Peptidoglycan synthesis in *Escherichia coli* from a PBP1b perspective

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Peptidoglycaan synthese in *Escherichia coli* uit het perspectief van PBP1b

(met een samenvatting in het Nederlands)

Proefschrift

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Table of contents

Chapter 1

General introduction

Partially based on:
Activities and regulation of peptidoglycan synthases
Alexander J. F. Egan, Jacob Biboy, **Inge van 't Veer**, Eefjan Breukink, Waldemar Vollmer
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Introduction

Bacteria possess a special structure in their cell envelope, which is composed of sugars and peptides, called peptidoglycan (PG). This PG surrounds the cytoplasmic membrane capturing it in a bag shaped macromolecule, which is referred to as the sacculus. This structure is essential for bacterial survival, because it gives the bacterium strength to withstand the difference in turgor pressure between the cells interior and its environment. It is also important in maintaining the specific cell shape of the bacteria. Furthermore, it serves as a scaffold for anchoring other cell envelope components such as proteins like Brauns lipoprotein and Pal, which link the outer membrane to the PG layer, serving cell envelope integrity [1]–[3]. Bacteria are divided in two main classes, gram positive and gram negative bacteria. This division is based on the chemical and physical properties of their cell envelopes. One of the main differences is, that gram positive bacteria have a thick, multilayered PG layer of 20 to 50 nanometer [4] and gram negative bacteria have a thin, mainly monolayered PG layer of 3 to 6 nanometers [5]. Another major difference is that the PG layer of gram negative bacteria is surrounded by an outer membrane, making the location where the PG is situated a defined and enclosed space, the periplasmic space (Fig 1).

The essentiality of this PG layer for bacterial cell survival, and the fact that humans do not possess this structure, and hence its synthesis machinery, makes that PG and its synthesis machinery has always been and still is a good target for antimicrobial compounds.

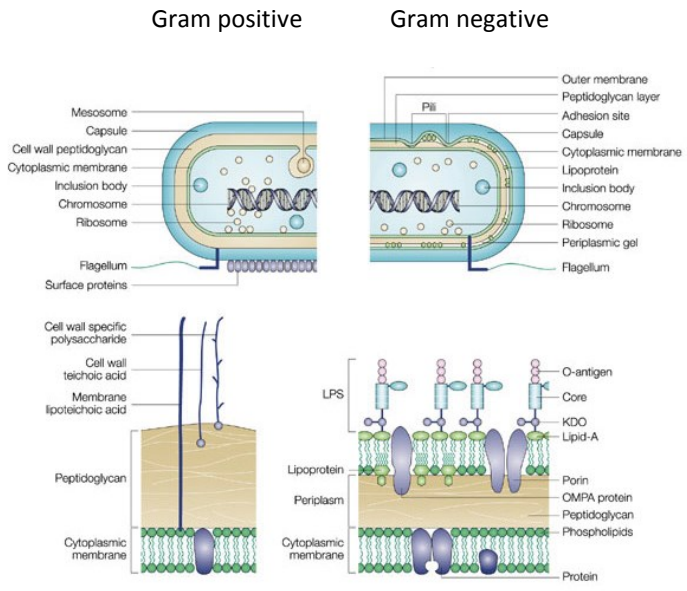

Figure 1. Gram positive and gram negative bacterial cell envelope composition.
Gram positive bacteria (left) have a thick, multilayered PG network, surrounding the cytoplasmic membrane, which is decorated with lipo- and wall teichoic acids.
Gram negative bacteria (right) have a thin, single layered PG network surrounding the cytoplasmic membrane, which is enclosed by an outer membrane. The location of PG is called the periplasmic space. The outer membrane is decorated with lipopolysaccharides. Adapted from [6].

Another great advantage of this target is that it is relatively easy to reach, located outside the cytoplasm. The emergence of resistance against available antibiotics now a days makes it of great importance to find new targets for the development of new antibiotics.
A vast amount of information on the mechanism and regulation of PG synthesis has been discovered so far. But still, new information about PG synthesis and its regulation (during cell division) is being unraveled, showing the high complexity of the interactions and interplay between the involved players. New insights in the processes of PG synthesis and its regulation can lead to the identification of new antibiotic targets, and eventually to the development of new antibiotics.

Peptidoglycan synthesis

To be able to designate new targets for the development of new antibiotics, it is of great importance to have an in depth understanding of the PG synthesis machinery and its acetylmuramic acid that are crosslinked by short peptides [7]. The building blocks of PG are synthesized as disaccharide pentapeptides, linked to a lipid carrier tail, called Lipid II (Fig 2). Lipid II production starts in the cytoplasm where the amino sugar subunits are synthesized. The first step in the process is the transfer of the enolpyruvyl moiety from phosphoenolpyruvate (PEP) to uridine diphosphate (UDP) N-acetylglycosamine (GlcNAc) by the enzyme MurA, generating enolpyruvyl UDP-GlcNAc [8]. Next, the PEP moiety is reduced to D-lactate by MurB, yielding UDP-N-acetylmuramate [9]. Subsequently the aminoligases MurC, MurD, MurE and MurF catalyze the stepwise addition of L-Ala, D-Glu, L-Lysine in most gram positive bacteria, or meso-diaminopimelic acid (m-DAP) in most gram negative bacteria,

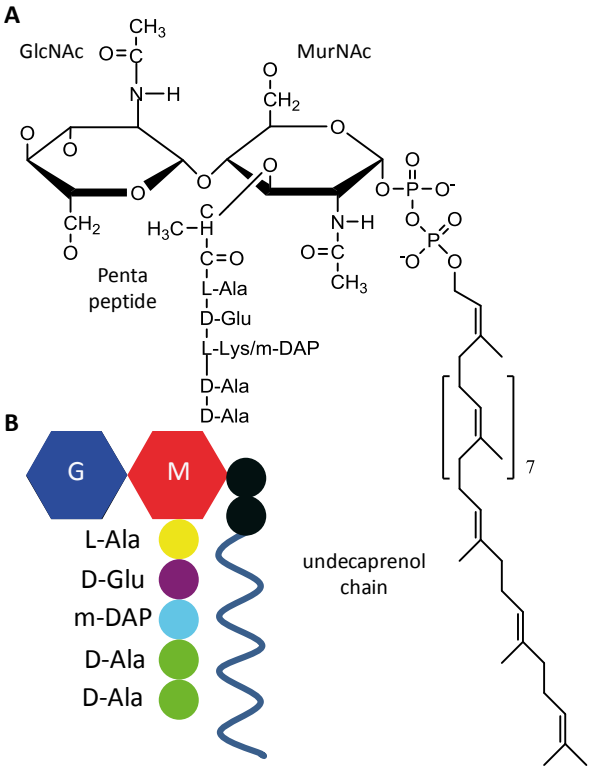

Figure 2. Structure of Lipid II.
A) Chemical structure of Lipid II.
B) Schematic representation of Lipid II.

and D-Ala-D-Ala to the D-lactate of UDP-N-acetylmuramate, respectively, creating the UDP-MurNAc-pentapeptide molecule [10](Fig 3).

The following steps are membrane associated, and take place on the cytoplasmic side of the inner membrane. First, the phosphor-MurNAc-pentapeptide of UDP-MurNAc-pentapeptide is transferred to a special membrane-bound lipid carrier, undecaprenyl-phosphate, by the integral membrane protein MraY, yielding Lipid I. Second, UDP-GlcNAc is coupled to Lipid I by the peripherally associated membrane protein MurG, resulting in the formation of Lipid II [11]. Third, Lipid II is flipped to the outer leaflet of the cytoplasmic membrane by RodA in the cell elongation PG synthesis complex, and by FtsW in the cell division PG synthesis complex [12]. Most recently MurJ has also been identified as a Lipid II flippase, of which it is not specified during which stage of the cell cycle it is mostly active [13] (Fig 3).

On the outer leaflet of the cytoplasmic membrane, membrane anchored glycosyltransferases (GTs) catalyze the polymerization of the sugar groups, forming linear chains of alternating amino sugars. The newly formed strands are incorporated into the existing PG network by transpeptidase (TP) reactions, the crosslinking of the peptide bonds [14] (Fig 3). Both monofunctional GTs and TPs exist, but the bifunctional PG

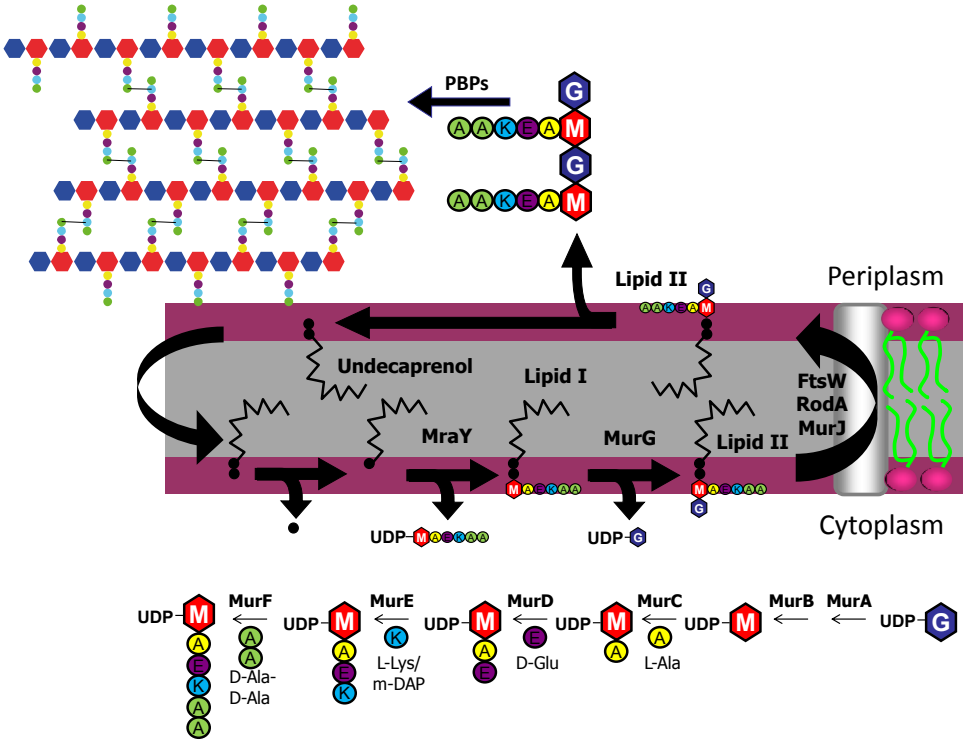

Figure 3. Schematic representation of the Lipid II synthesis pathway.

synthases, possessing both activities, are the most important proteins in PG synthesis. Proteins containing TP activity are called penicillin binding proteins (PBPs) as this functionality is the target of the antibiotic penicillin.
Thereafter, the lipid carrier is recycled by the dephosphorylation of undecaprenolpyrophosphate to reform undecaprenolphosphate. Several inner membrane proteins, facing the periplasmic side with their active site, have been identified as undecaprenolpyrophosphate dephosphatases [15]–[18]. Finally, the lipid carrier is flipped back to the cytoplasmic side of the membrane, ready for a new round of Lipid II synthesis and delivery to the outer leaflet of the cytoplasmic membrane [15], [19].

Variation in peptidoglycan structure

In this work the gram negative model bacterium *Escherichia coli* is used, therefore, the information in this thesis will be specific for this bacterium. However, there are many variations in PG structure between different species and growth stages, which are briefly discussed in this paragraph. Furthermore, the proteins involved in PG synthesis do also show variety between different species. The main difference in PG structure between bacterial species is in the composition of the stem peptide, with the largest variation at the third position. This amino acid is generally a meso-diaminopimalic acid (m-DAP) in most gram negative bacteria (including *E. coli*), Mycobacteria and Bacilli, or an L-Lysine in most gram positive bacteria, but still some more variations exist. Another type of diversity is introduced by modifications of the stem peptide amino acids at a later stage, mostly concerning positions two and three, taking place at the level of Lipid II. These modifications include amidation, hydroxylation and acetylation of specific amino acids. Next, the mode of crosslinkage between stem peptides, and the existence and composition of interpeptide bridges can differ between species and growth stages. The most common kind of crosslinkage is 3-4 crosslinkage, occurring directly in most gram negative bacteria, or through an interpeptide bridge, ranging from one to seven amino acids, in most gram positive bacteria. A second mode of crosslinkage, 2-4 crosslinkage, is found only in Corynebacteria, always containing an interpeptide bridge of a di-amino acid at position 2. There is furthermore a considerable variation in the degree of crosslinkage between species and growth stages, ranging from 20% in newly formed PG, up to 90% in older PG of some species. The glycan chain length on the other hand is inversely related with the age of the glycan strand, becoming shorter as a result of maturity. Besides, older PG can also contain 3-3 crosslinks, not seen in newly formed PG. The increase in percentage of peptides engaged in crosslinks and decrease in glycan chain length in older PG also holds true for stationary phase grown bacteria compared to exponentially grown [2]. These differences reflect the various needs in biophysical properties of different bacterial species during different growth stages in PG structure and composition.

Incorporation of newly formed peptidoglycan into the existing wall

After the polymerization of the monomeric building blocks, delivered to the periplasm in the form of Lipid II, these newly synthesized PG strands have to be incorporated into the existing cell wall. Attachment of newly synthesized PG to the existing layer would not result in sacculus expansion, it would only result in the PG layer becoming thicker [20]. Bonds in the existing wall need to be broken to allow for the incorporation of new material, resulting in (local) expansion of the sacculus [21]. There are three models for the completion of this. The first model is the make-before-break strategy, proposed by Koch, implying that the new peptide bonds between old and new strands are created before old bonds are hydrolyzed to release the stress [22]. In the second model, proposed by Burman and Park, bonds are broken first, before insertion of new material [23]. The third model is the three-for-one model, proposed by Höltje. Three newly synthesized strands, connected to each other in advance, are inserted to replace one old strand [24]. One thing is certain, these processes need to be tightly regulated in rate, space and time to maintain the shape and integrity of the cell wall. It is suggested that hydrolases and synthases work together in multi protein complexes, coordinating the action of both types of enzymes to guarantee the integrity of the cell wall [14], [25]. PG insertion by peptide crosslinking in *E. coli* extends mostly from the amino acid at position 3 to the residue at position 4, but 3-3 crosslinking also takes place, mainly in older PG. On average only 30% of pentapeptides are part of a crosslink, resulting in the existence of mainly monomers (not crosslinked peptides) or dimers (peptides taking part in one crosslink). Furthermore, slower growing cells (stationary phase) form less crosslinked PG than cells in the exponential phase (27-30% vs 36-42%), which is also composed of longer glycan strands (30-35 vs 15-18 disaccharides per chain) [2], [14]. These glycan strands are suggested to be positioned parallel in respect to the cell surface with a single layer of PG running perpendicular to the long axis of the cell, as observed by electron cryotomography on isolated *E. coli* sacculi [5].

There are two different modes of PG insertion, dispersed insertion during elongation, and localized mid cell incorporation during cell division, executed by different protein complexes. However, there also seems to be a period in between, in which proteins of both complexes localize at mid cell during pre septal elongation (Fig 4). During cell elongation, dispersed incorporation of PG in the cylindrical wall is seen, where new material is inserted in patches all over the cylindrical wall resulting in a mosaic like structure with isolated regions of new and old PG [27]. During this mode of PG synthesis, bonds are mainly formed between new and old glycan strands. During the zonal incorporation at mid cell during the process of cell division, the coordinated action of PG synthases, hydrolases, amidases and their regulators is even more important, because next to the synthesis of new PG, the cell gradually constricts, until it is divided into two

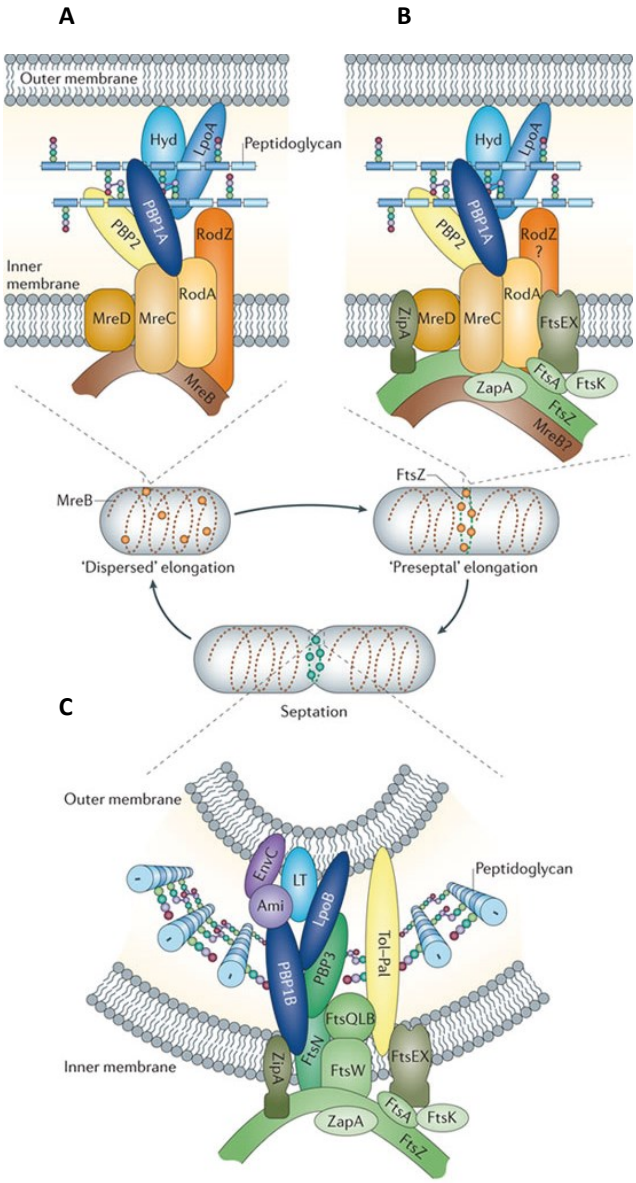

Figure 4. The different modes of peptidoglycan synthesis during cell cycle progression.
A) Dispersed PG incorporation during cell elongation, by the elongasome. B) Pre septal PG synthesis by the elongasome, accompanied by early cell division proteins. C) Septal PG synthesis by the cell division complex, composed of specialized PG synthases and their regulatory proteins. Adapted from [26].

daughter cells. This septal PG synthesis produces the new polar caps, where, for no known reason, no further PG synthesis, or turnover seems to take place. During septal PG synthesis, crosslinks are exclusively formed between newly synthesized glycan strands, of which 30% is accordingly removed during the septation process [14], [20], [27], [28].

Penicillin binding proteins

The polymerization of glycan strands and subsequent incorporation into the existing wall is executed by the penicillin binding proteins (PBPs), which work together with hydrolases in multi enzyme complexes for the removal and remodeling of old PG, and to break bonds to allow for the incorporation of the newly synthesized material. *E. coli* possesses twelve different PBPs, which are divided in two main categories, the high molecular weight (HMW) PBPs and low molecular weight (LMW) PBPs. The HMW PBPs are multi domain proteins responsible for the polymerization and insertion of newly synthesized PG into the existing wall [29]. These are further subdivided into class A PBPs, the bifunctional PBP1a, PBP1b and PBP1c, and class B PBPs the monofunctional PBP2 and PBP3. The class B PBPs have a specific role during different stages of the cell cycle, as PBP2 is essential during cell elongation and in maintenance of the rod shape, while PBP3 is essential during the cell division process [30].

Next to the HMW PBPs there are the LMW PBPs, or class C PBPs. These consist of PBP4 and 4b, PBP5, PBP6 and 6b, PBP7 and AmpH. They are D,D-caboxypeptidases and/ or endopeptidases, involved in cell separation, PG maturation and recycling. PBP4 and PBP7 are endopeptidases cleaving the cross bridges between two glycan strands. PBP5 is the most abundant carboxypeptidase, cleaving the terminal D-Ala-D-Ala bond, making the stem peptide only able to function as a donor during the TP reaction [31]. PBP6 and PBP6b have strong homology to PBP5 and both have carboxypeptidase activity. The role of PBP4b is unknown, and AmpH is associated with PG recycling [32]. Next to these twelve PBPs, *E. coli* has one monofunctional GT, MtgA, which is officially not a PBP, since it cannot bind penicillin because it has no TP domain, but is involved in the PG synthesis process by its capability to polymerize glycan strands, and hence often mentioned in respect to PG synthesis and its proteins [33].

The class A HMW PBPs PBP1a and PBP1b are the major PG synthases in *E. coli*, of which at least one is needed for cell survival [34]. Since PBP1b is studied in this work, this protein will be discussed in more detail below. Though PBP1a and PBP1b can take over each other's function, mutants lacking either PBP1a or PBP1b do show different phenotypes, indicating that they may play distinct roles during cell growth and division. The exact role of PBP1c is not well understood. This PBP is unaffected by most β-lactam antibiotics and overexpression of PBP1c cannot rescue the phenotype of PBP1a and PBP1b deletion [35].

It has been suggested to work as a periplasmic defense and repair system by repairing breaches in the PG network [36].

PBP1b

PBP1b is one of the two major PG synthases of *E. coli* found in approximately 127 (+/- 13) copies per cell [37]. *In vivo*, PBP1b exists in two isoforms, α, and γ, however, when analyzing membrane preparations, a third isoform is seen [38]. This β form is a 24 amino acids shorter degradation product, artificially created by cleavage of the α-isoform by OmpT [39], [40]. The α form is the full length, 844 amino acids long protein transcribed from the *PonB* (*MrcB*) gene. The γ form results from the translational start at an internal start codon at position M46, resulting in a protein of 799 amino acids [41]. Both isoforms are fully active, since they can complement a *PonB* deletion [42].
PBP1b is localized in foci diffusely spread over the cylindrical part of the membrane, as well as at the constriction sites. When measuring the fluorescence intensity of PBP1b over the cell, a higher intensity is found at mid cell, implying a more important role during cell division than elongation [43].
Besides its short cytoplasmic tail and transmembrane domain, anchoring it in the cytoplasmic membrane, PBP1b is largely located in the periplasmic space. Next to its GT and TP domains, PBP1b has a UB2H domain, which is not involved in the enzymatic function of PBP1b, but deletion of this domain does cause an aberrant growth rate. This domain has been shown to serve as a binding domain for the interaction with other proteins, like MltA [44], LpoB [45], [46] and YbgF (renamed into CpoB, this work and [47]).
Glu233 is the active site of the GT domain, which catalyzes the glycan strand polymerization. It is proposed that the lipid linked growing chain acts as a donor, which is transferred to the Lipid II precursor, functioning as an acceptor. Glu233 catalyzes the deprotonation of the GlcNAc 4-OH of Lipid II, and the activated nucleophile then directly attacks the C1 of the lipid linked MurNAc of the growing polysaccharide chain. Next to Glu233 there are several other conserved residues important for proper GT function. The residues Asp234, Phe237, His240 and Thr267 are proposed to be important for the stabilization of the helix containing the active site Glu233. Mutating these residues into alanines results in the production of inactive proteins, showing their importance in the proper functioning of the GT domain. Furthermore, Gln271 seems to interact directly with the side chain of Glu233, making this amino acid important for the positioning of Glu233 and therefore, resulting in an inactive protein when mutated. Gly242 and Gly264 are located upstream and downstream of the helix following the active site, mutating these amino acids results in low or no activity respectively, suggesting that flexibility of this region may be important for the activity of the enzyme. Finally, Lys274 is located in a positively charged pocket, which might be involved in the positioning of the negatively

charged pyrophosphate of the donor substrate. Mutation of this amino acid into an alanine also renders the protein inactive [48] (Fig 5). Not all of the amino acids important for proper positioning of the active site Glu233 are solved in the crystal structure of PBP1b and hence not highlighted in figure 5.

Ser510 marks the active site of the TP domain, catalyzing the peptide crosslinking. It attacks the carboxy-terminal D-Ala-D-Ala of a pentapeptide, leading to the formation of an acyl (peptidyl) enzyme. This is followed by the attack of the ester bond of the peptidyl enzyme by the amino group of the D-center of m-DAP of an acceptor peptide, leading to the creation of a crosslinked peptide [49]. This is the site blocked by β-lactam antibiotics. Their structure mimics the D-Ala-D-Ala bond of the pentapeptide. When the active site serine recognizes and binds this structure, a covalent bond is formed, resulting in inactivation of the protein (Fig 5).

PBP1b has been shown to form dimers *in vivo* and *in vitro,* which are not formed by disulfide bridges [50]–[52]. Dimerizing conditions result in a significant higher activity of PBP1b, in particular strongly increasing the percentage of peptide crosslinks [53]. PBP1b is known to interact with the essential cell division proteins FtsN [53] and PBP3 [43]. FtsN stimulates both the GT and TP activities of PBP1b and might have a role in the coordination of PG synthesis during septation. PBP3 is the essential TP during cell division, of which the septal localization is essential for the mid cell recruitment of PBP1b [43]. PBP1b also has an interaction with MipA, indirectly linking PBP1b to the PG hydrolase MltA [54]. Furthermore, the outer membrane lipoprotein LpoB has been shown to interact with PBP1b which is essential for PBP1b function *in vivo* [45], [55]. LpoB regulates the activity of PBP1b by stimulating both TP and GT activities, and inducing an increase in produced glycan chain length [45], [46], [55], [56]. This was the first outer membrane protein found to directly regulate the activity of a PG synthase. Since thus far it has been suggested that PG synthesis is a largely unregulated process only directed in space by cytoskeletal components [57]. Recently, CpoB, previously called YbgF, and TolA have also been shown to interact with PBP1b (this work and [47]) which are able to regulate the stimulatory effect of LpoB on the TP activity of PBP1b. All these interactions of PBP1b with many mainly cell division related proteins, together with the fact that PBP1b localizes at mid cell during cell division, suggests an important role for PBP1b in PG synthesis during cell division.

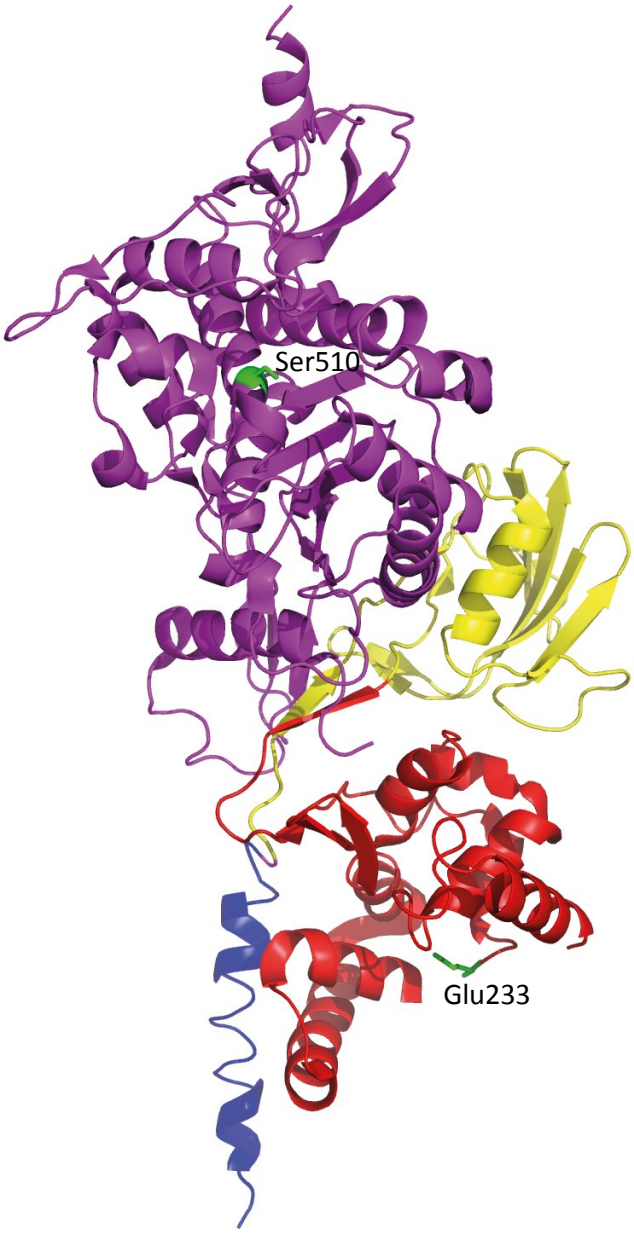

Figure 5. Crystal structure of PBP1b (3FWL).
PBP1b contains a transmembrane domain (blue), glycosyltransferase (GT) domain (red), transpeptidase (TP) domain (magenta) and a UB2H domain (yellow). The GT active site (Glu233) and TP active site (Ser510) are highlighted in green.

Cell division

The complexity and necessity of the tight coordination of the cell division process in space and time is reflected by the vast amount of proteins involved in this process. There are twelve essential proteins, and around fifteen accessory proteins taking part in *E. coli* cell division [58] (Fig 6).

Cytoplasmic steps of divisome formation

The GTP dependent polymerization of the tubulin like cytoplasmic protein FtsZ into filaments and bundles, which combine in a ring like structure, called the Z-ring, is the first step in the assembly of the cell division machinery (divisome) [59], [60]. The essential proteins FtsA and ZipA bind FtsZ and tether the Z-ring to the cytoplasmic membrane. The binding of these proteins furthermore contributes to the integrity of the Z-ring [61], [62].

To produce two equal daughter cells, and avoid septum formation at regions of nucleoid presence, Z-ring formation and subsequent cell division have to occur exactly at mid cell and for this, *E. coli* has two mechanisms. The first is the FtsZ polymerization inhibiting Min system, composed of the MinC/MinD complex which oscillates from pole-to-pole, and the membrane bound ATPase MinE, driving this oscillation process. This results in a lowest concentration of MinC/MinD at mid cell resulting in the lowest inhibition of Z-ring assembly there [63], [64]. The second mechanism is called nucleoid exclusion by the nucleoid exclusion factor SlmA. This prevents cell division near chromosomal DNA to avoid septum closing through the nucleoid, which would be lethal. SlmA dimers bind specific sites on the DNA and FtsZ to promote

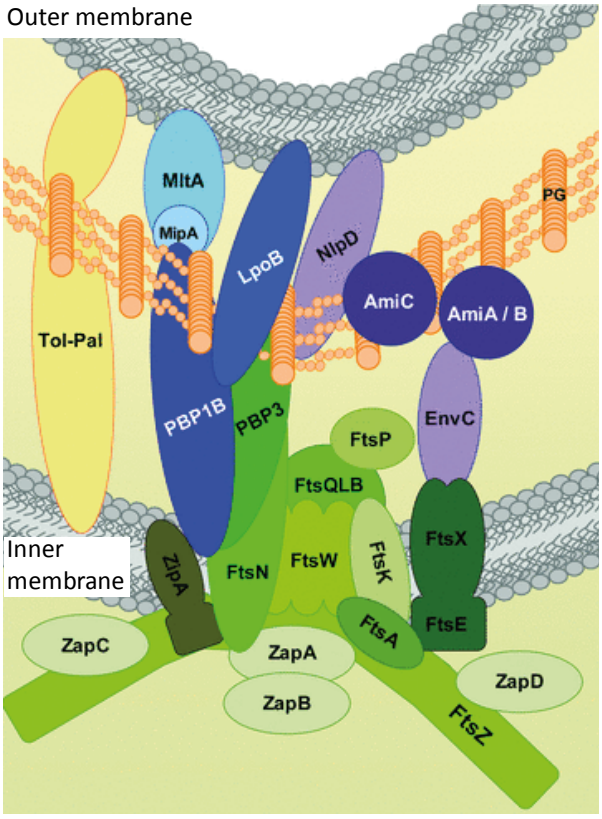

Figure 6. Schematic representation of the proteins of the cell division complex, with their localization and interactions indicated. Adapted from [67] .

protofilament disassembly, or inhibition of polymerization at these sites [65], [66].
The Z-ring formation is followed by the recruitment of the non-essential proteins ZapA, ZapB, ZapC and ZapD, which are involved in Z-ring stabilization. ZapA and ZapD execute their stabilizing function by stimulating protofilament formation [68], [69]. ZapC promotes FtsZ polymer bundling and suppresses its GTPase activity. The GTP hydrolysis by FtsZ has shown to lead to protofilament destabilization and disassembly, inhibition by ZapC results in stabilization of the Z-ring. GTP hydrolysis by FtsZ is however also implicated in the generation of the constrictive force in later stages of cell division [70], [71]. ZapB interacts with ZapA, and presumably stabilizes the Z-ring via this interaction [72]. Furthermore, ZapA and ZapB are also required for the anchoring of MatP, a protein involved in the structuring of the chromosomal terminus, required for proper nucleoid segregation [73]. Thereafter, the essential FtsE-FtsX complex is recruited to the divisome, which has no obvious role during this stage of cell division but is probably involved in assembly and stability of the septal ring. Later, it recruits and affects the activity of the amidase activator EnvC, activating the periplasmic amidases AmiA and AmiB [74]. The cytoplasmic part of the divisome is completed by the recruitment of FtsK, which is involved in the coordination of chromosome segregation by helping in untangling the DNA, allowing for correct sorting of the nucleoids [75]. Next to that, it presumably functions in the stabilization of late cell division proteins and the septal recruitment of the first periplasmic proteins; FtsQ, FtsL and FtsB, which arrive in a preformed complex [76], [77].

Periplasmic steps of divisome formation

The first arriving periplasmic proteins are FtsQ, FtsL and FtsB, recruited by FtsK in a preformed complex [76], [77]. These, and all following proteins recruited to the divisome are membrane proteins with at least a single membrane helix. The exact function of FtsQ is not known, but it is needed for correct divisome assembly, and the recruitment of FtsL, FtsB, FtsW and PBP3 (FtsI) to the site of cell division [78]. Though they are essential, both for FtsL and FtsB, their exact role in the division process is not known either. They might link upstream, cytoplasmic processes to the downstream periplasmic processes of cell division. FtsW is the cell division specific PG precursor transporter, moving Lipid II across the cytoplasmic membrane. Furthermore, FtsW is required for the recruitment of PBP3 (probably together with PBP1b) and is essential for cell survival [12], [43], [79], [80]. Then, FtsN is recruited, which is shown to be the last protein in the assembly of the divisome [81]. This essential protein has multiple interactions with both early and late, as well as cytoplasmic and periplasmic proteins (FtsA, ZapA, PBP3, FtsW and PBP1b) and is proposed to have a stabilizing function. Depletion of FtsN causes disassembly of already formed elements of the division ring [82]. Next to that it has been proposed to be a signal for completion of the division machinery assembly and start of the constriction process [83], a role supported by its interaction with both cytoplasmic and periplasmic proteins.

Furthermore, its stimulating activity on PBP1b implies a role in the coordination of PG synthesis during the septation process [53]. All these essential proteins, from FtsK to FtsN, localize at the septum site interdependently and simultaneously with the non-essential proteins PBP1b, AmiB and AmiC and their regulators LpoB, EnvC and NlpD, respectively. The ancillarity of these proteins is probably caused by the existence of redundant enzymes.

Outer membrane constriction

To finish cell division, both the inner and outer membrane, together with the PG layer in between them, need to invaginate and constrict, to eventually come together, and fuse with each other, to make the actual separation possible. This is gradually ongoing during the division process, in which FtsZ is the force generating protein, pulling the inner membranes slowly together [84]. Since proteins in this Z-ring complex have an interaction with proteins associated with the PG layer (FtsN, PBP1b and PBP3), this links the PG layer to the inner membrane and to the constriction it undergoes. However, the outer membrane also needs to follow this process.

It has long been thought that outer membrane invagination occurs passively via (lipo)protein linkages between the outer membrane and PG network [85]. Though it is not a specific component of the division machinery, the major murein binding lipoprotein Lpp, or Braun's lipoprotein, was thought to form this linkage, and in this way plays an important role in the outer membrane constriction process. Absence of Lpp causes defects in outer membrane invagination accompanied by the formation of large outer membrane blebs at constriction sites, and a mild cell chaining phenotype due to delayed daughter cell separation [86]. However, the energy transducing Tol-Pal system, which localizes at the division site during later stages of cell division, is also important for outer membrane integrity and constriction. Loss of any of its components leads to delayed outer membrane constriction and outer membrane integrity defects. It consists of the complex forming, cytoplasmic membrane proteins TolQ, TolR and TolA, the periplasmic proteins TolB and YbgF (later renamed into CpoB [47]), and the outer membrane protein Pal, which all localize to the division site in an FtsN dependent manner [47], [87]. Furthermore, the *tol-pal* operon encodes ybgC, a cytoplasmic protein with unknown function [88]. Pal is anchored to the outer membrane and can bind the periplasmic components PG and TolB and can furthermore interact with the inner membrane protein TolA in a proton motive force dependent manner. TolA and TolB can also interact with each other [89]–[92], and TolA interacts with YbgF as well, modulating its oligomeric status [93]. The exact role of YbgF is not completely clear though. It is highly conserved in most gram negative bacteria, but not essential for cell survival. Next to the Tol-Pal complex, the PBP1b-LpoB complex has been implicated to play a role in outer membrane constriction as well, since cells lacking a functional Tol-Pal system get more severe lysis phenotypes when LpoB is also not

functioning properly [45]. The interplay between all these proteins connect the cytoplasmic membrane, the PG network, and the outer membrane, creating a trans envelope complex, to form a physical link between PG synthesis and outer membrane constriction during cell division, and an intriguing network of connectivity of these processes.

Used techniques and procedures

In an effort to identify new antibiotic targets we attempted to reveal new interaction partners of the cell division specific bifunctional PG synthase PBP1b, or specify the interaction surface of known interaction partners of this highly important protein. For this, the site specific incorporation of unnatural amino acids by nonsense suppression mutagenesis was used, as well as Surface Plasmon Resonance studies. To verify the activity of the created genetically modified PBP1b variants, PG synthesis assays were performed using *in vitro* synthesized Lipid II substrate, with or without fluorescent tags. Below these techniques and procedures will be explained, to clarify the experimental chapters.

Nonsense suppression mutagenesis

For the *in vivo* incorporation of unnatural amino acids at specific sites in desired proteins a method has been developed called nonsense suppression mutagenesis [94]. This method makes it possible to site specifically encode amino acids with novel physical, chemical or biological properties, and has worked very well in our hands for the incorporation of unnatural amino acids with a photo-crosslinkable or azide containing functional group in *E. coli* PBP1b, and has shown to be very valuable for this research.

To be able to co-translationally incorporate unnatural amino acids at specific sites in a protein of interest in a living organism, it is required to have a unique tRNA-codon pair and a corresponding aminoacyl-tRNA synthase. The tRNA must be constructed in such a way that it is not recognized by endogenous aminoacyl-tRNA synthases and in this way loaded with natural amino acids, but also has to function efficiently in the translational system of the host (an orthogonal tRNA). Furthermore, this tRNA has to incorporate the unnatural amino acid in response to a unique codon that does not encode any of the natural 20 amino acids. The accompanying aminoacyl-tRNA synthase (the orthogonal synthase) should only aminoacylate the orthogonal tRNA and none of the endogenous tRNAs, and only with the desired unnatural amino acid, which on itself should not be a substrate for the endogenous synthases. Unique codons, that are not recognized by any endogenous tRNAs are the stop codons, also called nonsense codons. In *E. coli*, the amber stop codon UAG is used for this method, because it is the least used stop codon [95]. To evolve an orthogonal tRNA/aminoacyl-tRNA synthase pair from heterologous sources, a library of

tRNA mutants are subjected to a combination of negative and positive selection rounds in the absence and presence of the cognate synthase [96]. During negative selection, the tRNA library is introduced in *E. coli* together with a toxic bacterial ribonuclease gene, in which amber nonsense codons are introduced at specific sites. When the introduced tRNA is aminoacylated by an endogenous *E. coli* synthase, the amber codons are suppressed and the ribonuclease produced, which is fatal for the cells. Only cells that contain tRNAs that are not recognized by endogenous aminoacyl-tRNA synthases, or that are nonfunctional, will survive this selection round. The surviving tRNAs are subjected to a round of positive selection, in the presence of its cognate heterologous aminoacyl-tRNA synthase, together with a β-lactamase gene containing an amber codon. The tRNAs that function well in the translational system of the host strain, and are amino acylated by the cognate synthase will be able to produce the introduced β-lactamase and survive this selection round. Next, the substrate specificity of the tRNA synthase has to be changed, which is achieved by randomizing five or six residues in the substrate binding pocket, to only charge its cognate tRNA with the desired unnatural amino acid, and none of the common amino acids. Again rounds of positive and negative selection are applied to achieve this specificity. Positive selection was based on the resistance to chloramphenicol by suppression of an amber mutation in chloramphenicol acyl transferase. Cells with mutant synthases that can acylate the tRNA with the unnatural or a natural amino acid will survive. Next, these tRNAs are expressed in absence of the unnatural amino acid, in cells encoding the bacterial ribonuclease gene, containing amber mutations. If the synthase recognizes endogenous amino acids, they are incorporated in the bacterial ribonuclease, and the cells will die. In this way, mutant synthases that can specifically incorporate unnatural amino acids in response to the amber codon can be isolated [95], [97], [98].

Many tRNA-tRNA synthase pairs have been created that can incorporate unnatural amino acids, containing different chemical groups, fluorophores, crosslinkers or posttranslational modifications, in response to the amber codon. These unnatural amino acids can be incorporated site specifically in any protein, by mutating the codon of the amino acid to be substituted in the TAG amber codon in the DNA of the protein of interest (Fig 7A), and expressing this together with the tRNA- aminoacyl-tRNA synthase pair that will incorporate the desired unnatural amino acid in response to the altered codon (Fig 7B & C). In this way, the protein of interest with the unnatural amino acid of interest at the desired position can be generated (Fig 7D). In *E. coli*, for most unnatural amino acids, the suppression efficiencies range from 25-75% of wild type protein, and translational fidelity is >99%.

In this work, the nonsense suppression mutagenesis method was used to incorporate an unnatural amino acid with photo-crosslinking properties in PBP1b, to site specifically map interactions of PBP1b with other proteins. Furthermore, the incorporation of an unnatural

amino acid with a functional chemical group in the form of an azide was used to site specifically couple PBP1b to a Surface Plasmon Resonance chip surface using click chemistry.

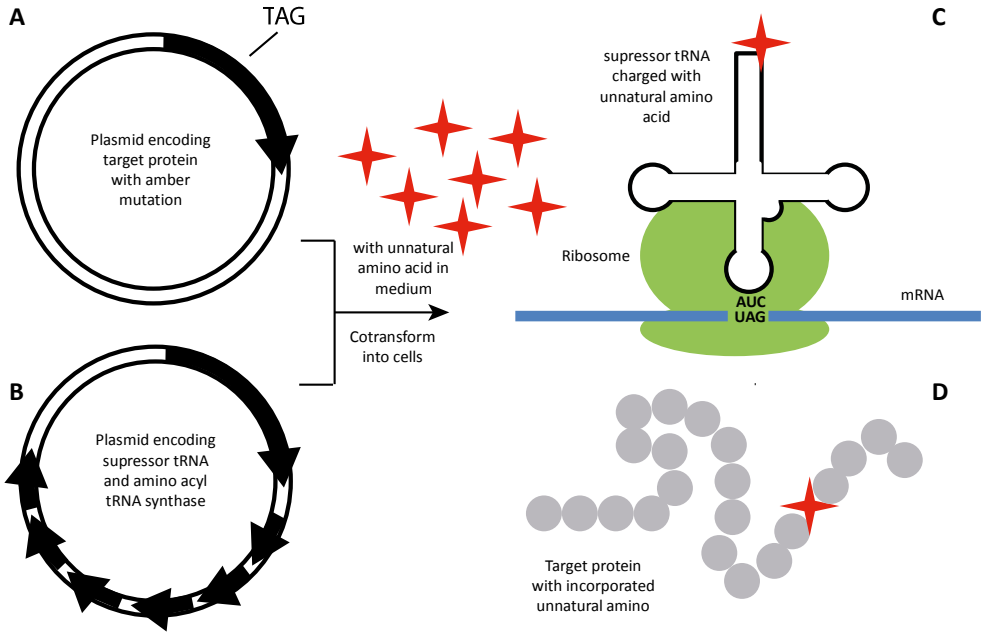

Figure 7. Schematic representation of the procedure of unnatural amino acid incorporation.
A) Expression of protein of interest with amber mutation replacing the codon of the amino acid to be substituted. B) Together with the plasmid encoding the orthogonal tRNA- tRNA synthase pair. C) Producing a tRNA that is charged with the unnatural amino acid and will recognize the amber codon. D) Resulting in the production of the target protein with the unnatural amino acid incorporated at the desired position.

Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) has shown to be one of the most powerful label free methods to determine specificity, affinity and kinetic parameters of protein-protein interactions in real-time and in a highly sensitive way [99], [100]. This optical technique measures a shift in reflectivity curve of a thin metal layer (eg. gold) in response to biomolecular interactions. Most commercial available SPR setups use a detection scheme called the Kretschmann configuration (Fig 8A). In this setup, a light source passes through a prism, reflects on the backside of the sensor chip, into the detector. At a certain incident angle, known as the resonance angle, light is absorbed by the electrons in the metal film of the sensor chip, causing them to resonate. These resonating electrons are also called surface plasmons, hence the name SPR. These surface plasmons are very sensitive to their surrounding environment. Consequently, biomolecular interactions will result in a shift in the reflectivity curve. Detection is based on measuring the changes in the reflected light

obtained by the detector. By measuring these changes over time, kinetic parameters of the interactions can be deduced [100]–[102].

Ligand molecules are immobilized on the sensor chip surface, which can be achieved in many different ways (see further). When the solution of analyte molecules is flown over the ligand immobilized surface, binding events occur, which induce an increase in the refractive index. Resonance or Response units (RUs) are used to describe this signal change. Initially many binding sites are available, resulting in a rapid increase in SPR response. In time, the binding sites will be occupied, resulting in an equilibrium of binding and unbinding events shown by leveling off of the SPR response signal. When introduction of the analyte into the system is ceased, the molecules will unbind, and a decrease in SPR response signal is observed (Fig 8B). The association constant, K_a, can be extracted from the binding response, and the dissociation constant K_d, from the unbinding response [100]–[102].

Covalent coupling is commonly used for ligand immobilization. Several functional groups such as amine (-NH_2) thiol (-SH) and aldehydes (-COOH) are most commonly used for the functionalization of the chip surface. Some drawbacks of covalent immobilization are that it may involve chemical modifications on the active site which can potentially affect the binding activity of the ligand, the reactive functional groups can be blocked by nonspecific binding of proteins and inappropriate blocking agents can inactivate the biomolecules. Next to covalent coupling to functional groups on the surface, affinity fusion tags such as gluthatione-s transferase specific for glutathione, maltose binding protein for amylose and hexahistidine for Ni-NTA can be used for the immobilization on an SPR chip.

In this research we use a click chemistry based approach to covalently couple PBP1b via an

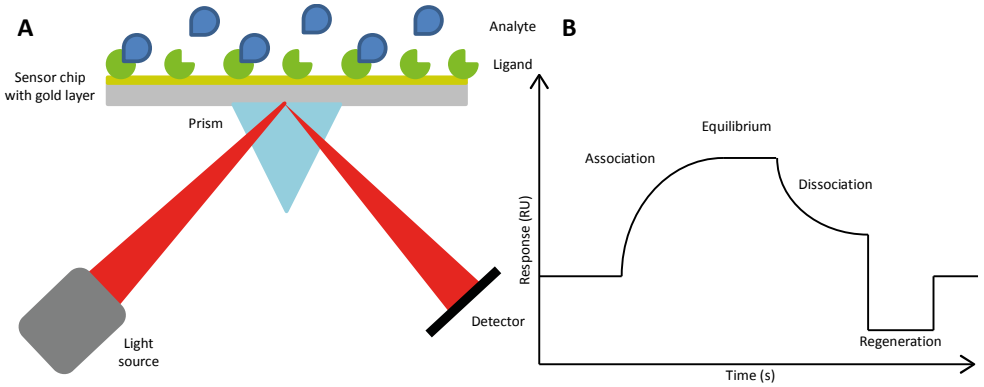

Figure 8. Surface Plasmon Resonance.

A) Schematic representation of the Kretschman configuration. B) When analyte binding occurs, this will be measured as an increase in RUs. From this increase the association constant K_a can be determined. This increase will continue until an equilibrium is reached where binding and unbinding events are equal. When analyte injection is stopped, unbinding will occur, measured as a drop in RUs. From this decrease, the dissociation constant K_d can be determined.

azide in its cytoplasmic tail to a cyclooctyne in a coating compound, reacting with its NHS ester to the NH_2 functionalized SPR chip surface. In this way we more approached the *in vivo* orientation of the protein, compared to the previously used immobilization technique composed of a covalent interaction with an ampicillin coated surface via its TP domain, and were able to perform experiments with fully active proteins.

