 in PBP3 [154] and amino acids 50 to 135 in FtsQ [155]. For the interaction with FtsL the N-terminal part of PBP3 extending up to amino acid 250 is required, while the interaction with FtsW requires only amino acids 1 to 70 [154]. Several amino acids of the nPB domain (G57, S61, L62, and R210) of PBP3 are involved in the interaction with other proteins, possibly FtsN. Residues V86, R141, and G188 in PBP3 are important for recruitment of FtsN to the septation site [86]. These results show that PBP3 interacts with several components of the divisome.

Cell elongation of most rod-shaped species requires the presence of the actin-like MreB which forms helical filaments coiling around the length of the cell underneath the cytoplasmic membrane [156]. Cells without MreB, or cells in which the formation of the MreB spiral is blocked by the A22 inhibitor lose their rod-shape and eventually lyse, similar to mutants lacking PBP2. A fluorescent derivative of vancomycin, presumably labeling murein precursor molecules and newly synthesized
murein, localizes on a helical path on the surface of *B. subtilis* cells, dependent on the presence of the helically arranged filaments of the MreB-homolog Mbl. Therefore, it was suggested that the intracellular cytoskeleton elements may direct the murein synthesis to maintain rod-shape during growth [157]. Another study used fluorescent ramoplanin or vancomycin to label *B. subtilis* cells showing similar helical patterns which, however, did not depend on the presence of Mbl [158]. The reason for these conflicting results are unclear. The membrane proteins MreC and MreD might couple the cytoplasmic MreB filament with the periplasmic murein syntheses [159]. In *E. coli*, MreB forms a membrane-bound complex with MreC and MreD, and the formation of intracellular MreB-filaments depends on the presence of MreC, MreD and RodA [160]. MreC has a membrane-anchor and a major periplasmic domain and was shown to form homodimers [161]. In *C. crescentus*, MreB and MreC form helices which do not co-localize, and the localization of MreC is independent of MreB, indicating that in this species there might not be a direct interaction between both proteins [162]. In an affinity chromatography experiment, immobilized MreC from *C. crescentus* was found to retain all murein synthases (PBP1A, PBP1B, PBP2A, PBP2B and PBP2) from a membrane fraction [163], and similarly, bacterial two hybrid system shows interactions between MreC from *B. subtilis* and all high-molecular weight PBPs of this bacterium [161].

### 11. Models for the growth of the murein sacculus in Gram-negative bacteria

Several models for the growth of the sacculus during elongation and division were proposed. According to most of these models the enlargement of the sacculus is achieved by the insertion of new glycan strands into the existing murein layer, and the synthesis of new murein and hydrolysis of bonds, are combined. The authors of the scaffold model (with a vertical arrangement of the glycan strands relative to the membrane) have presented a growth model which does not include any enlargement of the surface of the murein [164]. For the safe enlargement of the stress-bearing murein layer, Koch proposed, that first new covalent bonds have to be formed before hydrolysis of other bonds in the murein net takes place ('make-before-break' strategy) [30].

In the growth model of Burman and Park, local hydrolysis of cross-links by murein hydrolases precedes the incorporation of a newly synthesized glycan strand that becomes cross-linked to the neighboring strands in the existing murein layer [165,166]. Initially, cross-links are formed only between old and new material, but after a while, new glycan strands are inserted next to strands that were incorporated briefly before, resulting in cross-links between two new peptides. This model does not follow the ‘make-before-break’ strategy and it does not explain the observed murein turnover (the release of fragments from the sacculus during growth). It was proposed by Park, that murein turnover is a process of its own for sensing the structure of the sacculus [167].

According to the ‘three-for-one’ growth model, proposed by Höltje, three new glycan strands are attached in a relaxed conformation underneath one glycan strand (‘docking’ strand) in the sacculus [129,131]. Simultaneous removal of the docking strand allows the insertion of the new triplet into the sacculus, by this increasing its surface. During elongation, the middle strand of the new triplet would be pre-formed by a monofunctional transglycosylase, whereas during cell division, all three glycan strands would be synthesized simultaneously. The ‘three-for-one’ growth model explains the existence of a minor fraction of trimeric cross-links with a short half-life (these are the attachment sites of the new glycan strand triplet) and the significant degree of murein turnover (the removal of the docking strands), and it is in accordance with the ‘make-before-break’ strategy. If the glycan strands run perpendicular to the long axis of the cell, the diameter of the cell would remain constant if the inserted triplet has the same length as the removed docking strand. Also, if every second strand at the cylindrical part of the sacculus could serve as a docking strand, the length of the cell would exactly double in one generation. Höltje suggested a molecular mechanism for the removal of every second strand which involves DD- and LD-carboxypeptidase activities (catalyzed by PBP5, PBP6 and PBP6B). Höltje has also proposed that all enzymatic activities are coordinated by the formation of multi-enzyme complexes consisting of different murein synthases and hydrolases. This would then allow for murein synthesis multi-enzyme complexes that are specialized for certain functions, for example for cell elongation (with PBP2) or in cell division (with PBP3).