Isolation of Lipid II for in vitro assays

Unlabeled Lipid II

Lipid II can be completely or partially synthesized chemically as described by Schwartz *et al.* [103], Van Nieuwenhze *et al.* [104] an Xiang Yang *et al.* [105]. However, the biochemical way of Lipid II production is much easier, and more feasible for people who do not have a chemistry background, hence we here review the available biochemical methods of obtaining (labeled) Lipid II.

The biochemical production of Lipid II requires four ingredients, the lipid carrier undecaprenyl phosphate (11-p), UDP-Mur*N*Ac-pentapeptide (UMPP), UDP-Glc*N*Ac (UG) and the enzymes that catalyze the reactions forming Lipid II out of these substrates, MraY and MurG. The bottleneck in the (bio)chemical production of Lipid II has been the availability of the lipid tail of Lipid II. In 1967 Higashi *et al.* [106] showed that in gram positive bacteria, this tail of Lipid II is a derivative of C_{55} isoprenoid alcohol (11 prenoid units), also called bactoprenol or undecaprenol. Later, this was also shown for gram negative bacteria [107]. The early protocols for the biochemical Lipid II synthesis, used gram positive bacteria-derived membrane vesicles as the source of this lipid, which simultaneously provided the necessary enzyme activities of both MraY and MurG [108],[109].

Yet, although gram positive derived membranes contain more of this lipid opposed to membranes from gram negative bacteria [110], still only limited amounts of this lipid is present in the cell, as it is constantly being recycled during the cell wall synthesis cycle and consequently bacteria do not need bulk amounts [111], [112]. As a result, to obtain approximately 150 µg [109], large amounts of membranes and reaction/extraction volumes (1 L) and large anion exchange column sizes (2,5 x 25 cm) were needed for the production, extraction and purification of Lipid II. The yield of Lipid II in the biochemical synthesis could be considerably improved (by a factor of at least 100) by the external addition of undecaprenyl phosphate [113]. Moreover, the substrate specificity of MraY for polyisoprenoid phosphates turned out to be so broad that Lipid II variants could be prepared with polyisoprene tails that vary from two to more than 20 isoprene units long.

Plant leaves are the best source for polyisoprenoids [114], and there are two easily accessible sources for undecaprenol: Bay leaves (*Laurus nobilis*) and leaves from the Staghorn sumac (*Rhus typhia*), an indigenous plant in North America and ornamental in Europe. The extraction and purification of undecaprenol has been described [115], [116]

and is relatively straightforward only needing the extraction of the ground leafs with a mixture of acetone and hexane followed by silica column purification and if a uniform length is desired (the prenols are present in a mixture of polyisoprenoids of 10, 11 and 12 isoprene units) a second purification step using reversed phase HPLC (Fig 9A). Subsequent phosphorylation is again straightforward and performed in one step [117].

While UG can be bought from different chemical supplying companies like Sigma Aldrich, UMPP should be extracted from bacteria. The source of UMPP is dependent on the version of Lipid II that is desired with respect to the pentapeptide composition, where *Bacillus cereus* and *Staphylococcus simulans* are so far the best sources for the extraction of the m-DAP version or lysine version respectively [2]. The extraction of UMPPs was described in 1991 by Kohlrausch and Höltje [118] (Fig 9B) and is based on the accumulation of UMPP as a result of blocking the bacterial cell wall synthesis cycle by the addition of vancomycin. The UMPP is extracted by boiling the cells in water followed by centrifugation and lyophilization of the UMPP-containing supernatant. This UMPP-containing extract can directly be used for the synthesis of Lipid II without further purification.

The enzymes catalyzing the formation of Lipid II, MraY and MurG can come from any source ranging from the purified enzymes to isolated bacterial membranes from gram positive bacteria or even *E. coli* cells over expressing the two proteins [120]. Membranes from gram positive bacteria are easiest to work with and membranes from *Micrococcus flavus* are mostly used (Fig 9C). However some membrane preparations lack (sufficient) MurG activity, this is for instance the case when membranes from *Bacillus subtilis* are used, then only Lipid I is synthesized (E. Breukink, unpublished data).

To finally produce Lipid II, the four components mentioned above are mixed in a detergent containing buffer and stirred for 2-4 hours at room temperature (Fig 9D). Lipid II is extracted, according to the procedure originally developed in the Strominger lab [108], by Butanol/Pyridine at pH 4.2, and Lipid II can then be purified using a small anion exchange column in one step [113].

Labeled Lipid II

To be able to track Lipid II, or the products from e.g. the PBPs in functional assays, fluorescent groups or radioactive isotopes can be incorporated in both the UMPP and/or UG moiety. Fluorescently labeled Lipid II production can be achieved by the incorporation of fluorescently labeled UMPP, for example containing a pyrene [113], a dansyl [121] or an NBD label [122]. This approach requires purification of the UMPP which can then be used in a labeling reaction followed by purification of the labeled UMPP [113], [121]. For this procedure, mainly the lysine version of UMPP is used, which can be labeled at the amine of the side chain of the lysine. Next to that, radiolabeled UG can also be incorporated into Lipid II [121]. While producing radiolabeled Lipid II using [C^{14}]-UG, it was observed that

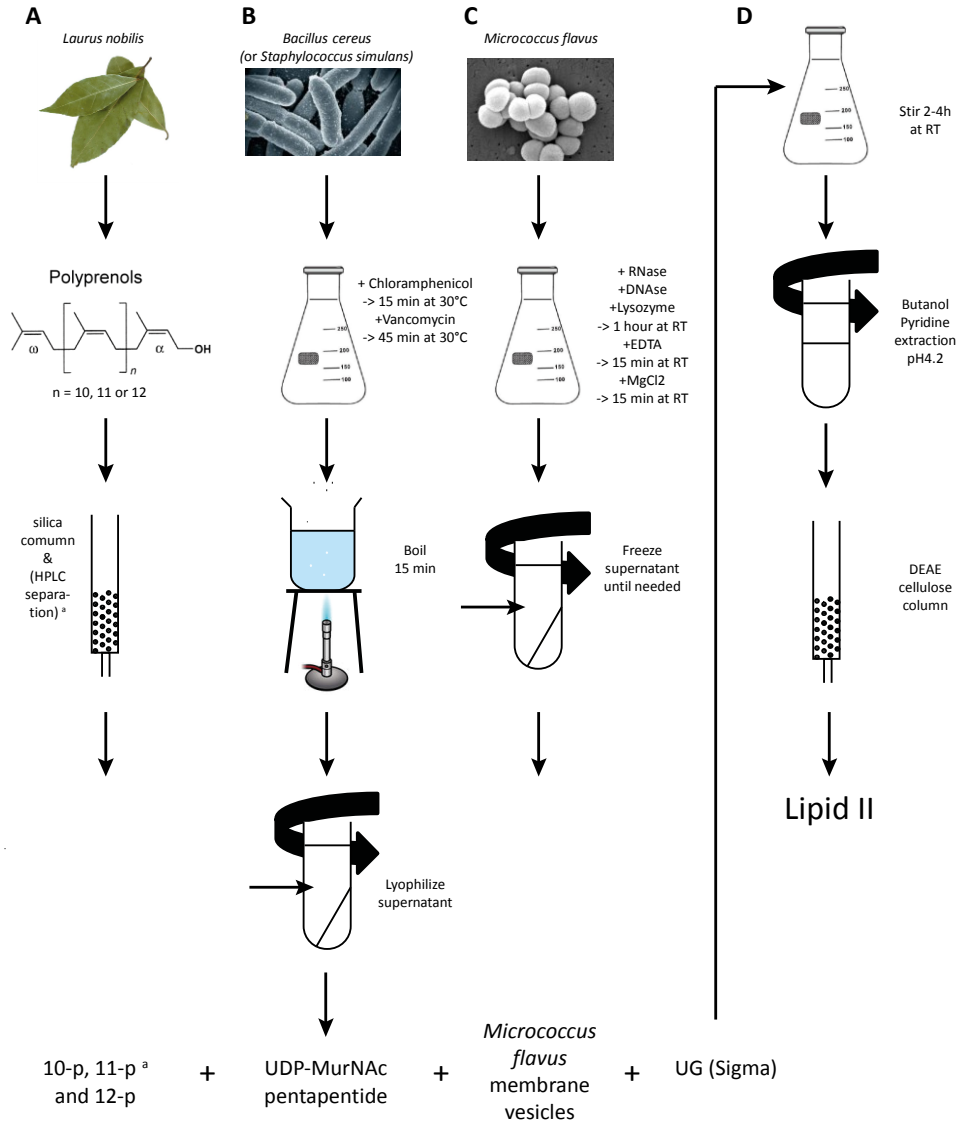

Figure 9. Biochemical synthesis of Lipid II.

A) The undecaprenol can be extracted from *Laurus nobilis* leaves using a mixture of acetone and hexane, followed by silica column purification, and if a uniform chain length is needed by reverse phase HPLC. B) UMPP can be extracted from *Bacillus cereus* (m-DAP) or *Staphylococcus simulans* (lysine) by first blocking the cell wall synthesis cycle by the addition of vancomycin, and the subsequent extraction by boiling the cells in water, followed by centrifugation and lyophilization of the UMPP containing supernatant. E) The enzymes (MraY and MurG) catalyzing the Lipid II formation come from *Micrococcus flavus* membranes, which are lysed and centrifuged to obtain the membrane vesicles in the supernatant. F) To produce Lipid II all the components are incubated for 2-4 hours at room temperature, the lipids are extracted using butanol/pyridine at pH 4,2, and Lipid II is purified using a DEAE cellulose column (published in [119]).

MurG is able to exchange the Glc*N*Ac of the Lipid II headgroup with the Glc*N*Ac of UG [121]. This property has been used to obtain Lipid II with a radiolabeled Glc*N*Ac by a simple exchange reaction using purified Lipid II and [C^{14}]-UG in the presence of MurG (E. Breukink, unpublished). The above approach for obtaining fluorescent Lipid II starting with fluorescent UMPP is rather inefficient as it first requires the purification of UMPP which is then labeled and subsequently used even in excess during the synthesis of the labeled Lipid II. To circumvent these disadvantages a novel approach has been developed that makes use of the biorthogonal click chemistry. For this, the amine of the lysine of the pentapeptide of Lipid II was converted into an azide via the incubation with imidazole-1-sulfonyl azide hydrochloride, which acts as a diazo donor in the conversion of primary amines into azides [124]. After complete conversion, the azido-Lipid II is extracted and purified from the reaction mixture, and can then be used in (copper catalyzed) click reactions with azide or cyclooctyne containing labels.

Biological variants of Lipid II

Next to the variations in amino acid composition of the pentapeptide, in some bacterial strains amino acids of the stem peptide are amidated at one of the carboxylates. In *Streptococcus Pneumoniae*, this amidation has been shown to be performed by the MurT/GatD protein complex [125] on the C-terminal carboxylate of the D-Glu, and by AsnB in *Bacillus subtilis* on the carboxylate of the DAP (van Bentum and Breukink, unpublished data). Lipid II carrying these amidations can be generated *in vitro* by incubating it in the presence of these enzymes in combination with ATP and a nitrogen donor such as glutamine or even ammonia. Using an amidated version of Lipid II it could be shown that the presence of the amidation was essential for the occurrence of the TP reaction by PBP1a, 2a, 2b and 2x of *Streptococcus pneumoniae* [126].

The existence of bridging peptides that provide the crosslinking between the pentapeptides of the different strands is another example of structural variation at the level of Lipid II. These inter peptide bridges are synthesized by branching enzymes and can be composed of different amounts of varying amino acid residues ranging from one to seven amino acids. The most well-known version is the pentaglycine bridging peptide that is present in the *S. aureus* PG layer. The bridging peptide is attached to Lipid II by the successive action of the Fem enzymes, FemX, FemA and FemB that use amino acid loaded t-RNAs as substrates [127]. FemX attaches the first glycine residue to Lipid II, the second and third residue are attached by FemA, which only recognizes Lipid II with a previously attached first glycine residue. The last two residues are attached by FemB, which also has acceptor specificity, and only uses Lipid II with three glycine residues attached. The branch attachment to Lipid II has also been achieved *in vitro* using purified Lipid II and the purified enzymes [128]. A similar system has been shown to exist for the bacterium *Weissella viridescens,* where Fem enzymes initially use UMPP as a substrate for the attachment of

the first alanine, which is followed by the attachment of a serine and alanine at the level of Lipid II by still unknown Fem-like proteins. The first step of this process has been reconstituted *in vitro* [129].
The successful generation of significant amounts of Lipid II even carrying reporter groups or necessary modifications such as amidation or bridging peptides has provided essential tools for studying the PG synthesis pathway and generated a wealth of knowledge, and has also been used in some experimental parts of this thesis.

Scope of this thesis

The aim of this thesis is to get more insight in the vast amount of protein-protein interactions in which PBP1b is involved, and the function of these interactions in the regulation of the PG synthesis activity of PBP1b. We tried to discover new interaction partners, and establish the interaction surface of these new and known interaction partners, which knowledge could lead to the identification of new antibiotic targets. In chapter 2 we use the nonsense suppression mutagenesis technique to site specifically incorporate an unnatural amino acid with photo-crosslinking properties in the UB2H domain of PBP1b, to identify new interaction partners. In this way we identified, YbgF (later renamed into CpoB) as a new interactor of PBP1b, binding in a cleft between its TP and UB2H domain. In these experiments, FtsN was also shown to interact with the same cleft between the TP and UB2H domain of PBP1b. In chapter 3 we show that a loop of the TP domain, covering the active site, is essential for PBP1b TP activity. In addition, we show that this loop is an interaction hot spot, forming several crosslinks as a result of the incorporation of the photo-crosslinkable unnatural amino acid. In chapter 4 we test a new immobilization strategy for PBP1b on an SPR chip surface. Instead of coupling it to an immobilized ampicillin molecule, rendering the TP domain inactive, and resulting in an orientation far from the *in vivo* situation, we introduced an azide in the cytoplasmic tail to couple it to a cyclooctyne coating, resulting in the immobilization of a fully active protein, with an orientation which is more comparable to the *in vivo* situation. This new immobilization strategy produced similar results when analyzing the already known interaction of PBP1b with LpoB showing that this is a good alternative to the ampicillin immobilization. It might even be a better way for immobilization when analyzing interactions that might involve the TP domain and allows for testing of the effects of TP domain substrate binding to possible interactions. In chapter 5 we describe the creation of Lipid II variants containing different fluorescent labels, to analyze their functionality in two different GT assays. We show that in the in gel GT assay, most of the fluorescently labeled Lipid II variants give a higher resolution result than when using the previously used dansylated Lipid II variant. However, unlabeled substrate needed to be added to the reaction mixtures to initiate (proper) GT activity. In the continuous GT assay, two of the

fluorescently labeled Lipid II variants did not have the properties of a changing fluorescence intensity as a result of changing environment, so could not be used in this assay. The other variants did not show a lot of difference in comparison to using dansylated Lipid II. A contradiction between these two assays is that in the in gel assay, unlabeled Lipid II had to be added to reach a proper reaction speed, which was not needed in the continuous GT assay. In chapter 6 an attempt to identify the dimer interface of PBP1b is presented. The results show that the region between amino acids Y498 and F480 is important for dimer formation, and that amino acid E492, on a protruding loop structure in this region, could be highly important in the dimerization of PBP1b. In chapter 7, all the found results are summarized and discussed and future perspectives for this research are presented.

References

[1] W. Weidel and H. Pelzer, “Bagshaped macromolecules--a new outlook on bacterial cell walls,” *Adv. Enzymol. Relat. Areas Mol. Biol.*, vol. 26, pp. 193–232, 1964.

[2] W. Vollmer, D. Blanot, and M. A. De Pedro, “Peptidoglycan structure and architecture,” *FEMS Microbiol. Rev.*, vol. 32, no. 2, pp. 149–167, 2008.

[3] S. Dramsi, S. Magnet, S. Davison, and M. Arthur, “Covalent attachment of proteins to peptidoglycan,” *FEMS Microbiol. Rev.*, vol. 32, no. 2, pp. 307–320, Mar. 2008.

[4] G. D. Shockman and J. F. Barren, “Structure, Function, and Assembly of Cell Walls of Gram-Positive Bacteria,” *Annu. Rev. Microbiol.*, vol. 37, no. 1, pp. 501–527, 1983.

[5] L. Gan, S. Chen, and G. J. Jensen, “Molecular organization of Gram-negative peptidoglycan,” *Proc. Natl. Acad. Sci.*, vol. 105, no. 48, pp. 18953–18957, Dec. 2008.

[6] E. Lolis and R. Bucala, “Therapeutic approaches to innate immunity: severe sepsis and septic shock,” *Nat. Rev. Drug Discov.*, vol. 2, no. 8, pp. 635–645, Aug. 2003.

[7] K. H. Schleifer and O. Kandler, “Peptidoglycan types of bacterial cell walls and their taxonomic implications.,” *Bacteriol. Rev.*, vol. 36, no. 4, pp. 407–477, Dec. 1972.

[8] E. D. Brown, E. I. Vivas, C. T. Walsh, and R. Kolter, “MurA (MurZ), the enzyme that catalyzes the first committed step in peptidoglycan biosynthesis, is essential in Escherichia coli,” *J. Bacteriol.*, vol. 177, no. 14, pp. 4194–4197, Jul. 1995.

[9] T. E. Benson, D. J. Filman, C. T. Walsh, and J. M. Hogle, “An enzyme-substrate complex involved in bacterial cell wall biosynthesis,” *Nat. Struct. Biol.*, vol. 2, no. 8, pp. 644–653, Aug. 1995.

[10] D. Mengin-Lecreulx, B. Flouret, and J. van Heijenoort, “Cytoplasmic steps of peptidoglycan synthesis in Escherichia coli.,” *J. Bacteriol.*, vol. 151, no. 3, pp. 1109–1117, Sep. 1982.

[11] A. Bouhss, A. E. Trunkfield, T. D. H. Bugg, and D. Mengin-Lecreulx, “The biosynthesis of peptidoglycan lipid-linked intermediates,” *Fems Microbiol. Rev.*, vol. 32, no. 2, pp. 208–233, Mar. 2008.

[12] T. Mohammadi, V. van Dam, R. Sijbrandi, T. Vernet, A. Zapun, A. Bouhss, M. Diepeveen-de Bruin, M. Nguyen-Disteche, B. de Kruijff, and E. Breukink, “Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane,” *Embo J.*, vol. 30, no. 8, pp. 1425–1432, Apr. 2011.

[13] L.-T. Sham, E. K. Butler, M. D. Lebar, D. Kahne, T. G. Bernhardt, and N. Ruiz, “MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis,” *Science*, vol. 345, no. 6193, pp. 220–222, Jul. 2014.

[14] W. Vollmer and U. Bertsche, “Murein (peptidoglycan) structure, architecture and biosynthesis in Escherichia coli,” *Biochim. Biophys. Acta BBA - Biomembr.*, vol. 1778, no. 9, pp. 1714–1734, Sep. 2008.

[15] L. D. Tatar, C. L. Marolda, A. N. Polischuk, D. van Leeuweni, and M. A. Valvano, “An Escherichia coli undecaprenyl-pyrophosphate phosphatase implicated in undecaprenyl phosphate recycling,” *Microbiol.-Sgm*, vol. 153, pp. 2518–2529, Aug. 2007.

[16] T. Touzé, D. Blanot, and D. Mengin-Lecreulx, “Substrate specificity and membrane topology of Escherichia coli PgpB, an undecaprenyl pyrophosphate phosphatase,” *J. Biol. Chem.*, vol. 283, no. 24, pp. 16573–16583, Jun. 2008.

[17] T. Touzé, A. X. Tran, J. V. Hankins, D. Mengin-Lecreulx, and M. S. Trent, “Periplasmic phosphorylation of lipid A is linked to the synthesis of undecaprenyl phosphate,” *Mol. Microbiol.*, vol. 67, no. 2, pp. 264–277, Jan. 2008.

[18] A. Hynninen, T. Touzé, L. Pitkänen, D. Mengin-Lecreulx, and M. Virta, “An efflux transporter PbrA and a phosphatase PbrB cooperate in a lead-resistance mechanism in bacteria,” *Mol. Microbiol.*, vol. 74, no. 2, pp. 384–394, Oct. 2009.

[19] M. El Ghachi, A. Derbise, A. Bouhss, and D. Mengin-Lecreulx, “Identification of multiple genes encoding membrane proteins with undecaprenyl pyrophosphate phosphatase (UppP) activity in Escherichia coli,” *J. Biol. Chem.*, vol. 280, no. 19, pp. 18689–18695, May 2005.

[20] F. Cava, E. Kuru, Y. V. Brun, and M. A. de Pedro, “Modes of cell wall growth differentiation in rod-shaped bacteria,” *Curr. Opin. Microbiol.*, vol. 16, no. 6, pp. 731–737, Dec. 2013.

[21] K. C. Huang, R. Mukhopadhyay, B. Wen, Z. Gitai, and N. S. Wingreen, “Cell shape and cell-wall organization in Gram-negative bacteria,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 49, pp. 19282–19287, Dec. 2008.

[22] A. Koch, *Bacterial Growth and Form*. Springer, 2001.

[23] L. G. Burman and J. T. Park, “Molecular model for elongation of the murein sacculus of Escherichia coli.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 81, no. 6, pp. 1844–1848, Mar. 1984.

[24] J.-V. Höltje, “Growth of the Stress-Bearing and Shape-Maintaining Murein Sacculus of Escherichia coli,” *Microbiol. Mol. Biol. Rev.*, vol. 62, no. 1, pp. 181–203, Mar. 1998.

[25] T. K. Lee and K. C. Huang, “The role of hydrolases in bacterial cell-wall growth,” *Curr. Opin. Microbiol.*, vol. 16, no. 6, pp. 760–766, Dec. 2013.

[26] A. Typas, M. Banzhaf, C. A. Gross, and W. Vollmer, “From the regulation of peptidoglycan synthesis to bacterial growth and morphology,” *Nat. Rev. Microbiol.*, vol. 10, no. 2, pp. 123–136, Feb. 2012.

[27] R. D. Turner, A. F. Hurd, A. Cadby, J. K. Hobbs, and S. J. Foster, “Cell wall elongation mode in Gram-negative bacteria is determined by peptidoglycan architecture,” *Nat. Commun.*, vol. 4, p. 1496, 2013.

[28] T. Uehara and J. T. Park, “Growth of Escherichia coli: Significance of Peptidoglycan Degradation during Elongation and Septation,” *J. Bacteriol.*, vol. 190, no. 11, pp. 3914–3922, Jun. 2008.

[29] C. Goffin and J. M. Ghuysen, “Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs,” *Microbiol. Mol. Biol. Rev. MMBR*, vol. 62, no. 4, pp. 1079–1093, Dec. 1998.

[30] B. G. Spratt, “Distinct penicillin binding proteins involved in the division, elongation, and shape of Escherichia coli K12,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 72, no. 8, pp. 2999–3003, Aug. 1975.

[31] B. Spratt and J. Strominger, “Identification of Major Penicillin-Binding Proteins of Escherichia-Coli,” *J. Bacteriol.*, vol. 127, no. 1, pp. 660–663, 1976.

[32] E. Sauvage, F. Kerff, M. Terrak, J. A. Ayala, and P. Charlier, “The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis,” *FEMS Microbiol. Rev.*, vol. 32, no. 3, pp. 556–556, 2008.

[33] H. Hara and H. Suzuki, “A novel glycan polymerase that synthesizes uncross-linked peptidoglycan in Escherichia coli,” *FEBS Lett.*, vol. 168, no. 1, pp. 155–160, Mar. 1984.

[34] H. Suzuki, Y. Nishimura, and Y. Hirota, “On the process of cellular division in Escherichia coli: a series of mutants of E. coli altered in the penicillin-binding proteins.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 75, no. 2, pp. 664–668, Feb. 1978.

[35] G. Schiffer and J. V. Höltje, “Cloning and characterization of PBP 1C, a third member of the multimodular class A penicillin-binding proteins of Escherichia coli,” *J. Biol. Chem.*, vol. 274, no. 45, pp. 32031–32039, Nov. 1999.

[36] A. Budd, S. Blandin, E. A. Levashina, and T. J. Gibson, “Bacterial α2-macroglobulins: colonization factors acquired by horizontal gene transfer from the metazoan genome?,” *Genome Biol.*, vol. 5, no. 6, pp. 1–13, May 2004.

[37] T. J. Dougherty, K. Kennedy, R. E. Kessler, and M. J. Pucci, “Direct quantitation of the number of individual penicillin-binding proteins per cell in Escherichia coli,” *J. Bacteriol.*, vol. 178, no. 21, pp. 6110–6115, Nov. 1996.

[38] J. Nakagawa and M. Matsuhashi, “Molecular divergence of a major peptidoglycan synthetase with transglycosylase - transpeptidase activities in Escherichia coli — Penicillin-binding protein 1Bs,” *Biochem. Biophys. Res. Commun.*, vol. 105, no. 4, pp. 1546–1553, Apr. 1982.

[39] H. Suzuki, J. Kato, Y. Sakagami, M. Mori, A. Suzuki, and Y. Hirota, “Conversion of the alpha component of penicillin-binding protein 1b to the beta component in Escherichia coli.,” *J. Bacteriol.*, vol. 169, no. 2, pp. 891–893, Feb. 1987.

[40] T. A. Henderson, P. M. Dombrosky, and K. D. Young, “Artifactual processing of penicillin-binding proteins 7 and 1b by the OmpT protease of Escherichia coli.,” *J. Bacteriol.*, vol. 176, no. 1, pp. 256–259, Jan. 1994.

[41] J. K. Broome-Smith, A. Edelman, S. Yousif, and B. G. Spratt, “The nucleotide sequences of the ponA and ponB genes encoding penicillin-binding proteins 1A and 1B of Escherichia coli K12,” *Eur. J. Biochem.*, vol. 147, no. 2, pp. 437–446, 3/1/85 1985.

[42] C. Chalut, X. Charpentier, M.-H. Remy, and J.-M. Masson, “Differential Responses of Escherichia coli Cells Expressing Cytoplasmic Domain Mutants of Penicillin-Binding Protein 1b after Impairment of Penicillin-Binding Proteins 1a and 3,” *J. Bacteriol.*, vol. 183, no. 1, pp. 200–206, Jan. 2001.

[43] U. Bertsche, T. Kast, B. Wolf, C. Fraipont, M. E. G. Aarsman, K. Kannenberg, M. Von Rechenberg, M. Nguyen-Distèche, T. Den Blaauwen, J.-V. Höltje, and W. Vollmer, “Interaction between two murein (peptidoglycan) synthases, PBP3 and PBP1B, in Escherichia coli,” *Mol. Microbiol.*, vol. 61, no. 3, pp. 675–690, Aug. 2006.

[44] M.-T. Sung, Y.-T. Lai, C.-Y. Huang, L.-Y. Chou, H.-W. Shih, W.-C. Cheng, C.-H. Wong, and C. Ma, “Crystal structure of the membrane-bound bifunctional transglycosylase PBP1b from Escherichia coli,” *Proc. Natl. Acad. Sci.*, vol. 106, no. 22, pp. 8824–8829, Jun. 2009.

[45] A. Typas, M. Banzhaf, B. van den Berg van Saparoea, J. Verheul, J. Biboy, R. J. Nichols, M. Zietek, K. Beilharz, K. Kannenberg, M. von Rechenberg, E. Breukink, T. den Blaauwen, C. A. Gross, and W. Vollmer, “Regulation of Peptidoglycan Synthesis by Outer-Membrane Proteins,” *Cell*, vol. 143, no. 7, pp. 1097–1109, Dec. 2010.

[46] A. J. F. Egan, N. L. Jean, A. Koumoutsi, C. M. Bougault, J. Biboy, J. Sassine, A. S. Solovyova, E. Breukink, A. Typas, W. Vollmer, and J.-P. Simorre, “Outer-membrane lipoprotein LpoB spans the periplasm to stimulate the peptidoglycan synthase PBP1B,” *Proc. Natl. Acad. Sci.*, vol. 111, no. 22, pp. 8197–8202, Jun. 2014.

[47] A. N. Gray, A. J. F. Egan, I. L. van’t Veer, J. Verheul, A. Colavin, A. Koumoutsi, J. Biboy, M. A. F. Altelaar, M. J. Damen, K. C. Huang, J.-P. Simorre, E. Breukink, T. den Blaauwen, A. Typas, C. A. Gross, and W. Vollmer, “Coordination of peptidoglycan synthesis and outer membrane constriction during Escherichia coli cell division,” *eLife*, p. e07118, May 2015.

[48] M. Terrak, E. Sauvage, A. Derouaux, D. Dehareng, A. Bouhss, E. Breukink, S. Jeanjean, and M. Nguyen-Distèche, “Importance of the Conserved Residues in the Peptidoglycan Glycosyltransferase Module of the Class A Penicillin-binding Protein 1b of Escherichia coli,” *J. Biol. Chem.*, vol. 283, no. 42, pp. 28464–28470, Oct. 2008.

[49] M. Terrak, T. K. Ghosh, J. van Heijenoort, J. Van Beeumen, M. Lampilas, J. Aszodi, J. A. Ayala, J. M. Ghuysen, and M. Nguyen-Distèche, “The catalytic, glycosyl transferase and acyl transferase modules of the cell wall peptidoglycan-polymerizing penicillin-binding protein 1b of Escherichia coli,” *Mol. Microbiol.*, vol. 34, no. 2, pp. 350–364, Oct. 1999.

[50] C. A. Zijderveld, M. E. Aarsman, T. den Blaauwen, and N. Nanninga, “Penicillin-binding protein 1B of Escherichia coli exists in dimeric forms.,” *J. Bacteriol.*, vol. 173, no. 18, pp. 5740–5746, Sep. 1991.

[51] C. Chalut, M.-H. Remy, and J.-M. Masson, “Disulfide Bridges Are Not Involved in Penicillin-Binding Protein 1b Dimerization in Escherichia coli,” *J. Bacteriol.*, vol. 181, no. 9, pp. 2970–2972, May 1999.

[52] U. Bertsche, E. Breukink, T. Kast, and W. Vollmer, “In Vitro Murein (Peptidoglycan) Synthesis by Dimers of the Bifunctional Transglycosylase-Transpeptidase PBP1B from Escherichia coli,” *J. Biol. Chem.*, vol. 280, no. 45, pp. 38096–38101, Nov. 2005.

[53] P. Müller, C. Ewers, U. Bertsche, M. Anstett, T. Kallis, E. Breukink, C. Fraipont, M. Terrak, M. Nguyen-Distèche, and W. Vollmer, “The Essential Cell Division Protein FtsN Interacts with the Murein (Peptidoglycan) Synthase PBP1B in Escherichia coli,” *J. Biol. Chem.*, vol. 282, no. 50, pp. 36394–36402, Dec. 2007.

[54] W. Vollmer, M. von Rechenberg, and J.-V. Höltje, “Demonstration of Molecular Interactions between the Murein Polymerase PBP1B, the Lytic Transglycosylase MltA, and the Scaffolding Protein MipA of Escherichia coli,” *J. Biol. Chem.*, vol. 274, no. 10, pp. 6726–6734, Mar. 1999.

[55] C. Paradis-Bleau, M. Markovski, T. Uehara, T. J. Lupoli, S. Walker, D. E. Kahne, and T. G. Bernhardt, "Lipoprotein Cofactors Located in the Outer Membrane Activate Bacterial Cell Wall Polymerases," *Cell*, vol. 143, no. 7, pp. 1110–1120, Dec. 2010.

[56] T. J. Lupoli, M. D. Lebar, M. Markovski, T. Bernhardt, D. Kahne, and S. Walker, "Lipoprotein Activators Stimulate Escherichia coli Penicillin-Binding Proteins by Different Mechanisms," *J. Am. Chem. Soc.*, vol. 136, no. 1, pp. 52–55, Jan. 2014.

[57] W. Margolin, "Sculpting the Bacterial Cell," *Curr. Biol.*, vol. 19, no. 17, pp. R812–R822, Sep. 2009.

[58] W. Margolin, "FtsZ and the division of prokaryotic cells and organelles," *Nat. Rev. Mol. Cell Biol.*, vol. 6, no. 11, pp. 862–871, Nov. 2005.

[59] E. F. Bi and J. Lutkenhaus, "FtsZ ring structure associated with division in Escherichia coli," *Nature*, vol. 354, no. 6349, pp. 161–164, Nov. 1991.

[60] A. Mukherjee and J. Lutkenhaus, "Dynamic assembly of FtsZ regulated by GTP hydrolysis.," *EMBO J.*, vol. 17, no. 2, pp. 462–469, Jan. 1998.

[61] S. Rueda, M. Vicente, and J. Mingorance, "Concentration and assembly of the division ring proteins FtsZ, FtsA, and ZipA during the Escherichia coli cell cycle," *J. Bacteriol.*, vol. 185, no. 11, pp. 3344–3351, Jun. 2003.

[62] S. Pichoff and J. Lutkenhaus, "Tethering the Z ring to the membrane through a conserved membrane targeting sequence in FtsA," *Mol. Microbiol.*, vol. 55, no. 6, pp. 1722–1734, Mar. 2005.

[63] P. A. de Boer, R. E. Crossley, and L. I. Rothfield, "A division inhibitor and a topological specificity factor coded for by the minicell locus determine proper placement of the division septum in E. coli," *Cell*, vol. 56, no. 4, pp. 641–649, Feb. 1989.

[64] D. M. Raskin and P. A. J. de Boer, "Rapid pole-to-pole oscillation of a protein required for directing division to the middle of Escherichia coli," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 96, no. 9, pp. 4971–4976, Apr. 1999.

[65] H. Cho, H. R. McManus, S. L. Dove, and T. G. Bernhardt, "Nucleoid occlusion factor SlmA is a DNA-activated FtsZ polymerization antagonist," *Proc. Natl. Acad. Sci.*, p. 201018674, Feb. 2011.

[66] N. K. Tonthat, S. T. Arold, B. F. Pickering, M. W. Van Dyke, S. Liang, Y. Lu, T. K. Beuria, W. Margolin, and M. A. Schumacher, "Molecular mechanism by which the nucleoid occlusion factor, SlmA, keeps cytokinesis in check," *EMBO J.*, vol. 30, no. 1, pp. 154–164, Jan. 2011.

[67] A. J. F. Egan and W. Vollmer, "The physiology of bacterial cell division," *Ann. N. Y. Acad. Sci.*, vol. 1277, pp. 8–28, Jan. 2013.

[68] T. Mohammadi, G. E. J. Ploeger, J. Verheul, A. D. Comvalius, A. Martos, C. Alfonso, J. van Marle, G. Rivas, and T. den Blaauwen, "The GTPase activity of Escherichia coli FtsZ determines the magnitude of the FtsZ polymer bundling by ZapA in vitro," *Biochemistry (Mosc.)*, vol. 48, no. 46, pp. 11056–11066, Nov. 2009.

[69] J. Durand-Heredia, E. Rivkin, G. Fan, J. Morales, and A. Janakiraman, "Identification of ZapD as a Cell Division Factor That Promotes the Assembly of FtsZ in Escherichia coli," *J. Bacteriol.*, vol. 194, no. 12, pp. 3189–3198, Jun. 2012.

[70] J. M. Durand-Heredia, H. H. Yu, S. De Carlo, C. F. Lesser, and A. Janakiraman, "Identification and characterization of ZapC, a stabilizer of the FtsZ ring in Escherichia coli," *J. Bacteriol.*, vol. 193, no. 6, pp. 1405–1413, Mar. 2011.

[71] C. A. Hale, D. Shiomi, B. Liu, T. G. Bernhardt, W. Margolin, H. Niki, and P. A. J. de Boer, "Identification of Escherichia coli ZapC (YcbW) as a component of the division apparatus that binds and bundles FtsZ polymers," *J. Bacteriol.*, vol. 193, no. 6, pp. 1393–1404, Mar. 2011.

[72] E. Galli and K. Gerdes, "FtsZ-ZapA-ZapB interactome of Escherichia coli," *J. Bacteriol.*, vol. 194, no. 2, pp. 292–302, Jan. 2012.

[73] O. Espéli, R. Borne, P. Dupaigne, A. Thiel, E. Gigant, R. Mercier, and F. Boccard, "A MatP-divisome interaction coordinates chromosome segregation with cell division in E. coli," *EMBO J.*, vol. 31, no. 14, pp. 3198–3211, Jul. 2012.

[74] T. Uehara, K. R. Parzych, T. Dinh, and T. G. Bernhardt, “Daughter cell separation is controlled by cytokinetic ring-activated cell wall hydrolysis,” *EMBO J.*, vol. 29, no. 8, pp. 1412–1422, Apr. 2010.

[75] G. Liu, G. C. Draper, and W. D. Donachie, “FtsK is a bifunctional protein involved in cell division and chromosome localization in Escherichia coli,” *Mol. Microbiol.*, vol. 29, no. 3, pp. 893–903, Aug. 1998.

[76] J. C. Chen and J. Beckwith, “FtsQ, FtsL and FtsI require FtsK, but not FtsN, for co-localization with FtsZ during Escherichia coli cell division,” *Mol. Microbiol.*, vol. 42, no. 2, pp. 395–413, Oct. 2001.

[77] N. Buddelmeijer and J. Beckwith, “A complex of the Escherichia coli cell division proteins FtsL, FtsB and FtsQ forms independently of its localization to the septal region,” *Mol. Microbiol.*, vol. 52, no. 5, pp. 1315–1327, Jun. 2004.

[78] G. Karimova, N. Dautin, and D. Ladant, “Interaction network among Escherichia coli membrane proteins involved in cell division as revealed by bacterial two-hybrid analysis,” *J. Bacteriol.*, vol. 187, no. 7, pp. 2233–2243, Apr. 2005.

[79] C. Fraipont, S. Alexeeva, B. Wolf, R. van der Ploeg, M. Schloesser, T. den Blaauwen, and M. Nguyen-Distèche, “The integral membrane FtsW protein and peptidoglycan synthase PBP3 form a subcomplex in Escherichia coli,” *Microbiol. Read. Engl.*, vol. 157, no. Pt 1, pp. 251–259, Jan. 2011.

[80] D. S. Boyle, M. M. Khattar, S. G. Addinall, J. Lutkenhaus, and W. D. Donachie, “ftsW is an essential cell-division gene in Escherichia coli,” *Mol. Microbiol.*, vol. 24, no. 6, pp. 1263–1273, Jun. 1997.

[81] S. G. Addinall, C. Cao, and J. Lutkenhaus, “FtsN, a late recruit to the septum in Escherichia coli,” *Mol. Microbiol.*, vol. 25, no. 2, pp. 303–309, 1997.

[82] A. I. Rico, M. García-Ovalle, P. Palacios, M. Casanova, and M. Vicente, “Role of Escherichia coli FtsN protein in the assembly and stability of the cell division ring,” *Mol. Microbiol.*, vol. 76, no. 3, pp. 760–771, May 2010.

[83] M. A. Gerding, B. Liu, F. O. Bendezú, C. A. Hale, T. G. Bernhardt, and P. A. J. de Boer, “Self-enhanced accumulation of FtsN at Division Sites and Roles for Other Proteins with a SPOR domain (DamX, DedD, and RlpA) in Escherichia coli cell constriction,” *J. Bacteriol.*, vol. 191, no. 24, pp. 7383–7401, Dec. 2009.

[84] H. P. Erickson, “Modeling the physics of FtsZ assembly and force generation,” *Proc. Natl. Acad. Sci.*, vol. 106, no. 23, pp. 9238–9243, Jun. 2009.

[85] L. I. Rothfield and S. S. Justice, “Bacterial cell division: the cycle of the ring,” *Cell*, vol. 88, no. 5, pp. 581–584, Mar. 1997.

[86] R. A. Weigand, K. D. Vinci, and L. I. Rothfield, “Morphogenesis of the bacterial division septum: a new class of septation-defective mutants,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 73, no. 6, pp. 1882–1886, Jun. 1976.

[87] M. A. Gerding, Y. Ogata, N. D. Pecora, H. Niki, and P. A. J. De Boer, “The trans-envelope Tol–Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in E. coli,” *Mol. Microbiol.*, vol. 63, no. 4, pp. 1008–1025, Feb. 2007.

[88] J. N. Sturgis, “Organisation and evolution of the tol-pal gene cluster,” *J. Mol. Microbiol. Biotechnol.*, vol. 3, no. 1, pp. 113–122, Jan. 2001.

[89] E. Cascales, M. Gavioli, J. N. Sturgis, and R. Lloubès, “Proton motive force drives the interaction of the inner membrane TolA and outer membrane Pal proteins in Escherichia coli,” *Mol. Microbiol.*, vol. 38, no. 4, pp. 904–915, Nov. 2000.

[90] J.-C. Lazzaroni and R. Portalier, “The excC gene of Escherichia coli K-12 required for cell envelope integrity encodes the peptidoglycan-associated lipoprotein (PAL),” *Mol. Microbiol.*, vol. 6, no. 6, pp. 735–742, Mar. 1992.

[91] E. Bouveret, R. Derouiche, A. Rigal, R. Lloubes, C. Lazdunski, and H. Benedetti, “Peptidoglycan-associated lipoprotein-TolB interaction. A possible key to explaining the formation of contact sites between the inner and outer membranes of Escherichia coli,” *J. Biol. Chem.*, vol. 270, no. 19, pp. 11071–11077, May 1995.

[92] A. Walburger, C. Lazdunski, and Y. Corda, “The Tol/Pal system function requires an interaction between the C-terminal domain of TolA and the N-terminal domain of TolB,” *Mol. Microbiol.*, vol. 44, no. 3, pp. 695–708, May 2002.

[93] A. M. Krachler, A. Sharma, A. Cauldwell, G. Papadakos, and C. Kleanthous, “TolA Modulates the Oligomeric Status of YbgF in the Bacterial Periplasm,” *J. Mol. Biol.*, vol. 403, no. 2, pp. 270–285, Oct. 2010.

[94] C. J. Noren, S. J. Anthony-Cahill, M. C. Griffith, and P. G. Schultz, “A General Method for Site-Specific Incorporation of Unnatural Amino Acids into Proteins,” *Science*, vol. 244, no. 4901, pp. 182–188, Apr. 1989.

[95] L. Wang, J. Xie, and P. G. Schultz, “Expanding the Genetic Code,” *Annu. Rev. Biophys. Biomol. Struct.*, vol. 35, no. 1, pp. 225–249, 2006.

[96] L. Wang and P. G. Schultz, “A general approach for the generation of orthogonal tRNAs,” *Chem. Biol.*, vol. 8, no. 9, pp. 883–890, Sep. 2001.

[97] L. Wang, A. Brock, B. Herberich, and P. G. Schultz, “Expanding the Genetic Code of Escherichia coli,” *Science*, vol. 292, no. 5516, pp. 498–500, Apr. 2001.

[98] L. Wang and P. G. Schultz, “Expanding the Genetic Code,” *Angew. Chem. Int. Ed.*, vol. 44, no. 1, pp. 34–66, Jan. 2005.

[99] A. Madeira, E. Vikeved, A. Nilsson, B. Sjögren, P. E. Andrén, and P. Svenningsson, “Identification of protein-protein interactions by surface plasmon resonance followed by mass spectrometry,” *Curr. Protoc. Protein Sci. Editor. Board John E Coligan Al*, vol. Chapter 19, p. Unit19.21, Aug. 2011.

[100] H. H. Nguyen, J. Park, S. Kang, and M. Kim, “Surface Plasmon Resonance: A Versatile Technique for Biosensor Applications,” *Sensors*, vol. 15, no. 5, pp. 10481–10510, May 2015.

[101] J. Homola, S. S. Yee, and G. Gauglitz, “Surface plasmon resonance sensors: review,” *Sens. Actuators B Chem.*, vol. 54, no. 1–2, pp. 3–15, Jan. 1999.

[102] J. Homola, “Present and future of surface plasmon resonance biosensors,” *Anal. Bioanal. Chem.*, vol. 377, no. 3, pp. 528–539, Jul. 2003.

[103] B. Schwartz, J. A. Markwalder, and Y. Wang, “Lipid II: total synthesis of the bacterial cell wall precursor and utilization as a substrate for glycosyltransfer and transpeptidation by penicillin binding protein (PBP) 1b of Escherichia coli,” *J. Am. Chem. Soc.*, vol. 123, no. 47, pp. 11638–11643, Nov. 2001.

[104] M. S. VanNieuwenhze, S. C. Mauldin, M. Zia-Ebrahimi, B. E. Winger, W. J. Hornback, S. L. Saha, J. A. Aikins, and L. C. Blaszczak, “The first total synthesis of lipid II: the final monomeric intermediate in bacterial cell wall biosynthesis,” *J. Am. Chem. Soc.*, vol. 124, no. 14, pp. 3656–3660, Apr. 2002.

[105] X.-Y. Ye, M.-C. Lo, L. Brunner, D. Walker, D. Kahne, and S. Walker, “Better Substrates for Bacterial Transglycosylases,” *J. Am. Chem. Soc.*, vol. 123, no. 13, pp. 3155–3156, Apr. 2001.

[106] Y. Higashi, J. L. Strominger, and C. C. Sweeley, “Structure of a lipid intermediate in cell wall peptidoglycan synthesis: a derivative of a C55 isoprenoid alcohol.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 57, no. 6, pp. 1878–1884, Jun. 1967.

[107] J. N. Umbreit and J. L. Strominger, “Isolation of the Lipid Intermediate in Peptidoglycan Biosynthesis from Escherichia coli,” *J. Bacteriol.*, vol. 112, no. 3, pp. 1306–1309, Dec. 1972.

[108] J. S. Anderson, M. Matsuhashi, M. A. Haskin, and J. L. Strominger, “LIPID-PHOSPHOACETYLMURAMYL-PENTAPEPTIDE AND LIPID-PHOSPHODISACCHARIDE-PENTAPEPTIDE: PRESUMED MEMBRANE TRANSPORT INTERMEDIATES IN CELL WALL SYNTHESIS*,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 53, no. 4, pp. 881–889, Apr. 1965.

[109] H. Brötz, G. Bierbaum, K. Leopold, P. E. Reynolds, and H. G. Sahl, “The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II,” *Antimicrob. Agents Chemother.*, vol. 42, no. 1, pp. 154–160, Jan. 1998.

[110] N. E. Kramer, E. J. Smid, J. Kok, B. de Kruijff, O. P. Kuipers, and E. J. Breukink, “Resistance of Gram-positive bacteria to nisin is not determined by Lipid II levels,” *FEMS Microbiol. Lett.*, vol. 239, pp. 157–161, 2004.

[111] R. J. Watkinson, H. Hussey, and J. Baddiley, “Shared Lipid Phosphate Carrier in the Biosynthesis of Teichoic Acid and Peptidoglycan,” *Nature*, vol. 229, no. 2, pp. 57–59, Jan. 1971.

[112] J. van Heijenoort, “Recent advances in the formation of the bacterial peptidoglycan monomer unit,” *Nat. Prod. Rep.*, vol. 18, no. 5, pp. 503–519, Jan. 2001.

[113] E. Breukink, H. E. van Heusden, P. J. Vollmerhaus, E. Swiezewska, L. Brunner, S. Walker, A. J. R. Heck, and B. de Kruijff, “Lipid II Is an Intrinsic Component of the Pore Induced by Nisin in Bacterial Membranes,” *J. Biol. Chem.*, vol. 278, no. 22, pp. 19898–19903, May 2003.

[114] E. Swiezewska, W. Sasak, T. Mańkowski, W. Jankowski, T. Vogtman, I. Krajewska, J. Hertel, E. Skoczylas, and T. Chojnacki, “The search for plant polyprenols,” *Acta Biochim. Pol.*, vol. 41, no. 3, pp. 221–260, 1994.

[115] M. D. Hartley, P. E. Schneggenburger, and B. Imperiali, “Lipid bilayer nanodisc platform for investigating polyprenol-dependent enzyme interactions and activities,” *Proc. Natl. Acad. Sci.*, vol. 110, no. 52, pp. 20863–20870, Dec. 2013.

[116] E. Świeżewska and T. Chojnacki, “Long-chain polyprenols from Potentilla aurea,” *Phytochemistry*, vol. 30, no. 1, pp. 267–270, 1991.

[117] L. L. Danilov, T. N. Druzhinina, N. A. Kalinchuk, S. D. Maltsev, and V. N. Shibaev, “Polyprenyl phosphates: synthesis and structure-activity relationship for a biosynthetic system of Salmonella anatum O-specific polysaccharide,” *Chem. Phys. Lipids*, vol. 51, no. 3–4, pp. 191–203, Nov. 1989.

[118] U. Kohlrausch and J.-V. Höltje, “One-step purification procedure for UDP-N-acetylmuramyl-peptide murein precursors from Bacillus cereus,” *FEMS Microbiol. Lett.*, vol. 78, no. 2–3, pp. 253–257, Mar. 1991.

[119] A. J. F. Egan, J. Biboy, I. van’t Veer, E. Breukink, and W. Vollmer, “Activities and regulation of peptidoglycan synthases,” *Phil Trans R Soc B*, vol. 370, no. 1679, p. 20150031, Oct. 2015.