### 12. Regulation of cell wall growth by cytoskeleton elements

Morphogenesis of *E. coli* seems rather simple with two phases in the cell cycle [168,169]. During elongation, the cell is enlarged by growth of the envelope along the cylindrical part with maintenance of a constant diameter. Once the length of the cell has doubled, there is a switch to a focused envelope synthesis at the site of septation for the synthesis of the new polar caps of the daughter cells.

Vancomycin-labeling of lipid II and nascent murein has been used in *B. subtilis* to localize the sites of new murein synthesis [157]. This method is not applicable for the Gram-negative *E. coli* because the outer membrane prevents access of the antibiotic to its binding sites. The topography of the enlargement of the sacculus of *E. coli* has been studied by in vivo labeling of murein with radioactive precursor, followed by high-resolution autoradiography of the isolated sacculi by electron microscopy [170]. An easier method with higher resolution is based on the in vivo incorporation of exogenous D-cysteine into murein by a yet unknown periplasmic enzyme, followed by biotinylation and immunodetection of the label by electron (or fluorescence) microscopy [171]. Pulse and pulse-chase experiments revealed that a focused murein synthesis takes place at the septation site during division. During cell elongation in the absence of exogenous D-cysteine, the label is diluted at the cylindrical part of the sacculus, indicating that new material is incorporated into the existing side wall. While there are areas of homogenous incorporation of new material into the sidewall, there appear to exist also patches and arcs of entirely new material [172]. At the
cell poles there is no incorporation of new material and no loss of old murein (turnover), even during long chase periods. These results on the murein segregation pattern are consistent with the observations that during elongation new cross-links are formed in the sacculus mainly between newly synthesized and old (already existing) material, whereas during cell division there is also cross-linkage between two newly made peptides [166,173]. The segregation pattern of the outer membrane is similar to that of the murein layer, indicating that the enlargement of both layers is coordinated [174]. Recent application of the D-cysteine labeling method in *C. crescentus* revealed a murein segregation pattern different to that in *E. coli* [175]. During the transition of the swarmer to the stalked cell there is initially a diffuse incorporation of new material into the side-wall. Unlike in *E. coli*, murein growth is re-located to the future division site early in the cell cycle and well before a constriction is visible, resulting in a preseptal growth from mid-cell for cell elongation. Mid-cell growth continues during the following constriction to form the new polar caps of the daughter cells. Interestingly, the appearance of the growth zone for cell elongation at mid-cell is dependent on the localization of FtsZ at mid-cell. Furthermore, MurG which synthesizes the lipid II precursor, localizes at mid-cell in an FtsZ-dependent way. Thus, FtsZ may have a function not only in cell division but also in cell elongation, which involves the localization of enzymes for lipid II synthesis. FtsZ-dependent preseptal growth is not observed in wild-type *E. coli* cells but in filamentous cells unable to constrict due to the lack of functional PBP3 [171]. An enhanced, penicillin-insensitive and PBP3-independent peptidoglycan synthesis activity has been reported at mid-cell before *E. coli* cells start septation, and it was named PIPS (penicillin-insensitive peptidoglycan synthesis) [176,177]. Although the molecular basis for this observation has remained unclear it was proposed that PBP2 might be involved [147]. Inhibition of FtsZ or production of an unstable FtsZ has severe effects on cell shape in certain mutants lacking PBP5, PBP4 and PBP7 (or certain combinations) with an increased percentage of pentapeptides in their murein [178,179]. These cells form branches or even grow in spiral-form. A recent study followed up this phenomenon and showed that, in these mutants, FtsZ contributes to the enlargement of the sacculus at non-mid-cell positions [178]. It is not yet clear whether the effect of FtsZ on murein synthesis away from mid-cell is relevant in wild-type cells, or whether it is the result of an imbalance between cell division and elongation systems in these mutants. In any case, these interesting results show that FtsZ has the potential to direct murein synthesis not only at mid-cell but also at other positions.