[120] L. E. Zawadzke, P. Wu, L. Cook, L. Fan, M. Casperson, M. Kishnani, D. Calambur, S. J. Hofstead, and R. Padmanabha, “Targeting the MraY and MurG bacterial enzymes for antimicrobial therapeutic intervention,” *Anal. Biochem.*, vol. 314, no. 2, pp. 243–252, Mar. 2003.

[121] G. Auger, J. van Heijenoort, D. Mengin-Lecreulx, and D. Blanot, “A MurG assay which utilises a synthetic analogue of lipid I,” *FEMS Microbiol. Lett.*, vol. 219, no. 1, pp. 115–119, Feb. 2003.

[122] V. van Dam, R. Sijbrandi, M. Kol, E. Swiezewska, B. de Kruijff, and E. Breukink, “Transmembrane transport of peptidoglycan precursors across model and bacterial membranes,” *Mol. Microbiol.*, vol. 64, no. 4, pp. 1105–1114, May 2007.

[123] G. Auger, M. Crouvoisier, M. Caroff, J. Van Heijenoort, and D. Blanot, “Synthesis of an analogue of the lipoglycopeptide membrane intermediate I of peptidoglycan biosynthesis,” *Int. J. Pept. Res. Ther.*, vol. 4, no. 4–6, pp. 371–376, 1997.

[124] T. Mohammadi, R. Sijbrandi, M. Lutters, J. Verheul, N. Martin, T. den Blaauwen, B. de Kruijff, and E. Breukink, “Specificity of the transport of Lipid II by FtsW in Escherichia coli,” *J. Biol. Chem.*, p. jbc.M114.557371, Apr. 2014.

[125] D. Münch, T. Roemer, S. H. Lee, M. Engeser, H. G. Sahl, and T. Schneider, “Identification and in vitro Analysis of the GatD/MurT Enzyme-Complex Catalyzing Lipid II Amidation in Staphylococcus aureus,” *PLoS Pathog*, vol. 8, no. 1, p. e1002509, Jan. 2012.

[126] A. Zapun, J. Philippe, K. A. Abrahams, L. Signor, D. I. Roper, E. Breukink, and T. Vernet, “In vitro Reconstitution of Peptidoglycan Assembly from the Gram-Positive Pathogen Streptococcus pneumoniae,” *ACS Chem. Biol.*, vol. 8, no. 12, pp. 2688–2696, Dec. 2013.

[127] S. S. Hegde and T. E. Shrader, “FemABX Family Members Are Novel Nonribosomal Peptidyltransferases and Important Pathogen-specific Drug Targets,” *J. Biol. Chem.*, vol. 276, no. 10, pp. 6998–7003, Mar. 2001.

[128] T. Schneider, M. M. Senn, B. Berger-Bächi, A. Tossi, H.-G. Sahl, and I. Wiedemann, “In vitro assembly of a complete, pentaglycine interpeptide bridge containing cell wall precursor (lipid II-Gly5) of Staphylococcus aureus,” *Mol. Microbiol.*, vol. 53, no. 2, pp. 675–685, Jul. 2004.

[129] M. Fonvielle, M. Chemama, R. Villet, M. Lecerf, A. Bouhss, J.-M. Valéry, M. Ethève-Quelquejeu, and M. Arthur, “Aminoacyl-tRNA recognition by the FemXWv transferase for bacterial cell wall synthesis,” *Nucleic Acids Res.*, vol. 37, no. 5, pp. 1589–1601, Apr. 2009.

Chapter 2

In vivo photo-crosslinking shows an interaction of *Escherichia coli* PBP1b with FtsN and YbgF/CpoB via a cleft between the UB2H domain and transpeptidase domain

Abstract

Penicillin binding protein (PBP) 1b is one of the two most important bifunctional peptidoglycan (PG) synthases of *E. coli*. PBP1b seems to be more important during cell divison than the other bifunctional PG synthase PBP1a, since it has a higher concentration at mid cell during the cell division process. To get more insight in the interaction profile of the UB2H domain of PBP1b, with known and new interacting proteins, we used the site specific incorporation of an unnatural amino acid with photo-crosslinkable properties, by the nonsense suppression mutagenesis method. In this way, we showed that the amino acids T118, E123, T751 and T753 of PBP1b, which line a cleft between the UB2H and transpeptidase (TP) domain, have an interaction with the same protein(s). By mass spectrometry analysis, we identified FtsN and YbgF as components of the crosslink products. Their presence was confirmed by immunoblotting with protein specific antibodies. These results show that PBP1b has an interaction with both FtsN and YbgF, via a cleft between the UB2H and TP domain.

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Coordination of peptidoglycan synthesis and outer membrane constriction during *Escherichia coli* cell division
Andrew N Gray, Alexander J F Egan, **Inge L van 't Veer**, Jolanda Verheul, Alexandre Colavin, Alexandra Koumoutsi, Jacob Biboy, Maarten A F Altelaar, Mirjam J Damen, Kerwyn Casey Huang, Jean-Pierre Simorre, Eefjan Breukink, Tanneke den Blaauwen, Athanassios Typas, Carol A Gross, Waldemar Vollmer.
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Introduction

The gram negative bacterial cell envelope is composed of an outer and an inner membrane, with a periplasmic space in between. In this periplasmic space, the peptidoglycan (PG) is situated, which provides the cell with strength to withstand turgor pressure and is involved in maintaining cell shape [1]–[3]. PG is a net-like structure, composed of alternating β-1,4-linked N-acetylglucosamine and N-acetylmuramic acid residues, crosslinked by peptides [4]. This net-like PG structure is created by two reactions. The first is the polymerization of the sugar moieties by the glycosyltransferase (GT) reaction. The second is the formation of crosslinks between the penta-peptides and subsequent insertion into the cell wall by the transpeptidation (TP) reaction [5].

E. coli has 3 bifunctional PG synthases, performing both reactions: the class A penicillin binding proteins (PBPs) PBP1a, PBP1b and PBP1c. Furthermore it possesses 2 monofunctional TPs the class B PBPs PBP2 and PBP3, and one monofunctional GT MtgA [6].

PBP1b is one of the two essential bifunctional PG synthases of *E. coli* (next to PBP1a) and is specifically localized at mid cell during cell division [7], [8]. *In vivo*, PBP1b exists in two isoforms, α, and γ, however, when analyzing membrane preparations, a third isoform is seen [9]. This β form is a 24 amino acids shorter degradation product, artificially created by cleavage of the α-isoform by OmpT [10], [11]. The α form is the full length, 844 amino acids long protein transcribed from the *PonB* (*MrcB*) gene. The γ form results from the translational start at an internal start codon at position M46, resulting in a protein of 799 amino acids [12]. Both isoforms are fully active, since they can complement a *PonB* deletion [13].

PBP1b has both GT and TP domains, and a transmembrane domain, anchoring it in the inner membrane. Furthermore, it has a UB2H domain, which is not involved in the enzymatic function of PBP1b, but deletion of this domain does cause an aberrant growth rate. This domain has been shown to serve as a binding domain for the interactions with other proteins, like MltA [14], LpoB [15], [16] and YbgF (later renamed into CpoB, this work and [17]).

PBP1b has been shown to form dimers *in vivo* by SDS-PAGE analysis of unboiled samples. These dimers are not formed by disulfide bridges, since the addition of β-mercaptoethanol did not dissociate the dimers, neither did the mutation of the cysteines in PBP1b [18], [19]. This dimerization has further been analyzed with surface plasmon resonance, resulting in a K_D value of 1,27 +/-0.96 $*10^{-7}$ for the dimerization of PBP1b [20]. The effect of dimerization on *in vitro* PBP1b activity is a significant higher activity of both the GT and TP domain of PBP1b. Dimerizing conditions increased the speed of glycan chain polymerization and the length of the produced glycan chains, furthermore, the percentage of peptide crosslinks increased as well [20]. PBP1b is known to interact with the essential

cell division proteins FtsN, which stimulates both GT and TP activity of PBP1b, and PBP3, which presence is necessary for the mid cell localization of PBP1b [8], [21]. PBP1b also interacts with the outer membrane lipoprotein LpoB, which regulates the activity of PBP1b, by stimulating its GT and TP activity and increasing glycan chain length produced [15], [16], [22], [23]. LpoB localizes at mid cell during cell division, and their interaction is essential for PBP1b function *in vivo* [15], [22]. This was the first outer membrane protein found to directly regulate the activity of a PG synthase. Since thus far it has been suggested that PG synthesis is a largely unregulated process only directed in space by cytoskeletal components [24]. These cell division specific interactions, together with the increased PBP1b concentration at mid cell during cell division, suggest a specific role for PBP1b and a higher order regulation of PG synthesis, by proteins in every layer of the cell envelope, during the cell division process.

In an effort to get more insight in the regulation of PBP1b, we aimed on identifying new interaction partners, as well as specifying the sites of known interactions of PBP1b. Since the UB2H domain has been shown to serve as a binding domain for several proteins interacting with PBP1b we focused on this domain in this study. We used the nonsense suppression mutagenesis technique to site specifically incorporate the photo-crosslinkable unnatural amino acid *p*-Benzoyl-L-phenylalanine (*p*Bpa) at different positions in the UB2H domain of PBP1b *in vivo*. By illumination with UV light, this photo-crosslinker will covalently couple to proteins in close proximity, and hence can reveal interaction partners of PBP1b.

In this way we show that the amino acids T118 and E123 of the UB2H domain, and T751 and T753 of the TP domain of PBP1b are involved in an interaction with both FtsN and YbgF. YbgF is a component of the Tol-Pal complex, which is as FtsN part of the cell division machinery and is involved in outer membrane constriction during cell division. These interactions could form a link between the processes of formation and completion of the divisome, PG synthesis and outer membrane constriction, which should be coordinated and tightly regulated with each other during the cell division process to ensure proper constriction of all three layers of the cell envelope without any leakage.

Materials and methods

Growth medium and conditions

Cultures were grown in LB at 37 °C, except when mentioned otherwise, supplemented with kanamycin (50 μg/mL) and/or chloramphenicol (34 μg/mL).

Bacterial strains and plasmids

E. coli DH5α cells were used for DNA amplification. *E. coli* BL21 (DE3) cells were used for protein expression and *in vivo* crosslinking experiments. *E. coli* EJ801 cells (a gift from M. Terrak) were used for the complementation assay.

Plasmid pDML924 carrying the *PonB* gene, encoding the N- terminal His_6-tagged PBP1bγ variant (gift from M. Terrak) was used for overexpression of PBP1b and as a template for the creation of PBP1b amber mutants. Plasmid pSub-BpaRS-6TRN encoding the orthogonal aminoacyl tRNA synthase-$tRNA_{CUA}$ pair was used for incorporation of *p*Bpa at the site of amber mutation (Gift from P.G. Schultz).

Site directed mutagenesis

The amber mutants were created by mutagenesis PCR using the primers listed in supplementary table 1. The reaction mixture contained 125 ng fwd and 125 ng rev primer, 1 µl dNTPs, 10 mM each, 0.5 µl 77ng/µl template and 1 µl Pfu turbo DNA polymerase (stratagene 2.5U/µl) in a total volume of 50 µl. 17 cycles of 30 sec @ 95°C, 1 min @ Tm (depending on used primers) and 9 min @ 68°C were performed. PCR products were digested with 10 U DpnI (Fermentas), per reaction and amplified in *E. coli* DH5α. Sequencing was performed to confirm the mutation in the DNA.

In vivo crosslinking experiments

E. coli BL21(DE3) cells were co-transformed with pDML924 containing the amber mutation and pSub-BpaRS-6TRN. Cells were grown till the culture reached an OD_{600} of 0,5-0,6. Protein production was induced with 10 µM IPTG, and 1 mM freshly prepared photo-crosslinker *p*-benzoyl-L-phenylalanine (Bachem) dissolved in 1M NaOH was added. After 2 hours of protein production, cells were harvested by centrifugation, washed with PBS and resuspended in 3 mL PBS. This was transferred into a petridish and exposed to UV light of 365 nm for 90 sec (photoMax Housing 200W, Oriel Instruments, model 60100, 30cm distance to sample). The sample was cooled on ice during UV illumination. A sample was taken and analyzed by western blot. Rest of the cells were pelleted and stored at -80°C.

Western blot analysis

Samples were separated by SDS-PAGE (8%) and blotted semi dry on nitrocellulose membrane (bio-rad) for 40 min @ 15 V. Used antibodies: monoclonal anti-polyhistidine peroxidase conjugate antibody (1:4000 dilution, Sigma Aldrich), rabbit-anti-YbgF/CpoB (1:40.000 dilution, a gift from W. Vollmer) and rabbit-anti-FtsN (1:10.000 dilution, a gift from W. Vollmer). As secondary antibody goat-anti-rabbit IgG(H+L)-HRP conjugate (1:3000 dilution, bio-rad) was used.

PBP1b and crosslink product purification

Cells from 20 mL culture were resuspended in 2 mL 20 mM Tris-HCL pH 8.0, 300 mM NaCL, 5 mM imidazole (Buffer A), supplemented with 25 µM phenylmethylsulphonyl fluoride (PMSF) and ¼ complete EDTA free protease inhibitor tablet (in total of 20 mL (Roche)). Cells were lysed by sonification using a sonicator with microtip (Branson Sonifier 250). Sonication was done for 6 rounds of 30 pulses. Intact cells were removed by centrifugation for 10 min at 3500 g (Sorvall legend RT, swinging buckets rotor 7500 6445). The cell lysate was centrifuged for 45 min at 200.000 g at 4°C (Sorvall WX 80 Ultra, Ti 60). Membrane fraction (= pellet) was solubilized in 1,2 mL buffer A supplemented with 2% Triton X-100, by stirring the membrane suspension for 2 h at 4°C. Insoluble material was removed by centrifugation for 45 min at 200.000 g at 4°C. Solubilized proteins were incubated with 50 µl Ni^{2+} Sepharose beads (GE healthcare) O/N at 4°C. Ni^{2+} Sepharose beads were transferred to a filter column and flow trough was collected. Columns were washed with 1.2 mL buffer A. Next washing steps were done with 1.2 mL buffer A with 20 mM imidazole and 0.1% Triton X-100 and 1.2 mL buffer A with 100 mM imidazole and 0.1% Triton X-100. Proteins were eluted with 600 µl buffer A with 150 mM imidazole and 0.1% Triton X-100 and 2x 300 µl buffer A with 500 mM imidazole and 0.1% Triton X-100. Fractions were stored at 4°C.

Mass spectrometry

Excised protein bands were reduced with DTT, alkylated with iodoacetamide and in-gel digested with trypsin [25]. Nanoflow liquid chromatography coupled to mass spectrometry was performed on an Agilent 1200 nanoflow system (Agilent technologies, Amstelveen, The Netherlands) connected to a MS LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany). The samples were trapped on a 20 mm ReproSil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany) trapping column (packed in-house, i.d., 100 µm; resin, 5 µm) with a flow-rate of 5 µL min^{-1}. Sequential elution of peptides was accomplished using an analytical column (Dr. Maisch GmbH, Germany; packed in-house, i.d., 50 µm; resin, 3 µm) with a 35 min gradient of 10–38% buffer B (buffer A, 0.1 M acetic acid; buffer B, 0.1 M acetic acid, 80% (v/v) acetonitrile) followed by 38–100% B in 3 min, 100% B for 2 min. The flow rate was passively split from 0.45 mL/min to 100 nL/[26]. Nanospray was achieved using a distally coated fused silica emitter (made in-house, o.d. 375 µm; i.d. 20 µm) biased to 1.7 kV. mass spectrometer was operated in the data dependent mode to automatically switch between MS and MS/MS. The high resolution survey full scan was acquired in the orbitrap from m/z 350 to m/z 1500 with a resolution of 30.000 (FHMW). The most intense ions at a threshold of above 500 were fragmented in the linear ion trap using collision-induced dissociation at a target value of 10.000.

Data analysis: Identification

Peak lists were generated from the raw data files using the Proteome Discoverer software package version 1.3.339 (Thermo Scientific, Bremen, Germany). Peptide identification was performed by searching the individual peak lists against a concatenated target-decoy database containing the *E. coli* sequences in the Uniprot database (release 2012_06) supplemented with a common contaminants database using the Mascot search engine version 2.3 (Matrix Science, London, United Kingdom) via the Proteome Discoverer interface. The search parameters included the use of trypsin as proteolytic enzyme allowing up to a maximum of 2 missed cleavages. Carbamidomethylation of cysteines was set as a fixed modification whereas oxidation of methionines were set as variable modifications. Precursor mass tolerance was initially set at 50 ppm, while fragment mass tolerance was set at 0.6 Da. Subsequently, the peptide identifications were filtered for an ion score of 20.

Complementation assay

O/N cultures of *E. coli* strain EJ801 co-transformed with pDML924 containing the amber mutations and pSub-BpaRS-6TRN were grown at 30°C. The O/N culture was diluted 1/50 in fresh LB medium. After 30 min, protein production was induced by the addition of 10 µM IPTG and 1 mM freshly dissolved *p*Bpa was added. Cells were grown at 30°C and OD_{600} was monitored. When OD_{600} reached 0.3, cultures were split and half continued growth at 30°C and the other half at 42°C. As a positive control *E. coli* EJ801 transformed with pDML924 was taken along in this experiment.

Results

Interaction profiling of different areas of PBP1bs UB2H domain

In a search for yet unidentified interaction partners of PBP1b we used the nonsense suppression mutagenesis technique to site specifically incorporate an unnatural amino acid, with photo-crosslinking properties in PBP1b *in vivo*. We focused on the UB2H domain, since it has been shown that this domain is involved in interactions of PBP1b with other proteins [14], [16]. We aimed on covering different areas of this domain, to be able to catch as many different interactions as possible. The chosen sites are depicted in red in figure 1A.

Plasmids encoding PBP1b, containing amber TAG mutations replacing the codon of the amino acids to be substituted for the photo-crosslinker *p*Bpa, were introduced in *E. coli* BL21 (DE3) cells. Additionally, a plasmid encoding the orthogonal tRNA-tRNA synthase pair, producing tRNAs loaded with the photo-crosslinker and suppressing the amber mutation, was introduced in these cells as well. Growing the cells in presence of *p*Bpa resulted in the incorporation of this unnatural amino acid, indicated by the presence of

full length PBP1b (Fig 1B, +IPTG & + *p*Bpa). The unnatural amino acid incorporation was highly efficient in most PBP1b mutants, producing similar amounts of full length protein. Only when the amber mutation was introduced at position N189, the unnatural amino acid was not incorporated, and hence no full length PBP1b was produced. In absence of *p*Bpa, a short fragment of the protein was produced ending at the site of the introduced mutation (not shown).

If these amino acids are involved in an interaction with other proteins, UV illumination will

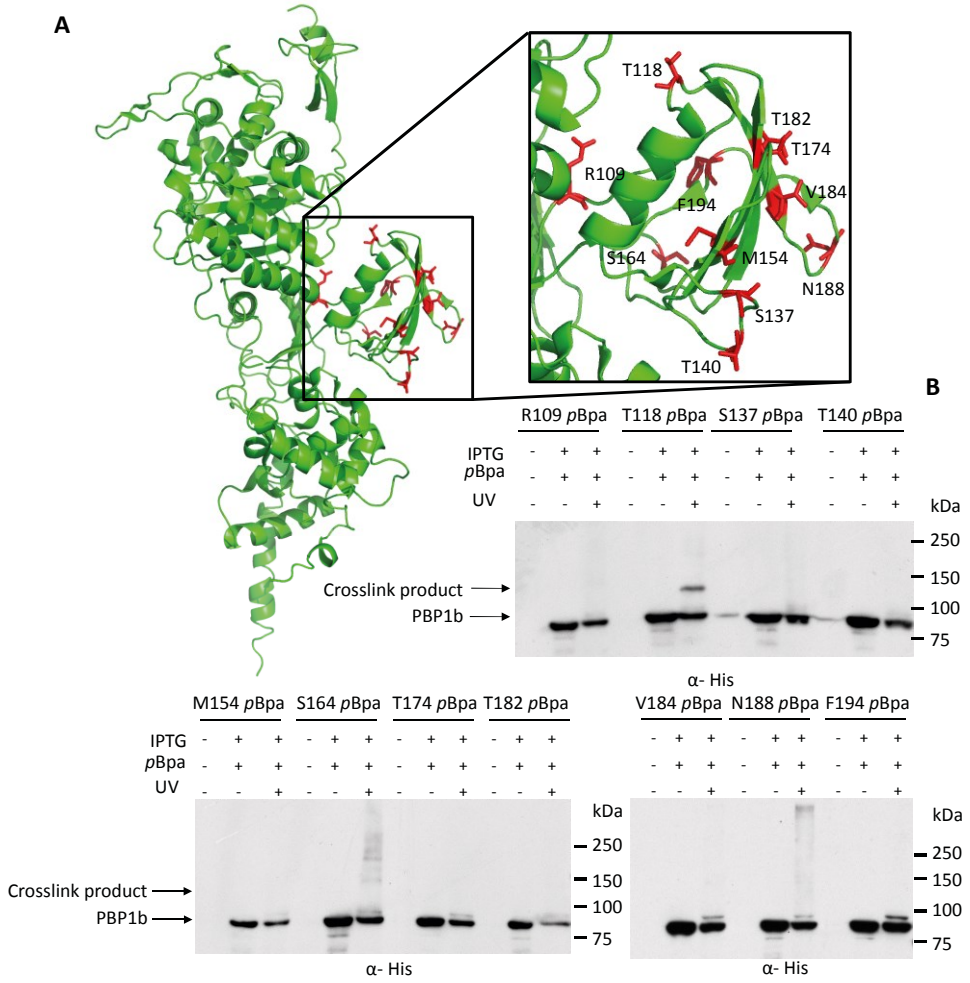

Figure 1. Incorporation of *p*Bpa in PBP1b, and production and detection of crosslink products.
Bacterial cell lysates of cells producing PBP1b, with incorporated photo-crosslinker at the indicated positions were separated on SDS-PAGE and analyzed by western blot. A) Crystal structure of PBP1b (3FWL), chosen sites for *p*Bpa incorporation are indicated in red. B) Photo-crosslinker incorporation at position T118, S164 and F194 produce crosslink product(s) as a result of UV illumination.

induce a covalent bond between PBP1b and its interacting proteins. This will be visible by the appearance of higher molecular weight bands in western blot analysis. Figure 1B shows that crosslinker incorporation and subsequent illumination with UV light at the positions R109, S137, T140, M154, T174, T182, V184 and N188 did not produce any (clear) crosslink products, indicating that these amino acids are not involved in an interaction of PBP1b with other proteins. Nevertheless, photo-crosslinker incorporation at position T118 showed a clear band at around 120 kDa, at position S164 multiple lighter bands (clearer after longer exposure Fig S1A) of higher molecular weight, and at position F194 a band slightly higher than PBP1b (which is also vaguely visible in the samples of S164*p*Bpa, T174*p*Bpa, V184*p*Bpa and N188*p*Bpa) as a result of UV illumination. This indicates the involvement of these amino acids in an interaction with other proteins (Fig 1B, +IPTG, + *p*Bpa & + UV).

Mapping of the interaction site

Incorporation of the crosslinker at positions T118 and F194 produced the most pronounced crosslink products, indicating that these sites specifically interact with another protein. Previously, the band slightly higher than PBP1b, produced as a result of crosslinker incorporation at position F194, has been observed as a result of crosslinker incorporation and UV illumination at different positions. However, we have never been able to determine the crosslinked protein in this band. Therefore we decided to focus on the crosslink product produced by T118*p*Bpa, to further map the interaction surface of this interaction, as the interaction surface between two proteins is likely to be larger than one amino acid. Subsequently, we introduced the photo-crosslinker at sites in close proximity to T118. We first substituted the amino acids N122, E123, K126 and E129 by the photo-crosslinker to test if the alpha helix close to T118 is involved in this interaction (Fig 2A, magenta). E123 points in the same direction as T118, N122 and E129 to the opposite side of the α helix, and K126 is oriented in the middle of these two positions, covering the exposed part of the α helix. Additionally, we introduced the photo-crosslinker at position D116 on the same loop, but pointing in a different direction, and D178 on a different loop, pointing in the same direction as T118, to investigate whether these loops at the top of the UB2H domain are involved in this interaction (Fig 2A, blue). Furthermore, we introduced the photo-crosslinker at the positions T751 and T753 in the TP domain, which, together with T118, seem to be part of a cleft between the UB2H domain and the TP domain (Fig 2A, Yellow). The result of *p*Bpa incorporation at these sites and subsequent UV illumination is shown in figure 2B. It can be seen that photo-crosslinker incorporation at positions N122, K126 and E129 do not produce any crosslink product, D116 *p*Bpa produces some not so bright crosslink products (clearer after long exposure Fig S1B), while photo-crosslinker incorporation at positions E123, T751 and T753 seem to produce a crosslink product of the same mass as produced by T118*p*Bpa (Fig 2B).

These results suggest that the amino acids T118, E123, T751 and T753 of the cleft between the UB2H domain and the TP domain are part of an interaction surface with the same protein.

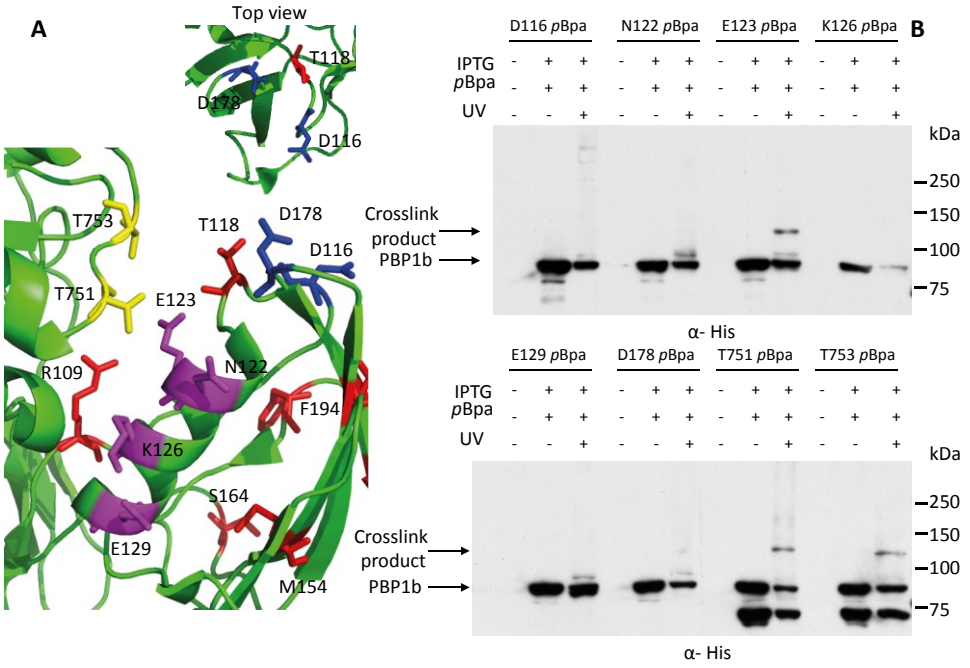

Figure 2. Incorporation of *p*Bpa in PBP1b, and production and detection of crosslink products.
Bacterial cell lysates of cells producing PBP1b, with incorporated photo-crosslinker at the indicated positions were separated on SDS-PAGE and analyzed by western blot. A) Crystal structure of PBP1b, additional sites for *p*Bpa incorporation are indicated in magenta (α helix in UB2H domain), blue (loops at top of UB2H domain) and yellow (TP domain). B) Photo-crosslinker incorporation at position D116 produces some vague crosslink products after UV illumination, and incorporation at positions E123, T751 and T753 seem to produce a crosslink product of the same size as the crosslink product produced by T118*p*Bpa.

Incorporation of *p*Bpa does not interfere with *in vivo* functionality of PBP1b

The *in vivo* functionality of the PBP1b mutants with incorporated photo-crosslinker was analyzed with a complementation assay using the *E. coli* EJ801 strain. These cells lack PBP1b and have a thermo sensitive PBP1a variant which becomes inactive at 42°C. As a result, cells can only survive if they are supplied with an active PBP1b protein. *E. coli* EJ801 cells co-transformed with PBP1bT118TAG, PBP1bE123TAG, PBP1bT751TAG or PBP1bT753TAG and the plasmid encoding the orthogonal tRNA-tRNA synthase pair were all able to grow at 42°C, indicating that these PBP1b mutants, with incorporated photo-crosslinker at the sites T118, E123, T751 and T753 can complement the absence of PBP1b, showing that these proteins are active *in vivo* (Fig 3). This also shows that the crosslinks

represent complexes between functional and correctly localized PBP1b variants and another protein.

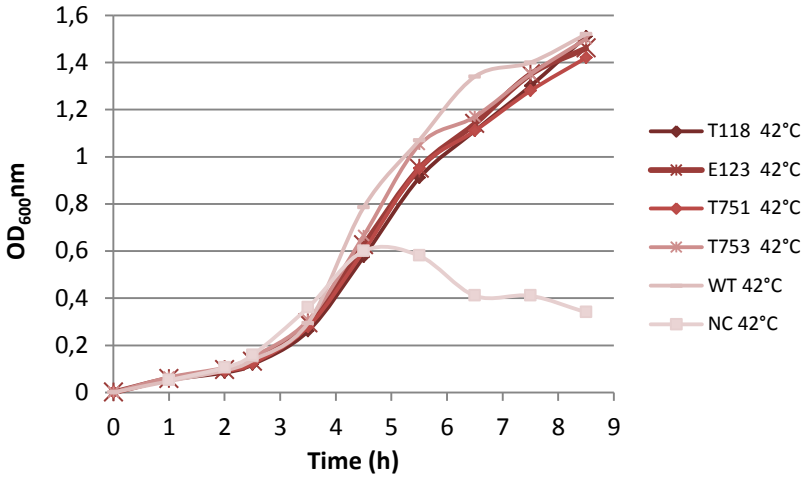

Figure 3. Complementation assay to analyze the i*n vivo* functionality of PBP1b proteins with photo-crosslinker incorporated at the indicated sites.
The PBP1b variants were expressed in *E. coli* EJ801 and growth was followed. The temperature was shifted to 42°C at OD 0.3. Cells containing PBP1b with photo-crosslinker incorporated at the four indicated positions grew as well as the positive control, expressing wild type PBP1b, indicating that these proteins are functional *in vivo*. WT= EJ801 cells expressing wild type PBP1b, NC = EJ801 cells lacking PBP1b.

Identification of the proteins in the crosslink product

As the amino acids T118, E123, T751 and T753 seem to be involved in an interaction with the same protein, we continued with the identification of the protein(s) in the crosslink product. The crosslink products of the mutants T118*p*Bpa, E123*p*Bpa, T751*p*Bpa and T753*p*Bpa were purified by nickel affinity chromatography using the his tag in PBP1b. The fractions, containing pure PBP1b and crosslink product, were separated by SDS-PAGE and stained with coomassie brilliant blue (Fig 4). The crosslink products were collected from the gel and subsequently analyzed by mass spectrometry. This resulted in a list of proteins of which peptides were identified in the crosslink products and were absent in a control sample. It appeared that the list contained a lot of proteins that have an interaction with PBP1b during its way to maturity. For example, SecF was found in all four crosslink products, which is part of the protein translocase complex, exporting PBP1b from the cytoplasm to the periplasm. Furthermore, it contained chaperones, like SurA and FkpA, helping in correct folding of periplasmic proteins. This likely indicates that crosslinks were obtained during all stages of the cell cycle, and with that all stages of PBP1b biogenesis.
This list was filtered according to size and cellular localization of the identified proteins, ending up with a list of possible interactors, including two interesting candidates; FtsN a protein known to interact with PBP1b [21], and YbgF, part of the Tol-Pal complex, for

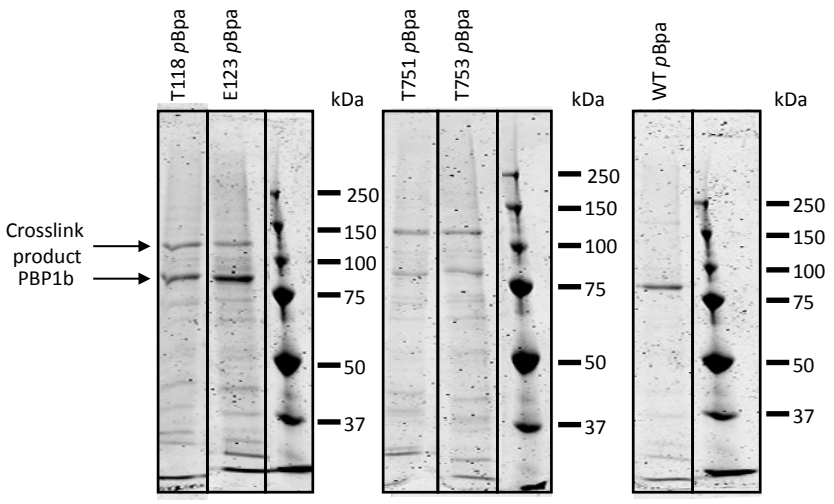

Figure 4. Purification of the crosslink products produced by the mutants T118, E123, T751 and T753. PBP1b and the produced crosslink product were purified using nickel affinity chromatography. The fractions containing the crosslink product were separated on SDS-PAGE, stained with coomassie brilliant blue, and analyzed by mass spectrometry.

which it is suggested that the PBP1b-LpoB complex can compensate in its absence [15] (highlighted in yellow (Table 1)). FtsN is an essential cell division protein of which it has already been shown that it interacts with PBP1b [21], [27]. We confirmed the presence of FtsN in the crosslink product by western blot analysis using FtsN specific antibodies, on the purified fractions, containing PBP1b and the crosslink product (Fig 5B). As a result, the crosslink product was detected. Unfortunately, this antibody has cross reactivity against PBP1b, resulting in PBP1b being detected as well. However, if the intensity of the bands on the blot probed with antibodies against FtsN is compared to the intensity of the bands on the blot stained with antibodies against the poly histidine tag, it can be seen that the pattern is different. The crosslink bands on the blot stained with antibodies against FtsN are more intensely stained in proportion to PBP1b. This points toward FtsN being present in the crosslink product. If only PBP1b in the crosslink product is recognized by the FtsN specific antibody, a similar pattern as when staining with antibodies against the poly histidine tag is expected.

The other interesting candidate identified by mass spectrometry, YbgF, is a protein encoded by the Tol-Pal operon [28]. The Tol-Pal complex is part of the cell division machinery and is involved in outer membrane constriction, but the role of YbgF in this process remains unclear. The complex, including YbgF, localizes at mid cell during cell division, and it is suggested that the PBP1b-LpoB complex, another mid cell localizing complex, can compensate for the absence of the Tol-Pal complex [15], [29]. YbgF is not an essential protein, since deletion mutants show no specific phenotype. However, it is

	T118				E123						
Protein	Score	Coverage	# Peptides	# PSMs	Score	Coverage	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
PBP1b	610744	68,96%	74	1995	53244,57	67,65%	56	1791	844	94,2	9,07
SecF	66,36	8,05%	2	2	163,54	10,22%	3	3	323	35,4	5,85
MreB	66,06	8,93%	2	2	84,92	11,53%	3	3	347	36,9	5,26
LpoB	65,94	6,57%	1	1	97,45	6,57%	1	2	213	22,5	6,93
Chain length determinant protein wzzB	62,88	3,38%	1	1	89,53	6,46%	2	2	326	36,4	5,59
DedD	54,48	5,45%	1	1	21,06	5,45%	1	1	220	22,9	8,21
FtsN	5126	6,90%	2	2	12156	16,93%	5	5	319	35,8	10,17
Uncharacterized protein YgiM	42,99	6,80%	1	2	63,57	11,17%	2	2	206	23,1	9,11
Uncharacterized protein YheO	42,23	2,92%	1	1	54,29	2,92%	1	2	240	26,8	5,66
YdgH	372,71	33,12%	8	12	55,34	8,60%	2	2	314	33,9	9,28
Lipoprotein 28	33,92	2,57%	1	1	38,18	8,46%	2	2	272	29,4	6,16
MurG	33,88	5,35%	1	1	124,37	15,21%	4	4	355	37,8	9,73
Acriflavine resistance protein A	32175	18,14%	6	9	357,52	16,88%	7	11	397	42,2	7,99
Uncharacterized protein YbgF	300,52	19,77%	7	12	79,99	11,41%	3	4	263	28,2	8,65
YgiW	186,18	26,15%	3	6	148,83	17,69%	2	5	130	14	5,26
DacA	163,54	13,65%	4	4	58,67	6,45%	2	2	403	44,4	8,28
Chaperone SurA	102,2	12,85%	5	5	43,15	4,21%	2	2	428	47,3	6,98
peptidyl-prolyl cis-trans isomerase FkpA	339	35,56%	8	11	259,55	20,74%	5	10	270	28,9	8,47
	T751				T753						
Protein	Score	Coverage	# Peptides	# PSMs	Score	Coverage	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
PBP1b	54508,48	67,65%	56	1796	62434,21	68,96%	74	1998	844	94,2	9,07
SecF	163,54	10,22%	3	3	66,36	8,05%	2	2	323	35,4	5,85
MreB	84,92	11,53%	3	3	66,06	8,93%	2	2	347	36,9	5,26
LpoB	97,45	6,57%	1	2	65,94	6,57%	1	1	213	22,5	6,93
Chain length determinant protein wzzB	97,54	6,44%	2	2	62,88	3,37%	1	1	326	36,4	5,59
DedD	2106	5,45%	1	1	54,48	5,45%	1	1	220	22,9	8,21
FtsN	124,85	16,93%	5	5	5126	6,90%	2	2	319	35,8	10,17
Uncharacterized protein YgiM	63,57	11,17%	2	2	42,99	6,80%	1	2	206	23,1	9,11
Uncharacterized protein YheO	54,29	2,92%	1	2	42,23	2,92%	1	1	240	26,8	5,66
YdgH	56,34	8,60%	2	2	387,34	33,12%	8	12	314	33,9	9,28
Lipoprotein 28	38,18	8,46%	2	2	33,92	2,57%	1	1	272	29,4	6,16
MurG	124,37	15,21%	4	4	33,88	5,35%	1	1	355	37,8	9,73
Acriflavine resistance protein A	379,68	16,88%	7	11	328,59	18,14%	6	9	397	42,2	7,99
Uncharacterized protein YbgF	79,99	11,41%	3	4	30164	19,77%	7	12	263	28,2	8,65
YgiW	148,83	17,69%	2	5	186,18	26,15%	3	6	130	14	5,26
DacA	58,67	6,45%	2	2	163,54	13,65%	4	4	403	44,4	8,28
Chaperone SurA	45,24	4,21%	2	2	110,05	12,85%	5	5	428	47,3	6,98
peptidyl-prolyl cis-trans isomerase FkpA	259,55	20,74%	5	10	339	35,56%	8	11	270	28,9	8,47

Table 1. Mass spectrometry analysis of the crosslink products.

Proteins of which peptides were identified by mass spectrometry in the crosslink products, and not in the control sample, filtered according to size and cellular localization are shown. Interesting candidates are highlighted in yellow. Score: addition of individual scores of the ion fragmentation spectra of the identified peptides. Coverage: percentage of protein sequence covered by the identified peptides. #peptides: number of unique peptides of the protein identified in the sample. #PMS: number of individual spectra in which the peptides were identified.

expected to have some importance in the regulation of the outer membrane invagination process, since YbgF is highly conserved among gram negative bacteria [29]. We also confirmed the presence of this protein in the crosslink product by western blot analysis (Fig 5B). This gave a similar result as for FtsN. The crosslink product is detected, however, these antibodies have cross reactivity against PBP1b as well. Again, the crosslink products are detected with higher intensity in proportion to the intensity of PBP1b, compared to the intensity as a result of staining with antibodies against the poly histidine tag.

These results show that PBP1b interacts with both FtsN and YbgF with the same interaction interface, including the amino acids T118, E123, T751 and T753 (Fig 5A). These interactions could serve as a link between the accomplishment of the assembly of the

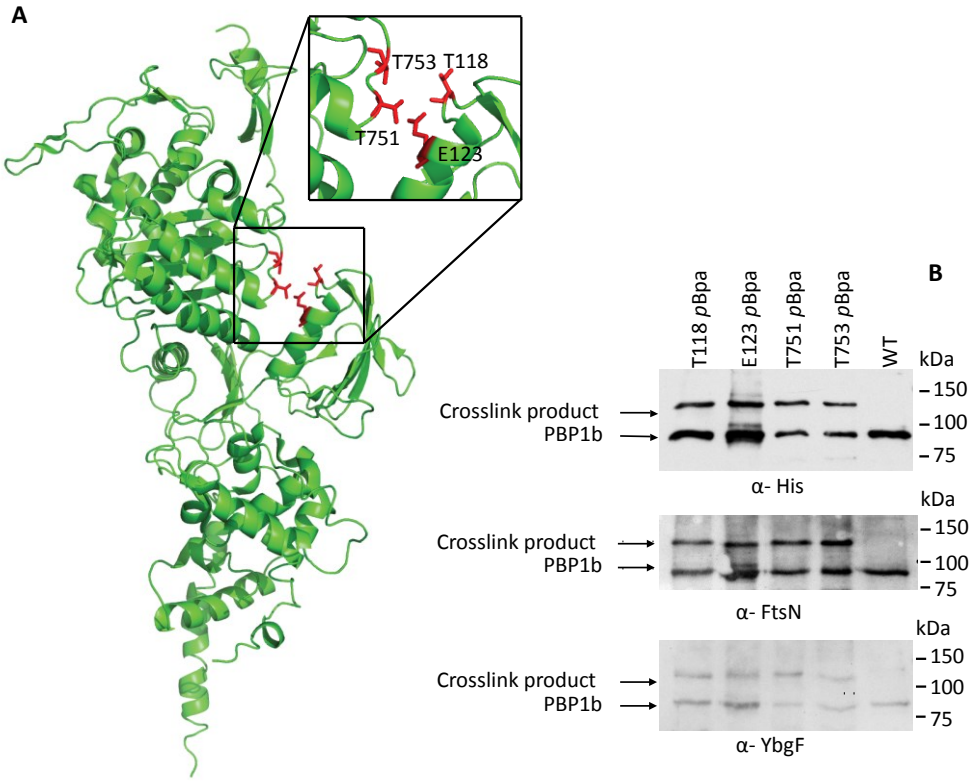

Figure 5. The amino acids T118, E123, T751 and T753 line a cleft that interacts with FtsN and YbgF.
PBP1b variants with *p*Bpa incorporated at the indicated sites was expressed in *E. coli* and illuminated with UV. PBP1b and produced crosslink products were purified using nickel affinity. The fractions containing the crosslink product were separated on SDS-PAGE, and analyzed by western blot with different antibodies. A) Crystal structure of PBP1b, sites crosslinked to FtsN and YbgF after *p*Bpa incorporation and UV illumination are indicated in red. B) Western blot analysis showed that crosslink products of ~30 kDa larger than PBP1b were produced as a result of incorporating *p*Bpa at the positions T118, E123, T751 and T753 and subsequent UV illumination. Staining with anti-FtsN and anti-YbgF antibodies confirmed the presence of both these proteins in the crosslink products.

division ring, of which FtsN is the last arriving proteins [30], the start of outer membrane constriction by the Tol-Pal complex, and the regulation of PG synthesis by PBP1b during these processes. These processes should be tightly regulated in respect to each other to ensure proper constriction of all three layers of the cell envelope, and completion of cell division without the loss of cell envelope integrity resulting in leakage.

Discussion

During cell division, all three layers of the cell envelope need to constrict to form two new daughter cells. This constriction needs to be tightly coordinated in space and time, to ensure integrity of all three layers during any moment of the constriction process. Here we show the interaction of PBP1b via a cleft between the UB2H domain and TP domain with FtsN, of which it has already been shown to interact with PBP1b, and with a new interaction partner YbgF (Fig 5). These interactions can form a link between the three cell envelope layers of the inner membrane (FtsN), the PG layer (PBP1b and FtsN) and the outer membrane (Tol-Pal complex via YbgF).

These results demonstrate that the *in vivo* photo-crosslinking approach is highly specific, displaying large differences in crosslink products produced as a result of crosslinker incorporation at positions in close proximity to each other. For example photo-crosslinker incorporation at position D116 showed a completely different pattern of produced crosslink products than T118, though these amino acids are very close to each other on the same loop of the UB2H domain (Fig S2).

It appeared that in all the results of PBP1b variants producing a crosslink product, there is still quite some not crosslinked, full length PBP1b present. This is caused by the fact that crosslinking is never 100% efficient. Not all PBP1b molecules are necessarily bound to an interacting protein at a certain time point. Furthermore, some interactions only take place during an explicit phase of the cell cycle, so will not be caught when the bacterium is not in that phase during crosslinking. Besides, there is also endogenous PBP1b in these cells, also participating in interactions, but not able to crosslink, nor to be detected by the anti-histidine antibody, neither to be purified. Nevertheless, an attractive feature of this method is, that different interaction partners, binding a similar surface at a different point of the cell cycle can be caught in the same experiment.

One of the identified interaction partners, FtsN is known to be the last protein in the assembly of the divisome, and to be involved in stability of the division complex [30], [31]. It also recruits the amidase AmiC and the Tol-Pal complex to the septation site [29], [32], and furthermore stimulates both the GT and TP activities of PBP1b [21]. The interaction between PBP1b and FtsN could ensure that after stabilization of the division ring by arrival of FtsN and subsequent start of Z-ring constriction (FtsN seems to initiate this process [33]) the amidase AmiC is recruited to the septum, to start cell separation. The PG

hydrolysis by AmiC should take place jointly with new PG synthesis by PBP1b (regulated by FtsN) and PBP3 to assure the integrity of the cell wall during the constriction process. The synthesis of septal PG and constriction of the inner membrane via PBP1b and FtsN, could be linked to successive outer membrane invagination by the Tol-Pal complex via the interaction of YbgF with PBP1b.

Recently it has been shown that YbgF, now renamed into CpoB, forms a complex with LpoB via its interaction with PBP1b as well, reducing the activating effect of LpoB on PBP1bs TP activity. Furthermore, it was shown that the Tol-Pal complex component TolA can also bind CpoB, alleviating this reducing effect [17]. This complex PG synthesis regulation by members of the outer membrane constriction complex represents a mechanism for the coordination of PG synthesis in accordance with outer membrane constriction during cell division.

That we found FtsN and YbgF both interacting with the same site of PBP1b does not necessarily mean that this also occurs simultaneously. Cells in these experiments are not synchronized and these interactions could take place during different stages of the cell cycle. The cross reactivity of the specific antibodies against FtsN and YbgF for PBP1b can be explained by the fact that these proteins form complexes with PBP1b, as shown here, but also in other work [17], [21]. It could be possible that the protein solutions used for the immunization of the rabbits contained some of these interacting proteins as well, and hence, antibodies against these proteins are produced as well.

Overall, *in vivo* photo-crosslinking, by the site specific incorporation of an unnatural amino acid with photo-crosslinking properties, has shown to be very useful to map the sites of protein-protein interactions. In this way we mapped the interaction site between the PG synthase PBP1b, the essential cell division protein FtsN, and the Tol-Pal complex protein YbgF to be in a cleft between the UB2H and TP domain of PBP1b. These interactions could form a physical link between all three cell envelope layers during the invagination and constriction in the course of cell division.

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References

[1] W. Weidel and H. Pelzer, “Bagshaped macromolecules--a new outlook on bacterial cell walls,” *Adv. Enzymol. Relat. Areas Mol. Biol.*, vol. 26, pp. 193–232, 1964.

[2] J. V. Höltje, “Growth of the stress-bearing and shape-maintaining murein sacculus of Escherichia coli,” *Microbiol. Mol. Biol. Rev. MMBR*, vol. 62, no. 1, pp. 181–203, Mar. 1998.

[3] W. Vollmer, D. Blanot, and M. A. De Pedro, “Peptidoglycan structure and architecture,” *FEMS Microbiol. Rev.*, vol. 32, no. 2, pp. 149–167, 2008.

[4] K. H. Schleifer and O. Kandler, “Peptidoglycan types of bacterial cell walls and their taxonomic implications.,” *Bacteriol. Rev.*, vol. 36, no. 4, pp. 407–477, Dec. 1972.

[5] W. Vollmer and U. Bertsche, “Murein (peptidoglycan) structure, architecture and biosynthesis in Escherichia coli,” *Biochim. Biophys. Acta BBA - Biomembr.*, vol. 1778, no. 9, pp. 1714–1734, Sep. 2008.

[6] E. Sauvage, F. Kerff, M. Terrak, J. A. Ayala, and P. Charlier, “The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis,” *FEMS Microbiol. Rev.*, vol. 32, no. 3, pp. 556–556, 2008.

[7] H. Suzuki, Y. Nishimura, and Y. Hirota, “On the process of cellular division in Escherichia coli: a series of mutants of E. coli altered in the penicillin-binding proteins.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 75, no. 2, pp. 664–668, Feb. 1978.

[8] U. Bertsche, T. Kast, B. Wolf, C. Fraipont, M. E. G. Aarsman, K. Kannenberg, M. Von Rechenberg, M. Nguyen-Distèche, T. Den Blaauwen, J.-V. Höltje, and W. Vollmer, “Interaction between two murein (peptidoglycan) synthases, PBP3 and PBP1B, in Escherichia coli,” *Mol. Microbiol.*, vol. 61, no. 3, pp. 675–690, Aug. 2006.

[9] J. Nakagawa and M. Matsuhashi, “Molecular divergence of a major peptidoglycan synthetase with transglycosylase - transpeptidase activities in Escherichia coli — Penicillin-binding protein 1Bs,” *Biochem. Biophys. Res. Commun.*, vol. 105, no. 4, pp. 1546–1553, Apr. 1982.

[10] H. Suzuki, J. Kato, Y. Sakagami, M. Mori, A. Suzuki, and Y. Hirota, “Conversion of the alpha component of penicillin-binding protein 1b to the beta component in Escherichia coli.,” *J. Bacteriol.*, vol. 169, no. 2, pp. 891–893, Feb. 1987.

[11] T. A. Henderson, P. M. Dombrosky, and K. D. Young, “Artifactual processing of penicillin-binding proteins 7 and 1b by the OmpT protease of Escherichia coli.,” *J. Bacteriol.*, vol. 176, no. 1, pp. 256–259, Jan. 1994.

[12] J. K. Broome-Smith, A. Edelman, S. Yousif, and B. G. Spratt, “The nucleotide sequences of the ponA and ponB genes encoding penicillin-binding proteins 1A and 1B of Escherichia coli K12,” *Eur. J. Biochem.*, vol. 147, no. 2, pp. 437–446, 3/1/85 1985.

[13] C. Chalut, X. Charpentier, M.-H. Remy, and J.-M. Masson, “Differential Responses of Escherichia coli Cells Expressing Cytoplasmic Domain Mutants of Penicillin-Binding Protein 1b after Impairment of Penicillin-Binding Proteins 1a and 3,” *J. Bacteriol.*, vol. 183, no. 1, pp. 200–206, Jan. 2001.

[14] M.-T. Sung, Y.-T. Lai, C.-Y. Huang, L.-Y. Chou, H.-W. Shih, W.-C. Cheng, C.-H. Wong, and C. Ma, “Crystal structure of the membrane-bound bifunctional transglycosylase PBP1b from Escherichia coli,” *Proc. Natl. Acad. Sci.*, vol. 106, no. 22, pp. 8824–8829, Jun. 2009.

[15] A. Typas, M. Banzhaf, B. van den Berg van Saparoea, J. Verheul, J. Biboy, R. J. Nichols, M. Zietek, K. Beilharz, K. Kannenberg, M. von Rechenberg, E. Breukink, T. den Blaauwen, C. A. Gross, and W. Vollmer, “Regulation of Peptidoglycan Synthesis by Outer-Membrane Proteins,” *Cell*, vol. 143, no. 7, pp. 1097–1109, Dec. 2010.

[16] A. J. F. Egan, N. L. Jean, A. Koumoutsi, C. M. Bougault, J. Biboy, J. Sassine, A. S. Solovyova, E. Breukink, A. Typas, W. Vollmer, and J.-P. Simorre, “Outer-membrane lipoprotein LpoB spans the periplasm to stimulate the peptidoglycan synthase PBP1B,” *Proc. Natl. Acad. Sci.*, vol. 111, no. 22, pp. 8197–8202, Jun. 2014.

[17] A. N. Gray, A. J. F. Egan, I. L. van’t Veer, J. Verheul, A. Colavin, A. Koumoutsi, J. Biboy, M. A. F. Altelaar, M. J. Damen, K. C. Huang, J.-P. Simorre, E. Breukink, T. den Blaauwen, A. Typas, C. A. Gross, and W. Vollmer, “Coordination of peptidoglycan synthesis and outer membrane constriction during Escherichia coli cell division,” *eLife*, p. e07118, May 2015.

[18] C. A. Zijderveld, M. E. Aarsman, T. den Blaauwen, and N. Nanninga, “Penicillin-binding protein 1B of Escherichia coli exists in dimeric forms.,” *J. Bacteriol.*, vol. 173, no. 18, pp. 5740–5746, Sep. 1991.

[19] C. Chalut, M.-H. Remy, and J.-M. Masson, “Disulfide Bridges Are Not Involved in Penicillin-Binding Protein 1b Dimerization in Escherichia coli,” *J. Bacteriol.*, vol. 181, no. 9, pp. 2970–2972, May 1999.

[20] U. Bertsche, E. Breukink, T. Kast, and W. Vollmer, “In Vitro Murein (Peptidoglycan) Synthesis by Dimers of the Bifunctional Transglycosylase-Transpeptidase PBP1B from Escherichia coli,” *J. Biol. Chem.*, vol. 280, no. 45, pp. 38096–38101, Nov. 2005.

[21] P. Müller, C. Ewers, U. Bertsche, M. Anstett, T. Kallis, E. Breukink, C. Fraipont, M. Terrak, M. Nguyen-Distèche, and W. Vollmer, “The Essential Cell Division Protein FtsN Interacts with the Murein (Peptidoglycan) Synthase PBP1B in Escherichia coli,” *J. Biol. Chem.*, vol. 282, no. 50, pp. 36394–36402, Dec. 2007.

[22] C. Paradis-Bleau, M. Markovski, T. Uehara, T. J. Lupoli, S. Walker, D. E. Kahne, and T. G. Bernhardt, “Lipoprotein Cofactors Located in the Outer Membrane Activate Bacterial Cell Wall Polymerases,” *Cell*, vol. 143, no. 7, pp. 1110–1120, Dec. 2010.

[23] T. J. Lupoli, M. D. Lebar, M. Markovski, T. Bernhardt, D. Kahne, and S. Walker, “Lipoprotein Activators Stimulate Escherichia coli Penicillin-Binding Proteins by Different Mechanisms,” *J. Am. Chem. Soc.*, vol. 136, no. 1, pp. 52–55, Jan. 2014.

[24] W. Margolin, “Sculpting the Bacterial Cell,” *Curr. Biol.*, vol. 19, no. 17, pp. R812–R822, Sep. 2009.

[25] A. Shevchenko, H. Tomas, J. Havlis, J. V. Olsen, and M. Mann, “In-gel digestion for mass spectrometric characterization of proteins and proteomes,” *Nat. Protoc.*, vol. 1, no. 6, pp. 2856–2860, 2006.

[26] A. I. Nesvizhskii, A. Keller, E. Kolker, and R. Aebersold, “A statistical model for identifying proteins by tandem mass spectrometry,” *Anal. Chem.*, vol. 75, no. 17, pp. 4646–4658, Sep. 2003.

[27] K. Dai, Y. Xu, and J. Lutkenhaus, “Cloning and characterization of ftsN, an essential cell division gene in Escherichia coli isolated as a multicopy suppressor of ftsA12(Ts).,” *J. Bacteriol.*, vol. 175, no. 12, pp. 3790–3797, Jun. 1993.

[28] J. N. Sturgis, “Organisation and evolution of the tol-pal gene cluster,” *J. Mol. Microbiol. Biotechnol.*, vol. 3, no. 1, pp. 113–122, Jan. 2001.

[29] M. A. Gerding, Y. Ogata, N. D. Pecora, H. Niki, and P. A. J. De Boer, “The trans-envelope Tol–Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in E. coli,” *Mol. Microbiol.*, vol. 63, no. 4, pp. 1008–1025, Feb. 2007.

[30] S. G. Addinall, C. Cao, and J. Lutkenhaus, “FtsN, a late recruit to the septum in Escherichia coli,” *Mol. Microbiol.*, vol. 25, no. 2, pp. 303–309, 1997.

[31] A. I. Rico, M. García-Ovalle, P. Palacios, M. Casanova, and M. Vicente, “Role of Escherichia coli FtsN protein in the assembly and stability of the cell division ring,” *Mol. Microbiol.*, vol. 76, no. 3, pp. 760–771, May 2010.

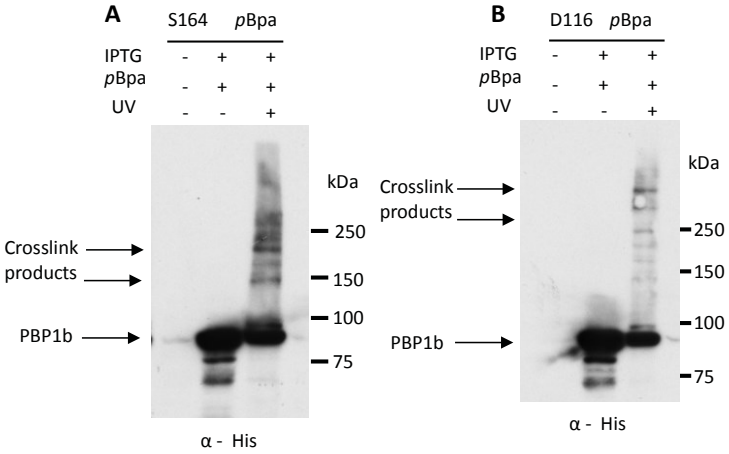
[32] T. G. Bernhardt and P. A. J. de Boer, “The Escherichia coli amidase AmiC is a periplasmic septal ring component exported via the twin-arginine transport pathway,” *Mol. Microbiol.*, vol. 48, no. 5, pp. 1171–1182, Jun. 2003.

[33] M. A. Gerding, B. Liu, F. O. Bendezú, C. A. Hale, T. G. Bernhardt, and P. A. J. de Boer, “Self-enhanced accumulation of FtsN at Division Sites and Roles for Other Proteins with a SPOR domain (DamX, DedD, and RlpA) in Escherichia coli cell constriction,” *J. Bacteriol.*, vol. 191, no. 24, pp. 7383–7401, Dec. 2009.

Supplementary information

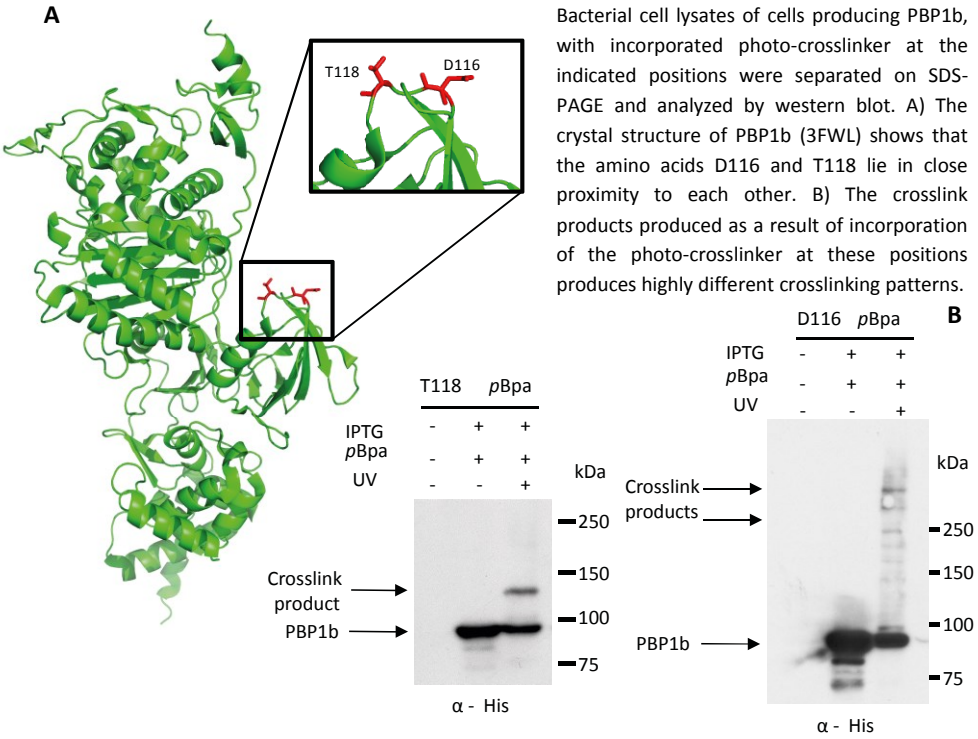
Supplementary table 1.		
R109 → TAG	fwd	GGCAGTTTATGGCTAGATGGTCAATCTTGAGCC
	rev	GGCTCAAGATTGACCATCTAGCCATAAACTGCC
D116 → TAG	fwd	GGTCAATCTTGAGCCATAGATGACCATCAGC
	rev	GCTGATGGTCATCTATGGCTCAAGATTGACC
T118 → TAG	fw	CTTGAGCCAGACATGTAGATCAGCAAGAACG
	rev	CGTTCTTGCTGATCTACATGTCTGGCTCAAG
N122 → TAG	fwd	CCATCAGCAAGTAGGAGATGGTGAAGC
	rev	GCTTCACCATCTCCTACTTGCTGATGG
E123 → TAG	fwd	GCAAGAACTAGATGGTGAAGC
	rev	GCTTCACCATCTAGTTCTTGC
K126 → TAG	fwd	CGAGATGGTGTAGCTGCTGGAGGCG
	rev	CGCCTCCAGCAGCTACACCATCTCG
E129 → TAG	fwd	CGAGATGGTGAAGCTGCTGTAGGCGACCC
	rev	GGGTCGCCTACAGCAGCTTCACCATCTCG
S137 → TAG	fw	GTATCGTCAGGTGTAGAAAATGACCCG
	rev	CGGGTCATTTTCTACACCTGACGATAC
T140 → TAG	fw	CAGGTGTCGAAAATGTAGCGTCCTGGCGAA
	rev	TTCGCCAGGACGCTACATTTTCGACACCTG
M154→ TAG	fw	GCCAACAGCATTGAGTAGATTCGCCGTCCG
	rev	CGGACGGCGAATCTACTCAATGCTGTTGGC
S164 → TAG	fw	GATTTCCCGGACTAGAAAGAAGGACAGGTG
	rev	CACCTGTCCTTCTTTCTAGTCCGGGAAATC
T174→ TAG	fw	GCGCGCGCGTCTGTAGTTTGATGGCGATCATCTGG
	rev	CCAGATGATCGCCATCAAACTACAGACGCGCGCGC
D178 → TAG	fwd	CCTTTGATGGCTAGCATCTGGCGACG
	rev	CGTCGCCAGATGCTAGCCATCAAAGG
T182 → TAG	fw	GCGATCATCTGGCGTAGATCGTCAATATGG
	rev	CCATATTGACGATCTACGCCAGATGATCGC
V184→ TAG	fw	GGCGACGATCTAGAATATGGAGAACAACC
	rev	GGTTGTTCTCCATATTCTAGATCGTCGCC
N188 → TAG	fwd	CGTCAATATGGAGTAGAACCGTCAGTTCGG
	rev	CCGAACTGACGGTTCTACTCCATATTGACG
F194 → TAG	fw	CCGTCAGTTCGGCTAGTTCCGTCTTGATC
	rev	GATCAAGACGGAACTAGCCGAACTGACGG
T751→ TAG	fwd	GGCTAACCAGTAGCCAACGCCGC
	rev	GCGGCGTTGGCTACTGGTTAGCC
T753→ TAG	fwd	GGCTAACCAGACGCCATAGCCGC
	rev	GCGGCTATGGCGTCTGGTTAGCC

Supplementary table 1. Used primers for amber mutant formation.



Supplementary figure 1. Production of multiple not so prominent crosslink products by incorporation of photo-crosslinker at position S164 and D116.

Bacterial cell lysates of cells producing PBP1b, with incorporated photo-crosslinker at the indicated positions were separated on SDS-PAGE and analyzed by western blot. A) Multiple crosslink products, produced by incorporation of photo-crosslinker at position S164, become visible after prolonged exposure. B) Multiple crosslink products, produced by incorporation of photo-crosslinker at position D116, become visible after prolonged exposure.



Supplementary figure 2. Specificity of *in vivo* photo-crosslinking.

Bacterial cell lysates of cells producing PBP1b, with incorporated photo-crosslinker at the indicated positions were separated on SDS-PAGE and analyzed by western blot. A) The crystal structure of PBP1b (3FWL) shows that the amino acids D116 and T118 lie in close proximity to each other. B) The crosslink products produced as a result of incorporation of the photo-crosslinker at these positions produces highly different crosslinking patterns.

Chapter 3

Identification of an essential interaction site in the transpeptidase domain of PBP1b from *Escherichia coli*.

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Abstract

In bacteria cell elongation and division are organized by membrane-anchored protein complexes called elongasome and divisome. These multi enzyme complexes comprise peptidoglycan synthases, hydrolases and cell morphogenesis proteins which co-localize to specific sites in the cell envelope. These complexes perform the enlargement of the peptidoglycan layer in a spatial and temporal controlled manner, involving multiple protein-protein interactions. Here we identified a hairpin loop protruding from the *E. coli* penicillin binding protein (PBP) 1b transpeptidase domain to be essential for the transpeptidase activity of the protein. Furthermore, we show that this loop is an interaction hotspot, being involved in multiple protein-protein interactions. This structural element is conserved among high molecular weight PBPs, and partly shields the active site of the transpeptidase domain. This site participates in the interactions of PBP1b with PBP1a, PBP5, FtsN and MreC, suggesting that it is an important regulatory element for PBP1b function.

Manuscript in preparation

Introduction

The peptidoglycan (PG) layer is an essential component of the bacterial cell envelope that maintains cell shape and protects the cell from rupture caused by the inner osmotic pressure [1]. PG is a mesh-like macromolecule which is constantly synthesized and turned over by penicillin binding proteins (PBPs), lytic glycosyltransferases (GTs) and amidases during the cell cycle [1], [2]. It is composed of glycan strands of alternating N-acetylglucosamine and N-acetylmuramic acid residues crosslinked by peptides, resulting in a stable structure. Because of the importance of the PG layer for cell viability, its precursors as well as the enzymes catalyzing its synthesis are excellent targets for antibiotics, including major classes like the β-lactams and glycopeptides [3].

Based on known localization patterns and protein-protein interactions, it was proposed that distinct multi-enzyme complexes, called divisome and elongasome, are active during cell elongation and division respectively, which consist of PG synthases, hydrolases and cell morphogenesis proteins [1], [4], [5]. The existance of these complexes may allow for the coordination and regulation of enzyme activities through protein-protein interactions. PBPs participate in these complexes and can have different activities, including the polymerization of glycan strands by glycosyltransferase (GT) and the formation of peptide crosslinks by transpeptidation (TP), but also the cleavage of peptides by carboxy- or endopeptidases. All of these activities require a tight regulation in space and time to maintain a stable PG layer and to prevent cell lysis due to uncontrolled PG hydrolysis [6]. Here we focused on PBP1b which is, next to PBP1a, the major bifunctional PG synthase in *E. coli* [7], [8]. PBP1b is a high molecular weight (HMW) PBP, able to perform both, the glycan strand polymerization (GT) and the subsequent crosslinking of the stem peptides (TP) [9]–[11]. The crystal structure of PBP1b has been determined and shows the GT domain, TP domain and, in between them, a small UB2H domain [12]. The proteins localization in the inner membrane is defined by a single transmembrane helix and a membrane-associated site of the GT domain [12], [13].

PBP1b and PBP1a are partly redundant although they do exhibit a different localization pattern during the cell cycle [8], [9], [14], [15]. PBP1b localizes at the lateral wall during cell elongation, as well as at the division site during cell division. This mid cell localization is dependent on the presence of PBP3, where they are both part of the divisome [9], [16]. PBP1a localizes to the lateral wall and is only enhanced at the division site in a PBP1b mutant [15]. PBP1b interacts directly with the cell division proteins PBP3 and FtsN [9], [17]. Furthermore it interacts with MipA, which bridges the interaction of PBP1b with MltA, a PG hydrolyzing enzyme [18]. Next, PBP1b interacts with LpoB, which binds to the UB2H domain to stimulate both the TP and GT activities of PBP1b and is required for its proper functioning in the cell [14], [16], [19], [20]. Recently, PBP1b has been shown to interact with TolA and CpoB as well, which regulate the stimulatory effect of LpoB on

PBP1b [21]. Besides, affinity chromatography with immobilized proteins in combination with crude membrane extracts revealed other direct or indirect interactions of PBP1b with Slt70, MltB, PBP1c and AmiC. In addition, a two hybrid assay suggested an interaction between PBP1b and FtsW [17], [22]–[24].

By comparing crystal structures of HMW PBPs from different bacteria we identified a conserved loop located on the surface of the TP domain in close proximity to the active site. This loop, ranging from amino acids D535 to S558, is important for PBP1b TP activity. We used a site-specific *in vivo* photo-crosslinking approach for the identification of interactions involving this loop [25], which has previously been shown to be a very powerful method in the identification of protein-protein interactions [21]. This approach is based on the replacement of single amino acids in the TP domain hairpin by the UV inducible photo-crosslinker *p*-benzoyl-L-phenylalanine (*p*Bpa), and resulted in the detection of known and novel interaction partners that could be involved in the localization and/or regulation of the activity of PBP1b.

Materials and methods

Bacterial strains and plasmids

E. coli DH5α was used for DNA amplification. Protein expression and *in vivo* photo-crosslinking experiments were performed in *E. coli* strain BL21(DE3) [F^-, dcm, ompT, hsdS($r_B^-m_B^-$), gal, (λ DE3)] which was also used for the preparation of inner membrane, outer membrane and periplasmic fractions. *E. coli* EJ801 [*ponA* 1104(ts), *mrcB* 1085, *dacA* 1191, *dacB* 12, *lac*, *strA*, *tonA*, *metB*, *proA*, *tsx*] was used in the complementation assay [11]. Plasmid pDML924 carrying the *mrcB* gene encoding the N-terminal His_6-tagged PBP1bγ(M46-N844) under regulation of P_{T7lac} was used for the mild overexpression of PBP1b [11]. Plasmid pSub-BpaRS-6TRN encodes the orthogonal aminoacyl tRNA synthetase-$tRNA_{CUA}$ pair for the incorporation of *p*Bpa at the amber codon [26].

Construction of pDML924Δloop coding His_6-tagged PBP1bΔD535-S558

A ScaI restriction site was introduced in *mrcB* by site directed mutagenesis using pDML924 as template. The resulting plasmid pDML924ScaI served as template in an overlap extension PCR [27] whereby the nucleotides coding D535-S558 were deleted. The generated insert and pDML924ScaI were double digested with PstI/ScaI followed by ligation, giving rise to pDML924Δloop. Used primers are listed in supplementary table 1.

Construction of pDML924-Xamber

pDML924-Xamber encoding amber mutants of His_6-tagged PBP1b were generated by replacing specific codons at position X by the amber stop codon TAG using the

QuikChange site directed mutagenesis kit (Stratagene) with pDML924 as template. Used primers are listed in supplementary table 1.

Complementation assay

E. coli EJ801, either transformed with pDML924Δloop or co-transformed with pDML924-Xamber and pSub-BpaRS-6TRN, was grown in LB supplemented with 25 µg/ml kanamycin, 10 µM IPTG and in LB supplemented with 25 µg/ml kanamycin, 17 µg/ml chloramphenicol, 10 µM IPTG at 37°C, respectively. When required, photo-crosslinker *p*Bpa (Bachem, Switzerland) was added to a final concentration of 1 mM. At an OD_{600} of 0.1-0.25 the cultures were shifted to 42°C. The OD was measured for the next 4 hours. As positive control *E. coli* EJ801 transformed with pDML924 was cultured at 42°C.

In vitro peptidoglycan synthesis assay

[^{14}C]GlcNAc-labeled Lipid II (15 µM, 12,000 dpm) was dried under vacuum and dissolved on ice in 1% Triton X-100. The reaction was performed in 10 mM HEPES/NaOH pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 2% glycerol, 0.2% Triton X-100, in the presence of 1 µM PBP1b or PBP1bΔD535-S558. The samples were incubated in a thermal shaker at 37°C with 900 rpm for 60 min. To stop the reaction the samples were acidified with 0.1 M HCl to pH 4.8 and heated at 100°C for 10 min. Samples were cooled to room temperature and incubated O/N with 10 µg of cellosyl at 37°C (provided by Hoechst AG, Frankfurt, Germany). The samples were heated at 100°C for 10 min and centrifuged in a microfuge. The supernatant was collected and incubated with an equal volume of 0.5 M sodium borate, pH 9.0 and 10 mg/ml $NaBH_4$ for 30 min at room temperature. Excess of $NaBH_4$ was destroyed by adjusting the pH to 3.5 - 4.5 with 20% phosphoric acid, and the muropeptides were analyzed by HPLC as described [28], [29].

The in gel analysis was performed in a similar way, using 10 µM ATTO550 labeled Lipid II and 200 µM m-DAP Lipid II, in the presence or absence of 1 µg Penicillin G. Samples were taken at different time intervals, the reaction was stopped by heating the samples for 5 min at 99°C. Samples were dried using a speedvac, and dissolved in 4 µl sample buffer containing 60 mM Tris-HCl ph 8.8, 25% glycerol and 2% SDS. These samples were analyzed on Tris/Tricine SDS-PAGE gels as described [30], [31]. Gels were prepared in a final concentration of 9%T, 2.6%C, where T is total percentage of both acrylamide and bisacrylamide and C the percentage of bisacrylamide relative to T. This was prepared in 1x gel buffer containing 0.5 M Tris, 0.13% SDS, pH 8.45. Gels were runned using an anode buffer of 0.1 M Tris pH 8.8 and cathode buffer of 0.1 M Tris, 0.1 M Tricine and 0.1% SDS, pH8.25 at a constant current of 30 mA with a maximum voltage of 200 V. Gels were visualized with a Typhoon 9400 (GE healthcare) fluorescence scanner using an excitation wavelength of 532 nm and emission filter of 580/30.

Protein expression and in vivo photo-crosslinking of PBP1b and PBP1bΔD535-S558

E. coli BL21(DE3) cells containing pDML924 or pDML924Δloop, encoding His_6-tagged PBP1b or His_6-tagged PBP1bΔD535-S558, were grown in LB with 25 µg/ml kanamycin at 37°C. At OD_{600} of 0.8, protein expression was induced with 100 µM IPTG. After 2 h induction, cells were harvested and washed with PBS. *E. coli* BL21(DE3) cells containing pSub-BpaRS-6TRN and pDML924-Xamber encoding His_6-tagged PBP1b amber mutant, were grown in LB with 25 µg/ml kanamycin and 17 µg/ml chloramphenicol at 37°C. At OD_{600} of 0.5-0.6 protein expression was induced with 10 µM IPTG and 1 mM *p*Bpa, freshly dissolved in 1 M NaOH, was added to the medium. After 2 h induction, cells were harvested and washed with PBS. Cell pellets of a 20 ml culture were resuspended in 3 ml PBS, transferred into a petri dish, and exposed to UV light at 366 nm for 60 min (UVGL-58, 6 W from UVP Inc., California) 1 cm above the cell suspension. The cell suspension was cooled on ice and slowly stirred during photo-crosslinking. Cell pellets were stored at -80°C.

Protein purification of PBP1b and PBP1bΔD535-S558

Cell pellets of 20 ml cell cultures expressing His_6-tagged PBP1b or His_6-tagged PBP1bΔD535-S558 were resuspended in 2 ml 20 mM Tris-HCl pH 8.0, 300 mM NaCl supplemented with 25 µM phenylmethylsulphonyl fluoride (PMSF). Cells were lysed by sonication at 4°C using a sonicator with microtip (Branson Sonifier 250) 6 times for 30 sec each at 65 W. Cell debris was removed by centrifugation at 3500 x *g* for 15 min at 4°C. The membranes were sedimented by centrifugation for 45 min at 200000 x *g* at 4°C, membrane proteins were solubilized in 1.4 ml of 20 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM imidazole, 0.5% LAPAO (Anatrace, USA) and stirring the membrane suspension for 4 h or overnight at 4°C. Insoluble material was removed by centrifugation for 45 min at 200000 x *g*. Solubilized proteins were incubated with 50 µl Ni^{2+} sepharose (GE Healthcare) for 7 h or overnight at 4°C. The Ni^{2+} sepharose suspension was poured into a column and washed with 1 ml 20 mM Tris-HCl pH 8.0, 300 mM NaCl (buffer A) containing 5 mM imidazole. The column was then washed with 1 ml buffer A containing 20 mM imidazole and 0.5 ml buffer A containing 100 mM imidazole, 0.1% Triton-X-100. PBP1b and PBP1bΔD535-S558 were eluted in two steps with 0.5 ml buffer A containing 0.1% Triton-X-100 and 150 mM imidazole, and 0.2 ml buffer A containing 0.1% Triton-X-100 and 500 mM imidazole. Proteins were stored at 4°C.

Protein purification of PBP1b amber mutants and crosslink products

Cell pellets of 200 ml UV irradiated cell cultures expressing His_6-tagged PBP1b variants were resuspended in 20 ml PBS supplemented with 25 µM PMSF and complete EDTA free protease inhibitor cocktail (Roche). Cells were passed twice through a cooled cell disruptor at 1.9 kbar. Cell debris was removed by centrifugation at 12000 x *g* for 10 min at 4°C.

Membranes were collected by centrifugation (45 min, 200000 x *g*, 4°C), membrane proteins were solubilized in 1.8 ml of 20 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM imidazole, 0.5% LAPAO and stirring the membrane suspension for 4 h at 4°C. Insoluble material was removed by centrifugation for 45 min at 200000 x *g*. Solubilized proteins were incubated with 100 µl Ni^{2+} sepharose overnight at 4°C. The Ni^{2+} sepharose suspension was poured into a column and washed with 1.5 ml 20 mM Tris-HCl pH 8.0, 300 mM NaCl (buffer A) containing 5 mM imidazole. The column was then washed with 2 ml buffer A containing 20 mM imidazole and 1.5 ml buffer A containing 100 mM imidazole, 0.1% Triton-X-100. PBP1b mutants and crosslink products were eluted in three steps with 1.5 ml buffer A containing 0.1% Triton-X-100 and 150 mM, 300 mM or 500 mM imidazole. The elution fractions were concentrated to a final volume of ~100-150 µl using a centrifugal filter device with a 100 kDa cut-off (Amicon) and stored at 4°C.

Cell fractionation

E. coli Bl21(DE3) cells were grown in 1L LB at 37°C to mid-log phase. Cells were cooled on ice for 30 min and harvested by centrifugation at 7300 x *g* for 30 min at 4°C. The cells were washed with PBS and centrifuged at 7900 x *g* for 10 min at 4°C, which resulted in a wet cell pellet of ~3 g. The cells were resuspended in 10 ml 50 mM Tris-HCl pH 7.5, 250 mM sucrose, 1 mM EDTA, 25 µM PMSF supplemented with lysozyme (1.5 mg / 20 ml) and incubated for 10 min at room temperature, inducing spheroplast formation and release of the periplasmic fraction into the buffer. After centrifugation at 5800 x *g* for 10 min at 4°C the periplasmic fraction (supernatant) was collected. The spheroplasts were resuspended in 20 ml 50 mM Tris-HCl pH 7.5, 25 µM PMSF supplemented with DNase and RNase. The suspension was passed twice through a pre-cooled French press. Cell debris was removed by centrifugation at 9700 x *g* for 10 min at 4°C. The supernatant was centrifuged at 48000 x *g* for 30 min at 4°C to collect the outer membrane fraction. Next, the inner membrane vesicles were collected by centrifugation at 200000 x *g* for 45 min at 4°C. Proteins of both membrane fractions were solubilized by incubation in 5 ml 50 mM Tris-HCl pH 7.5, 0.5% LAPAO for 3 h at 4°C. After centrifugation at 200000 x *g* for 45 min aliquots of the supernatants were stored at -20°C.

Synthesis of the loop peptide

The loop peptide D_{535}APIALRQPNGQVWSPQN_{552}, whereby the first and the last amino acid were replaced by cysteines, was synthesized automatically on Rink resin (1 g; 0.25 mmol) using an Applied Biosystems synthesizer. A small amount of peptide was cleaved from the resin and analyzed by MALDI-TOF mass spectrometry. The N-terminal 9-fluorenylmethoxycarbonyl (Fmoc) protecting group was removed using 20% piperidine in N-methylpyrrolidone (NMP) (3 × 10 ml/gram of resin, 10 min) followed by washing with NMP (3 × 10 ml, 2 min), dichloromethane (DCM) (3 × 10 ml, 2 min) and NMP (3 × 10 ml, 2

min). Next, the spacer was coupled (3 h) by adding a mixture of spacer (530.6 mg; 1 mmol), N-hydroxy-benzotriazol (135.1 mg; 1 mmol), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (379.2 mg; 1 mmol) and *N*,*N*-diisopropyl-*N*-ethylamine (DiPEA) (174.2 µl; 1 mmol) in NMP (5 ml) to the resin. The N-terminal Fmoc protecting group was removed, followed by coupling of pentynoic acid (98.1 mg; 1 mmol) using benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (442.3 mg; 1 mmol) and DiPEA (174.2 µl) in NMP (5 ml). The resin was washed with NMP (3 × 10 ml, 2 min) and DCM (3×10ml, 2 min) after every coupling and deprotection step. These steps were monitored using the Kaiser test [32] or bromophenol blue test in case of secondary amines [33]. The linear peptide was deprotected and cleaved from the resin using trifluoroacetic acid/1,2-ethanedithiol/triisopropyl silane/water (90/2.5/2.5/5; v/v/v/v; 10 ml), during 5 hours. The resin beads were removed by filtration and the filtrate was precipitated in methyl-tert-butyl ether/hexanes (3x) and lyophilized. The linear peptide (150 mg; 63.4 µmol) was dissolved in aq. 2.5% acetic acid (90 ml) and the pH was adjusted to 6.0 with aq. ammonia (25%). 25 mL of dimethylsulfoxide (DMSO) was added and the reaction mixture was stirred over the weekend. The mixture was partially concentrated *in vacuo* and remaining DMSO was removed using a speedvac O/N at 40°C. The crude peptide was purified by preparative HPLC and after lyophilisation the cyclic peptide (60 mg; 10% overall yield) was obtained as a white solid. Purity was confirmed by analytical HPLC and was found to be higher than 99%.

Preparation of PBP1b loop peptide C535-C552 column

In methanol resuspended Magnabind-NH_2 beads (Pierce) were converted into the azide form by adding 100 µmol diazotransfer reagent, imidazole-1-sulfonyl azide hydrochloride, 10 µmol $CuSO_4$ and 600 µmol K_2CO_3 [34]. After 4 h at room temperature the beads were washed with water and methanol. The formation of the azide was confirmed by coupling a TAMRA alkyne onto a small fraction of the azide beads. The loop peptide was clicked onto Magnabind-N_3 beads in 1 mM $CuSO_4$, 400 µM tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA), 3 mM sodium ascorbate and 1.5 µmol peptide in a Tris-HCl pH 8.5 buffer. After 2 h at 37°C the beads were washed with 20 mM Tris-HCl pH 8.5, 1 M NaCl and 1 M NH_4HCO_3. Beads with conjugated peptide were stored in Tris-HCl buffer pH 8.5 at 4°C.

Affinity chromatography using C535-C552-column

Three columns were made from 100 µl Magnabind-loop C535-C552 suspension each. 100 µl periplasmic, inner membrane and outer membrane fraction, was applied to the columns. After 5 min incubation, columns were washed with 0.5 ml 20 mM Tris-HCl pH 7.5, 0.1% Triton-X-100, and with 100 µl 50 mM Tris-HCl pH 7.5, 0.1% Triton-X-100 (buffer B) supplemented with 100 mM, 300 mM and 500 mM NaCl. Bound proteins were eluted

with 50 µl buffer B supplemented with 1 M NaCl and 50 µl 1 M NH_4HCO_3 pH 9.0. This purification was repeated twice, in between the purification steps the columns were washed three times with 200 µl 20 mM Tris-HCl pH 7.5 and equilibrated in the same buffer. The fractions of the three purifications were pooled. The periplasmic protein fractions were concentrated 3x using centrifugal filter devices (Amicon) with a 3 kDa cutoff.

Protein detection

For the Bocillin binding test purified PBP1b and PBP1bΔD535-S558 were incubated for 30 min at 35°C with 20 µM Bocillin 650/665 (Invitrogen) [35]. SDS-PAGE sample buffer was added and the samples were boiled. The proteins were separated by SDS-PAGE and fixed in the gel with 40% EtOH, 10% acetic acid, 50% H_2O. Bocillin-labeling was detected on an Odyssey imaging system (LI-COR) at 700 nm. To visualize total protein, gels were incubated with Flamingo fluorescent gel stain (Bio-Rad) O/N and analyzed with a Typhoon 9410 imager using an excitation of 488 nm and the emission filter 560 LP.

For immunodetection of proteins, samples were separated by SDS-PAGE and blotted semidry on nitrocellulose membrane (Bio-Rad). His_6-tagged PBP1b and PBP1b mutants were detected by the mouse monoclonal anti-polyhistidine peroxidase conjugate antibody (Sigma Aldrich, 1:4000). PBP5 was identified with rabbit anti-PBP5 serum (1:10000, A. Piette, Université de Liège, Belgium). Antibodies against FtsN and MreC were diluted 1:2000, and that against PBP1a 1:5000. Goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad) was used as secondary antibody (1:3000).

In-gel digestion and mass spectrometric analysis

The in-gel digest was done as described with slight modifications [36]. Gel slices were stored in 1% formic acid before proteins were reduced with 50 µl 10 mM dithiothreitol in 100 mM NH_4HCO_3 and alkylated with 30 µl 55 mM iodacetamide in 100 mM NH_4HCO_3. Gel slices were dehydrated in acetonitrile and swollen in digestion buffer containing 3 ng/µl trypsin in 50 mM NH_4HCO_3. After the samples were incubated for 45 min on ice, the supernatant was replaced with 20 µl 50 mM NH_4HCO_3. The digest was performed at 37°C O/N. The supernatant was collected and combined with the peptides extracted from the gel with 5% (v/v) formic acid. Samples were stored at -20°C. The mass spectrometric analysis was done with an Agilent 1200 HPLC system coupled to a Thermo LTQ-Orbitrap mass spectrometer.

Criteria for protein identification

Mascot (Matrix Science, London, UK) was used for the database search in SwissProt_56.2 selected for *E. coli* (13109 entries) with a parent ion tolerance of 15 ppm and a fragment ion tolerance of 0.90 Da. As fixed modification the iodoacetamide derivative of cysteine

was specified. The oxidation of methionine was set as variable modification. Maximal two missed cleavages were allowed.
Scaffold_3_00_05 (Proteome Software Inc., Portland, OR) was used to validate the peptide and protein identification. Peptide identifications were accepted as true when the probability was greater than 95%. For the protein identifications a probability of at least 99% was set and at least 2 peptides of one protein had to be identified.

Results

Identification of a loop structure shared among transpeptidase domains of high molecular weight PBPs.

During the last decade several crystal structures of PBPs were solved allowing further insight in the organization of active sites of GTs, TPs and carboxy/endopeptidases [12], [37]–[43]. Comparing the crystal structures of the TP domain of different PBPs, we noticed that HMW PBPs share a common structural feature. They all contain a structured loop of 19 – 24 amino acids long, which is part of a cleft, reaching from the transpeptidase active site to the surface. This loop partially covers the active site (Fig 1A-F), limiting the direct access for incoming substrates and/or leaving products. In contrast, the peptidase domain of low molecular weight (LMW) PBPs is characterized by a relatively distant loop that is not part of the cleft surrounding the active site (Fig 1G, H).

The TP domain loop of *E. coli* PBP1b is important for proper TP activity.

To examine the relevance of this intriguing structural feature for enzymatic activity and protein function we focused our investigations on PBP1b of *E. coli* (Fig 1A). First, we generated a loop deletion mutant (PBP1bΔD535-S558, Fig 2A) and tested its functionality in a complementation assay using *E. coli* EJ801. Strain EJ801 is expressing a labile, thermosensitive PBP1a and lacks PBP1b and thereby is not viable at elevated (non-permissive) temperature, unless complemented with a functional PBP1b variant [11]. Shortly after the temperature shift to 42°C, EJ801 cells expressing PBP1bΔD535-S558 showed less growth compared to cells expressing wild type PBP1b. Negative control cells stopped growing and began to lyse as expected (Fig 2B). Furthermore, we failed to obtain an *E. coli* mutant that expresses PBP1bΔD535-S558 in a PBP1a deletion background. These results indicate that PBP1bΔD535-S558 is only partially functional, and that the loop, consisting of amino acids 535-558 is essential for proper functioning of PBP1b *in vivo*.
Previous *in vitro* peptidoglycan synthesis assays showed that PBP1b is most active at concentrations that favor dimerization [28]. Therefore, we tested the ability of PBP1bΔD535-S558 to dimerize, to investigate the possibility of its lower activity by an inability to form dimers. When PBP1b protein samples are not boiled, a 180kD dimer band is visible on SDS-PAGE [44]. PBP1bΔD535-S558, like wild type PBP1b, was capable of

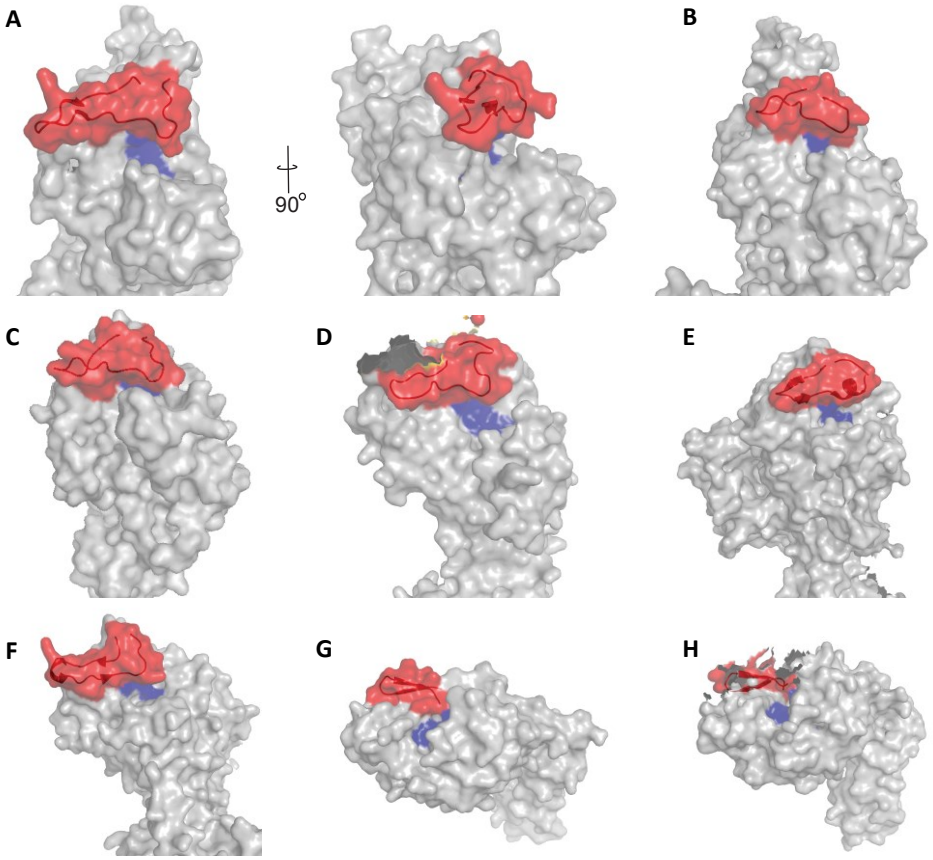
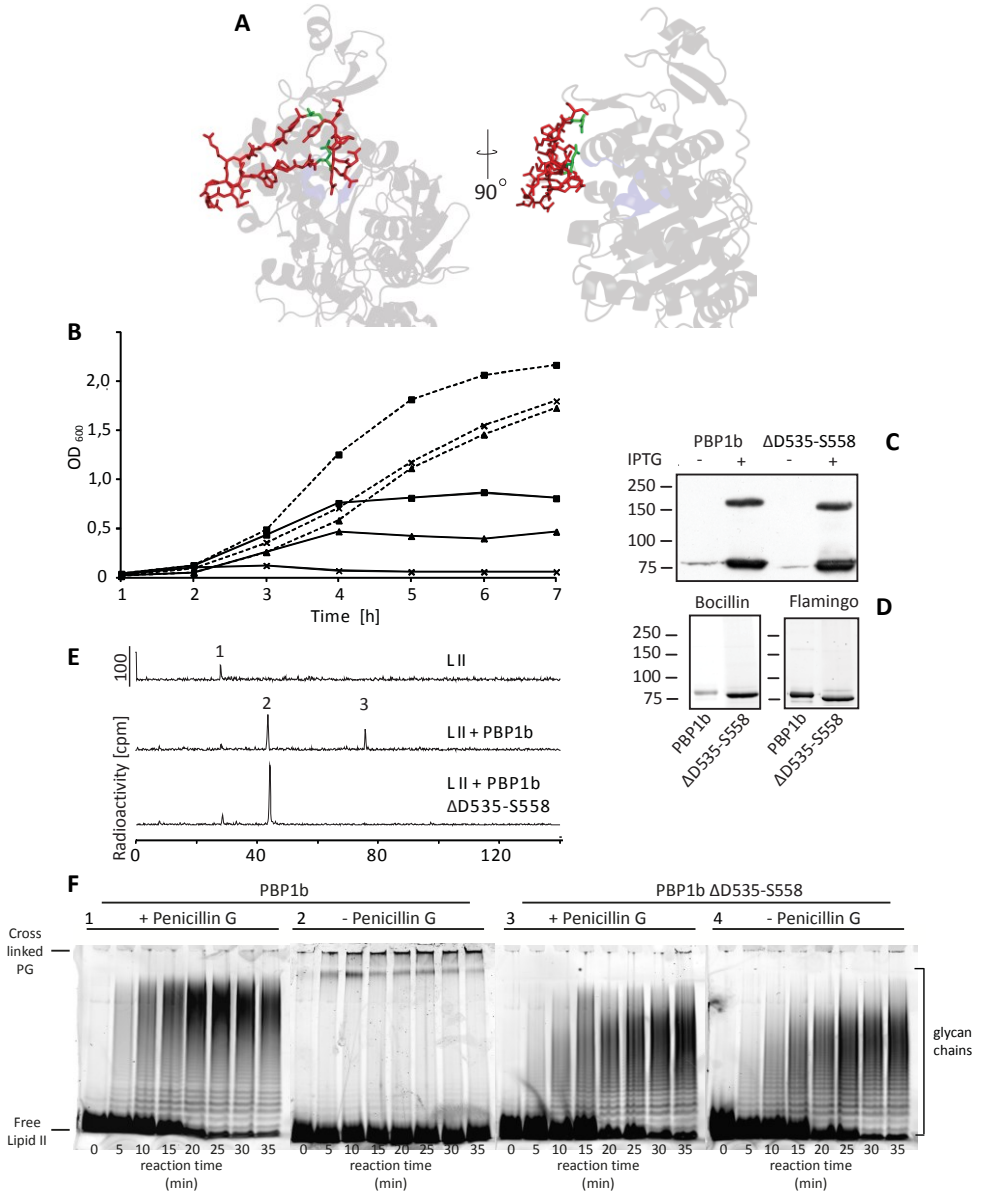


Figure 1. Surface representation of peptidase domains of high and low molecular weight PBPs.
All depicted crystal structures of HMW PBPs have a loop (red) shielding the active site (blue). In contrast the peptidase domains of LMW PBPs are characterized by a loop distant from the active site. HMW class A PBPs: A) PBP1b *E. coli* (3fwl), B) PBP1b *S. pneumoniae* (2bg3), C) PBP1a *S. pneumoniae* (2c6w), HMW class B PBPs: D) PBP2b *S. pneumoniae* (2wad), E) PBP2x *S. pneumoniae* (1rp5), F) PBP2a *S. aureus* (1mws). LMW PBPs: G) PBP5 *E. coli* (1nzo), H) PBP6 *E. coli* (3itb) [12], [37]–[43].

Figure 2 (right page). Functionality of PBP1bΔD535-S558.
Crystal structure of the PBP1b TP domain showing the D535-S558 loop (red) on the surface of the protein. The residues D535 and N552 are adjacent to each other and are depicted in green. B) Complementation assay showing growth of *E. coli* EJ801 (PBP1a (ts), ΔPBP1b) expressing PBP1bΔD535-S558 after the temperature shift to 42°C (at OD 0.1-0.2) (solid line, triangle). The cells grew less than the positive control, expressing wild type PBP1b (solid line, rectangle) indicating that PBP1bΔD535-S558 is partly functional. EJ801 was used as negative control (cross). Solid line, cultures at 42°C; dashed line, control cultures at 37°C. C) His_6-PBP1bΔD535-S558, like His_6-PBP1b, forms a dimer as detected with anti-His-tag antibody as a ~ 180 kDa band. D) The penicillin-binding capacity of His_6-PBP1bΔD535-S558 was determined by SDS-PAGE after purified His_6-PBP1b and His_6-PBP1bΔD535-S558 were incubated with 20 µM Bocillin 650/665 for 30 min at 35°C. Both proteins bind Bocillin, detected at 700 nm (left). The total protein was detected by Flamingo (right). E) In an *in vitro* PG synthesis assay PBP1b and PBP1bΔD535-S558 were incubated with radiolabeled Lipid II. The reaction products were analyzed by HPLC allowing the detection of GT [2] and GT/TP products [3], [27].