The gene order in the *dcw* gene cluster encoding for enzymes for peptidoglycan precursor synthesis and cell division proteins is conserved in rod-shaped bacteria [180]. Based on this observation, the model of genomic channeling has been proposed according to which there is a selective pressure to maintain the cluster to efficiently coordinate the processes of cell elongation and cell division [181]. The proposed mechanism includes (i) the limitation in the amount of peptidoglycan precursor, (ii) the co-translational assembly of complexes of cell division proteins and enzymes for murein precursor synthesis, and (iii) the alternation in cellular localization of the complexes to participate in murein synthesis either at the lateral wall or at the division site. In addition, the conservation of the *dcw* cluster could be required to maintain an optimum ratio of the numbers of molecules per cell of cell division proteins and peptidoglycan precursor synthesis enzymes.

Based on genetic studies, intracellular localization of proteins and on the identification of protein–protein interactions a new

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**Fig. 7.** Model for the temporal and spatial organisation of cytoskeleton elements and murein synthesis complexes during the cell cycle. Upper part: Young cells elongate by a ‘dispersed’ incorporation of precursors by elongation-specific murein synthesis complexes (green circles) at filaments of the actin-like MreB (green dotted line). Formation of the FtsZ-ring (together with other early cell division proteins, blue dotted line) redirects the elongation complexes to mid-cell resulting in preseptal elongation at mid-cell. Constriction begins once the elongation complexes have been replaced from the FtsZ-ring by cell division-specific murein synthesis complexes (blue circles). The lower part shows the composition of the sub-complexes for the different modes of growth.

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view about how bacteria enlarge their cell wall has emerged in the recent years: Bacteria have a dynamic cytoskeleton which, among other functions, directs (or regulates) cell wall growth performed by murein synthesis multi-enzyme complexes [131,182,183]. We could envision the following model for cell wall growth during the cell cycle (Fig. 7): In newborn cells membrane-bound multi-protein complexes for cell wall growth are formed along the dynamic filaments of the actin-like MreB protein. These complexes contain enzymes for precursor (lipid II) synthesis (such as MraY and MurG), the cell elongation-specific murein synthesis complex (with RodA, MreD, MreC, PBP2 and, presumably, PBP1A), and the murein hydrolase sub-complex (with lytic transglycosylases, amidases and/or endopeptidases). During cell elongation, the cell wall synthesis complex would either remain localized at the MreB filament to use it as a track. Alternatively, the periplasmic sub-complexes might assemble at the MreB filament and would then move away from the filament for murein synthesis. This complex is responsible for the incorporation of new material along the side-wall by a ‘dispersed’ or ‘diffuse’ mode during cell elongation. In young cells, the bacterial tubulin FtsZ appears to rapidly oscillate on helical paths through the cytoplasm [184]. Later in the cell cycle, FtsZ assembles together with other cell division proteins to a ring-like structure at mid-cell. This happens well before a constriction is visible [147]. We propose that the localized, pre-divisional FtsZ-ring ‘captures’ the cell wall elongation machinery from the MreB filament, redirecting murein growth to a zone at mid-cell. In E. coli, this second, septal phase of cell elongation occurs only during a short transition period between elongation and constriction. In contrast, preseptal growth is much more relevant in C. crescentus such that a large part of the sidewall is synthesized by this mechanism [175]. The switch from elongation to constriction requires remodeling of the complex. Constriction starts once the division-specific murein synthesis sub-complex (with FtsW, FtsQ, FtsL, FtsB, FtsN, PBP1B and PBP3) has assembled and replaced the elongation complex from the FtsZ ring. When septation progresses the multi-protein ring operates at the leading edge of constriction to attach new murein in ever smaller concentric circles. Once division is completed, the FtsZ-ring complex disassembles and murein growth is redirected to the MreB filaments to start another elongation cycle. This model would be in accordance with the earlier speculation that cell wall growth involves the competition between two systems, one for elongation and another for cell division [185]. Morphogenesis of non-spherical bacteria might be driven by a competition between different cytoskeleton elements for controlling the murein synthesis multi-enzyme complexes.

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