PBP1bΔD535-S558 is able to catalyze the formation of glycan chains (GT) but is not able to perform TP. Peak 1, phosphorylated disaccharide pentapeptide (resulting from Lipid II and chain ends); peak 2, reduced disaccharide pentapeptide (monomer); peak 3, reduced bis-disaccharide tetrapentapeptide (dimer). F) In an *in vitro* PG synthesis assay PBP1b and PBP1bΔD535-S558 were incubated with fluorescently labeled Lipid II. The reaction products were separated by Tris/Tricine SDS-PAGE and scanned with a fluorescence scanner. Both proteins produce glycan chains with increasing amount of monomers per chain in time (gel 1 and 3). PBP1b also produces crosslinked PG by TP activity when penicillin G (penG) is not added to the reaction mixture, visible as a high molecular weight band, at the top of the gel (gel 2). This high molecular weight band of crosslinked PG is absent in the reaction of PBP1bΔD535-S558, indicating the inability to perform the TP reaction (gel 4).

forming a dimer which was stable on SDS-PAGE, indicating that the folding of PBP1bΔD535-S558 is sufficiently retained for dimer formation and that the loop is not primarily involved in dimer formation. This shows that the diminished function of PBP1bΔD535-S558 is not caused by the inability of dimer formation (Fig 2C).
Next, we tested the capacity of PBP1bΔD535-S558 to bind the substrate of the TP domain in the form of a β-lactam. The loop mutant was still able to bind Bocillin, which is a Bodipy 650/665-labeled penicillin V, a structural analog of the donor substrate D-Ala-D-Ala moiety of the pentapeptide (Fig 2D) [35]. This indicates that the TP domain of this loop mutant is still able to bind its substrate and hence correctly folded. Furthermore, it shows that the loop itself is not involved in the binding of the D-Ala-D-Ala moiety by the TP domain. However, this does not exclude involvement of the loop in the attachment of the donor peptide to the Meso-diaminopimelic acid (m-DAP) in the acceptor peptide.
To elucidate the defect of the loop mutant, an *in vitro* PG synthesis assay using radiolabeled Lipid II as substrate and subsequent HPLC analysis on the formed products was performed. This showed that PBP1bΔD535-S558 was capable to polymerize glycan chains by GT reactions, but was totally devoid of TP activity (Fig 2E). This was also observed, when using fluorescently labeled Lipid II as a substrate in the *in vitro* assay, and subsequent separation of the produced PG by Tris/Tricine SDS-PAGE (Fig 2F). A pattern of glycan chains of different lengths, increasing in length in time, was produced by both PBP1bΔD535-S558 and wild type PBP1b. High molecular weight crosslinked PG, was visible as a band at the top of the gel in case of wild type PBP1b. This product disappeared when the TP domain was inactivated by the addition of Penicillin G to the reaction mixture. This high molecular weight crosslinked PG was not produced by PBP1bΔD535-S558 in the absence of Penicillin G (Fig 2F). This shows that the D535-S558 loop of PBP1b is required for the TP function of this HMW PBP.

Identification of the TP loop of PBP1b as an interaction hot spot.
Since the loop structure of the TP domain of PBP1b turned out to be essential for the function of this domain, but was not involved in its substrate binding capability, we hypothesize that this loop might be involved in the regulation of this domain, which could be accomplished via protein-protein interactions. To gain more insights in the existence of loop-specific interactions, we used an *in vivo* photo-crosslinking approach, which is a powerful method for the detection of direct protein-protein interactions in a cellular context. This method is based on the introduction of the unnatural, photo reactive amino acid *p*Bpa at unique sites in the protein sequence as a result of nonsense suppression mutagenesis. Subsequent UV illumination induces crosslinking of the *p*Bpa-containing protein with its interaction partners within the cell. This can be analyzed by SDS-PAGE and western blotting. Crosslinking of the *p*Bpa-containing protein with other proteins will result in the formation of higher molecular weight complexes, visualized as higher running

bands in western blot analysis. Upon UV-illumination, several PBP1b mutants with *p*Bpa present in the loop produced additional protein bands of higher molecular weights, containing PBP1b and one or more interaction partners (Fig 3A). This shows that PBP1b interacts with several proteins via the loop in the TP domain. The mutant with *p*Bpa at position 545 (Q545*p*Bpa) produced three crosslink bands; at 150 kDa, 250 kDa and a band of >250 kDa, representing PBP1b with crosslinked interaction partners. These bands are also present in the sample from G546*p*Bpa, which furthermore produced a band of around 140 kDa. The mutants W548*p*Bpa and S549*p*Bpa show two similar crosslink bands, at 150

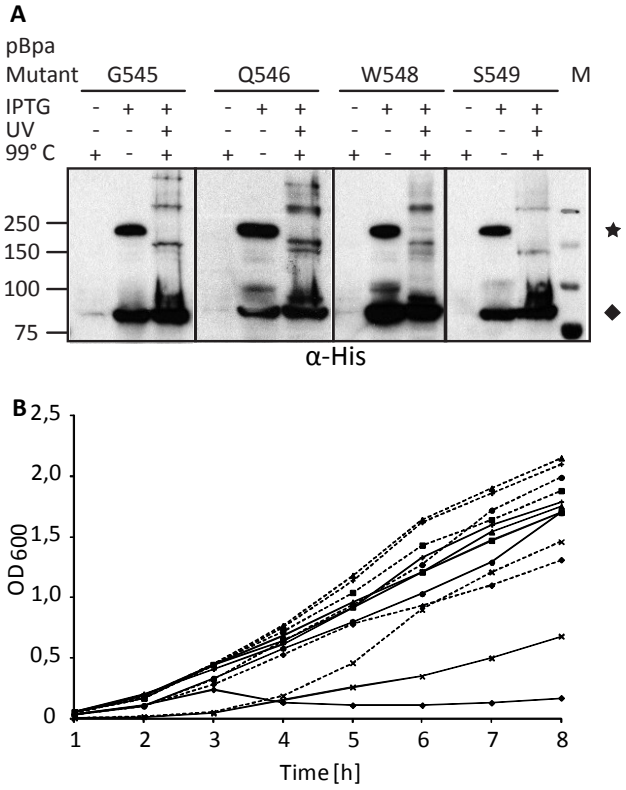


Figure 3. Characterization of PBP1b crosslink mutants.

A) Cells expressing PBP1b mutants containing the artificial amino acid *p*Bpa at position G545, Q546, W548 or S549 were irradiated with UV light to initiate cross linking reactions. Cell lysates were analyzed by SDS-PAGE and western blotting with protein detection by MαHis. All four mutants showed several crosslink products of high molecular weight after UV illumination. In the unboiled induced samples, a PBP1b dimer was detected at ~180 kDa. Star: PBP1b dimer, diamond: PBP1b monomer. B) Growth complementation assay using EJ801 cells expressing PBP1b loop mutants after shifting the cultures from 37°C to 42°C at an OD_{600} of 0.1-0.2. All PBP1b mutants with incorporated *p*Bpa are functional except the mutant W548*p*Bpa (diamond). EJ801 pDML924 expressing PBP1b was used as positive control (rectangle). The negative control was EJ801 (cross). PBP1b mutants: G545*p*Bpa, dot; Q546*p*Bpa, triangle; W548*p*Bpa, diamond; S549*p*Bpa, plus. Solid line, cultures at 42°C; dashed line, control cultures at 37°C.

kDa and at 250 kDa which are at the same height as in the other mutants. Somewhat surprisingly, crosslink bands well over 250 kDa were visible for the mutants G545*p*Bpa and Q546*p*Bpa, suggesting that either very big proteins have been crosslinked or complexes were captured that somehow are stabilized to withstand boiling in SDS (Fig 3A). Interestingly, differences in the crosslinking pattern were observed even for adjacent positions (for example, G545 and Q546). This shows the specificity of this method, and furthermore indicates that different interaction interfaces are present and may even overlap in this region. It should be taken into account that these cells are not synchronized during the procedure, meaning that the overlap may also be due to different interactions (resulting in different crosslinks) during different growth stages.

To investigate the functionality of the PBP1b mutants with incorporated *p*Bpa we tested them in the complementation assay using *E. coli* strain EJ801. At nonpermissive temperature the mutants G545*p*Bpa, Q546*p*Bpa and S549*p*Bpa grew like the positive control; EJ801 expressing unmodified PBP1b (Fig 3B), indicating that the *in vivo* incorporated artificial amino acid *p*Bpa at these positions did not prevent the cellular function of the protein. In contrast, the W548*p*Bpa substitution rendered PBP1b non-functional, probably by interfering with the essential function of the loop.

Identification of the PBP1b loop interacting proteins.

The crosslink products produced by the PBP1b loop mutants (Fig 3A) were purified using nickel affinity, binding the his-tag in PBP1b and the crosslink products. These samples were run on SDS-PAGE, the bands containing the crosslink products were excised, and analyzed by mass spectrometry. These results showed an interaction of PBP1b with the essential cell division protein FtsN, the endopeptidases PBP5 and PBP6, the other major bifunctional PG synthase PBP1a, the Tol-Pal complex protein TolB, which is involved in outer membrane constriction during cell division, and the cell morphogenesis protein MreC (table1). As a control, cells expressing wild type PBP1b and the tRNA-tRNA synthase pair, were grown under the same conditions, in the presence of the crosslinker *p*Bpa and illuminated with UV light as well. No crosslink products were produced in this case. This thus shows that the crosslink products seen after illumination of the mutants are a result of the incorporation of the crosslinker at the specific sites in the protein. PBP1b was also purified from these cells, and this sample was run next to the crosslink samples of the mutants, on SDS-PAGE. Gel slices at the similar heights as the crosslink products, generated by illuminating the cells expressing the mutant proteins, were cut out of the wild type lane and analyzed by mass spectrometry as well. None of the proteins described above were found in these gel slices, proving that the proteins were found as a result of a covalent bond with the crosslinker in the PBP1b mutants. Also, wild type cells, not overexpressing any protein were transfected with the plasmid encoding the tRNA-tRNA synthase pair, and grown in the presence of the photo-crosslinker. UV illumination of

these cells did result in the appearance of some faint bands of higher molecular weight, which did not run on the same height as the crosslink products produced by illuminating the mutant proteins (data not shown).

The identified interactions were further validated by immunodetection with specific antibodies against PBP5, FtsN, MreC and PBP1a. PBP5 was stained by the specific antibody in a band of 250 kDa for all crosslinked PBP1b variants G545*p*Bpa, Q546*p*Bpa, W548*p*Bpa, and S549*p*Bpa (Fig 4A). This was in accordance with the mass spectrometry results, where PBP5 was also found back in the 250 kDa gel slice of all mutants. FtsN was shown to be present in a ~230 kDa band of the crosslink sample of mutants G545*p*Bpa, Q546*p*Bpa and S549*p*Bpa. This band was specific for the mutants and not seen in the control (Fig 4B).

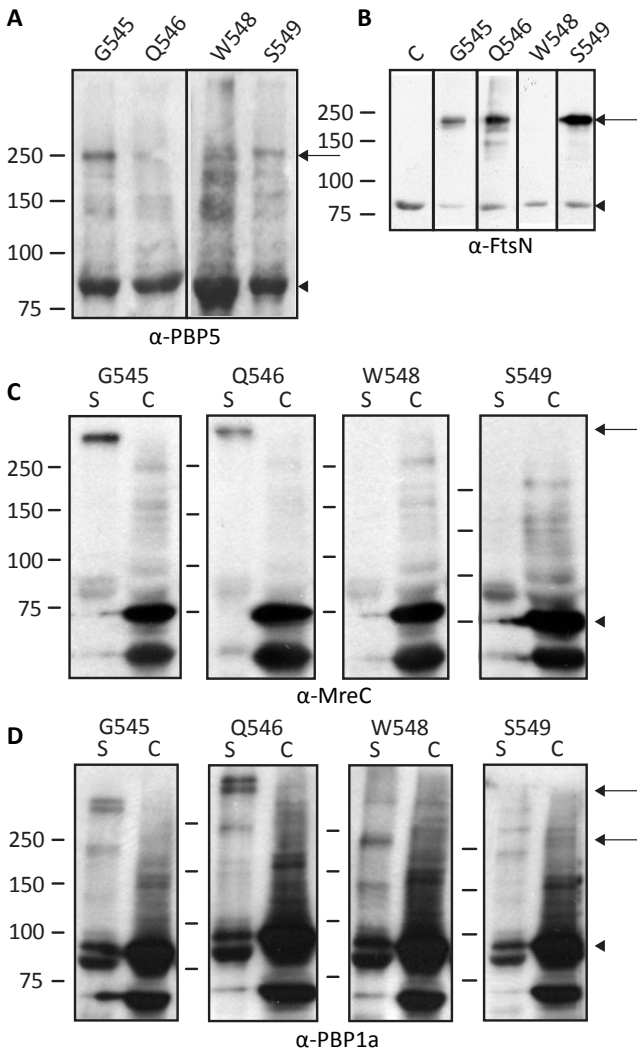


Figure 4. Immuno–blot analysis of the crosslink products of the PBP1b *p*Bpa versions G545, Q546, W548 and S549 using antibodies against PBP5, FtsN, MreC and PBP1a.

PBP1b variants with *p*Bpa incorporated at the indicated sites was expressed in *E. coli* and illuminated with UV. PBP1b and produced crosslink products were purified using nickel affinity. The fractions containing the crosslink product were separated on SDS-PAGE, and analyzed by western blot with different antibodies. A) The protein complexes of ~250 kDa of PBP1b mutants G545*p*Bpa, Q546*p*Bpa, W548*p*Bpa, and S549*p*Bpa all contain PBP5. B) The ~230 kDa protein complexes of PBP1b mutants G545*p*Bpa, Q546*p*Bpa and S549*p*Bpa contain FtsN. C) MreC is part of complexes larger than 250 kDa of PBP1b mutants G545*p*Bpa and Q546*p*Bpa. D) PBP1a was detected in samples PBP1b mutants G545*p*Bpa and Q546*p*Bpa as double bands of >250 kDa and in a band of ~250 kDa, of which the latter was also found back in the sample of W548*p*Bpa, and S549*p*Bpa. S, purified PBP1b *p*Bpa mutant sample; C, control, i.e. the total cell lysate of His_6-PBP1b expressing cells; arrowhead, unspecific band of the PBP1b monomer, arrow, crosslink product.

These results are also in accordance with the mass spectrometry analysis, where FtsN was detected in crosslink products of G545*p*Bpa, Q546*p*Bpa and S549*p*Bpa, but not in W548*p*Bpa. Of note is that the interaction of PBP1b with FtsN has been reported previously [17]. We now identify the PBP1b TP domain loop as part of the FtsN binding site. Immunostaining with antibodies against MreC confirmed the presence of this cell shape protein in high molecular weight (>250 kDa) protein complexes in PBP1b mutants G545*p*Bpa and Q546*p*Bpa (Fig 4C) which is also in agreement with the mass spectrometry results. Immunostaining with antibodies against PBP1a resulted in detection of two bands of >250 kDa, and in a band of ~250 kDa in samples of mutants G545*p*Bpa and Q546*p*Bpa, of which the ~250 kDa band was also present in the sample of W548*p*Bpa and S549*p*Bpa (Fig 4D). It is a bit odd though, that the same protein is immunostained in bands of different sizes. This could be explained by the possible crosslinking of PBP1a to PBP1b as part of different protein complexes. However, these results were not in accordance with the mass spectrometry results. PBP1a was only detected in the >250 kDa band of the samples of Q546*p*Bpa and S549*p*Bpa, and not in the samples of G545*p*Bpa and W548*p*Bpa by mass spectrometry.

We had no opportunity to obtain specific antibodies against PBP6 and TolB, hence these interactions were not further verified in this way.

Table 1. Summary of mass spectrometry results								
mutant	G545			Q546				
protein	coverage	# peptides	# spectra	coverage	# peptides	# spectra	AAs	MW (kDa)
PBP1a	X	X	X	21%	11	11	850	93,636
PBP5 (DacA)	43%	14	18	36%	11	13	403	44,44
PBP6 (dacC)	46%	14	19	36%	11	13	400	43,601
FtsN	6,9%	2	2	11%	3	3	319	37,793
MreC	12%	4	4	12%	4	4	367	39,53
TolB	29%	10	10	30%	11	12	430	45,956
mutant	W548			S549				
protein	coverage	# peptides	# spectra	coverage	# peptides	# spectra	AAs	MW (kDa)
PBP1a	X	X	X	17%	8	8	850	93,636
PBP5 (DacA)	20%	6	7	40%	14	18	403	44,44
PBP6 (dacC)	X	X	X	14%	4	4	400	43,601
FtsN	X	X	X	21%	6	6	319	37,793
MreC	X	X	X	X	X	X	367	39,53
TolB	X	X	X	15%	5	5	430	45,956

Table 1. Summary of mass spectrometry results for the proteins described in the text.
Coverage: percentage of protein sequence covered by the identified peptides. #peptides: number of unique peptides of the protein identified in the sample. # spectra: number of individual spectra in which the peptides were identified.

Affinity chromatography

Next, we tested if we could trace back the proteins identified by *in vivo* photo-crosslinking, by using affinity chromatography with a synthetic peptide corresponding to the loop structure. This loop peptide was chemically synthesized and used to prepare an affinity column. As the D535 and N552 side chains of this loop are in close proximity to each other and presumably linked by a hydrogen bond, according to the PBP1b crystal structure (Fig 2A), we replaced these residues by cysteines resulting in a cyclic peptide with a disulfide bridge (C-A_{536}PIALRQPNGQVWSPQ$_{551}$-C). This cyclized peptide was covalently bound to Magnabind-N_3 beads via an alkyne at the N-terminal cysteine residue. It should be taken into account that the isolated loop might be part of a larger interface between PBP1b and interacting proteins, which could lead to reduced affinities of binding partners to the immobilized loop-peptide. We applied fractions enriched for periplasmic, inner- or outer- membrane proteins to this column, and separated samples of different wash and elution fractions by SDS-PAGE (Fig 5 and Fig S1). The method turned out to be not very specific, since proteins eluted in every step, and none were highly concentrated in the last elution step. However, the previously identified PBP1b interaction partners PBP5 and 6 could be identified in inner membrane fraction band 7 and PBP1a and b in outer membrane fraction band 2. FtsN was found in inner membrane fraction band 10 and TolB in outer membrane fraction band 5. The identification of PBP1a and b and TolB was not expected to be in the outer membrane fraction. However, this could be caused by

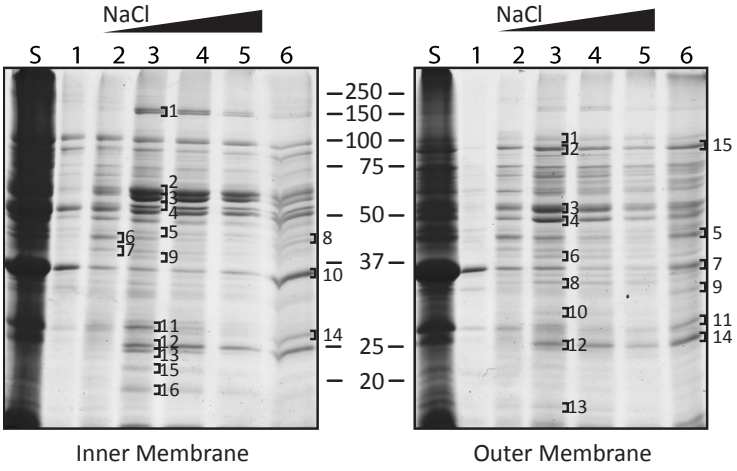


Figure 5. Coomassie blue stained SDS-PAGE gels with inner- and outer membrane fractions from affinity chromatography with PBP1b loop peptide C535-C552.

Proteins were eluted with buffer containing increasing NaCl concentration or by a pH shift. S, sample; 1, wash 50 mM Tris/HCl pH 7.5, 0.1% Triton X-100 (buffer A); 2, elution with buffer A containing 100 mM NaCl; 3, elution with buffer A containing 300 mM NaCl; 4, elution with buffer A containing 500 mM NaCl; 5, elution with buffer A containing 1 M NaCl; 6, elution with 1 M NH_4HCO_3 pH 9.0. Protein bands excised for MS analysis are marked and numbered.

the interactions of these proteins with outer membrane proteins (LpoA, LpoB and Pal respectively [14], [16], [19], [45]–[47]). In addition, the amidase AmiC, which is involved in septum cleavage during cell division, was detected in outer membrane fraction band 5 by this procedure. Common for these found proteins is that they have a periplasmic part that could potentially interact with the TP domain of PBP1b. The identification of some of these proteins in the same bands is due to their comparable sizes.
Based on these analysis methods, we conclude that the loop of the TP domain of PBP1b may play a role in various protein-protein interactions and therefore might be required for the regulation of PBP1b activity in protein complexes. The inability to detect MreC by the affinity chromatography method could be caused by the fact that this interaction may need a larger part of PBP1b than only the loop peptide.
In summary our results demonstrate that the loop D535-S558 in the TP domain of *E. coli* PBP1b is essential for its functionality, probably due to participation in interactions with PBP5, PBP1a, FtsN and MreC.

Discussion

In this study we identified the PBP1b TP domain loop D535-S558 as an important part of the TP domain. Based on assays for *in vivo* complementation and *in vitro* PG synthesis with a deletion mutant, we deduce that the loop is important for PBP1b TP activity. This, together with the multiple different proteins that were identified in the *in vivo* crosslinking studies, suggests that this loop might be a regulatory handle for the TP activity of this protein.
Our loop mutant PBP1bΔD535-S558, lacking the amino acids D535-S558 is not able to perform the TP reaction, tough it remained active in binding the donor peptide analog Bocillin (Fig 2). Therefore, we envision that the loop is likely important for acceptor peptide binding. Furthermore, the PBP1b mutant having W548 replaced by the photo-crosslinker *p*Bpa was nonfunctional, as shown in the complementation assay, though other mutants having flanking residues replaced were still active (Fig 3B). This confirms the importance of the loop in PBP1b activity, and pointing to a specific role for W548 herein. This is supported by the finding of Triboulet *et al.* that a single tryptophan (W425) in a comparable loop structure of the *Enterococcus Faecium* transpeptidase Ldt_{fm} is responsible for acceptor peptide binding in this protein [48]. If the tryptophan in this loop structure of PBP1b has a similar function, this would be an explanation of the inability of the loop mutant to perform the TP reaction, though it was still able to bind the D-Ala-D-Ala donor substrate. Analysis of the structure of PBP1b shows that W548 is positioned close to and facing towards the active site serine (S510) as is the case for W425 in respect to the active site cysteine (C442) in Ldt_{fm}. Interestingly, when comparing the crystal structure of 20 proteins with transpeptidase activity of different bacteria, they all seem to

have a loop structure covering the active site. These loop structures do not all contain a tryptophan, but do contain a tyrosine in structures without a tryptophan, which all face the active site serine or cysteine (Table S2). This suggests that TP domains need an aromatic residue in close proximity to the active site, which is probably involved in acceptor peptide binding.

Furthermore, *in vivo* photo-crosslinking experiments, using site specific incorporation of an unnatural amino acid with photo-crosslinking properties, showed that this loop is also an interaction hot spot, involved in several interactions with other proteins. We identified PBPs 1a, 5 and 6, FtsN, MreC and TolB as proteins that possibly interact with the loop of the TP domain of PBP1b. Some of these interactions were later verified by immunoblot using specific antibodies. We were able to trace back a number of these proteins with affinity chromatography using the loop C-A_{536}PIALRQPNGQVWSPQ$_{551}$-C as bait. We hypothesize that the interactions of this loop with the identified proteins might be important for the regulation of the state of this loop, making it more or less accessible for acceptor peptide binding. Furthermore, the interacting proteins could also control the degree of crosslinkage by regulating the availability of donor and acceptor peptides (PBP5 and PBP6) and/or the site in the sacculus where it is active (MreC) in a multi enzyme complex.

From a sterical perspective, it is not likely that all the identified proteins form one single complex with PBP1b. Rather, the detected proteins may reflect the range of interactions that the loop of PBP1b has during the different stages of cell wall synthesis and the cell cycle, which are required for the control of TP activity during these different stages. This is corroborated by the fact that the binding partners are part of proposed multi enzyme complexes like the divisome and elongasome that are formed at different stages of PG synthesis during the cell cycle [4], [5].

Of note is that the detected crosslink products migrate in SDS-PAGE between 150 and 250 kDa molecular weight. All crosslink products that were detected with immuno-blot (Fig 3) are too large to be a complex of one PBP1b (89 kDa) monomer and one interacting partner like PBP5 (41 kDa), FtsN (36 kDa), MreC (40 kDa) or PBP1a (94 kDa). Several possibilities might explain these results. One is that the complexed proteins behave differently on SDS-PAGE because of a decreased accessibility of the proteins for SDS after crosslinking and therefore, incomplete unfolding. It was described before that the position of a crosslink between two proteins can influence the electrophoretic mobility of the protein complex, resulting in 10-20 kDa shifts between expected and detected molecular weights [49], [50]. Furthermore, it is feasible that two monomers of PBP1b sandwich the interacting proteins, resulting in larger products. This would imply that the interacting partner possesses two binding sites for PBP1b.

The interaction between PBP1b and PBP1a, the second major bifunctional PG synthase in *E. coli,* was surprising. Both, PBP1a and PBP1b form homodimers, but a heterodimer was

never detected [51]. Furthermore, there are indications that PBP1b and PBP1a might be part of different protein complexes: PBP1b interacts with PBP3, FtsN and LpoB in the divisome, and PBP1a with PBP2 and LpoA in the elongasome [9], [14]–[17]. Nevertheless, they can substitute for each other, indicating at least a partial redundancy in their function [8]. Besides, specific TPs of the elongasome (PBP2) and divisome (PBP3) interact during a preparative phase of cell division as well [52]. Therefore, it is possible that PBP1a and PBP1b also interact during this phase in the cell cycle.

PBP1b localizes at the cell periphery as well as at the division site, where PG synthesis is organized by the actin homolog MreB and the tubulin homolog FtsZ, respectively [9], [53]. It is suggested that MreB might deliver PBP1b to the division site after maturation of the divisome [53]. MreB interacts with MreC in the cytoplasm and our results suggest that MreC also interacts with the TP loop of PBP1b by its large periplasmic portion. Perhaps this interaction is involved in the regulation of PBP1b activity at the lateral wall and its recruitment at mid cell during cell division. A similar role of MreC in organizing PBPs has been proposed in *B. subtilis* and in *C. crescentus* [54]–[56].

The DD-carboxypeptidase PBP5 is localized in both, the lateral wall and the division site, depending on active PG synthesis, which gives rise to the presence of its substrate: the pentapeptides [57]. Its interaction with PBP1b (Fig 4A) supports the suggestion that PBP5 might have a regulatory role on the number of peptide crosslinks formed in the PG, by the removal of terminal D-alanines from defined positions in newly synthesized PG. This reaction results in the formation of tetrapeptides that can only act as acceptors in the TP reaction [2].

During cell division the growth and invagination of the inner- and outer- membrane, and PG network located in between, has to occur in a coordinated manner to prevent cell lysis. It was demonstrated that the Tol-Pal system is essential for proper outer membrane constriction by tethering the three envelope layers [47]. This complex comprises the periplasmic proteins TolB and CpoB, the PG-associated outer membrane lipoprotein Pal, the inner membrane proteins TolA, TolQ, TolR and the cytoplasmic YbgC [58]. The outer membrane protein Pal binds the peptide moieties of PG, linking these two layers. Next, Pal also binds TolA, which forms a complex with the inner membrane proteins TolQ/R, providing a link between the three envelope layers. Recently it has been found that TolA and CpoB also interact with PBP1b, regulating the intensity of the stimulatory activity of LpoB on PBP1bs TP activity [21]. In the here presented experiments we identified TolB as another putative interacting partner of PBP1b, providing an additional link between PG synthesis machinery and outer membrane constriction, and with that an extra layer of regulation of these processes during cell division.

In conclusion the PBP1b TP domain loop consisting of D535-S558 is conserved in HMW PBPs, required for enzyme activity and function, and is involved in multiple protein-protein interactions. These multiple identified interactions presumably enable the

formation of protein complexes and could regulate the substrate availability and TP activity. We speculate that this loop structure is involved in acceptor peptide binding, with a direct role of the tryptophan at position 548. Generating novel antibiotics, targeting the function of this loop, or preventing essential interactions between PBPs and their regulators, could be a new therapeutic opportunity for treatment of gram negative bacterial infections.

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References

[1] J. V. Höltje, "Growth of the stress-bearing and shape-maintaining murein sacculus of Escherichia coli," *Microbiol. Mol. Biol. Rev. MMBR*, vol. 62, no. 1, pp. 181–203, Mar. 1998.

[2] W. Vollmer, B. Joris, P. Charlier, and S. Foster, "Bacterial peptidoglycan (murein) hydrolases," *FEMS Microbiol. Rev.*, vol. 32, no. 2, pp. 259–286, Mar. 2008.

[3] J.-L. Mainardi, R. Villet, T. D. Bugg, C. Mayer, and M. Arthur, "Evolution of peptidoglycan biosynthesis under the selective pressure of antibiotics in Gram-positive bacteria," *FEMS Microbiol. Rev.*, vol. 32, no. 2, pp. 386–408, Mar. 2008.

[4] T. den Blaauwen, M. A. de Pedro, M. Nguyen-Distèche, and J. A. Ayala, "Morphogenesis of rod-shaped sacculi," *FEMS Microbiol. Rev.*, vol. 32, no. 2, pp. 321–344, Mar. 2008.

[5] A. Typas, M. Banzhaf, C. A. Gross, and W. Vollmer, "From the regulation of peptidoglycan synthesis to bacterial growth and morphology," *Nat. Rev. Microbiol.*, vol. 10, no. 2, pp. 123–136, Feb. 2012.

[6] E. Sauvage, F. Kerff, M. Terrak, J. A. Ayala, and P. Charlier, "The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis," *FEMS Microbiol. Rev.*, vol. 32, no. 3, pp. 556–556, 2008.

[7] J. M. Ghuysen, "Serine beta-lactamases and penicillin-binding proteins," *Annu. Rev. Microbiol.*, vol. 45, pp. 37–67, 1991.

[8] S. Y. Yousif, J. K. Broome-Smith, and B. G. Spratt, "Lysis of Escherichia coli by beta-lactam antibiotics: deletion analysis of the role of penicillin-binding proteins 1A and 1B," *J. Gen. Microbiol.*, vol. 131, no. 10, pp. 2839–2845, Oct. 1985.

[9] U. Bertsche, T. Kast, B. Wolf, C. Fraipont, M. E. G. Aarsman, K. Kannenberg, M. Von Rechenberg, M. Nguyen-Distèche, T. Den Blaauwen, J.-V. Höltje, and W. Vollmer, "Interaction between two murein (peptidoglycan) synthases, PBP3 and PBP1B, in Escherichia coli," *Mol. Microbiol.*, vol. 61, no. 3, pp. 675–690, Aug. 2006.

[10] J. Nakagawa, S. Tamaki, S. Tomioka, and M. Matsuhashi, "Functional biosynthesis of cell wall peptidoglycan by polymorphic bifunctional polypeptides. Penicillin-binding protein 1Bs of Escherichia coli with activities of transglycosylase and transpeptidase," *J. Biol. Chem.*, vol. 259, no. 22, pp. 13937–13946, Nov. 1984.

[11] M. Terrak, T. K. Ghosh, J. van Heijenoort, J. Van Beeumen, M. Lampilas, J. Aszodi, J. A. Ayala, J. M. Ghuysen, and M. Nguyen-Distèche, "The catalytic, glycosyl transferase and acyl transferase modules of the cell wall peptidoglycan-polymerizing penicillin-binding protein 1b of Escherichia coli," *Mol. Microbiol.*, vol. 34, no. 2, pp. 350–364, Oct. 1999.

[12] M.-T. Sung, Y.-T. Lai, C.-Y. Huang, L.-Y. Chou, H.-W. Shih, W.-C. Cheng, C.-H. Wong, and C. Ma, "Crystal structure of the membrane-bound bifunctional transglycosylase PBP1b from Escherichia coli," *Proc. Natl. Acad. Sci.*, vol. 106, no. 22, pp. 8824–8829, Jun. 2009.

[13] R. A. Nicholas, D. R. Lamson, and D. E. Schultz, "Penicillin-binding protein 1B from Escherichia coli contains a membrane association site in addition to its transmembrane anchor," *J. Biol. Chem.*, vol. 268, no. 8, pp. 5632–5641, Mar. 1993.

[14] A. Typas, M. Banzhaf, B. van den Berg van Saparoea, J. Verheul, J. Biboy, R. J. Nichols, M. Zietek, K. Beilharz, K. Kannenberg, M. von Rechenberg, E. Breukink, T. den Blaauwen, C. A. Gross, and W. Vollmer, "Regulation of Peptidoglycan Synthesis by Outer-Membrane Proteins," *Cell*, vol. 143, no. 7, pp. 1097–1109, Dec. 2010.

[15] M. Banzhaf, B. van den Berg van Saparoea, M. Terrak, C. Fraipont, A. Egan, J. Philippe, A. Zapun, E. Breukink, M. Nguyen-Distèche, T. den Blaauwen, and W. Vollmer, "Cooperativity of peptidoglycan synthases active in bacterial cell elongation," *Mol. Microbiol.*, vol. 85, no. 1, pp. 179–194, Jul. 2012.

[16] C. Paradis-Bleau, M. Markovski, T. Uehara, T. J. Lupoli, S. Walker, D. E. Kahne, and T. G. Bernhardt, "Lipoprotein Cofactors Located in the Outer Membrane Activate Bacterial Cell Wall Polymerases," *Cell*, vol. 143, no. 7, pp. 1110–1120, Dec. 2010.

[17] P. Müller, C. Ewers, U. Bertsche, M. Anstett, T. Kallis, E. Breukink, C. Fraipont, M. Terrak, M. Nguyen-Distèche, and W. Vollmer, "The Essential Cell Division Protein FtsN Interacts with the Murein (Peptidoglycan) Synthase PBP1B in Escherichia coli," *J. Biol. Chem.*, vol. 282, no. 50, pp. 36394–36402, Dec. 2007.

[18] W. Vollmer, M. von Rechenberg, and J.-V. Höltje, "Demonstration of Molecular Interactions between the Murein Polymerase PBP1B, the Lytic Transglycosylase MltA, and the Scaffolding Protein MipA of Escherichia coli," *J. Biol. Chem.*, vol. 274, no. 10, pp. 6726–6734, Mar. 1999.

[19] T. J. Lupoli, M. D. Lebar, M. Markovski, T. Bernhardt, D. Kahne, and S. Walker, “Lipoprotein Activators Stimulate Escherichia coli Penicillin-Binding Proteins by Different Mechanisms,” *J. Am. Chem. Soc.*, vol. 136, no. 1, pp. 52–55, Jan. 2014.

[20] A. J. F. Egan, N. L. Jean, A. Koumoutsi, C. M. Bougault, J. Biboy, J. Sassine, A. S. Solovyova, E. Breukink, A. Typas, W. Vollmer, and J.-P. Simorre, “Outer-membrane lipoprotein LpoB spans the periplasm to stimulate the peptidoglycan synthase PBP1B,” *Proc. Natl. Acad. Sci.*, vol. 111, no. 22, pp. 8197–8202, Jun. 2014.

[21] A. N. Gray, A. J. F. Egan, I. L. van’t Veer, J. Verheul, A. Colavin, A. Koumoutsi, J. Biboy, M. A. F. Altelaar, M. J. Damen, K. C. Huang, J.-P. Simorre, E. Breukink, T. den Blaauwen, A. Typas, C. A. Gross, and W. Vollmer, “Coordination of peptidoglycan synthesis and outer membrane constriction during Escherichia coli cell division,” *eLife*, p. e07118, May 2015.

[22] C. Fraipont, S. Alexeeva, B. Wolf, R. van der Ploeg, M. Schloesser, T. den Blaauwen, and M. Nguyen-Distèche, “The integral membrane FtsW protein and peptidoglycan synthase PBP3 form a subcomplex in Escherichia coli,” *Microbiol. Read. Engl.*, vol. 157, no. Pt 1, pp. 251–259, Jan. 2011.

[23] M. von Rechenberg, A. Ursinus, and J. V. Höltje, “Affinity chromatography as a means to study multienzyme complexes involved in murein synthesis,” *Microb. Drug Resist. Larchmt. N*, vol. 2, no. 1, pp. 155–157, 1996.

[24] G. Schiffer and J. V. Höltje, “Cloning and characterization of PBP 1C, a third member of the multimodular class A penicillin-binding proteins of Escherichia coli,” *J. Biol. Chem.*, vol. 274, no. 45, pp. 32031–32039, Nov. 1999.

[25] J. W. Chin, A. B. Martin, D. S. King, L. Wang, and P. G. Schultz, “Addition of a photocrosslinking amino acid to the genetic code of Escherichia coli,” *Proc. Natl. Acad. Sci.*, vol. 99, no. 17, pp. 11020–11024, Aug. 2002.

[26] Y. Ryu and P. G. Schultz, “Efficient incorporation of unnatural amino acids into proteins in Escherichia coli,” *Nat. Methods*, vol. 3, no. 4, pp. 263–265, Apr. 2006.

[27] R. Higuchi, B. Krummel, and R. K. Saiki, “A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions,” *Nucleic Acids Res.*, vol. 16, no. 15, pp. 7351–7367, Aug. 1988.

[28] U. Bertsche, E. Breukink, T. Kast, and W. Vollmer, “In Vitro Murein (Peptidoglycan) Synthesis by Dimers of the Bifunctional Transglycosylase-Transpeptidase PBP1B from Escherichia coli,” *J. Biol. Chem.*, vol. 280, no. 45, pp. 38096–38101, Nov. 2005.

[29] J. Biboy, N. K. Bui, and W. Vollmer, “In vitro peptidoglycan synthesis assay with lipid II substrate,” *Methods Mol. Biol. Clifton NJ*, vol. 966, pp. 273–288, 2013.

[30] A. J. Lesse, A. A. Campagnari, W. E. Bittner, and M. A. Apicella, “Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis,” *J. Immunol. Methods*, vol. 126, no. 1, pp. 109–117, Jan. 1990.

[31] D. Barrett, T.-S. A. Wang, Y. Yuan, Y. Zhang, D. Kahne, and S. Walker, “Analysis of Glycan Polymers Produced by Peptidoglycan Glycosyltransferases,” *J. Biol. Chem.*, vol. 282, no. 44, pp. 31964–31971, Nov. 2007.

[32] E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, “Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides,” *Anal. Biochem.*, vol. 34, no. 2, pp. 595–598, Apr. 1970.

[33] T. Vojkovsky, “Detection of secondary amines on solid phase,” *Pept. Res.*, vol. 8, no. 4, pp. 236–237, Aug. 1995.

[34] E. D. Goddard-Borger and R. V. Stick, “An Efficient, Inexpensive, and Shelf-Stable Diazotransfer Reagent: Imidazole-1-sulfonyl Azide Hydrochloride,” *Org. Lett.*, vol. 9, no. 19, pp. 3797–3800, Sep. 2007.

[35] K. R. Gee, H. C. Kang, T. I. Meier, G. Zhao, and L. C. Blaszcak, “Fluorescent Bocillins: synthesis and application in the detection of penicillin-binding proteins,” *Electrophoresis*, vol. 22, no. 5, pp. 960–965, Mar. 2001.

[36] M. Wilm, A. Shevchenko, T. Houthaeve, S. Breit, L. Schweigerer, T. Fotsis, and M. Mann, “Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry,” *Nature*, vol. 379, no. 6564, pp. 466–469, Feb. 1996.

[37] P. Macheboeuf, A. M. Di Guilmi, V. Job, T. Vernet, O. Dideberg, and A. Dessen, “Active site restructuring regulates ligand recognition in class A penicillin-binding proteins,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 3, pp. 577–582, Jan. 2005.

[38] C. Contreras-Martel, C. Dahout-Gonzalez, A. D. S. Martins, M. Kotnik, and A. Dessen, “PBP active site flexibility as the key mechanism for beta-lactam resistance in pneumococci,” *J. Mol. Biol.*, vol. 387, no. 4, pp. 899–909, Apr. 2009.

[39] C. Contreras-Martel, V. Job, A. M. Di Guilmi, T. Vernet, O. Dideberg, and A. Dessen, “Crystal structure of penicillin-binding protein 1a (PBP1a) reveals a mutational hotspot implicated in beta-lactam resistance in Streptococcus pneumoniae,” *J. Mol. Biol.*, vol. 355, no. 4, pp. 684–696, Jan. 2006.

[40] L. Pernot, L. Chesnel, A. Le Gouellec, J. Croizé, T. Vernet, O. Dideberg, and A. Dessen, “A PBP2x from a clinical isolate of Streptococcus pneumoniae exhibits an alternative mechanism for reduction of susceptibility to beta-lactam antibiotics,” *J. Biol. Chem.*, vol. 279, no. 16, pp. 16463–16470, Apr. 2004.

[41] D. Lim and N. C. J. Strynadka, “Structural basis for the beta lactam resistance of PBP2a from methicillin-resistant Staphylococcus aureus,” *Nat. Struct. Biol.*, vol. 9, no. 11, pp. 870–876, Nov. 2002.

[42] R. A. Nicholas, S. Krings, J. Tomberg, G. Nicola, and C. Davies, “Crystal structure of wild-type penicillin-binding protein 5 from Escherichia coli: implications for deacylation of the acyl-enzyme complex,” *J. Biol. Chem.*, vol. 278, no. 52, pp. 52826–52833, Dec. 2003.

[43] Y. Chen, W. Zhang, Q. Shi, D. Hesek, M. Lee, S. Mobashery, and B. K. Shoichet, “Crystal Structures of Penicillin-Binding Protein 6 from Escherichia coli,” *J. Am. Chem. Soc.*, vol. 131, no. 40, pp. 14345–14354, Oct. 2009.

[44] C. A. Zijderveld, M. E. Aarsman, T. den Blaauwen, and N. Nanninga, “Penicillin-binding protein 1B of Escherichia coli exists in dimeric forms.,” *J. Bacteriol.*, vol. 173, no. 18, pp. 5740–5746, Sep. 1991.

[45] N. L. Jean, C. M. Bougault, A. Lodge, A. Derouaux, G. Callens, A. J. F. Egan, I. Ayala, R. J. Lewis, W. Vollmer, and J.-P. Simorre, “Elongated structure of the outer-membrane activator of peptidoglycan synthesis LpoA: implications for PBP1A stimulation,” *Struct. Lond. Engl. 1993*, vol. 22, no. 7, pp. 1047–1054, Jul. 2014.

[46] A. Walburger, C. Lazdunski, and Y. Corda, “The Tol/Pal system function requires an interaction between the C-terminal domain of TolA and the N-terminal domain of TolB,” *Mol. Microbiol.*, vol. 44, no. 3, pp. 695–708, May 2002.

[47] M. A. Gerding, Y. Ogata, N. D. Pecora, H. Niki, and P. A. J. De Boer, “The trans-envelope Tol–Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in E. coli,” *Mol. Microbiol.*, vol. 63, no. 4, pp. 1008–1025, Feb. 2007.

[48] K. Plath, W. Mothes, B. M. Wilkinson, C. J. Stirling, and T. A. Rapoport, “Signal sequence recognition in posttranslational protein transport across the yeast ER membrane,” *Cell*, vol. 94, no. 6, pp. 795–807, Sep. 1998.

[49] M. Berg, A. Michalowski, S. Palzer, S. Rupp, and K. Sohn, “An In Vivo Photo-Cross-Linking Approach Reveals a Homodimerization Domain of Aha1 in S. cerevisiae,” *PLoS ONE*, vol. 9, no. 3, p. e89436, Mar. 2014.

[50] X. Charpentier, C. Chalut, M.-H. Rémy, and J.-M. Masson, “Penicillin-binding proteins 1a and 1b form independent dimers in Escherichia coli,” *J. Bacteriol.*, vol. 184, no. 13, pp. 3749–3752, Jul. 2002.

[51] R. van der Ploeg, J. Verheul, N. O. E. Vischer, S. Alexeeva, E. Hoogendoorn, M. Postma, M. Banzhaf, W. Vollmer, and T. den Blaauwen, “Colocalization and interaction between elongasome and divisome during a preparative cell division phase in Escherichia coli,” *Mol. Microbiol.*, vol. 87, no. 5, pp. 1074–1087, Mar. 2013.

[52] A. K. Fenton and K. Gerdes, “Direct interaction of FtsZ and MreB is required for septum synthesis and cell division in Escherichia coli,” *EMBO J.*, vol. 32, no. 13, pp. 1953–1965, Jul. 2013.

[53] F. van den Ent, M. Leaver, F. Bendezu, J. Errington, P. de Boer, and J. Löwe, “Dimeric structure of the cell shape protein MreC and its functional implications,” *Mol. Microbiol.*, vol. 62, no. 6, pp. 1631–1642, Dec. 2006.

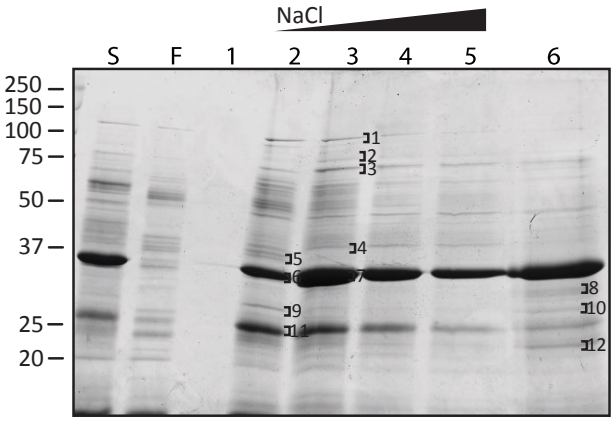
[54] A. V. Divakaruni, R. R. O. Loo, Y. Xie, J. A. Loo, and J. W. Gober, “The cell-shape protein MreC interacts with extracytoplasmic proteins including cell wall assembly complexes in Caulobacter crescentus,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 51, pp. 18602–18607, Dec. 2005.

[55] C. L. White, A. Kitich, and J. W. Gober, “Positioning cell wall synthetic complexes by the bacterial morphogenetic proteins MreB and MreD,” *Mol. Microbiol.*, vol. 76, no. 3, pp. 616–633, May 2010.

[56] L. Potluri, A. Karczmarek, J. Verheul, A. Piette, J.-M. Wilkin, N. Werth, M. Banzhaf, W. Vollmer, K. D. Young, M. Nguyen-Distèche, and T. den Blaauwen, “Septal and lateral wall localization of PBP5, the major D,D-carboxypeptidase of Escherichia coli, requires substrate recognition and membrane attachment,” *Mol. Microbiol.*, vol. 77, no. 2, pp. 300–323, Jul. 2010.

[57] R. Godlewska, K. Wiśniewska, Z. Pietras, and E. K. Jagusztyn-Krynicka, “Peptidoglycan-associated lipoprotein (Pal) of Gram-negative bacteria: function, structure, role in pathogenesis and potential application in immunoprophylaxis,” *FEMS Microbiol. Lett.*, vol. 298, no. 1, pp. 1–11, Sep. 2009.

Supplementary information



Supplementary figure 1: Coomassie blue stained SDS-PAGE gel with periplasmic fraction from affinity chromatography with PBP1b loop peptide C535-C552.
Proteins were eluted with buffer containing increasing NaCl concentration or by a pH shift. S, sample; F, flow through; 1, wash 50 mM Tris/HCl pH 7.5, 0.1% Triton X-100 (buffer A); 2, elution with buffer A containing 100 mM NaCl; 3, elution with buffer A containing 300 mM NaCl; 4, elution with buffer A containing 500 mM NaCl; 5, elution with buffer A containing 1 M NaCl; 6, elution with 1 M NH_4HCO_3 pH 9.0. Protein bands excised for MS analysis are marked and numbered.

supplementary table 1.			
Δloop	ScaI	fwd	CATTGACGGCAGTACTGTGACCATCACC
	introduction	rev	GGTGATGGTCACAGTACTGCCGTCAATG
	overlap	fwd A	GAGCTGCAGGCAAAACTGGGCG
	extension	rev B	CACTCTGCCGCTTTCCGCAATCCAGC
	PCR	fwd C	GAATACGTGGATTGCGGAAAGCGGCAG
		rev D	CACAGTACTGCCGTCAATGCCCG
G545 → TAG		fwd	GTCAGCCGAATTAGCAGGTCTGGTC
		rev	GACCAGACCTGCTAATTCGGCTGAC
Q546→ TAG		fwd	GCCGAATGGCTAGGTCTGGTCAC
		rev	GTGACCAGACCTAGCCATTCGGC
W548 → TAG		fwd	GGCCAGGTCTAGTCACCGCAG
		rev	CTGCGGTGACTAGACCTGGCC
S459 → TAG		fwd	CAGGTCTGGTAGCCGCAGAATG
		rev	CATTCTGCGGCTACCAGACCTG

Supplementary table 1. Used primers for the creation of Δloop and amber mutants.

Supplementary table 2.				
Enterococcus Faecium				
PDB code	protein	active site	loop	sequence
3ZG4	Ldtfm	C442	D422-L433	DSDWQPEYGGDL
1ZAT	Ldt_{fm217}	C442	T392-P406	TLKGTNGTPYESP
Bacillus subtilis				
PDB code	protein	active site	loop	sequence
3ZQD	Ldt_{Bs}	C142	I101-L118	INRQRNPGGPFGAYWLSL
4A1K	Ykud	C139	G109-N127	GAYWLSLSAAHYGIHGTNN
Streptococcus pneumoniae				
PDB code	protein	active site	loop	sequence
2C6W	PBP1a	S370	H395-G417	HDEPYNYPGTNTPVYNWDRGYFG
2FFF	PBP1b	S460	N485-M506	NYPTNFANGNPIMYANSKGTGM
2WAD	PBP2B	S386	L408-F432	LTDQPIVFQGSAPIYSWYKLAYGSF
1QME	PBP2X	S337	S361-R384	SSELKIADATIRDWDVNEGLTGGR
MRSA				
PDB code	protein	active site	loop	sequence
1VQQ	PBP2a	S403	I427-G451	IDFKGWQKDKSWGGYNVTRYEVVNG
3VSK	PBP3	S392	E412-N440	ETMVDEPLHFQGGLTKRSYFNKNGHVSIN
Acinetobacter baumannii				
PDB code	protein	active site	loop	sequence
3UDF	PBP1a	S434	M449-P479	MTPYSMVNDSPITIGKWTPKNSDGRYLGMIP
3UE3	PBP3	S336	N359-A380	NTAPGTMRLGWHTIRDTHNYGA
pseudomonas aerginosa				
PDB code	protein	active site	loop	sequence
3OC2	PBP3	S294	D317-Q339	DVYPGTLQIGRYTIRDVSRNSRQ
Escherichia coli				
PDB code	protein	active site	loop	sequence
4BJP	PBP3	S307	N330-S348	NTIPYRINGHEIKDVARYS
3VMA	PBP1b	S510	D535-S558	DAPIALRQPNGQVWSPQNDDRRYS
Mycobacterium tuberculosis				
PDB code	protein	active site	loop	sequence
4JMX	LdtMt1	S203	D176-L191	DSRTIGIPLNSSDGYL
4GSR	LdtMt2	S331	D304-R319	DSSTYGVPVNSPNGYR
4Z7A	LdtMt5	C360	F314-E328	FYMSNPAAGYSHIHE
4PPR	dacB1	S121	G206-S226	GGRDTRVATPSGLDGPGMSTS
3UN7	PBPA	S222	T244-T271	TAAPTIPLPGSTAQLENYGGAPCGDEPT

Supplementary table 2. Analysis of TP domains of different proteins of different species shows that they all have a loop structure covering the active site, that all contain an aromatic residue in this structure, close to and facing towards the active site (underlined).

Chapter 4

Site specific immobilization of PBP1b on an SPR chip surface

Inge van 't Veer, Nadia Leloup, Alexander Egan, Bert Janssen, Waldemar Vollmer, Nathaniel Martin and Eefjan Breukink

Abstract

Surface Plasmon Resonance (SPR) has shown to be one of the most powerful label free methods to determine kinetic parameters of protein-protein interactions as well as protein-substrate interactions in real time and in a highly sensitive way. To analyze interactions of penicillin binding proteins (PBPs) by SPR, immobilization is accomplished by coating the chip surface with ampicillin, to have a covalent interaction with the active site of the transpeptidase (TP) domain of the PBPs. However, in this way interactions are measures with PBPs with a substrate bound TP domain, which can have an effect on the interaction profile, and may obscure important interaction sites in the TP domain near the active site. Furthermore, they are immobilized in an orientation far from the *in vivo* situation. Therefore, we designed a new immobilization method in which an azide, incorporated in the cytoplasmic tail, in combination with copper free click chemistry, is used for the immobilization of PBP1b on an SPR chip surface. This method has shown to be useful for the analysis of the already known interaction of PBP1b with LpoB, and can be applied for the analysis of any protein-protein interaction with a homogeneous immobilization and a chosen protein orientation.

Manuscript in preparation

Introduction

For the kinetic characterization of protein-protein interactions, as well as protein-substrate interactions, surface plasmon resonance (SPR) studies are often performed. In this assay, one part of an interacting couple is immobilized on a sensor chip, and the interacting analyte is injected over the chip surface. This will generate an SPR sensorgram, from which the association and dissociation rate constant, and hence the affinity, as well as the equilibrium constant of the interaction can be deduced. By using this technique, the interaction of penicillin binding protein (PBP) 1b with MltA, indirectly via MipA [1], with LpoB [2] and with itself [3] have been investigated. Furthermore, the interaction of PBP3 with PBP1b was also investigated in this way, though, here PBP3 was immobilized [4]. For investigating interactions of PBPs with other proteins using SPR, immobilization was realized via their transpeptidase (TP) domain to the sensor chip. This was achieved by the coupling of ampicillin by its amine to an NHS activated chip surface and subsequent covalent interaction of this β-lactam antibiotic with the active site of the TP domain of the PBP. However, this immobilization method results in a situation in which measurements are performed with PBPs with an occupied active site, which can have effects on the interaction profile of the protein. Besides, the proteins also have an orientation far from the *in vivo* situation, where it is inserted in the membrane with its transmembrane domain. In addition, in chapter 3 we have shown that the loop covering the entrance of the active site of the TP domain of PBP1b, is involved in several protein-protein interactions. Such interactions may be sterically hindered, and hence cannot be investigated via SPR using the above mentioned ampicillin-based immobilization strategy. Furthermore, we also showed that this loop is essential for the TP activity of PBP1b, which implicates a role for this loop in the regulation of the TP reaction and possibly in the conformational change caused by or needed for the aforementioned protein-protein interactions. To be able to analyze interactions of PBP1b with its interaction partners in a way that more resembles the *in vivo* situation we designed a new immobilization method in which we incorporated an azide in the cytoplasmic tail of PBP1b to use copper free click chemistry for the immobilization of PBP1b on an SPR chip surface. This experimental setup ensures a correct topological orientation of the protein with an accessible and fully active TP domain, thereby allowing characterization of interactions with this domain.

Materials and methods

Bacterial strains and plasmids

E. coli DH5α cells were used for DNA amplification. *E. coli* BL21 (DE3) cells were used for protein expression. Plasmid pDML924 carrying the *PonB* gene, encoding the N- terminal His_6-tagged PBP1bγ variant (gift from M. Terrak) was used for overexpression of PBP1b

and as a template for the creation of PBP1b amber mutants. Plasmid pEvol-*p*AzF encoding the orthogonal aminoacyl tRNA synthase-$tRNA_{CUA}$ pair was used for incorporation of *p*-azidophenylalanine at the site of amber mutation.

Site directed mutagenesis

The amber mutants were created by mutagenesis PCR using the primers listed in supplementary table 1. The reaction mixture contained 125 ng fwd and 125 ng rev primer, 1 µl dNTPs, 10 mM each, 1 µl 61.6 ng/µl template and 0.5 µl Phusion DNA polymerase (Thermo Scientific 2U/µl) in a total volume of 50 µl. 17 cycles of 30 sec @ 98°C, 1 min @Tm (depending on used primers) and 5 min @ 72°C were performed. PCR products were digested with 10 U DpnI (Fermentas), per reaction and amplified in *E. coli* DH5α. Sequencing was performed to confirm the mutation in the DNA.

Expression and purification of azide containing PBP1b

E. coli BL21(DE3) cells were co-transformed with pDML924 containing the amber mutation and pEvol-*p*AzF. Cells were grown till the culture reached an OD_{600} of 0.5-0.6. tRNA/tRNA synthase production was induced with 0.04% arabinose, and 0.1 mM freshly prepared *p*-azidophenylalanine, dissolved in 1 M NaOH, was added. After 30 min, protein production was induced with 1 mM IPTG. After 2 hours of protein production, cells were harvested by centrifugation.

Cells from 1 L culture were resuspended in 18 mL 20 mM Tris-HCL pH 8.0, 300 mM NaCL, 5 mM imidazole (Buffer A), supplemented with 0.1 mM PMSF and 1 complete EDTA free protease inhibitor tablet. Cells were lysed by sonification using a sonicator with microtip (Branson Sonifier 250). Sonication was done for 6 rounds of 30 pulses. Intact cells were removed by centrifugation for 10 min at 3500 g (Sorvall legend RT, swinging buckets rotor 7500 6445). The cell lysate was centrifuged for 90 min at 200000 g, at 4°C (Sorvall WX 80 Ultra, T865). Membrane fraction (= pellet) was solubilized in 12 mL buffer A supplemented with 2% Triton X-100, by stirring the membrane suspension for 2 h at 4°C. Insoluble material was removed by centrifugation for 90 min at 200000 g, at 4°C. Solubilized proteins were incubated with 300 µl Ni^{2+} Sepharose beads (GE healthcare) O/N at 4°C. Ni^{2+} Sepharose beads were centrifuged at 3500 g for 3 min at 4°C, and flow trough was collected. Beads were washed with 5 X 10 mL buffer A with 50 mM imidazole and 0.1% Triton X-100 and 3 X 2 mL buffer A with 100 mM imidazole and 0.1% Triton X-100. Proteins were eluted with 4 X 2 mL buffer A with 500 mM imidazole and 0.1% Triton X-100. Fractions were dialyzed using a 500 Da membrane against 20 mM Tris/HCL pH 8.0, 300 mM NaCl, 10 mM $MgCl_2$, 0.1% Triton X-100 and 10% glycerol at 4°C O/W. Protein content was analyzed with a BSA range on a coomassie stained gel to be +/- 0.2 µg/µl for all tree mutants, which is +/- 2.25 µM. Protein was stored at -20°C.

Confirmation of the presence of the azide in PBP1b mutants by coupling of cyclooctyne containing fluorescent dye and SDS-PAGE analysis

10 µl 2.25 µM protein solution was incubated with 2.25 or 22.5 µM Megastokes dye 608 (sigma Aldrich) O/N at RT. Laemmli sample buffer without DTT was added, and samples were run on an 8% SDS-PAGE gel. Fluorescence signal was analyzed using a Typhoon 9400 scanner (GE healthcare), and protein content by coomassie staining.

Protein activity test using in vitro PG synthesis assay and visualization on Tris/Tricine SDS-PAGE

1 µM PBP1b was incubated with 10 µM ATTO550 Lipid II and 100 µM m-DAP Lipid II, in 20 mM HEPES pH7.5, 150 mM NaCl, 10 mM $MgCl_2$ and 0.05% Triton X-100 at 30°C. 1 µg Penicillin G was added to some reactions to be able to only analyze the GT reaction. After various time intervals, 15 µl samples were taken, and the protein was inactivated by boiling for 5 min at 99°C. Samples were dried using a speedvac, and dissolved in 4 µl sample buffer containing 60 mM Tris-HCl pH 8.8, 25% glycerol and 2% SDS. Samples were analyzed by Tris/Tricine SDS-PAGE. Gels were prepared in a final concentration of 9%T, 2.6%C, where T is total percentage of both acrylamide and bisacrylamide and C the percentage of bisacrylamide relative to T. This was prepared in 1x gel buffer containing 0.5 M Tris, 0.13% SDS, pH 8.45. Gels were run using anode buffer of 0.1 M Tris pH 8.8 and cathode buffer of 0.1 M Tris, 0.1 M Tricine and 0.1% SDS, pH8.25 at a constant current of 30 mA with a maximum voltage of 200 V. Gels were scanned using a Typhoon 9400 (GE healthcare).

Surface Plasmon Resonance studies

An IBIS MX96 (IBIS technologies) machine was used for Surface Plasmon Resonance studies. PBP1b variants with an azide incorporated, replacing Gly53, Lys54 or Gly55 were immobilized on the surface of a Sulfo-Dibenzylcyclooctyne-NHS ester (Jena biosciences) coated sensEye P-NH2 sensor. After activation of the chip, the spots were coated for 60 min with 1, 0.5 or 0.25 mM Sulfo-Dibenzylcyclooctyne-NHS ester in 20 mM HEPES pH 7.5. After a rinse with PBS, 0.5, 0.2 or 0.04 µM PBP1b in running buffer (10 mM Tris/Maleate pH 7.5, 150 mM NaCl and 0.05% Triton X 100) was spotted, for 30 min. After a rinse with running buffer, the excess of Sulfo-Dibenzylcyclooctyne-NHS ester was blocked with 0.5 M Azido Ethanol in running buffer, for 10 min. The amount of immobilized protein ranged from +/- 1000 - 3000 resonance units (RUs, 1RU corresponds to approximately 1pg of protein per mm2). Some spots were selected as control surfaces, having different treatments, which are indicated in supplementary table 2. Analytes were injected at different concentrations, diluted in running buffer. 2 min baseline, 10 min association, 5 min dissociation and 2 times 30 sec of regeneration with running buffer, containing 1 M NaCl were recorded. The sensorgrams were evaluated with SPRintX, and the parameters

of the interaction were calculated using Sigmaplot, using the simple ligand binding tool and one site saturation.

Results

Confirmation of the presence of the azide in the PBP1b mutants by coupling to a cyclooctyne containing fluorescent dye

For the immobilization of PBP1b on a chip surface, for the performance of SPR experiments, we designed a new method, using the cytoplasmic tail for immobilization rather than the TP domain used in the past. A cyclooctyne coated chip surface is used, to react with an azide, incorporated in the cytoplasmic tail of PBP1b. Since this is a new method, and there is no knowledge about the efficiency of this immobilization method and the dependence on the position of the azide, we decided to substitute three different, sequential amino acids in the cytoplasmic tail of PBP1b for the unnatural amino acid *p*-azidophenylalanine. We used nonsense suppression mutagenesis to incorporate the azide containing unnatural amino acid at the chosen sites. By site directed mutagenesis the codons for Gly53, Lys54, or Gly55 of PBP1b were mutated into an amber TAG codon. When simultaneously expressing these mutated PBP1b variants with a tRNA-tRNA

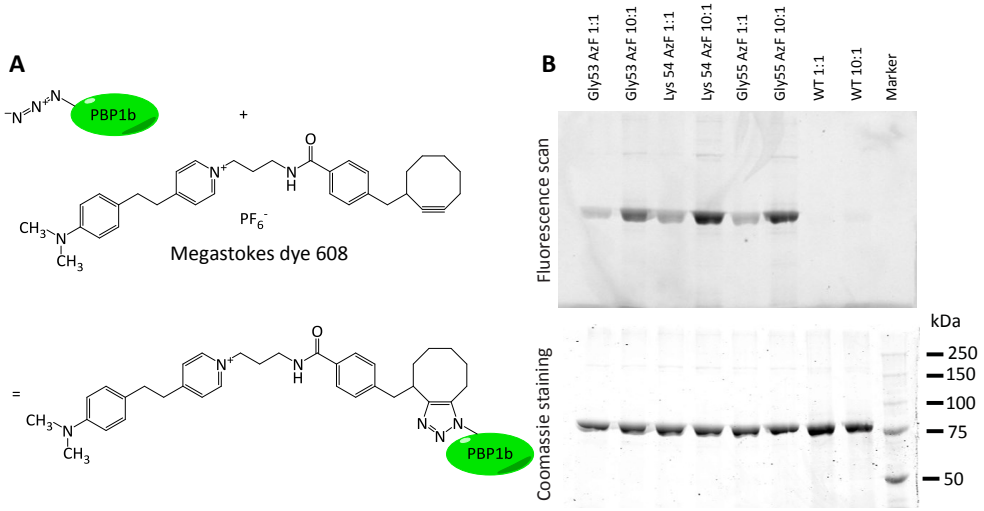


Figure 1. The three PBP1b variants have an azide incorporated, which specifically reacts with a cyclooctyne containing fluorescent dye.

PBP1b with azidophenylalanine incorporated at the indicated positions was expressed in *E. coli* and purified using nickel affinity. Purified protein was incubated O/N at RT with the cyclooctyne containing fluorescent dye Megastokes dye 608. Samples were separated on SDS-PAGE and analyzed by fluorescence scanning and coomassie staining. A) Reaction scheme of azide containing PBP1b and cyclooctyene containing fluorescent dye. B) All three PBP1b variants have an azide incorporated and react specifically with the cyclooctyne; WT protein is not labeled by a cyclooctyne linked to a fluorescent dye (upper), while similar amounts of the different proteins were loaded on the gel (lower). Number represents ratio of dye to protein.

synthase pair, which recognizes the TAG codon, and is specific for the incorporation of the unnatural amino acid *p*-azidophenylalanine, three mutant proteins were produced, having an azidophenylalanine replacing either Gly53, or Lys54, or Gly55. To verify whether the azide was indeed incorporated in the three mutant proteins, we incubated the purified proteins with a fluorescent dye containing a cyclooctyne group, spontaneously reacting with the azide, forming a triazole (Fig 1A). In this way, azide containing proteins will become fluorescently tagged. By running this reaction mixture on an SDS-PAGE gel, and scanning the gel with a fluorescence scanner, the reactive proteins were visualized. In figure 1B it can be seen that the azide was indeed incorporated in all three mutant proteins by the fluorescence signal of the proteins. An excess of cyclooctyne containing dye was needed for an efficient reaction to occur under these conditions (1:1 vs. 10:1). Furthermore, incubation of wild type protein with the dye did not result in any fluorescence signal, showing that the reaction is specific.

The azide containing PBP1b proteins show both glycosyltransferase as well as transpeptidase activity in an *in vitro* peptidoglycan synthesis assay

For the implementation of our method in the SPR experiments we aim on using fully active PBP1b proteins possessing both glycosyltransferase (GT) as well as transpeptidase (TP) activity. To verify whether the azide containing proteins still possess both activities, we performed an *in vitro* peptidoglycan (PG) synthesis assay. In this assay, the proteins are incubated in a buffer containing all the ingredients needed for activity, supplemented with fluorescently labeled Lipid II. As a result of GT activity, the sugar moieties of Lipid II will be polymerized into glycan strands. These glycan strands can be separated using a Tris/Tricine SDS-PAGE system. The non-incorporated Lipid II monomers will be visible at the bottom of the gel, glycan polymers will be separated by size, smaller ones visible as separated bands in the lower region, and longer strands as a smear in the higher region of the gel. Since unlabeled Lipid II is incorporated in the reaction mixture as well, Penicillin G has to be added when exclusively analyzing glycan polymerization, because the unlabeled Lipid II can also be used in the TP reaction. When Penicillin G is not incorporated in the reaction mixture, peptide crosslinks will be formed, if the protein possesses TP activity. This will be visible as a band at the bottom of the slot, because the crosslinked PG network is too large to enter the gel. Since part of the Lipid II used in the reaction is fluorescently labeled, the products can be visualized using a fluorescence scanner. Performing this assay, using the three mutant proteins containing an azide at positions Gly53, Lys54 or Gly55, showed that all three proteins are still active. They show both GT activity, visible by the long glycan strands produced in time, as well as TP activity, shown by the appearance, and increase of intensity of a band at the top of the gel, in the bottom of the slot (Fig 2).

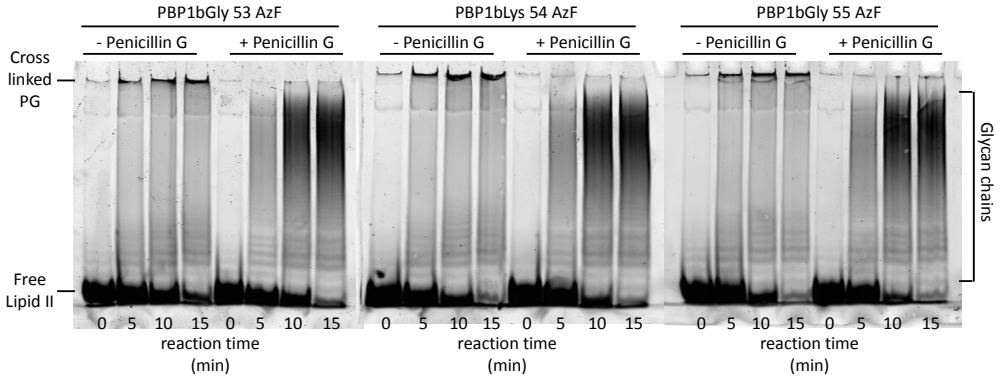


Figure 2. *In vitro* PG synthesis assay shows that all three proteins are fully active, both in GT as well as TP activity.

Purified PBP1b with the azidophenylalanine incorporated at the indicated positions was incubated at 30 °C with a mixture of labeled and unlabeled Lipid II, and samples were taken at the indicated time points. Produced glycan chains were separated by size on a Tris/Tricine SDS-PAGE gel. Apparent is the overall increase in chain length and decrease in intensity of the free Lipid II band in time, showing GT activity of the three mutant proteins. TP activity is seen by the high intensity band at the top of the gel in penicillin G - reactions (which inhibits the TP reaction). This is crosslinked PG, unable to enter the gel.

The successful use of the azide incorporated in PBP1b for the site specific immobilization on an SPR chip surface

Optimization of immobilization conditions.

For the immobilization of the azide containing PBP1b variants, we used an amine containing chip surface to perform the SPR experiments, first coated with a cyclooctyne containing NHS-ester. Because the efficiency of this coating and the subsequent reaction with the azide in the proteins is not known, we tested the efficiency at different conditions. Different cyclooctyne coating concentrations of 1, 0.5 or 0.25 mM were tested, as well as different protein concentrations of 0.5, 0.2 or 0.04 µM, of all three protein variants having the azide incorporated at different positions. A blocking step using ethanolamine is included in the ampicillin immobilization method. Therefore, we analyzed the necessity of a blocking step with azido ethanol, to block the unbound cyclooctyne molecules, to obtain a more comparable measurement environment with respect to the ampicillin immobilization method. To analyze the most optimal conditions for PBP1b immobilization and interaction measurements, we used the well characterized interaction of LpoB with PBP1b as a test system, because it is known to interact with PBP1b [5], [6], and the kinetic parameters of this interaction have been well established [2].

The amount of protein bound to the chip surface is represented by the response of local ligand (RLL) value, expressed in RUs. 1RU corresponds to approximately 1pg of protein per mm^2, and the binding capacity Rmax depends on the amount of protein immobilized on the chip surface according to the following formula: Rmax= (analyte MW / ligand MW) * RL * Sm (stoichiometric ratio). Typical RLL values are at least 1000 or more. In

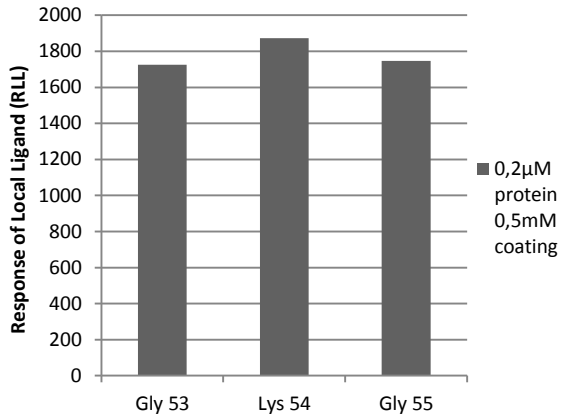


Figure 3. Immobilization of the three azide containing PBP1b variants on the SPR surface.

The amount of immobilized protein is represented by the response of local ligand (RLL) value, which shows that all thee PBP1b variants were well immobilized on the SPR chip surface (values > 1000).

supplementary table 2 all the combined results of the optimization experiments are shown. For ease of interpretation the data is subdivided into separate graphs below.

In figure 3 it can be seen that all three protein variants with the azide at different positions are well immobilized onto the chip surface, so in respect to immobilization efficiency, the location of the azide is not important in this case.

For clarity, the protein and cyclooctyne concentration variables will be discussed in respect to one PBP1b version (Gly 55). There is an obvious correlation between higher RLL values (amount of protein immobilization) and used protein concentration. A higher protein concentration results in a higher amount of immobilized protein on the chip surface (Fig 4A). The highest concentration of protein (0.5 µM) did not result in protein aggregation on the chip surface (data not shown). Furthermore, the highest amount of

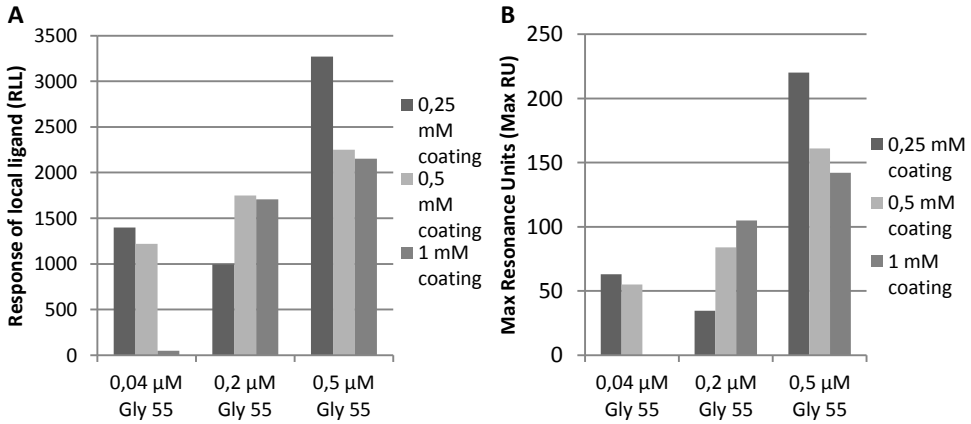


Figure 4. Effect of protein and cyclooctyne concentration on immobilization efficiency and signal produced as a result of analyte injection.

The amount of immobilized protein is represented by the response of local ligand (RLL), and the response generated as a result of analyte injection by maxRU value. The maxRU value generated by injection of the highest concentration (4 µM LpoB) of analyte is shown in the table. A) A higher protein concentration results in a higher amount of protein immobilization. B) The higher amounts of protein immobilization does not always result in higher maxRU values.

protein immobilization also resulted in higher maxRU values as a result of analyte injection (Fig 4B). This value is more important when analyzing the results, since higher maxRU values result in a higher signal to noise ratio. Sometimes, the highest amount of protein immobilized on the chip surface is not necessarily the best situation for the protein-protein interactions measured. When the chip surface is to crowded with immobilized ligand, the analyte can have problems with "floating through" the proteins and hence with binding. In figure 4 it can also be seen that the amount of cyclooctyne coating used does not have any influence on the amount of protein immobilized on the chip surface, nor on the maxRU values reached.

The last variable tested was the effect of a blocking step of unbound cyclooctyne molecules with azido ethanol. Blocking does not have a very striking effect on the results, but it seems to slightly increased maxRU values obtained when using 0.5 µM protein, of which it was shown in figure 4 to be the most important variable (Fig 5).

When comparing the obtained responses rather than the amount of immobilized protein for the different mutants, when using 0.5 µM of protein, the PBP1b variant with the azide replacing Lys54, on average produced SPR curves with lower signals, making this the least attractive variant for future use in SPR experiments (Fig 6). Since the PBP1b variant with the azide replacing Gly53 showed to be slightly less active in the *in vitro* PG synthesis assay, than when replacing Gly55 (Fig 2), we decided to use the PBP1b variant with the azide replacing Gly55 for use in future experiments, since it bound well to the surface, and gave reliable response curves as a result of LpoB injection. Furthermore, a protein concentration of 0.5 µM, and a cyclooctyne coating concentration of 1 mM were used, because in most situations these conditions produced the best results. Finally we decided to include the blocking step in future experiments, because it did result in a slightly higher response when using the aforementioned conditions, but more importantly to block possible hydrophobic interactions of proteins with free cyclooctyne groups on the chip surface.

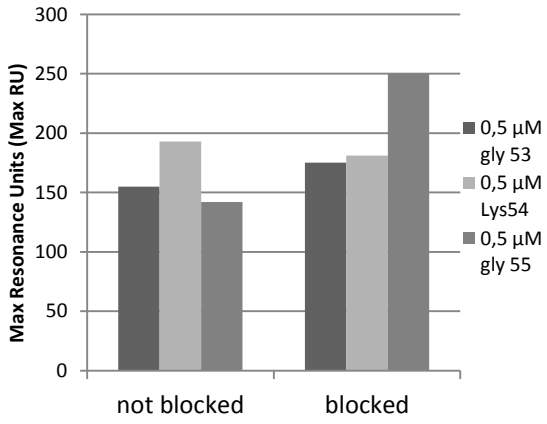


Figure 5. Effect of free cyclooctyne blocking with azido ethanol on produced maxRU values.
Blocking of free cyclooctyne molecules with azido ethanol does not have a very striking effect on the results. It seems to slightly increase the obtained maxRU values.

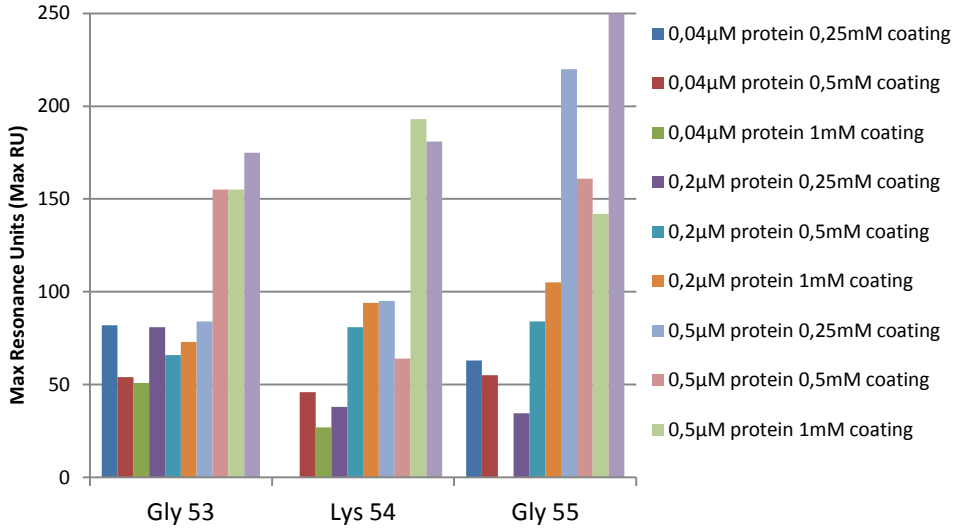


Figure 6. The maxRU values produced by the different PBP1b variants as a result of LpoB injection.
The maxRU value generated by injection of the highest concentration (4 µM LpoB) of analyte is shown in the table. PBP1b variant with azide replacing Lys54 produced SPR curves with lower signals on average, making this the least attractive variant. PBP1b variants with azide replacing Gly53 or Gly 55 produced similar results.

Do these immobilized PBP1b variants still interact with LpoB in a similar way?

When injecting the PBP1b binding analyte LpoB over the PBP1b immobilized SPR surfaces, the binding is measured as an increase in RUs. This value increases until an equilibrium of binding and unbinding events is reached. When injection is ceased, the interacting molecules are released, seen as a decrease in RUs. Commonly, in SPR experiments, higher concentrations of analyte are injected, until the maximum amount of binding is reached and the maxRU at equilibrium does not increase as a result of injection of higher concentration of analyte anymore. Non-linear regression, using one site saturation in the simple ligand binding tool of Sigmaplot was used to plot the maxRU values of the different analyte concentration injections to determine the equilibrium constant.

When analyzing the SPR curves produced by the injection of LpoB over the PBP1b immobilized surfaces, the expected pattern was observed (Fig 7A). LpoB has a very quick association with PBP1b, as published before [2], seen as an immediate rise in RUs to the equilibrium, making it impossible to determine the association constant. The same holds for the dissociation of LpoB from PBP1b when injection is ceased. The equilibrium rate constant K_D was determined for all spots except spot number 1 and 4 since these signals were to low and noisy to determine the maxRU of every injected concentration. As shown in supplementary table 2, K_D values ranging from 0.37 to 0.75 µM were found (when ignoring the outlier of 1.41 µM calculated from the measurements of spot number 16), which is very close to the 0.81 +/- 0.08 µM found by Egan *et al* [2]. This experiment was

repeated with a chip on which all the spots were treated with the optimal conditions found in the previous paragraph, resulting in found K_D values ranging from 0.71 to 0.97 µM. These small differences in equilibrium constant, can be easily caused by a slight change in buffer composition, pH or temperature at which the measurements are performed.

These results show that our new immobilization technique for PBP1b, via an azide incorporated in its cytoplasmic tail using non sense suppression mutagenesis for the site specific incorporation of unnatural amino acids, for the use in SPR experiments is a good alternative for the ampicillin immobilization method. And that this new immobilization technique produced similar results when analyzing the interaction of PBP1b with LpoB. A difference in results for this interaction was not expected, since the TP domain of PBP1b is not involved in the interaction with LpoB, which has shown to bind the UB2H domain. This new immobilization method can also be used for the investigation of any desired protein, and creates the possibility to determine the orientation of the protein, by the site specific incorporation of the azide. This will result in a homogeneous orientation of the immobilized proteins and creates a possibility, by replacing an amino acid facing the inside or outside of the membrane by the azide containing unnatural amino acid, to study binding differences in respect to the topology of membrane proteins.

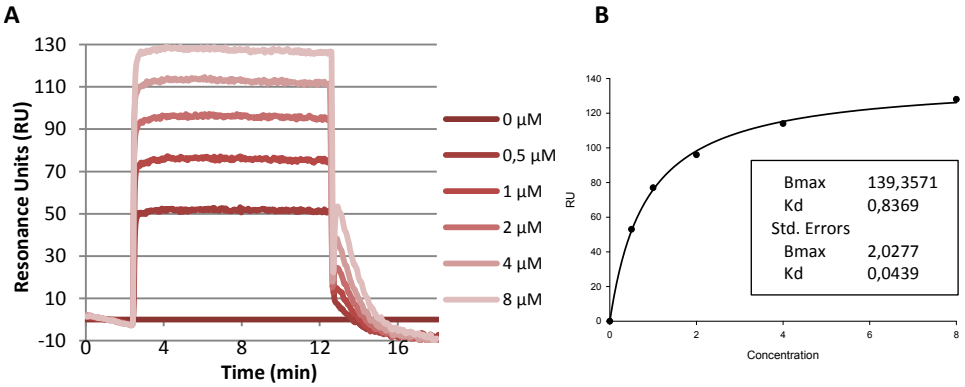


Figure 7. SPR curves produced as a result of the injection of LpoB over a PBP1b immobilized chip surface. A) Sensorgram of injection of different concentrations of LpoB over PBP1b immobilized SPR surface. B) Analysis of the SPR data, using nonlinear regression with a simple one site enzyme binding hypothesis in Sigmaplot, results in a K_d of 0,83 +/- 0,0439 µM, which is close to the 0,81 +/- 0,08 µM found by Egan *et al.*[2].

Acknowledgements

We thank P.G. Schultz for providing us the pEvol-*p*AzF plasmid.

References

[1] W. Vollmer, M. von Rechenberg, and J.-V. Höltje, "Demonstration of Molecular Interactions between the Murein Polymerase PBP1B, the Lytic Transglycosylase MltA, and the Scaffolding Protein MipA of Escherichia coli," *J. Biol. Chem.*, vol. 274, no. 10, pp. 6726–6734, Mar. 1999.

[2] A. J. F. Egan, N. L. Jean, A. Koumoutsi, C. M. Bougault, J. Biboy, J. Sassine, A. S. Solovyova, E. Breukink, A. Typas, W. Vollmer, and J.-P. Simorre, "Outer-membrane lipoprotein LpoB spans the periplasm to stimulate the peptidoglycan synthase PBP1B," *Proc. Natl. Acad. Sci.*, vol. 111, no. 22, pp. 8197–8202, Jun. 2014.

[3] U. Bertsche, E. Breukink, T. Kast, and W. Vollmer, "In Vitro Murein (Peptidoglycan) Synthesis by Dimers of the Bifunctional Transglycosylase-Transpeptidase PBP1B from Escherichia coli," *J. Biol. Chem.*, vol. 280, no. 45, pp. 38096–38101, Nov. 2005.

[4] U. Bertsche, T. Kast, B. Wolf, C. Fraipont, M. E. G. Aarsman, K. Kannenberg, M. Von Rechenberg, M. Nguyen-Distèche, T. Den Blaauwen, J.-V. Höltje, and W. Vollmer, "Interaction between two murein (peptidoglycan) synthases, PBP3 and PBP1B, in Escherichia coli," *Mol. Microbiol.*, vol. 61, no. 3, pp. 675–690, Aug. 2006.

[5] A. Typas, M. Banzhaf, B. van den Berg van Saparoea, J. Verheul, J. Biboy, R. J. Nichols, M. Zietek, K. Beilharz, K. Kannenberg, M. von Rechenberg, E. Breukink, T. den Blaauwen, C. A. Gross, and W. Vollmer, "Regulation of Peptidoglycan Synthesis by Outer-Membrane Proteins," *Cell*, vol. 143, no. 7, pp. 1097–1109, Dec. 2010.

[6] C. Paradis-Bleau, M. Markovski, T. Uehara, T. J. Lupoli, S. Walker, D. E. Kahne, and T. G. Bernhardt, "Lipoprotein Cofactors Located in the Outer Membrane Activate Bacterial Cell Wall Polymerases," *Cell*, vol. 143, no. 7, pp. 1110–1120, Dec. 2010.

Supplementary information

Supplementary table 1		
(G) Gly53	fwd	GGTAAGGGCAAATAGAAAGGGCGTAAGC
	rev	GCTTACGCCCTTTCTATTTGCCCTTACC
(K) Lys54	fwd	GGTAAGGGCAAAGGCTAGGGGCGTAAGC
	rev	GCTTACGCCCCTAGCCTTTGCCCTTACC
(G) Gly55	fwd	GGCAAAGGCAAATAGCGTAAGC
	rev	GCTTACGCTATTTGCCTTTGCC

Supplementary table 1. Used primers for the creation of amber mutants.

Supplementary table 2											
spot	protein	coating	RLL	max RU	kD (µM)	spot	protein	coating	RLL	max RU	kD (µM)
23	0,04µM gly53	0,25mM	1401	82	0,3752	4	0,04µM lys54	0,25mM	363	-	-
24	0,04µM gly53	0,5mM	1098	54	0,387	6	0,04µM lys54	0,5mM	1041	46	0,6712
2	0,04µM gly53	1mM	1387	51	0,5745	8	0,04µM lys54	1mM	657	27	0,7544
7	0,04µM gly53	1mM	1175	44	0,5447	14	0,04µM lys54	1mM	1138	32,5	0,8229
20	0,2µM gly53	0,25mM	1798	81	0,5032	28	0,2µM lys54	0,25mM	1094	38	0,5718
21	0,2µM gly53	0,5mM	1725	66	0,6093	29	0,2µM lys54	0,5mM	1872	81	0,5165
22	0,2µM gly53	1mM	1367	73	0,5205	30	0,2µM lys54	1mM	1976	94	0,5616
10	0,2µM gly53	1mM	1526	80	0,6459	16	0,2µM lys54	1mM	1335	59	1,4046
17	0,5µM gly53	0,25mM	1929	84	0,5245	25	0,5µM lys54	0,25mM	1971	95	0,5095
18	0,5µM gly53	0,5mM	2618	155	0,6574	26	0,5µM lys54	0,5mM	1323	64	0,4533
19	0,5µM gly53	1mM	2413	155	0,6737	27	0,5µM lys54	1mM	2408	193	0,6399
3	0,5µM gly53	1mM	1878	175	0,8172	5	0,5µM lys54	1mM	2345	181	0,7312
spot	protein	coating	RLL	max RU	kD (µM)	spot	protein	coating	RLL	max RU	kD (µM)
31	0,04µM gly55	0,25mM	1399	63	0,3774	39	0	0	32		
32	0,04µM gly55	0,5mM	1219	55	0,3748	9	0	0	45		
1	0,04µM gly55	1mM	48	-	-	40	0	0	-54		
11	0,04µM gly55	1mM	949	52	0,6937	12	0	0	-172		
36	0,2µM gly55	0,25mM	995	34,5	0,509	13	0	0	-179		
37	0,2µM gly55	0,5mM	1747	84	0,5535	15	0	0	-247		
38	0,2µM gly55	1mM	1708	105	0,6815	41	0	0,25mM	317		
48	0,2µM gly55	1mM	1640	58	0,6363	42	0	0,5mM	751		
33	0,5µM gly55	0,25mM	3271	220	0,5662	43	0	1mM	868		
34	0,5µM gly55	0,5mM	2251	161	0,5316	44	0	0,25mM	721		
35	0,5µM gly55	1mM	2151	142	0,5555	45	0	0,5mM	746		
47	0,5µM gly55	1mM	3522	250	0,7265	46	0	1mM	613		

Supplementary table 2. Different conditions tested for PBP1b immobilization. Yellow lanes are blocked with azido ethanol.

Chapter 5

The use of different fluorescently labeled Lipid II variants for the improvement of glycosyltransferase assays

Inge van 't Veer, Alexander Egan, Jacob Biboy, Waldemar Vollmer and Eefjan Breukink

Abstract

The peptidoglycan (PG) layer of the bacterial cell envelope is an essential structure for bacterial cell survival. PG is produced by the polymerization of disaccharide moieties of Lipid II by glycosyltransferase (GT) reactions, and subsequent incorporation into the existing wall by transpeptidase (TP) reactions. These reactions are performed by penicillin binding proteins (PBPs) and can be monitored by using functionalized substrates in different assays. This can for example be done by in gel visualization of the reaction product after different time intervals, or with the continuous GT assay following the glycan strand polymerization over time. An often used fluorescently labeled Lipid II variant in these assays is dansylated Lipid II. However, dansyl does not have a very high quantum yield compared to other fluorophores. Therefore, we created different fluorescently labeled Lipid II variants, using a click chemistry based approach. This labeling technique, on the level of Lipid II, showed to be highly efficient, and resulted in hardly any loss of the Lipid II starting material during the procedure. Furthermore, these fluorescently labeled Lipid II variants improved the resolution and sensitivity of the in gel analysis of PG production. One of these dye labeled Lipid II variants (Megastokes dye 608) was also useful in the continuous GT assay. These different fluorescently labeled Lipid II variants can be used for analysis of PBP activities under different conditions with a higher resolution.

Manuscript in preparation

Introduction

Peptidoglycan (PG) is produced by the polymerization of the disaccharide moieties of Lipid II by glycosyltransferase (GT) reactions, and the subsequent incorporation into the existing bacterial cell wall by crosslinking of the peptide moieties by the transpeptidase (TP) reactions performed by penicillin binding proteins (PBPs). Deletions or mutations in these proteins, the addition of different regulatory proteins, as well as different reaction conditions can have effects on the speed of these reactions, the lengths of glycan chains produced, and the percentage of crosslinking that occurs. For example, the addition of LpoA to a reaction with PBP1A increases both its GT, as well as the TP activity [1]. A similar effect has been shown for the addition of LpoB [1]–[3] and FtsN [4] to a reaction with PBP1b. Next to that, an influence on the length of produced glycan chains has been shown for the presence or absence of the membrane anchor of PBP2a from *Streptococcus pneumonia* [5].

To analyze the PG produced by PBPs under different conditions, an in gel electrophoresis assay is often used [2], [5]–[9]. In this assay PG is produced in an *in vitro* assay and subsequently separated by size with a modified Tris/Tricine SDS-PAGE system. Glycan chains are separated by size, running higher or lower on the gel, depending on the length of the glycan chain. Active transpeptidation will generate high mass products that do not migrate into the gel and will be observed as a band in the bottom of the slot. This assay is often performed using radiolabeled or dansylated Lipid II. However, the quantum yield of dansyl is not very high compared to other fluorophores, therefore, the use of a different fluorophore coupled to Lipid II could result in a much higher resolution. Recent advances in the technology to label Lipid II allow us to fluorescently label it with the use of a click chemistry based approach. In this way we can easily produce different fluorescently labeled Lipid II variants. Hence we decided to fluorescently label Lipid II, containing a range of different (high quantum-yield) fluorophores that cover a large part of the visible light spectrum (+/- 550-700 nm (Fig 1).

This allowed us to improve the sensitivity of the in gel GT assay, which suffers from low signal to noise ratios due to the use of a suboptimal fluorescent probe. The optimization of this assay (e.g. with regards to sensitivity and resolution), gave the best result when using Lipid II labeled with ATTO 550 and 647N fluorophores. The use of these fluorescently labeled Lipid II variants improved the sensitivity and resolution of this assay, and therefore makes it possible to observe more subtle changes in the GT and TP activities of PBPs.

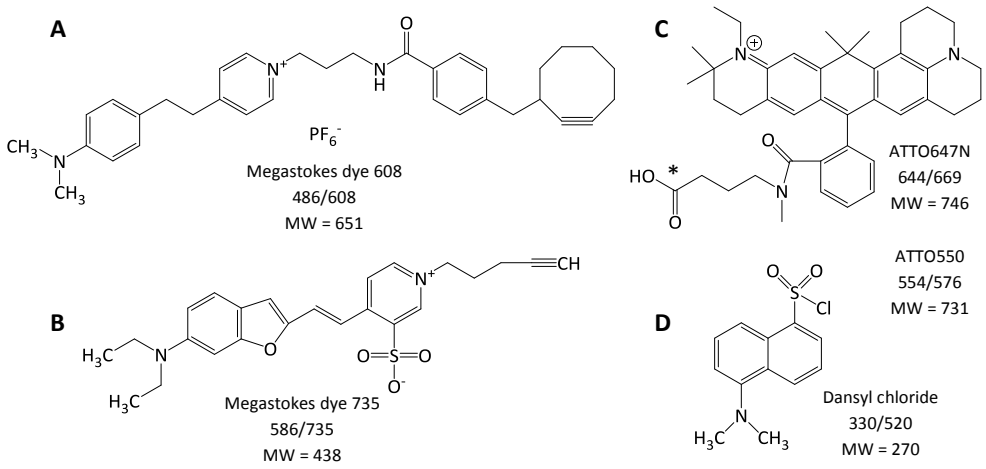


Figure 1. Chemical structure of the fluorescent dyes used for Lipid II labeling.
For the labeling of Lipid II we used Megastokes dye 608 (A), Megastokes dye 735 (B), ATTO647N (C) here represented as the carboxylic acid variant as the structure of the alkyne functionalized variant is not published, which is probably at the same position (indicated with *) and results in a molecule with a MW of 783. ATTO550, of which the structure is not published but is likely comparable to the structure of ATTO647N. These fluorophores are compared to Lipid II labeled with dansyl (D).

Furthermore, we tested these newly created Lipid II variants for their performance in the continuous GT assay developed by Schwartz *et al.* [10], in which dansylated Lipid II is used for detection as well. This continuous GT assay is based on the property that the fluorescence intensity of dansyl is higher in a more hydrophobic environment (i.e. connected to the lipid carrier tail) than in a more hydrophilic environment (incorporated into the glycan chain and released from its lipid tail). Hence, a decrease in fluorescence intensity is observed over time, as a result of the incorporation of Lipid II into glycan chains. Unfortunately, both ATTO dyes did not possess this property of fluorescence decrease as a result of a changing environment and therefore were not useful in this assay. Both Megastokes dyes 608 and 735 did have this property and were subsequently tested for their performance in this assay. Megastokes dye 735 produced a very noisy signal for an unknown reason and consequently is not very useful in this assay when measured with our fluorimeter. Megastokes dye 608 showed similar behavior as dansylated Lipid II in this assay, making it possible to also use this labeled Lipid II variant for the continuous analysis of GT activity of PBPs as well.

These results show that the click chemistry based labeling of Lipid II is highly efficient, which allowed us to produce Lipid II labeled with different fluorophores. All these variants showed to be useful in the in gel analysis of PG synthesis and produced results with a higher resolution, of which Megastokes dye 608 can also be used in the continuous GT assay.

Materials and methods

Bacterial strains and plasmids.

For the production of UDP-MurNAc-pentapeptide, *Bacillus cereus* (meso- diaminopimelic version) or *Staphylococcus simulans* (lysine version) were used. For protein production, *Escherichia coli* BL21 (DE3) was used, grown in LB medium at 37°C supplemented with 50µg/mL kanamycin. Protein was expressed from pDML924 producing His_6-PBP1b-γ. Production was started by IPTG induction with 1 mM for 2 hours, activating the T7 promoter of this plasmid.

Production of Lipid II

For the production of Lipid II, 4 ingredients are needed: undecaprenol phosphate, UDP-MurNAc-pentapeptide, UDP-Glc-*N*Ac (Sigma Aldrich) and the enzymes that catalyze the reactions forming Lipid II out of these substrates, MraY and MurG.

Undecaprenol was extracted from *Laurus nobilis* leaves by grounding the material and incubating this with acetone-hexane (9:1) in a Soxhlet for 48 hours. After evaporation of solvent, the residue obtained was dissolved in hexane:ethanol:15%potassium hydroxide in water (3:15:2), and the solution with the lipids was subjected to alkaline hydrolysis for 1 hour at 90°C. The alkaline hydrolysate was diluted with water:ether (1:1) and the ether layer was separated and dried with sodium sulfate. The unsaponifiable lipids were dissolved in hexane, and purified using a silica column, eluted with 5% ether in 95% hexane and 15% ether in 85% hexane, the elution of the polyprenols was followed using C18 TLC. These polyprenols were phosphorylated to form undecaprenol phosphate in one step as described by Danilov *et al.* [11]. The resulting polyprenyl-phosphates were dissolved in acetone.

UDP-MurNAc-pentapeptide was extracted from *Bacillus cereus* (meso- diaminopimelic version) or *Staphylococcus simulans* (lysine version). Cells were grown at 30°C in medium containing 5 g/L tryptone, 2.5 g/L yeast extract, 5 g/L NaCl, 0.8 g/L K_2HPO_4, 0.2 g/L KH_2PO_4, 0.05 g/L $CaSO_4$* $2H_2O$ and 0.025 g/L $MgSO_4$*$7H_2O$. When OD_{600} reached 0.4, 0.13 mg/mL chloramphenicol was added and cells were incubated for 15 min at 30°C after which 0.018 mg/mL vancomycin was added, and cells were incubated for 45 min at 30°C. Cells were harvest by centrifugation for 30 min at 3500 g (Beckman J6HC) at 4°C and resuspend in 2 mL MiliQ per Liter culture. 25 mL MiliQ per Liter culture was boiled and the resuspended pellets were slowly added, and boiled for 15 min. The boiled suspension was centrifuged for 15 min at 27000 g (Sorval RC5B plus, SS-34). Supernatants were collected and freeze dried. The freeze dried powder containing the UDP-MurNAc-pentapeptide was rehydrated in MiliQ (+/- 1.5 mL per Liter culture), and stored at -20°C until later use.

The enzymes catalyzing the reactions forming Lipid II were used in the form of *Micrococcus flavus* membrane vesicles. *Micrococcus flavus* is grown in TSB medium

containing 17 g/L pancreatic digest of casein, 3.0 g/L papaic digest of soybean meal, 5.0 g/L NaCl, 2.5 g/L Na_2HPO_4 and 2.5 g/ glucose. Fresh medium was inoculated at the end of the day (1:40), and this was incubated O/N at 30°C to an OD_{600} = 5.0-7.0. Cells were harvest by centrifugation for 30 min at 3500 g (Sorvall legend RT, swinging buckets rotor 7500 6445) at 4°C and washed with 50 mL 50 mM Tris-HCL pH 7.5 per Liter culture. Cells were centrifuged for 15 min at 15000 g (Sorval RC5B plus, SLA1500) at 4°C and resuspend in 50 mL 50 mM Tris-HCL pH 7.5 per gram cells, and 0.5 mg RNAse and DNAse and 20 mg lysozyme per gram of cells was added. This mixture was passed 5 times through a cell disruptor. The supernatant, containing the membrane vesicles was collected and centrifuged for 30 min at 27000 g (Sorval RC5B plus, SS-34) at 4°C. The pellets were resuspended in an as small as possible volume of 50 mM Tris-HCL pH 7.5, and frozen with liquid N_2 and stored at -20°C.

The amount of poyprenyl-phosphates necessary for optimal Lipid II synthesis was determined via a small scale Lipid II synthesis test (150 µl) that results in maximal Lipid II synthesis (in this instance this was 2 µl). This amount can be linearly extrapolated to the amount needed for the large scale synthesis of Lipid II. For large scale synthesis in 150 mL, 2 mL poyprenyl-phosphate stock solution, 10 mL UDP-MurNAc-pentapeptide extract, 0.5% Triton X-100, 50 mM Tris pH8, 6.67 mM UDP-Glc-*N*Ac, 6.67 mM $MgCl_2$, and 30 mL *Micrococcus flavus* membrane vesicles (obtained from 2 L culture) were incubated while stirring in an Erlenmeyer, O/N at RT. The lipids were extracted from this reaction mixture by Butanol/Pyridine (pH 4.2) extraction. The Lipid II was purified from this mixture using a DEAE-cellulose column and eluted using a gradient of 0-500 mM NH_4HCO_3 in water in a mixture of $CHCL_3$:MeOH:NH_4HCO_3 (2:3:1). All fractions containing Lipid II were pooled and dried using a rotavap. The dried Lipid II was dissolved in $CHCL_3$:MeOH 1:1. Lipid concentrations were determined using phosphorus determination [12].

The conversion of lysine form of Lipid II into the azidolysine form.

Lipid II (lysine form, 1 µmol) was dissolved in 2 ml water containing 1 mM $CuSO_4$ and 0.5% Triton X-100 (w/v). Subsequently, 10 µmol of imidazole-1-sulfonyl azide hydrochloride was added, followed by 20 µmol of diisopropylethylamine. Complete conversion could be observed after 4 hours of incubation, upon which the lipids were extracted from this reaction mixture by Butanol/Pyridine (pH 4.2) extraction. The butanol phase was applied to a DEAE-cellulose column, and the azido-Lipid II (Lipid II-N_3) was purified as described above and stored at -20°C in $CHCl_3$:MeOH (1:1) until use.

Coupling of different fluorescent groups onto azidolysine Lipid II

1.75 µmol Lipid II-N_3 was dissolved in 500 µl 10 mM Tris pH8, 0.1% Triton X-100. Three fold molar excess of alkyne Megastokes dye 735 (Sigma Aldrich), ATTO550-alkyne dye or ATTO647N alkyne dye (ATTO tec) was added to this solution, together with 1 mM $CuSO_4$

and 5 mM sodium ascorbate and stirred at RT. The reaction was followed by silica gel 60 TLC. When all Lipid II-N_3 was converted to Lipid II-dye, the lipids were extracted from the reaction mixture by Butanol/Pyridine (pH 4.2) extraction, and purified using a DEAE-cellulose column. The butanol phase was applied to the column (2 x 1 cm), and washed with 8 mL $CHCl_3$:MeOH:H_2O (2:3:1), 16 mL $CHCl_3$:MeOH:200mM NH_4HCO_3 (2:3:1) and 16 mL $CHCl_3$:MeOH:500mM NH_4HCO_3 (2:3:1) and eluted with 16 mL $CHCl_3$:MeOH:1000mM NH_4HCO_3 (2:3:1). The dye labeled Lipid II containing fractions were pooled and dried using a rotavap. The dried Lipid II was dissolved in CHCl3:MeOH (1:1). Lipid II concentrations were determined using phosphorus determination.

For the coupling of cyclooctyne Megastokes dye 608 (Sigma Aldrich) the same procedure was followed, except for the addition of $CuSO_4$ and sodium ascorbate, which are not necessary since the reaction of N_3 with a cyclooctyne occurs spontaneously.

Purification of PBP1b

Cells from 6 L culture were resuspended in 60 mL 20 mM Tris-HCL pH 8.0, 300 mM NaCL, 5 mM imidazole (Buffer A), supplemented with 0.1 mM PMSF and 1 complete EDTA free protease inhibitor tablet. Cells were lysed by sonification using a sonicator with microtip (Branson Sonifier 250). Sonication was done for 6 rounds of 30 pulses. Intact cells were removed by centrifugation for 10 min at 3500 g (Sorvall legend RT, swinging buckets rotor 7500 6445). The cell lysate was centrifuged for 90 min at 200000 g (Sorvall WX 80 Ultra, T865) at 4°C. Membrane fraction (= pellet) was solubilized in 32 mL buffer A supplemented with 2% Triton X-100, by stirring the membrane suspension for 2 hours at 4°C. Insoluble material was removed by centrifugation for 1 hour at 200000 g (Sorvall WX 80 Ultra, T865) at 4°C. Solubilized proteins were incubated with 1 ml Ni^{2+} Sepharose beads (GE healthcare) O/N at 4°C. Ni^{2+} Sepharose beads were centrifuged at 3500 g for 3 min at 4°C, and flow trough was collected. Beads were washed with 5 X 10 mL mL buffer A with 50 mM imidazole and 0.1% Triton X-100 and 3 X 3 mL buffer A with 100 mM imidazole and 0.1% Triton X-100. Proteins were eluted with 5 X 2 mL buffer A with 500 mM imidazole and 0.1% Triton X-100. Fractions were dialyzed using a 500 Da membrane against 20 mM Tris/HCL pH8, 300 mM NaCl, 10 mM $MgCl_2$ 0.1% Triton X-100 and 10% glycerol at 4°C O/W. Protein content was analyzed with a BSA range on a coomassie stained gel to be +/- 0.5 µg/µl which is +/- 5 µM. Protein was stored at -20°C.

In vitro PG synthesis assay and visualization on Tris/Tricine SDS-PAGE

1 µM PBP1b was incubated with 10 µM fluorescently labeled Lipid II and 200 µM m-DAP Lipid II, in 20 mM HEPES pH7.5, 150 mM NaCl, 10 mM $MgCl_2$ and 0.05% Triton X-100 at 30°C. 1 µg Penicillin G was added to some reactions. After various time intervals, 15 µl samples were taken, and the protein was inactivated by boiling for 5 min at 99°C. Samples were dried using a speedvac, and dissolved in 4 µl sample buffer containing 60 mM Tris-

HCl pH 8.8, 25% glycerol and 2% SDS. These samples were analyzed on Tris/Tricine SDS-PAGE gels. Gels were prepared in a final concentration of 9% T, 2.6% C, where T is total percentage of both acrylamide and bisacrylamide and C the percentage of bisacrylamide relative to T. This was prepared in 1x gel buffer containing 0.5 M Tris, 0.13% SDS, pH 8.45. Running buffer consisted of the anode buffer of 0.1 M Tris pH 8.8 and cathode buffer of 0.1 M Tris, 0.1 M Tricine and 0.1% SDS, pH8.25. Electrophoresis was performed at a constant current of 30mA with a maximum voltage of 200V. Gels were scanned using a Typhoon 9400 (GE healthcare).

Continuous in vitro GT assay

1 µM PBP1b was incubated in 50 mM HEPES pH7.5, 150 mM NaCl, 25 mM $MgCl_2$, 0.14 µg/µl muramidase and 0.02% Triton X-100 at 30°C, +/- 50 µM Moenomycin. Right before starting the measurement, 10 µM fluorescently labeled Lipid II and to some reactions 10 or 100 µM non labeled m-DAP-Lipid II was added, in a total volume of 60 µl. Measurements were performed for 60-120 cycles of 20 seconds with orbital shaking, 4mm width for 5 seconds before each cycle in a BMG biotech FLUOstar OPTIMA, or in a spectraMax i3 flurometer in Greiner Bio-One black microplates.

Results

Coupling of different fluorophores onto Lipid II

For the coupling of fluorescent dyes onto Lipid II the amine (NH_2) that is located on the lysine of the pentapeptide of Lipid II was converted into an azide (N_3), to form azido-Lipid II, which is suitable for click reactions with alkynes (copper catalyzed) and cyclooctynes (copper free) (Fig 2). To perform these reactions, Lipid II-N_3 was dried and dissolved in 500 µl 10 mM Tris pH8, 0.1% Triton X-100 to a final concentration of 3.5 mM. Subsequently, the dyes, Megastokes dye 608, 735 and ATTO 550 and 647N, were added to this mixture, and copper sulfate and sodium ascorbate were added to the latter three to catalyze the reaction. The reactions were stirred at room temperature, and the progress of the conversion was followed by TLC. The progress of the reaction, creating Megastokes dye 608 Lipid II, is shown here as an example (Fig 3). After 1 hour of incubation time almost all the dye was consumed from the reaction mixture (Fig 3A arrow 1), and labeled Lipid II had been produced (Fig 3A arrow 2). However, there was still a large amount of unlabeled Lipid II in the mixture (Fig 3A arrow 3). Therefore, more dye was added, and the reaction was continued for four more hours. After this period, still some unlabeled Lipid II was present in the reaction mixture (Fig 3B arrow 3), and the dye was almost fully consumed from the reaction (Fig 3C arrow 1). Therefore, more dye was added, and the reaction was continued over night at 4°C. Hereafter, all the Lipid II-N_3 was converted into Megastokes dye 608 labeled Lipid II (Fig 3C arrow 2). The Lipid II containing fractions were pooled,

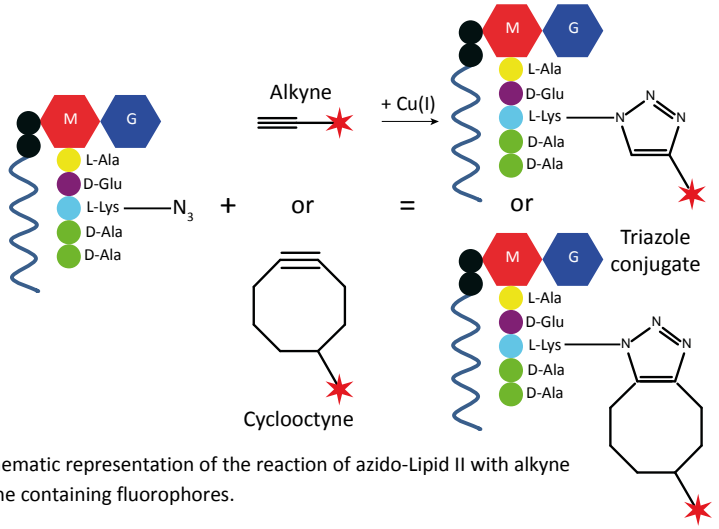


Figure 2. Schematic representation of the reaction of azido-Lipid II with alkyne or cyclooctyne containing fluorophores.

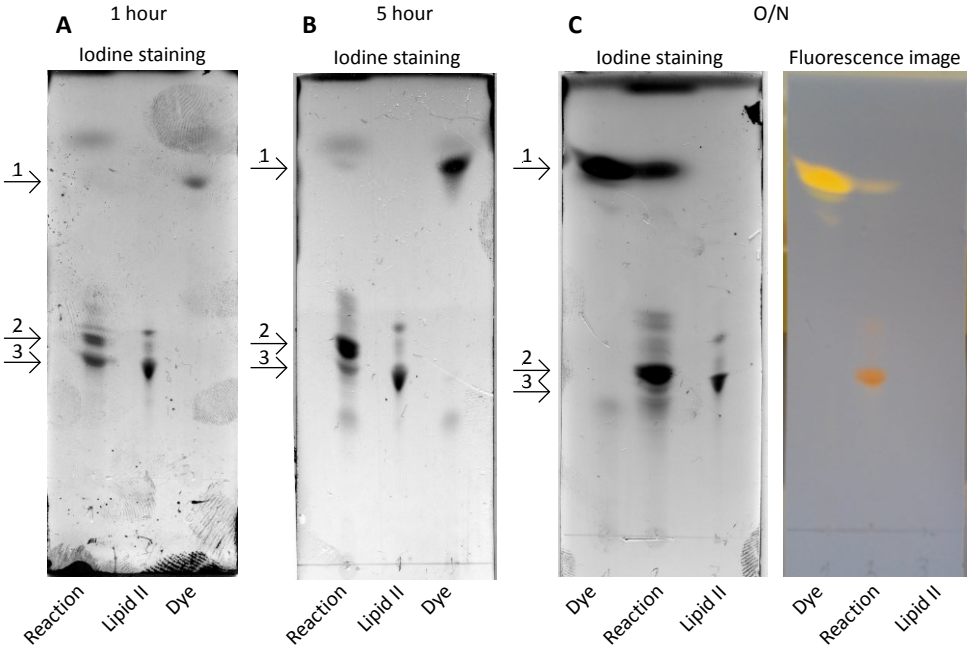


Figure 3. Coupling of cyclooctyne Megastokes 608 dye to azido-Lipid II followed by TLC.
Azido-Lipid II was incubated with cycloocytyne Megastokes dye 608 at RT. A) After 1 hour of incubation time, all the dye seems to be consumed (1), and some labeled Lipid II has been produced (2). Though, still a lot of unlabeled Lipid II is present (3). B) After adding more dye and continuation of the reaction for 4 hours, again all the dye seems to be consumed (1), and more labeled Lipid II had been produced (2). Though, still some unlabeled Lipid II (3) is left. C) After adding more dye and continuation of the reaction O/N at 4 °C, the reaction is completed. All the unlabeled Lipid II (3) has been converted into labeled Lipid II (2).

dried and resolved in 1.5 mL $CHCl_3$:MeOH. The concentration was found to be 1.10 mM by phosphate determination which is almost 95% recovery of the 500 µl of 3.5 mM Lipid II-N_3 of starting material. This shows that there was barely any loss of Lipid II during this labeling procedure, and that this is a highly efficient way of fluorescent labeling of Lipid II.

The incorporation of different fluorescently labeled Lipid II variants into glycan chains and crosslinked PG by *Escherichia coli* PBP1b, visualized in gel.

To analyze the GT and TP activity of PBPs under different conditions or as a result of deletions or mutations, an in gel electrophoresis assay is often used. This assay is based on the incorporation and visualization of fluorescently labeled Lipid II into the PG network. During an *in vitro* PG synthesis assay, samples are taken at different time intervals, and the content is separated by size with a modified Tris/Tricine SDS-PAGE system. Subsequently, the results are visualized with a fluorescence scanner. Glycan chains are separated by size, running higher or lower on the gel, depending on the length of the glycan chain. Active transpeptidation will generate high mass products that do not migrate into the gel electrophoresis system and will be observed as a band in the bottom of the slot. This assay was up till now often performed using radiolabeled or dansylated Lipid II [2], [5]–[9]. However, dansyl does not have a very high quantum yield, opening up the possibility to highly improve the resolution and sensitivity of this assay by using different fluorophores with better properties. Therefore, we used click chemistry to attach different (high quantum yield) fluorophores to Lipid II as described in the previous paragraph, to investigate their performance in this assay.

The *in vitro* PG synthesis by PBP1b, using slightly adapted reaction conditions as previously found to be optimal for this protein [13], only seemed to marginally work for the incorporation of Lipid II labeled with Megastokes dye 608 into glycan chains (Fig 4A). When using this Lipid II variant, some short chains are produced, which become slightly more intense in time. Though, this is not near expectation, e.g. much more production (and hence labeled Lipid II consumption) was expected after this period of reaction time, since it has been shown by HPLC analysis of the PG produced by PBP1b in a similar *in vitro* assay, that after one hour of incubation time (almost) all radiolabeled Lipid II was used and incorporated into glycan strands [4], [13]. Furthermore, when using a fluorescence assay to analyze the GT activity of PBP1b, complete utilization was already seen after 20 min of incubation [3], [10]. In the reaction with Megastokes dye 608 labeled Lipid II, free Lipid II consumption was calculated to be around 80% after 120 min of reaction time (supplementary Fig 1). For the other Lipid II variants, labeled with ATTO550 and ATTO647N, no production of glycan chains was observed at all (Fig 5A).

Hereupon we tested if the addition of unlabeled substrate to the reaction mixture changed the ability of PBP1b to incorporate the dye labeled substrates into glycan chains. The addition of unlabeled Lipid II to the reaction is only necessary for the production of

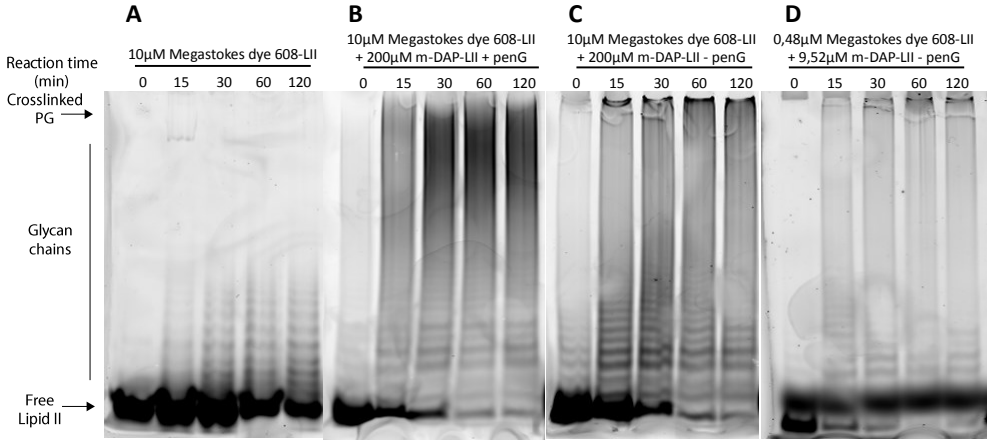


Figure 4. The incorporation of Megastokes dye 608 labeled Lipid II into glycan chains and crosslinked PG by PBP1b.

PBP1b was incubated with Megastokes dye 608 labeled Lipid II (A) and unlabeled Lipid II (B, C and D) in the presence or absence of PenG, samples were taken at the indicated time points, and glycan chain production was analyzed by Tris/Tricine SDS-PAGE. A) Some production of short glycan chains was observed when only using Megastokes dye 608 labeled Lipid II in the reaction. B) The addition of unlabeled Lipid II drastically increased the reaction speed (and hence free Lipid II consumption) and glycan chain length. C) PBP1b is able to incorporate Megastokes dye 608 labeled Lipid II into crosslinked PG as well. D) The increase of the reaction speed and glycan chain length produced is not caused by the addition of a higher total amount of substrate.

crosslinks in the PG, since the amino acid on the third position of the pentapeptide of dye labeled Lipid II variants is not available as acceptor in the TP reaction since the fluorophores are attached to this position. Furthermore, *E. coli* PBP1b needs the meso-diaminopimelic acid (m-DAP) form of Lipid II for the production of crosslinks, because the m-DAP at the third position of the pentapeptide functions as the acceptor in the TP reaction of this enzyme [14]. To be able to evaluate changes in glycan chain production under these conditions, penicillin G (PenG) was added to the reactions, to inhibit crosslinking of PG by the TP activity of the protein. As can be seen in figure 5B, this addition of unlabeled substrate made PBP1b able to incorporate both ATTO550 and ATTO647N labeled Lipid II into glycan chains. Furthermore, this addition increased the reaction speed and length of chains produced in the reaction with Megastokes dye 608 labeled Lipid II (Fig 4B). When performing the reaction without penicillin G, all three dyes were also incorporated in crosslinked PG networks, seen by the appearance of a high intensity band in the bottom of the slot and a decrease in long glycan chains (Fig 4 and 5C). It seemed that Lipid II labeled with Megastokes dye 735 was also incorporated in both glycan strands and crosslinked PG under these conditions, but the fluorescence scanner used for visualization did not have the capacity to clearly visualize this fluorophore (data not shown).

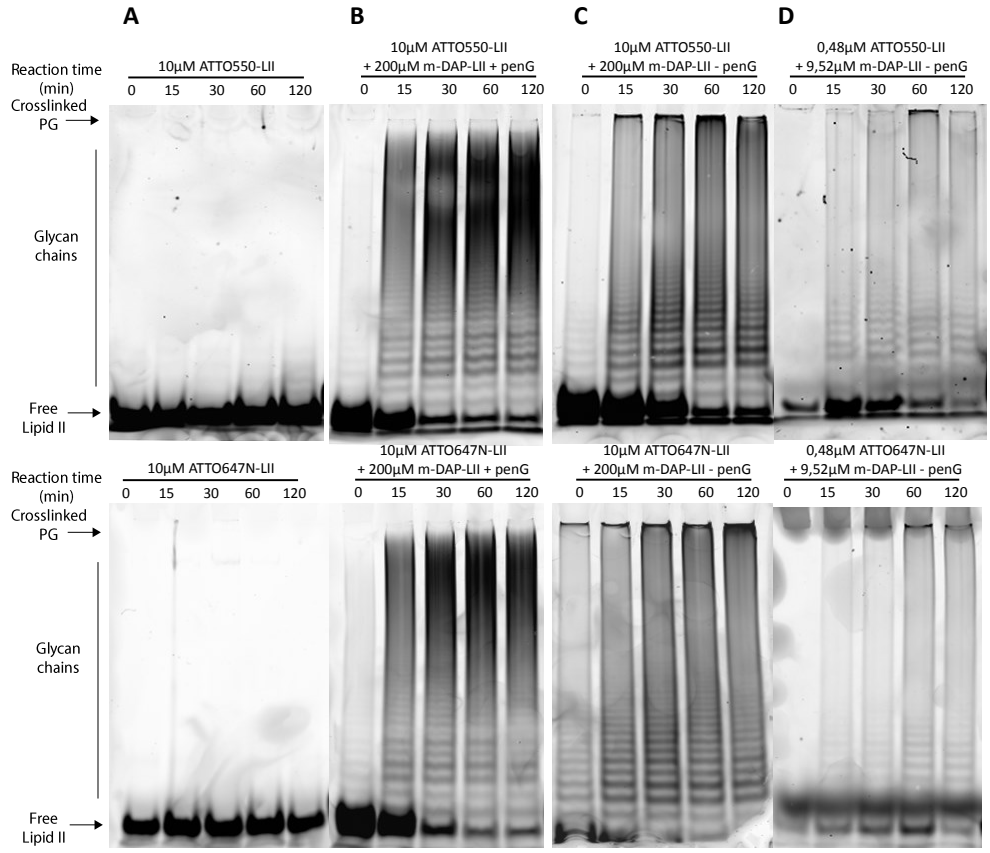


Figure 5. The incorporation of ATTO550 and ATTO647N labeled Lipid II into glycan chains and crosslinked PG by PBP1b.

PBP1b was incubated with ATTO550 or ATTO647N labeled Lipid II (A) and unlabeled Lipid II (B, C and D) in the presence or absence of PenG, samples were taken at the indicated time points, and glycan chain production was analyzed by Tris/Tricine SDS-PAGE. A) No production of glycan chains was observed when only using ATTO550 or ATTO647N labeled Lipid II in the reaction. B) The addition of unlabeled Lipid II initiated the production of glycan chains. C) PBP1b is able to incorporate ATTO550 and ATTO647N labeled Lipid II into crosslinked PG as well. D) The initiation of the glycan chain production is not caused by the addition of a higher total amount of

The necessity of the addition of unlabeled substrate to the reactions with ATTO550 and ATTO647N labeled Lipid II, to initiate the glycan chain production and to the reaction with Megastokes dye 608 labeled Lipid II to reach a proper reaction rate and glycan chain length, was unexpected, since when using radiolabeled or dansylated Lipid II this was not reported to be necessary. We also investigated whether the initiation and increase of PG production after addition of the unlabeled substrate to the reactions might be due to the addition of a higher total amount of substrate. In a separate reaction, a total of 10 µM Lipid II was used, of which 5% was labeled and 95% unlabeled. PG production was still visible in these reactions, nevertheless with a much lower intensity, showing that the

initiation and increase of PG production was caused by the addition of unlabeled Lipid II and not by the addition of a higher total amount of substrate in these reactions (Fig 4 and 5D).

The use of all these dyes in this assay resulted in a much clearer result with a higher intensity and resolution, as compared to experiments performed with dansylated Lipid II [5], [8]. Clearly separated bands were visible, especially in the lower region of the gel. Using a larger size gel would even improve this. The ATTO550 and ATTO647N dyes performed the best, as they produced the neatest and most clearly separated bands in these experiments, and hence will be used in future experiments.

Analysis of the performance of these new fluorescently labeled Lipid II variants in the continuous glycosyltransferase assay.

As shown in the previous paragraph, our newly synthesized fluorescently labeled Lipid II variants, with high quantum yield properties, showed an excellent performance in the in gel analysis of PG synthesis activity of PBP1b. Therefore, we decided to analyze their performance in the continuous GT assay, developed by Schwartz *et al.* [10], in which dansylated Lipid II is used for detection as well. This continuous GT assay is based on the property that the fluorescence intensity of dansyl is higher in a more hydrophobic environment (connected to the lipid carrier tail) than in a more hydrophilic environment (incorporated into the glycan chain and released from its lipid tail). Hence, a decrease in fluorescence intensity is observed over time, as a result of the incorporation of Lipid II into glycan chains. When using either of the ATTO dye labeled Lipid II variants in this assay, no intensity decrease was observed as a result of incorporation into glycan chains (Fig 6). This is caused by the fact that these fluorophores' intensities show only a minor dependency on their environment, and therefore, the intensities do not decrease as a result of release form the lipid carrier tail, nor incorporation into glycan chains. Both the Megastokes dyes 608 and 735 Lipid II variants did show a decrease in fluorescence intensity as a result of incorporation into glycan chains. The result using Megastokes dye 608 Lipid II was slightly less noisy then when using dansylated Lipid II. When using Megastokes dye 735 Lipid II, the results were very noisy due to a so far unknown reason which is regretful, since the total fluorescence decrease in this reaction is significantly higher than when using dansylated Lipid II (Fig 6). Remarkably, in this assay, Megastokes dye 608 Lipid II was incorporated into glycan strands with a comparable speed as dasyl Lipid II, having even a slightly faster incorporation at the start of the measurement, without the addition of unlabeled Lipid II. The addition of unlabeled Lipid II did slightly increase the incorporation speeds of all three fluorescent dyes tough, but did not result in the significant increase of the incorporation speed as seen in the in gel assay (Fig 7, and data not shown). Furthermore, these graphs show that the reaction, when analyzed with dansylated Lipid II, shows a lag phase at the start of the measurement, which is not seen when using

Megastokes dye 608 Lipid II. These results show that Megastokes dye 608 Lipid II can well be used in this continuous GT assay as well, but does not result in a significant improvement of the results when compared to the use of dansylated Lipid II.

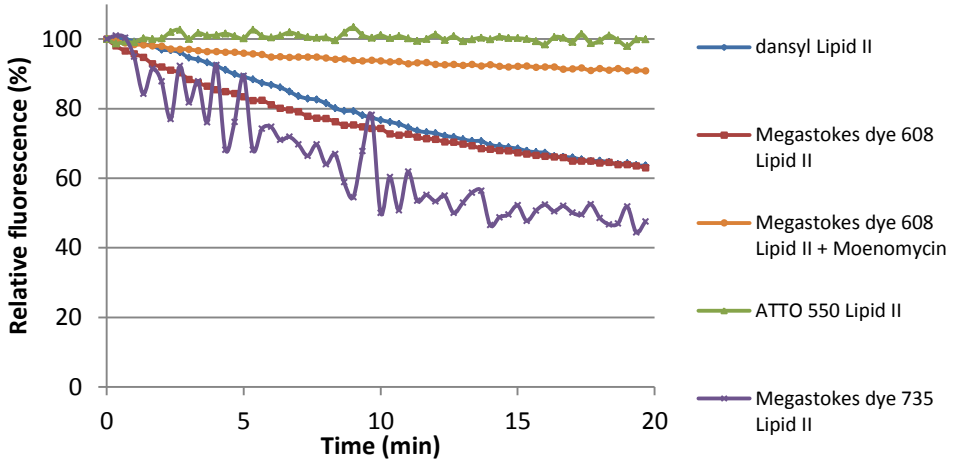


Figure 6. Comparison of performance of Lipid II labeled with different fluorescent dyes in the *in vitro* continuous GT assay.

PBP1b was incubated with different fluorescently labeled Lipid II variants, and substrate consumption was measured continuously with a fluorimeter as a decrease in signal. Megastokes dye 608 Lipid II (red) shows a similar result as dansyl Lipid II (blue). ATTO550 Lipid II (green) does not show the property of decreased fluorescence intensity as a result of environmental change. Megastokes dye 735 Lipid II (purple) shows a higher total decrease of fluorescence signal, but gives a very noisy result. The fluorescent decrease is a result of GT activity, since almost no fluorescence decrease is observed in the presence of the GT inhibitor Moenomycin (orange, only shown for the reaction with Megastokes dye 608 Lipid II, similar results observed with other Lipid II variants).

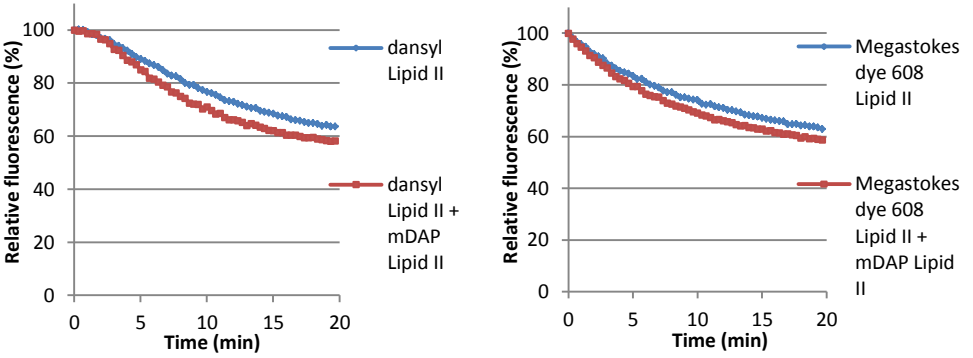


Figure 7. Effect of the addition of unlabeled Lipid II on the incorporation speed as measured by the *in vitro* continuous GT assay.

PBP1b was incubated with dansyl or Megastokes dye 608 labeled Lipid II, in the presence or absence of 10 µM unlabeled substrate, and substrate consumption was measured continuously with a fluorimeter as a decrease in signal. For both dansyl Lipid II and Megastokes dye 608 Lipid II, the addition of unlabeled Lipid II slightly increased the reaction speed.

Comparison of the in gel and continuous GT assays

To investigate why in the in gel visualization of PG synthesis, unlabeled Lipid II had to be added to the reaction mixture to increase the polymerization speed, which did not significantly change the reaction speed in the continuous GT assay, we compared the components of the reaction mixture of both assays. One difference is that in the continuous assay lysozyme is added, to cut the glycan chains into small pieces, which should increase the drop in fluorescence as a result of the environmental change. We analyzed the effect of this difference on the reaction speed in two different ways. We performed the continuous GT assay, and the *in vitro* PG synthesis reaction, taking a sample at different time points, and performing buthanol/pyridine extraction to analyze the amount of lipids that are left, e.g. not incorporated into glycan strands, by TLC both in the presence and absence of lysozyme.

The results are displayed in figure 8, which shows that the addition of lysozyme to the reaction mixture with Megastokes dye 608 Lipid II actually decreases the reaction speed in both the continuous GT assay and the TLC analysis of the remaining lipids in the *in vitro* assay (Fig 8 A and C). It also shows that in both assays, the addition of unlabeled Lipid II increases the reaction speed under most conditions, except for the reaction without lysozyme measured via the continuous GT assay. We do not have a good explanation for this, but it may be caused by the build up of cell wall material (in the presence of m-DAP Lipid II) that causes a background signal that increases in time. The observed appearance of a large amount of scattering is likely also related to the formation of (insoluble) cell wall material. Both the TLC analysis of the remaining lipids, as well as the in gel analysis of glycan chain production showed an increase in reaction speed and glycan chain length as a result of the addition of unlabeled Lipid II. The idodine stained TLC plate also shows that the absence of lysozyme both increased the turnover of labeled Lipid II as well as unlabeled, m-DAP Lipid II, with an apparent similar rate, also showing that there is no substrate preference of PBP1b under these conditions. In this image the detachment of the disaccharide pentapeptide from the lipid tail, due to PG synthesis by PBP1b, can also be seen by the appearance of undecaprenyl pyrophosphate (11-PP) on TLC. This is only seen in the reactions containing unlabeled Lipid II, because the other reactions do not produce enough 11-PP to visualize it. Since the amount of labeled Lipid II is very low, and the 11-PP spot is running at a similar hight, the decrease of Megastokes dye 608 Lipid II is not clearly visible in the iodine stained image.

The results when using dansylated Lipid II in the continuous GT assay are unfortunately not very clear, but do show that addition of unlabeled Lipid II increases the reaction speed both in the presence and absence of lysozyme (Fig 8B). However, the expected increase in reaction speed by the absence of lysozyme is not observed in the measurement that did not contain unlabeled Lipid II (Fig 8B purple). A second difference between the continuous and in gel PG synthesis assay is that in the continuous GT assay, Lipid II is dissolved in

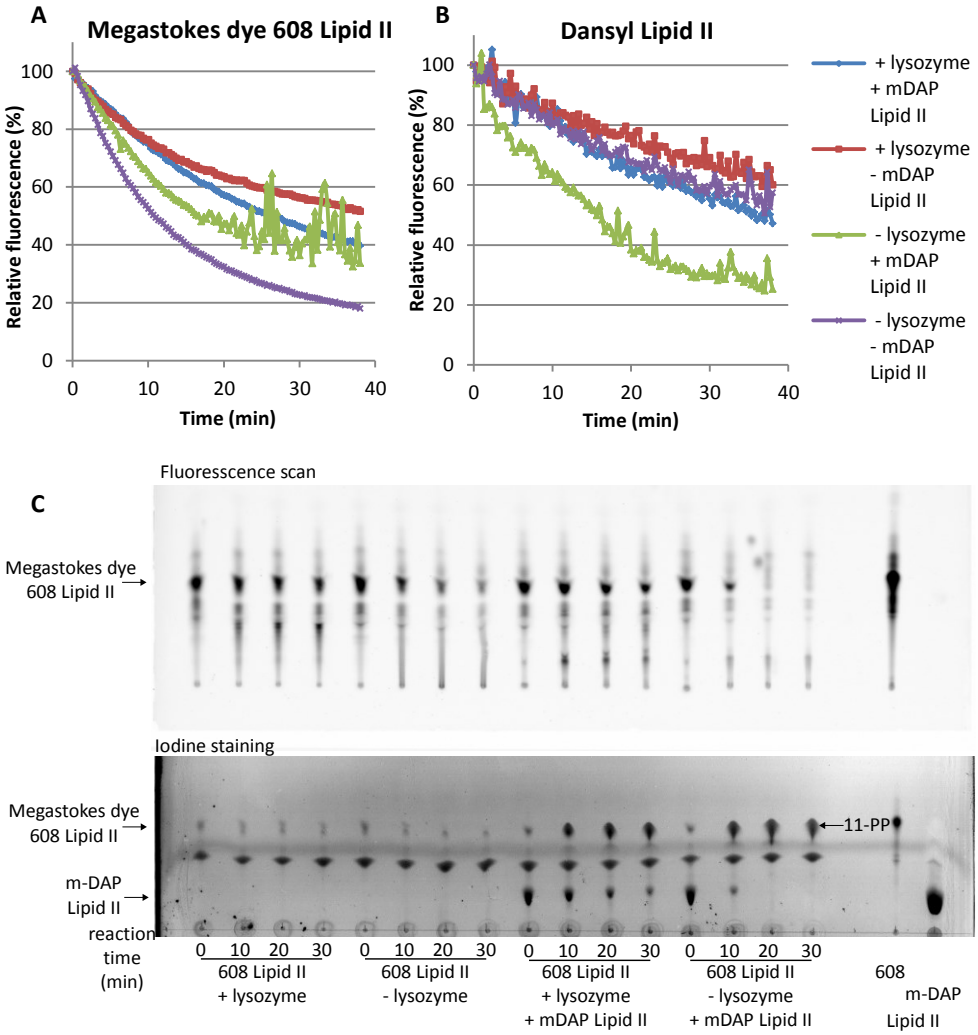


Figure 8. Effect of the addition of lysozyme and unlabeled Lipid II on the *in vitro* PG synthesis by PBP1b.
PBP1b was incubated with Megastokes dye 608 labeled Lipid II or dansyl labeled Lipid II, in the presence or absence of lysozyme and 100 µM unlabeled Lipid II. Substrate consumption was measured continuously with a fluorimeter as a decrease of signal, or by analyzing the lipids that are left after a certain reaction period by TLC. A) When using Megastokesdye 608 Lipid II, the addition of unlabeled Lipid II increases the reaction speed only in the presence of lysozyme. The reaction without lysozyme is faster than with lysozyme.B) When using dansyl Lipid II , the addition of unlabeled Lipid II increases the reaction speed both in the presence and absence of lysozyme. But the reaction without lysozyme and unlabeled Lipid II had not the expected higher rate. C) TLC analysis of remaining lipids, when using Megastokes dye 608 Lipid II in the *in vitro* PG synthesis assay, shows a similar result as in the continuous GT assay: the absence of lysozyme increases the reaction speed. The addition of unlabeled Lipid II slightly increases the reaction speed.

water, and in the in gel assay, Lipid II is dissolved in 5% Triton X 100, before addition to the reaction mixture. This will result in the formation of micelles of Lipid II when dissolved in water, and the formation of Triton micelles containing Lipid II in the other situation. This could lead to a difference in substrate presentation, which might influence the reaction speed. Figure 9 shows however, that there is no influence on the incorporation speed between lipids that are dissolved in water or in Triton X 100 prior to addition to the reaction mixture.

These results still do not explain why Megastokes dye 608 Lipid II is barely incorporated into glycan strands when no unlabeled Lipid II is added to the reaciton mixture when samples are analyzed by Tris/Tricin SDS-PAGE, but almost all of it seems to be incorporated in 20-30 minutes when this reaction is folowed by the fluorescence decrease in the continous GT assay. We do not have a solid solution to this contradiction, but hypothesize that it might have something to do with the way the polymerization is analyzed in both assays. Maybe there is a high amount of glycan chain polymerization termination (for an unknown reason) in the absence of unlabeled Lipid II, which would result in the production of short chains resembling no, or barely no production when analyzed by the in gel assay, which will be interpreted as (normal) polymerization when analyzed by the continuous GT assay as a constant drop in fluorescence, since the environment of the fluorophores still changes as a result of detachment of the lipid tail in this situation.

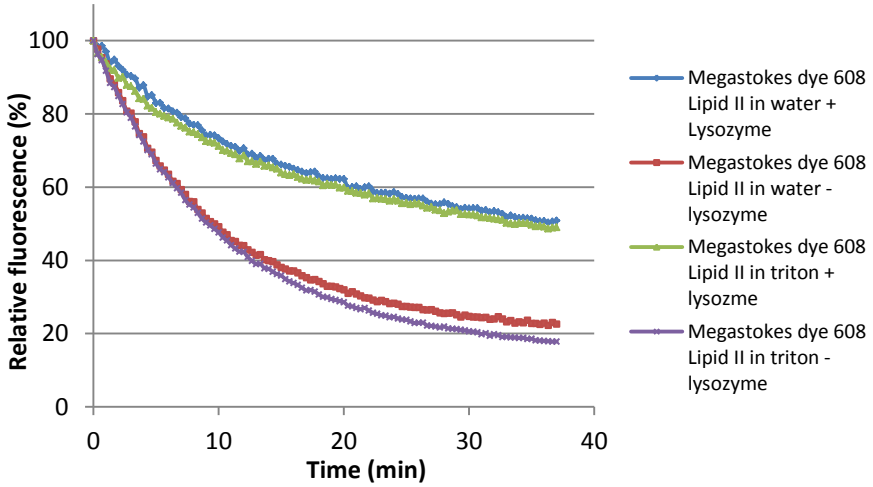


Figure 9. Effect of the addition of Lipid II dissolved in water or Triton X 100 on the *in vitro* PG synthesis speed by PBP1b.

PBP1b was incubated with Megastokes dye 608 labeled Lipid II, dissolved in water or in Triton X 100 prior to addition. Substrate consumption was measured continuously with a fluorimeter as a decrease in signal. These results show that dissolving Lipid II in water or in Triton X 100 prior to addition to the reaction mixture does not have an effect on the incorporation speed. These results also show the large increase in reaction speed in the absence of lysozyme.

Discussion

Here we show that the use of Lipid II labeled with other, high quantum yield, fluorophores in the in gel PG synthesis assay highly improved the resolution and intensity of the results, compared to the use of Lipid II labeled with dansyl. This leads to a much clearer result, making it possible to observe more subtle changes in these activities of PBPs. However, to be able to observe incorporation of these new fluorescently labeled Lipid II variants into glycan chains, unlabeled Lipid II had to be added to the reaction mixture. This was not reported before, when dansylated Lipid II was used. A possible explanation for this could be the difference in molecular weight of the fluorophores. Since the molecular weight of dasyl is only 270 g/mol, and that of ATTO550 and 647N is 731 g/mol and 783 g/mol respectively, and that of Megastokes dye 608 and 735 is 651 g/mol and 438 g/mol respectively. This is, except for Megastokes dye 735, at least two times the weight of dansyl. It could be that PBP1b had problems in initiating glycan chain formation via the incorporation of Lipid II labeled with the ATTO dyes, where no glycan chain polymerization is observed when no unlabeled Lipid II is added, because of steric hindrance. PBP1b has two substrate binding sites, the acceptor and donor site. During glycan chain polymerization, Lipid II is bound to the acceptor site, which later reacts with the growing chain in the donor site [15]–[17]. The elongated chain is subsequently translocated to the donor site for a new round of polymerization. It could be that due to the size of the attached dyes it is not possible to have both sites occupied by the ATTO labeled Lipid II variants, and the addition of unlabeled Lipid II solved this problem.

For Megastokes dye 608 labeled Lipid II the situation is slightly different. This Lipid II variant was incorporated in glycan chains without the addition of unlabeled Lipid II, as visualized by the in gel assay, but not as fast, and did not produce long glycan chains, as expected from previous results [3], [10], [13]. In the continuous GT assay, there seemed to be no problem in the utilization of Megastokes dye 608 Lipid II for the GT reaction. We hypothesize that there might be an increased glycan chain polymerization termination when this Lipid II variant is used, resulting in the production of short chains which resembles no, or barly no production when analyzed by the in gel assay, but will be interpreted as (normal) polymerization when analyzed by the continuous GT assay as a constant drop in fluorescence.

The use of Megastokes dye 608 does not result in a larger decrease in fluorescence signal (e.g. increase in resolution) in the continuous GT assay, when comparing to the use of dansylated Lipid II. However, there is a small difference at the start of the reaction. When using dansylated Lipid II, there seems to be a lag phase at the start of the measurement, which is not seen when Megastokes dye 608 is used. This could point to a phase of polymerization initiation, in which substrate association is slower for dansylated Lipid II than for Megastokes dye 608 Lipid II. One would expect the association of substrate

labeled with a smaller fluorophore (dansyl) to be easier though, but maybe the more extended structure of Megastokes dye 608 results in quicker enzyme substrate association. Furthermore, Megastokes dye 608 is a more hydrophobic structure than dansyl, which could make it easier to form a complex with the partially membrane associated GT domain of PBP1b. Finally, Megastokes dye 735 Lipid II also showed a drop in fluorescence signal in the continuous GT assay, however, this result was very noisy, and therefore this Lipid II variant is not useful in this assay.

Still, Megastokes dye 608 Lipid II can very well be used as a substrate for PBPs in both these assays. In the in gel assay unlabeled Lipid II needs to be added to the reaction to obtain proper glycan chain lengths and polymerization speed, however, this even more resembles the *in vivo* situation so should not cause any problems. When using the ATTO labeled Lipid II variants the results of the in gel assay were even more clear, with a higher resolution band separation. These fluorophores cannot be used in the continuous GT assay though. Furthermore, this method of fluorescent labeling on the level of Lipid II, using click chemistry showed to be very straightforward and highly efficient, showing hardly any product loss during the procedure. Taken together, these fluorescently labeled Lipid II variants showed to be very useful for the analysis of GT and TP activities of PBP1b.

References

[1] T. J. Lupoli, M. D. Lebar, M. Markovski, T. Bernhardt, D. Kahne, and S. Walker, "Lipoprotein Activators Stimulate Escherichia coli Penicillin-Binding Proteins by Different Mechanisms," *J. Am. Chem. Soc.*, vol. 136, no. 1, pp. 52–55, Jan. 2014.

[2] C. Paradis-Bleau, M. Markovski, T. Uehara, T. J. Lupoli, S. Walker, D. E. Kahne, and T. G. Bernhardt, "Lipoprotein Cofactors Located in the Outer Membrane Activate Bacterial Cell Wall Polymerases," *Cell*, vol. 143, no. 7, pp. 1110–1120, Dec. 2010.

[3] A. J. F. Egan, N. L. Jean, A. Koumoutsi, C. M. Bougault, J. Biboy, J. Sassine, A. S. Solovyova, E. Breukink, A. Typas, W. Vollmer, and J.-P. Simorre, "Outer-membrane lipoprotein LpoB spans the periplasm to stimulate the peptidoglycan synthase PBP1B," *Proc. Natl. Acad. Sci.*, vol. 111, no. 22, pp. 8197–8202, Jun. 2014.

[4] P. Müller, C. Ewers, U. Bertsche, M. Anstett, T. Kallis, E. Breukink, C. Fraipont, M. Terrak, M. Nguyen-Distèche, and W. Vollmer, "The Essential Cell Division Protein FtsN Interacts with the Murein (Peptidoglycan) Synthase PBP1B in Escherichia coli," *J. Biol. Chem.*, vol. 282, no. 50, pp. 36394–36402, Dec. 2007.

[5] N. Helassa, W. Vollmer, E. Breukink, T. Vernet, and A. Zapun, "The membrane anchor of penicillin-binding protein PBP2a from Streptococcus pneumoniae influences peptidoglycan chain length," *FEBS J.*, vol. 279, no. 11, pp. 2071–2081, Jun. 2012.

[6] A. J. Lesse, A. A. Campagnari, W. E. Bittner, and M. A. Apicella, "Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis," *J. Immunol. Methods*, vol. 126, no. 1, pp. 109–117, Jan. 1990.

[7] D. Barrett, T.-S. A. Wang, Y. Yuan, Y. Zhang, D. Kahne, and S. Walker, "Analysis of Glycan Polymers Produced by Peptidoglycan Glycosyltransferases," *J. Biol. Chem.*, vol. 282, no. 44, pp. 31964–31971, Nov. 2007.

[8] A. Zapun, J. Philippe, K. A. Abrahams, L. Signor, D. I. Roper, E. Breukink, and T. Vernet, "In vitro Reconstitution of Peptidoglycan Assembly from the Gram-Positive Pathogen Streptococcus pneumoniae," *ACS Chem. Biol.*, vol. 8, no. 12, pp. 2688–2696, Dec. 2013.

[9] Y. Rebets, T. Lupoli, Y. Qiao, K. Schirner, R. Villet, D. Hooper, D. Kahne, and S. Walker, "Moenomycin Resistance Mutations in Staphylococcus aureus Reduce Peptidoglycan Chain Length and Cause Aberrant Cell Division," *ACS Chem. Biol.*, vol. 9, no. 2, pp. 459–467, Feb. 2014.

[10] B. Schwartz, J. A. Markwalder, S. P. Seitz, Y. Wang, and R. L. Stein, "A Kinetic Characterization of the Glycosyltransferase Activity of Eschericia coli PBP1b and Development of a Continuous Fluorescence Assay," *Biochemistry (Mosc.)*, vol. 41, no. 41, pp. 12552–12561, Oct. 2002.

[11] L. L. Danilov, T. N. Druzhinina, N. A. Kalinchuk, S. D. Maltsev, and V. N. Shibaev, "Polyprenyl phosphates: synthesis and structure-activity relationship for a biosynthetic system of Salmonella anatum O-specific polysaccharide," *Chem. Phys. Lipids*, vol. 51, no. 3–4, pp. 191–203, Nov. 1989.

[12] G. Rouser, S. Fkeischer, and A. Yamamoto, "Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots," *Lipids*, vol. 5, no. 5, pp. 494–496, May 1970.

[13] U. Bertsche, E. Breukink, T. Kast, and W. Vollmer, "In Vitro Murein (Peptidoglycan) Synthesis by Dimers of the Bifunctional Transglycosylase-Transpeptidase PBP1B from Escherichia coli," *J. Biol. Chem.*, vol. 280, no. 45, pp. 38096–38101, Nov. 2005.

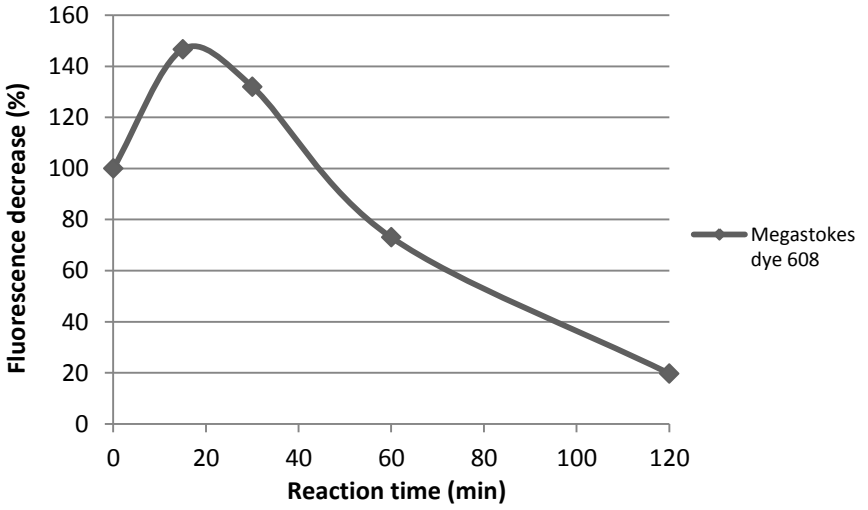
[14] "Van Heijenoort, J. (1996) Murein synthesis. In Escherichia coli and Salmonella. Neidhardt, F.C. (ed.).Washington, DC: American Society for Microbiology Press, pp. 1025–1034."

[15] M. Terrak, T. K. Ghosh, J. van Heijenoort, J. Van Beeumen, M. Lampilas, J. Aszodi, J. A. Ayala, J. M. Ghuysen, and M. Nguyen-Distèche, "The catalytic, glycosyl transferase and acyl transferase modules of the cell wall peptidoglycan-polymerizing penicillin-binding protein 1b of Escherichia coli," *Mol. Microbiol.*, vol. 34, no. 2, pp. 350–364, Oct. 1999.

[16] D. L. Perlstein, Y. Zhang, T.-S. Wang, D. E. Kahne, and S. Walker, "The Direction of Glycan Chain Elongation by Peptidoglycan Glycosyltransferases," *J. Am. Chem. Soc.*, vol. 129, no. 42, pp. 12674–12675, Oct. 2007.

[17] M. Terrak, E. Sauvage, A. Derouaux, D. Dehareng, A. Bouhss, E. Breukink, S. Jeanjean, and M. Nguyen-Distèche, "Importance of the Conserved Residues in the Peptidoglycan Glycosyltransferase Module of the Class A Penicillin-binding Protein 1b of Escherichia coli," *J. Biol. Chem.*, vol. 283, no. 42, pp. 28464–28470, Oct. 2008

Supplementary information



Supplementary figure 1. Fluorescence decrease of free Lipid II in the in gel assay when using Megastokes dye 608 Lipid II.

The intensity of the free Lipid II bands were analyzed using Image J. There is probably a pipetting error in the first sample, making the intensity lower than in the second sample. Starting at the second sample, the fluorescence signal decreases in time to approximately 20% of the starting intensity.

Chapter 6

Mapping of the PBP1b dimer interface

Inge van ’t Veer & Eefjan Breukink

Abstract

Peptidoglycan (PG) is an essential structure of the bacterial cell envelope, composed of glycan strands, which are crosslinked by peptides. The synthesis of PG from its precursor, Lipid II, is performed by penicillin binding proteins (PBPs), of which PBP1b is one of the two major bifunctional PG synthases of *E. coli*. It possesses both glycosyltransferase (GT) activity, polymerizing the glycan strands, and transpeptidase (TP) activity, forming the peptide crosslinks. PBP1b has shown to form dimers, and under dimerizing conditions its GT and TP activity are increased. Because of the effect of dimer formation on its activity, it is highly interesting to elucidate the region of PBP1b involved in dimer formation. This can very well be achieved using an *in vivo* photo-crosslinking approach, by the site specific incorporation of an unnatural amino acid with photo-crosslinkable properties. Analyzing the ability of truncated versions to form dimers, indicated that the amino acids between F480 and Y498 are part of an important region for dimer formation. *In vivo* photo-crosslinking studies showed that the amino acid E492 on a protruding loop in this region could be part of the dimer interface of PBP1b.

Introduction

The bacterial peptidoglycan (PG) layer is an essential structure of their cell envelope, giving them strength to withstand turgor pressure and is important in maintaining their cell shape [1], [2]. This structure is composed of glycan strands containing alternating N-acetylmuramic acid and N- acetylglucosamine residues, which are interlinked by peptide bridges [3]. The disaccharide-pentapeptide building blocks are formed and transported to the outer leaflet of the cytoplasmic membrane, linked to an undecaprenol pyrophosphate lipid carrier tail, in the form of Lipid II. The polymerization of the sugar moieties by glycosyltransferase (GT) reactions and the incorporation into the existing wall by crosslinking of the peptides by transpeptidase (TP) reactions is performed by penicillin binding proteins (PBPs).

The two major bifunctional PG synthases of *E. coli* are the high molecular weight class A PBPs PBP1a and PBP1b [4], of which at least one is needed for cell survival [5]. These proteins can take over each other's function, since only one of the two is needed for cell survival, but they do seem to have distinct roles when both are present. For PBP1b this is reflected for example by the higher mid cell concentration during cell division, implying a more important role for PBP1b during cell division [6]. The crystal structure of *E. coli* PBP1b has been solved, also in complex with the only inhibitor of the GT domain, moenomycin [7]. PBP1b has a short cytoplasmic tail, followed by the transmembrane helix with which it is inserted in the cytoplasmic membrane. The largest part of PBP1b resides in the periplasmic space and consists of a GT domain, a TP domain and the non-enzymatic UB2H domain. This domain is important for proper functioning of PBP1b though, since deletion does cause an aberrant growth rate. This is most likely related to the involvement of the UB2H domain in the interaction of PBP1b with other proteins, like MltA [8], LpoB [9], [10]and YbgF (renamed into CpoB, this work and [11]). Furthermore, PBP1b interacts with the essential cell division protein FtsN, which stimulates both its GT and TP activity [12], and PBP3 of which the septal localization is essential for the mid cell recruitment of PBP1b [6]. *In vivo*, PBP1b exists in two isoforms, α, and γ, however, when analyzing membrane preparations, a third isoform is seen [13]. This β form is a 24 amino acids shorter degradation product, artificially created by cleavage of the α-isoform by OmpT [14], [15]. The α form is the full length, 844 amino acids long protein transcribed from the *PonB* (*MrcB*) gene. The γ form results from the translational start at an internal start codon at position M46, resulting in a protein of 799 amino acids [16]. Both isoforms are fully active, since they can complement a *PonB* deletion [17].

PBP1b has shown to form dimers *in vivo*, by SDS-PAGE analysis [18]. These dimers are highly heat stable, and stay intact until incubation at 80°C for 10 min [19], and are not formed by disulfide bridges, as the addition of β-mercaptoethanol does not dissociate the dimers [18], [20]. *In vitro* analysis of the kinetic parameters of PBP1b dimerization by

surface plasmon resonance showed a K_D value of 1,27+/- 0,96 $*10^{-7}$ M [21]. Analysis of the reaction products formed during an *in vitro* synthesis assay displayed large differences between experiments performed using PBP1b concentrations below, around or higher than the dimerization concentration. Below dimerization concentrations, the GT reaction speed was very low, and no TP activity was observed. Dimerizing concentrations resulted in an increase in GT reaction speed and the occurrence of TP reactions. Higher concentrations even resulted in a further increase of the reaction speed, the production of longer glycan chains and an increase in crosslinked material [21]. Because the PBP1b concentration, in respect to dimer formation, had such a dramatic effect on the reaction speed of both domains, the chain length and amount of crosslinks in the product, it was proposed that the amount of dimers may serve a regulatory function. It was suggested that *in vivo*, dimerization is a well monitored event, inversely correlated with the amount of PBP1a present (and vice versa) [18]. Since only homo dimers (α-α and γ-γ) are found, it was suggested that the cytoplasmic tail might play a role in dimerization [18]. However, later Chalut *et al.* showed that the cytoplasmic tail is not involved in dimer formation [17]. Instead, from experiments using tail deletion mutants, they proposed that this region is involved in the interaction of PBP1b with PBP3 [17].

Zijderveld *et al.* partially mapped the dimer interface by the expression of truncated PBP1b variants and analysis of isolated cell envelopes by SDS-PAGE and immunoblotting. They showed that the carboxy-terminal half of PBP1b (amino acids 405 to 844) was not needed for dimer formation [18]. To map the dimer interface region in a similar way, but more precisely, we created truncated N-terminal His tagged version of PBP1b, lacking different sizes of the carboxy-terminal part of the protein. Because these proteins are His tagged, the used monoclonal anti poly Histidine antibody has identical antigenicity for all different versions, which was a problem in the experiments of Zijderveld *et al.* who used polyclonal anti PBP1b antibodies which lost their antigenicity against short (< 405 amino acids) truncated versions. Furthermore, we used more versions, with different truncations to perform the SDS-PAGE analysis to more precisely track the ability of dimer formation. In this way we mapped the region between F480 and Y498 to be important for PBP1b dimer formation.

To analyze the dimer formation involvement of specific amino acids in this region of PBP1b in an *in vivo* situation, we used the nonsense suppression mutagenesis technique to site specifically incorporate the photo-crosslinkable unnatural amino acid *p*-Benzoyl-L-phenylalanine (*p*Bpa) at different positions. By illumination of cells expressing these proteins with UV light, the photo-crosslinker will covalently couple to proteins in close proximity. In this way the involvement of the replaced amino acids in dimer formation can be revealed, since crosslinking would result in the formation of a heat stable dimer. The incorporation of the photo-crosslinker at different positions in the region between F480

and Y498 showed that the amino acid E492 might be involved in the dimer formation of PBP1b.

Materials and methods

Growth medium and conditions

Cultures were grown in LB at 37°C, supplemented with kanamycin (50 µg/mL) and/or chloramphenicol (34 µg/mL).

Bacterial strains and plasmids

E. coli DH5α cells were used for DNA amplification. *E. coli* BL21 (DE3) cells were used for protein expression and *in vivo* photo-crosslinking experiments.

Plasmid pDML924 carrying the *PonB* gene, encoding the N- terminal His_6-tagged PBP1bγ variant (gift from M. Terrak) was used for overexpression of PBP1b and as a template for the creation of PBP1b amber mutants. Plasmid pSub-BpaRS-6TRN encoding the orthogonal aminoacyl tRNA synthase-$tRNA_{CUA}$ pair was used for incorporation of *p*Bpa at the site of amber mutation (Gift from P.G. Schultz).

Site directed mutagenesis

The amber mutants were created by mutagenesis PCR using the primers listed in supplementary table 1. The reaction mixture contained 125 ng fwd and 125 ng rev primer, 1 µl dNTPs, 10 mM each, 0,5 µl 77ng/µl template and 1 µl Pfu turbo DNA polymerase (stratagene 2,5U/µl) in a total volume of 50 µl. 17 cycles of 30 sec @ 95°C, 1 min @ Tm (depending on used primers) and 9 min @ 68°C were performed. PCR products were digested with 10 U DpnI (Fermentas), per reaction and amplified in *E. coli* DH5α. Sequencing was performed to confirm the mutation in the DNA.

Expression of truncated proteins

E. coli BL21(DE3) cells were transformed with pDML924 containing the amber mutation. Cells were grown till the culture reached an OD_{600} of 0,5-0,6. Protein production was induced with 10 µM IPTG. After 2 hours of protein production, samples were taken and diluted according to their OD_{600} value. Samples were boiled or not boiled and analyzed by western blot.

In vivo photo- crosslinking experiments

E. coli BL21(DE3) cells were co-transformed with pDML924 containing the amber mutation and pSub-BpaRS-6TRN. Cells were grown till the culture reached an OD_{600} of 0,5-0,6. Protein production was induced with 10 µM IPTG, and 1 mM freshly prepared photo-crosslinker *p*Bpa (Bachem) dissolved in 1M NaOH was added. After 2 hours of protein

production, cells were harvested by centrifugation, washed with PBS and resuspended in 3 mL PBS. This was transferred into a petridish and exposed to UV light of 366 nm for 60 min (UVGL-58, 6 W from UVP Inc., California) 1 cm above the cell suspension or to UV light of 365 nm for 90 sec (photoMax Housing 200W, Oriel Instruments, model 60100, 30cm distance to sample). The samples were cooled on ice during UV illumination. A sample was taken and analyzed by western blot. Rest of the cells were pelleted and stored at -80 °C.

Western blot analysis

Samples were separated by SDS-PAGE (8% or 12%) and blotted semi dry on nitrocellulose membrane (bio-rad) for 18, 30 or 40 min @ 15 V. Monoclonal anti-polyhistidine peroxidase conjugate antibody (1:4000 dilution, Sigma Aldrich) was used for detection.

Results and discussion

The region between amino acids F480 and Y498 is important for dimer formation

To map the dimer interface of PBP1b, we created truncated PBP1b variants with a TAG (stop) mutations at the positions F774, Y690, D601, Y498, Y391, Y301, F194 and F76, to roughly map the region important for dimer formation. When *E. coli* samples are not boiled prior to SDS-PAGE and western blot analysis, a dimer band of PBP1b is visible, which disappears after boiling of the samples [18]. The (in)ability of dimer formation of these truncated PBP1b versions should show which part of the protein is important for the dimerization.

When performing SDS-PAGE and western blot analysis on the samples of these bacteria, expressing truncated versions of PBP1b it can be seen that after IPTG addition (Fig 1, + samples) the protein production is induced, and as a result shorter fragments of PBP1b are produced, as expected. Looking at the non-boiled samples (+2 samples) a clear dimer band is observed in the samples of mutants producing PBP1b up to amino acids F774, Y690, D601 and Y498 (star), which is not present in the samples of mutants producing PBP1b up to amino acids Y391, Y301, F194 and F76. This indicates that the region of dimer formation should lie between the amino acids Y391 and Y498.

To further map the region important for dimer formation in more detail, we performed a similar experiment, expressing PBP1b up to amino acids V430 and F480. In figure 2 it can be seen that dimer bands were hardly visible for these truncates, implying that the amino acids between V430 and F480 are not (very) important for dimer formation either.

These results imply that the amino acids between F480 and Y498 form an important region for dimerization of PBP1b.

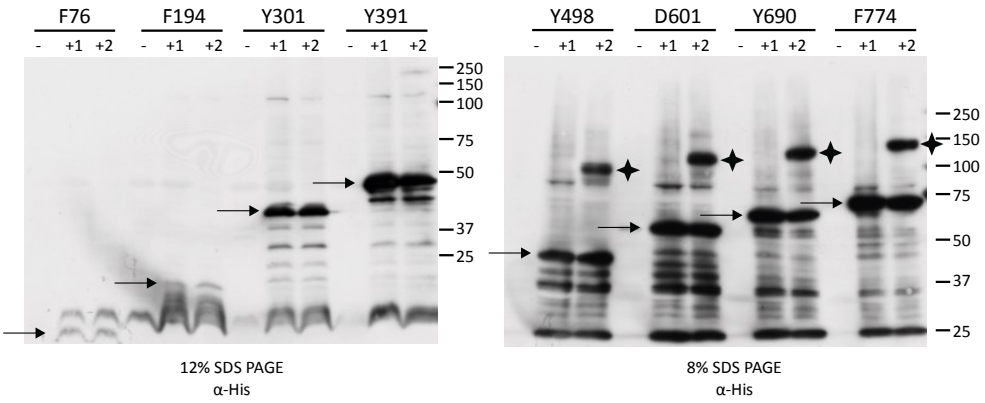


Figure 1. Mapping of the dimer interface of PBP1b.
Bacterial cell lysates of cells producing short PBP1b fragments, up to the indicated amino acid were separated on SDS-PAGE and analyzed by western blot. These results shows that the amino acids between Y391 and Y498 are involved in dimerization.- = protein production not induced. +1= protein production induced, samples boiled. +2 = protein production induced, samples not boiled. Arrow is PBP1b monomer, star is PBP1b dimer.

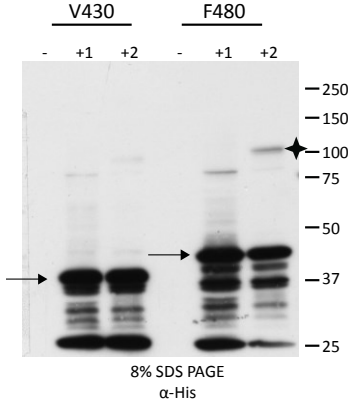


Figure 2. Reducing the PBP1b dimer interface region.
Bacterial cell lysates of cells producing short PBP1b fragments, up to the indicated amino acid were separated on SDS-PAGE and analyzed by western blot. These results show that the amino acids between V430 and F480 are not involved in dimerization. - = protein production not induced. +1 = protein production induced, samples boiled. +2 = protein production induced, samples not boiled. Arrow is PBP1b monomer, star is PBP1b dimer.

In vivo photo-crosslinking of the PBP1b dimer interface

In the previous paragraph we showed that the dimer interface of PBP1b should lie between the amino acids F480 and Y498. When analyzing this region in the crystal structure of PBP1b (Fig 3 indicated in red), it is remarkable, that most of the amino acids of this region are located in the interior of the protein, and therefore, not likely to be involved in dimer formation. However, there is one loop in this region, containing the amino acids E492, Q494 and F495 that does clearly point outwards of the protein, and could be part of an interaction interface (Fig 3, enlargement).

Therefore, we decided to mutate these amino acids into the amber stop codon TAG, for the *in vivo* incorporation of an unnatural amino acid with photo-crosslinking properties. If these amino acids are involved in dimer formation, UV illumination of cells expressing these proteins should result in the formation of a covalent bond between the dimers, that is heat resistant and should therefore still be visible on SDS-PAGE after boiling of the samples. In

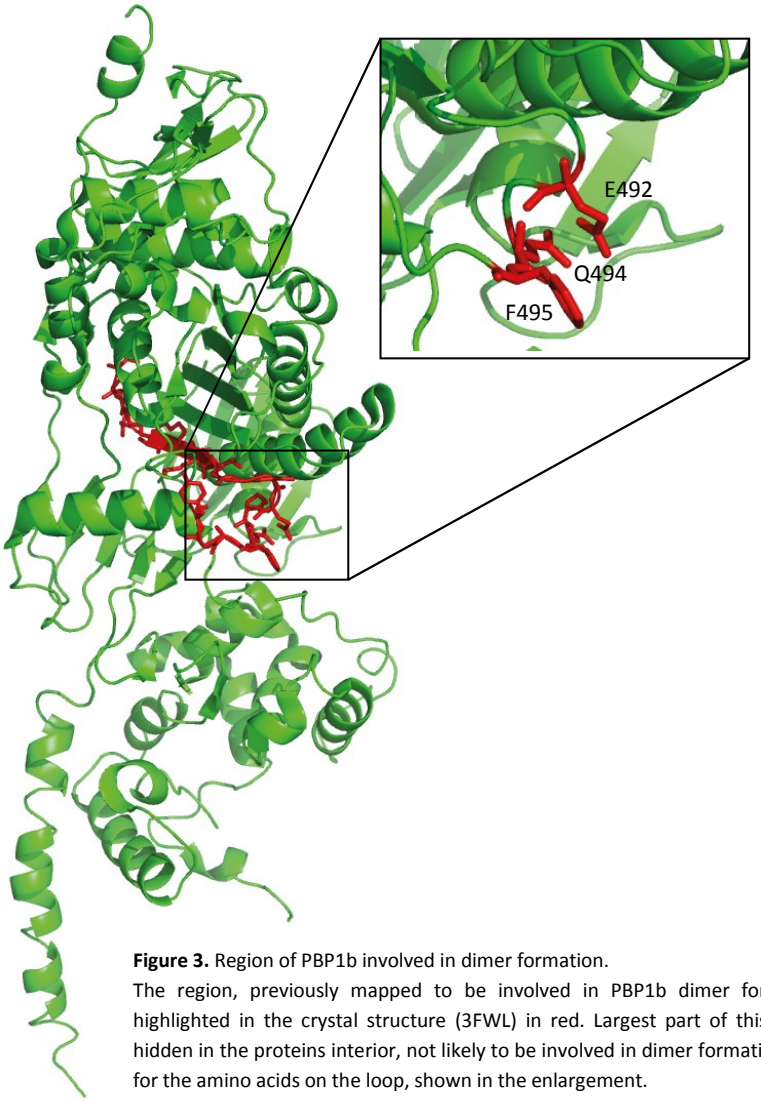


Figure 3. Region of PBP1b involved in dimer formation.
The region, previously mapped to be involved in PBP1b dimer formation is highlighted in the crystal structure (3FWL) in red. Largest part of this region is hidden in the proteins interior, not likely to be involved in dimer formation, except for the amino acids on the loop, shown in the enlargement.

figure 4 it can be seen that the amino acid Q494 is not part of any interaction, since no crosslink products are formed as a result of UV illumination (two types of UV illumination are tested next to each other in this experiment: X-low, 1 hour exposure to low intensity (6W) UV lamp and X-high, 90 seconds exposure to UV light with high intensity (200W), which results in production of comparable results, only a bit more crosslink product formation by E492*p*Bpa as a result of exposure to UV light of high intensity). PBP1b variant F495*p*Bpa does show some crosslink formation, but these products are too large to be a dimer of PBP1b (dimer bands are visible in samples +2, at the star). PBP1b version

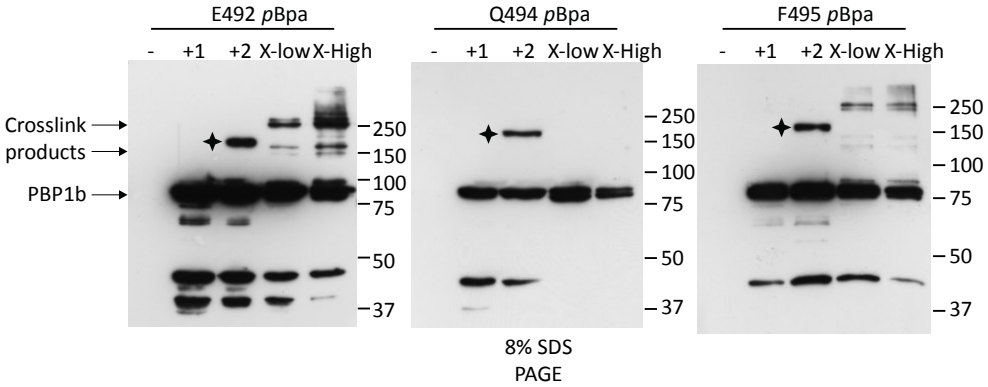


Figure 4. Amino acid E492 could be involved in dimer formation, as shown by *in vivo* photo-crosslinking. Bacterial cell lysates of cells producing PBP1b, with incorporated photo-crosslinker at the indicated positions were separated on SDS-PAGE and analyzed by western blot. Photo-crosslinker incorporation at positions E492 and F495 produced crosslink products after UV illumination. - = protein production not induced. +1= protein production induced, samples boiled. +2 = protein production induced, samples not boiled. X-low = sample crosslinked 1 hour with low intensity UV. X-high = sample crosslinked for 90 seconds with high intensity UV. Arrow is PBP1b monomer and crosslink products, star is PBP1b dimer.

E492*p*Bpa produced the same, too large to be a dimer of PBP1b crosslink product as F495*p*Bpa, but also a smaller one, which runs just slightly lower than the dimer band in the non-boiled (+2) sample. These results imply that the amino acid E492 could be part of the dimer interface of PBP1b, however, on this moment we do not have an additional method to prove these results in a different experimental setup. It is a bit peculiar though, that especially replacement of F495, pointing in the same direction as E492, does not produce this crosslink product as well. In this respect, it is worthwhile to note that in an approach to determine the SecA dimer interface, using the same *in vivo* photo-crosslinking technique similar results were obtained. Here, also only one amino acid position in a domain was found to render an SDS-PAGE stable dimer, though flanking residues did not [22].

Finding the most important part of PBP1b in dimer formation opens up possibilities for future experiments. These could comprise the creation of a PBP1b variant hampered or devoid in the dimer formation ability. By changing or removing residues in the dimer interface and expression of this variant in a PBP1b deficient strain, the importance of dimerization for the *in vivo* activity of PBP1b and the regulation of PG synthesis during cell division could be investigated. Furthermore, by using this dimer formation hampered variant in an *in vitro* activity assay, the necessity of dimerization for the interaction with and effect of different regulatory proteins on its activity can be analyzed. This would give a wealth of information on the role of dimerization in the regulation of PG synthesis by PBP1b *in vivo*.

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References

[1] W. Weidel and H. Pelzer, “Bagshaped macromolecules--a new outlook on bacterial cell walls,” *Adv. Enzymol. Relat. Areas Mol. Biol.*, vol. 26, pp. 193–232, 1964.

[2] W. Vollmer, D. Blanot, and M. A. De Pedro, “Peptidoglycan structure and architecture,” *FEMS Microbiol. Rev.*, vol. 32, no. 2, pp. 149–167, 2008.

[3] K. H. Schleifer and O. Kandler, “Peptidoglycan types of bacterial cell walls and their taxonomic implications.,” *Bacteriol. Rev.*, vol. 36, no. 4, pp. 407–477, Dec. 1972.

[4] B. Spratt and J. Strominger, “Identification of Major Penicillin-Binding Proteins of Escherichia-Coli,” *J. Bacteriol.*, vol. 127, no. 1, pp. 660–663, 1976.

[5] H. Suzuki, Y. Nishimura, and Y. Hirota, “On the process of cellular division in Escherichia coli: a series of mutants of E. coli altered in the penicillin-binding proteins.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 75, no. 2, pp. 664–668, Feb. 1978.

[6] U. Bertsche, T. Kast, B. Wolf, C. Fraipont, M. E. G. Aarsman, K. Kannenberg, M. Von Rechenberg, M. Nguyen-Distèche, T. Den Blaauwen, J.-V. Höltje, and W. Vollmer, “Interaction between two murein (peptidoglycan) synthases, PBP3 and PBP1B, in Escherichia coli,” *Mol. Microbiol.*, vol. 61, no. 3, pp. 675–690, Aug. 2006.

[7] M.-T. Sung, Y.-T. Lai, C.-Y. Huang, L.-Y. Chou, H.-W. Shih, W.-C. Cheng, C.-H. Wong, and C. Ma, “Crystal structure of the membrane-bound bifunctional transglycosylase PBP1b from Escherichia coli,” *Proc. Natl. Acad. Sci.*, vol. 106, no. 22, pp. 8824–8829, Jun. 2009.

[8] W. Vollmer, M. von Rechenberg, and J.-V. Höltje, “Demonstration of Molecular Interactions between the Murein Polymerase PBP1B, the Lytic Transglycosylase MltA, and the Scaffolding Protein MipA of Escherichia coli,” *J. Biol. Chem.*, vol. 274, no. 10, pp. 6726–6734, Mar. 1999.

[9] A. Typas, M. Banzhaf, B. van den Berg van Saparoea, J. Verheul, J. Biboy, R. J. Nichols, M. Zietek, K. Beilharz, K. Kannenberg, M. von Rechenberg, E. Breukink, T. den Blaauwen, C. A. Gross, and W. Vollmer, “Regulation of Peptidoglycan Synthesis by Outer-Membrane Proteins,” *Cell*, vol. 143, no. 7, pp. 1097–1109, Dec. 2010.

[10] C. Paradis-Bleau, M. Markovski, T. Uehara, T. J. Lupoli, S. Walker, D. E. Kahne, and T. G. Bernhardt, “Lipoprotein Cofactors Located in the Outer Membrane Activate Bacterial Cell Wall Polymerases,” *Cell*, vol. 143, no. 7, pp. 1110–1120, Dec. 2010.

[11] A. N. Gray, A. J. F. Egan, I. L. van’t Veer, J. Verheul, A. Colavin, A. Koumoutsi, J. Biboy, M. A. F. Altelaar, M. J. Damen, K. C. Huang, J.-P. Simorre, E. Breukink, T. den Blaauwen, A. Typas, C. A. Gross, and W. Vollmer, “Coordination of peptidoglycan synthesis and outer membrane constriction during Escherichia coli cell division,” *eLife*, p. e07118, May 2015.

[12] P. Müller, C. Ewers, U. Bertsche, M. Anstett, T. Kallis, E. Breukink, C. Fraipont, M. Terrak, M. Nguyen-Distèche, and W. Vollmer, “The Essential Cell Division Protein FtsN Interacts with the Murein (Peptidoglycan) Synthase PBP1B in Escherichia coli,” *J. Biol. Chem.*, vol. 282, no. 50, pp. 36394–36402, Dec. 2007.

[13] J. Nakagawa and M. Matsuhashi, “Molecular divergence of a major peptidoglycan synthetase with transglycosylase - transpeptidase activities in Escherichia coli — Penicillin-binding protein 1Bs,” *Biochem. Biophys. Res. Commun.*, vol. 105, no. 4, pp. 1546–1553, Apr. 1982.

[14] H. Suzuki, J. Kato, Y. Sakagami, M. Mori, A. Suzuki, and Y. Hirota, “Conversion of the alpha component of penicillin-binding protein 1b to the beta component in Escherichia coli.,” *J. Bacteriol.*, vol. 169, no. 2, pp. 891–893, Feb. 1987.

[15] T. A. Henderson, P. M. Dombrosky, and K. D. Young, “Artifactual processing of penicillin-binding proteins 7 and 1b by the OmpT protease of Escherichia coli.,” *J. Bacteriol.*, vol. 176, no. 1, pp. 256–259, Jan. 1994.

[16] J. K. Broome-Smith, A. Edelman, S. Yousif, and B. G. Spratt, “The nucleotide sequences of the ponA and ponB genes encoding penicillin-binding proteins 1A and 1B of Escherichia coli K12,” *Eur. J. Biochem.*, vol. 147, no. 2, pp. 437–446, 3/1/85 1985.

[17] C. Chalut, X. Charpentier, M.-H. Remy, and J.-M. Masson, “Differential Responses of Escherichia coli Cells Expressing Cytoplasmic Domain Mutants of Penicillin-Binding Protein 1b after Impairment of Penicillin-Binding Proteins 1a and 3,” *J. Bacteriol.*, vol. 183, no. 1, pp. 200–206, Jan. 2001.

[18] C. A. Zijderveld, M. E. Aarsman, T. den Blaauwen, and N. Nanninga, “Penicillin-binding protein 1B of Escherichia coli exists in dimeric forms.,” *J. Bacteriol.*, vol. 173, no. 18, pp. 5740–5746, Sep. 1991.

[19] C. A. Zijderveld, M. E. Aarsman, and N. Nanninga, “Differences between inner membrane and peptidoglycan-associated PBP1B dimers of Escherichia coli.,” *J. Bacteriol.*, vol. 177, no. 7, pp. 1860–1863, Apr. 1995.

[20] C. Chalut, M.-H. Remy, and J.-M. Masson, “Disulfide Bridges Are Not Involved in Penicillin-Binding Protein 1b Dimerization in Escherichia coli,” *J. Bacteriol.*, vol. 181, no. 9, pp. 2970–2972, May 1999.

[21] U. Bertsche, E. Breukink, T. Kast, and W. Vollmer, “In Vitro Murein (Peptidoglycan) Synthesis by Dimers of the Bifunctional Transglycosylase-Transpeptidase PBP1B from Escherichia coli,” *J. Biol. Chem.*, vol. 280, no. 45, pp. 38096–38101, Nov. 2005.

[22] D. Yu, A. J. Wowor, J. L. Cole, and D. A. Kendall, “Defining the Escherichia coli SecA Dimer Interface Residues through In Vivo Site-Specific Photo-Cross-Linking,” *J. Bacteriol.*, vol. 195, no. 12, pp. 2817–2825, Jun. 2013.

Supplementary information

supplementary table 1.		
F76 → TAG	fw	CTGGCTATCGTTTAGGCCGTGCTGATCG
	rev	CGATCAGCACGGCCTAAACGATAGCCAG
F194 → TAG	fw	CCGTCAGTTCGGCTAGTTCCGTCTTGATC
	rev	GATCAAGACGGAACTAGCCGAACTGACGG
Y301 → TAG	fwd	GACGCGCGTTAGAGCAAAGACCGTATTCTAGAGCTGTATATG
	rev	CATATACAGCTCTAGAATACGGTCTTTGCTCTAACGCGCGTC
Y391 → TAG	fwd	GATTATTGATCAAGAGCTCTAGGACATGTTGAGTGCCC
	rev	GGGCACTCAACATGTCCTAGAGCTCTTGATCAATAATC
V430 → TAG	fwd	CTGGCGATAAGTAGAAAGATCTCTCCGGCG
	rev	CGCCGGAGAGATCTTTCTACTTATCGCCCAG
F480 → TAG	fwd	GGTCGTCGACCGGTAGAGTGGTGAAGTTCG
	rev	CGAACTTCACCACTCTACCGGTCGACGACC
Y498 → TAG	fwd	CGCAGTTTGCCGGCTAGAACCGTGCGATGC
	rev	GCATCGCACGGTTCTAGCCGGCAAACTGCG
D601 → TAG	fwd	CGTACCGAAATAGCAGTTGCATCC
	rev	GGATGCAACTGCTATTTCGGTACG
Y690 → TAG	fwd	GTACGGGTCGACAGCTTGGGGCGAAATAGCCGAACCTGC
	rev	GCAGGTTCGGCTATTTCGCCCCAAGCTGTCGACCCGTAC
F774 → TAG	fw	CGACGGCAACTAGGTTTGCAGCG
	rev	CGCTGCAAACCTAGTTGCCGTCG
E492 → TAG	fwd	GGAGGTTCTTAGCCGCAGTTTGCGGGCTACAACC
	rev	GGTTGTAGCCCGCAAACTGCGGCTAAGAACCTCC
Q494 → TAG	fwd	GGTTCTGAGCCGTAGTTTGCGGGCTACAACC
	rev	GGTTGTAGCCCGCAAACTACGGCTCAGAACC
F495 → TAG	fwd	GGTTCTGAGCCGCAGTAGGCGGGCTACAACC
	rev	GGTTGTAGCCCGCCTACTGCGGCTCAGAACC

Supplementary table 1. Used primers for amber mutant formation.

Chapter 7

Summarizing discussion

The spatiotemporal regulation of cell wall synthesis, especially during cell division, is an intriguing process, involving interactions between a vast amount of proteins. Because of the emergence of a growing amount of resistant bacteria, attempts are taken to get more insight in these interaction networks, to be able to rationally develop new antibiotics. In this perspective, we focused on the cell division specific bifunctional peptidoglycan (PG) synthase PBP1b of *E. coli*, to find new interaction partners, specify the interaction surface of known interactions, and the development of new and improved techniques for the analysis of the kinetics of these interactions and the activity of PBP1b under different conditions.

The involvement of PBP1b in various protein-protein interactions

In chapter 2 we described the use of site specific unnatural amino acid incorporation for the mapping of interaction sites with known and new interaction partners of PBP1b *in vivo*. By the incorporation of an unnatural amino acid with photo-crosslinkable properties, we identified a cleft between the UB2H domain and TP domain of PBP1b, to interact with FtsN, of which it has already been shown to interact with PBP1b [1], and with a new interaction partner YbgF (later renamed into CpoB [2]).

In chapter 3 we furthermore identified PBPs 1a, 5 and 6, FtsN, MreC and TolB as proteins that possibly interact with a TP domain loop consisting of the amino acids D535-S558 of PBP1b, using similar *in vivo* photo-crosslinking experiments. Some of these interactions were later verified by immunoblot using specific antibodies. Furthermore, we were able to trace back a number of these proteins with affinity chromatography using the TP domain loop C-A_{536}PIALRQPNGQVWSPQ_{551}-C as bait.

FtsN is the last arriving protein of the divisome, and is shown to be involved in stability of the division complex [3], [4]. It recruits the amidase AmiC and the Tol-Pal complex to the septation site [5], [6]. However, the relation between FtsN and AmiC could also be the other way around in light of the finding that SPOR domain containing proteins (where FtsN belongs to) are recruited to the division site by the interaction with glycan strands that lack stem peptides, which are enriched in septal PG and formed by the action of amidases like AmiC [7]. The interaction between PBP1b and FtsN could ensure that the cell separation by PG hydrolysis by AmiC, which seems to be recruited by FtsN, takes place jointly with new PG synthesis by PBP1b and PBP3 to assure the integrity of the cell wall during the constriction process.

This occurs in concert with the recruitment of the Tol-Pal complex, which performs the outer membrane invagination. The Tol-Pal complex comprises the periplasmic proteins TolB and CpoB, the PG-associated outer membrane lipoprotein Pal, the inner membrane proteins TolA, TolQ, TolR and the cytoplasmic YbgC [6], [8]. The outer membrane lipoprotein Pal associates with the peptide moieties of PG, and also binds TolA, which forms a complex with the inner membrane proteins TolQ/R. In this way, the Tol-Pal

complex provides a link between the three envelope layers [9]–[11]. The interaction of PBP1b with both FtsN, and the Tol-Pal complex via CpoB and TolB could regulate the correct timing of these processes in respect to each other. The synthesis of septal PG, the hydrolysis by AmiC needed for cell separation, and the constriction of the inner membrane, could be linked to successive outer membrane invagination by the Tol-Pal complex via the interactions of FtsN, CpoB and TolB with PBP1b.

One could speculate about the sequence and interplay of these events, starting with the interaction of FtsN with PBP1b at the septation site, followed by the recruitment of the Tol-Pal complex. When the outer membrane constriction complex is in position, the Tol-Pal complex component CpoB binds PBP1b and removes FtsN from their mutual interaction site in the cleft between the TP domain and UB2H domain. This could function as a signal that the last player involved in the invagination of the three cell envelope layers, the outer membrane constriction complex, is in place and cell constriction can start (initiated by FtsN [12]). The identification of TolB as putative interaction partner of the PBP1b TP domain loop, could function as an extra layer of PG synthesis regulation during the cell division process.

Recently, it has been shown that another protein of the Tol-Pal complex, TolA, interacts with PBP1b as well, and that they, together with CpoB can form a ternary complex [2]. LpoB, the outer membrane lipoprotein, which can stimulate both the glycosyltransferase (GT) and transpeptidase (TP) activity of PBP1b [13], has also been shown to be part of this complex, but only in the presence of PBP1b. The interaction of TolA with PBP1b increases the GT activity of PBP1b by 1,9 +/- 0,5 fold, which is not near the 8 fold increase caused by the interaction of PBP1b with LpoB. When both proteins are present, their stimulatory effect is additive and the GT activity of PBP1b is increased to 11,3 +/- 0,5 fold. No effect on PBP1b TP activity has been observed as a result of addition of CpoB or TolA to the reaction. An interesting observation was however, that the increase in TP activity caused by the addition of LpoB is decreased in the presence of CpoB, which can be alleviated by the addition of TolA. This shows that TolA can prevent CpoB to associate with LpoB and therefore can interfere with the stimulatory activity of LpoB on the TP activity of PBP1b. This reduction in TP stimulation by LpoB, caused by the presence of CpoB actually results in a level of peptide crosslinking comparable to the *in vivo* level, suggested to be the default cellular state, also supported by the finding that CpoB is present in 10 fold excess over PBP1b and TolA. This makes CpoB the first regulatory protein that can control TP activity independently of GT activity [2]. The Tol-Pal complex performs the outer membrane constriction in an energy driven way (by a proton motive force [11]). Mutants deficient in different components of the Tol-Pal complex showed that this complex should be in the energized state for TolA to prevent CpoB from interacting with LpoB and inhibit PBP1b TP stimulation by LpoB. This links the degree of PG crosslinking during cell division to outer membrane invagination by the Tol-Pal complex. Highly crosslinked PG is only

produced when the outer membrane is brought into close proximity of the PG synthesis machinery, allowing TolA to counteract CpoB function, required at certain stages of septal PG synthesis. The importance of the regulation of the degree of crosslinking by CpoB is shown by the phenotype of a strain lacking CpoB. A higher degree of outer membrane blebbing is observed, which is highly increased under osmotic stress, by inhibition of PBP1a, or in absence of LpoA (eg. cells that more rely on the function of PBP1b) [2].

These interactions form a link between the three cell envelope layers of the inner membrane (FtsN), the PG layer (PBP1b and FtsN) and the outer membrane (Tol-Pal complex via CpoB, TolB and TolA and the interaction of PBP1b with LpoB), forming a complex PG synthesis regulation network, to ensure tight coordination of PG synthesis in accordance with the constriction of all three layers of the cell envelope in space and time, during the cell division process.

The interaction between the TP domain loop of PBP1b and PBP1a was surprising, since both proteins have shown to form homodimers, but a heterodimer was never detected [14]. However, it has been shown that the cell elongation specific protein PBP2 and the cell division specific protein PBP3 interact during a preseptal elongation phase, creating the possibility that PBP1a and PBP1b also interact during this phase of the cell cycle [15]. PBP1b localizes at the cell periphery during cell elongation, and at mid cell during cell division, where PG synthesis is organized by the actin homolog MreB and the tubulin homolog FtsZ, respectively. It is suggested that MreB might deliver PBP1b to the division site after maturation of the divisome. In our *in vivo* crosslinking studies using PBP1b mutants having the photo-crosslinker incorporated in the TP domain loop structure, we found MreC as an interaction partner of PBP1b, suggesting that the delivery of PBP1b by MreB to the division site could be achieved via an interaction of MreB with MreC. A similar role of MreC in the organization of PBPs has been proposed in *B. subtilis* and in *C. crescentus* [17]–[19]. The localization of the DD-carboxypeptidase PBP5 is dependent on active PG synthesis, which gives rise to the presence of its substrate, the pentapeptides [20]. In this respect it is logical that we find evidence for an interaction of PBP5 with PBP1b, which actively performs PG synthesis, and hence is responsible for the presence of the pentapeptides. PBP5 could have a regulatory role on the number of peptide crosslinks that are formed in the PG network, by the removal on the terminal D-alanines, resulting in the formation of tetrapeptides, that can only function as acceptors in the TP reaction. This could also hold true for the other DD-carboxypeptidase found, PBP6.

All the interactions described in this section are schematically represented in Figure 1. From sterical perspective, it is not likely that all the identified proteins form one single complex with PBP1b. Rather, the detected proteins may reflect the range of interactions that the cleft between the UB2H domain and the TP domain loop of PBP1b have during the different stages of the cell cycle, required for PG synthesis regulation during these different stages. Since cells in these experiments are not synchronized, we were able to

catch these different interaction in one experiment. The possibility of the occurrence of these interactions during different stages of the cell cycle is furthermore corroborated by the fact that the identified proteins are part of proposed multi enzyme complexes like the divisome and elongasome that are formed at different stages of PG synthesis during the cell cycle.

The interactions of PBP1b via its TP domain loop structure together with the interactions via the UB2H domain ensure proper regulation of PG synthesis by PBP1b, and especially of PBP1b TP activity during cell division. These results of different domains of PBP1b being involved in multiple interactions do well fit the idea of the existence of multi enzyme PG synthesis (and hydrolysis) complexes for the spatio temporal regulation of PG synthesis during different stages of the cell cycle.

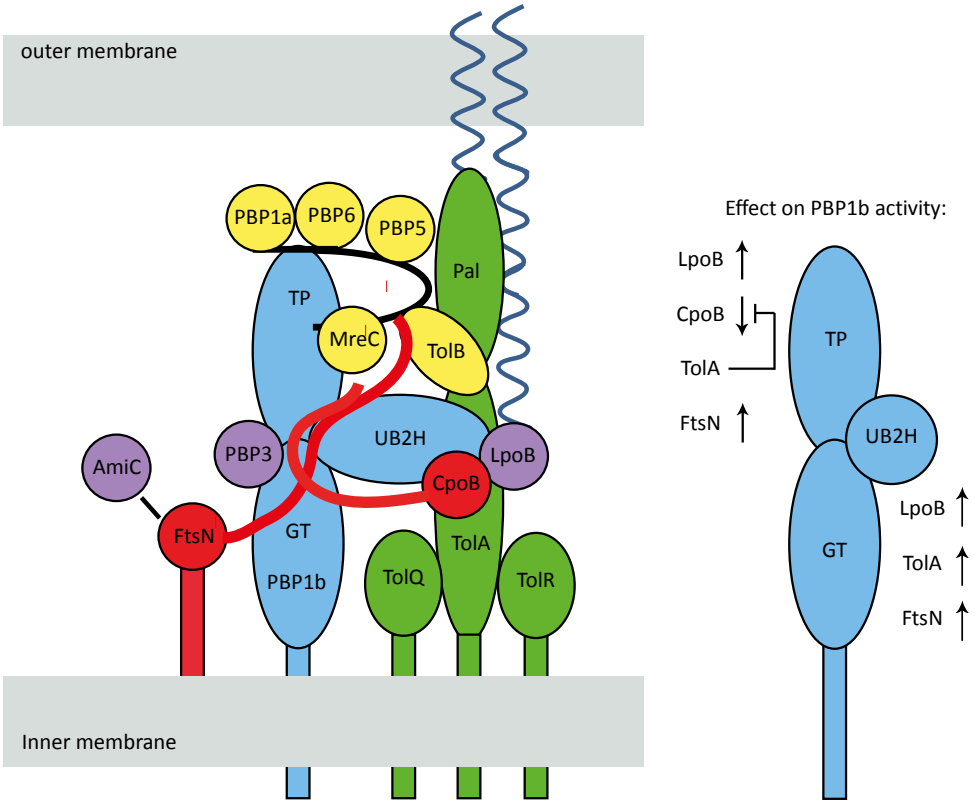

Figure 1. Schematic representation of the interactions of PBP1b described in the text, and their effect on PBP1b activity.

* PBP1a, PBP3 and MreC are inner membrane proteins. PBP5 and PBP6 are membrane associated. Pal and FtsN also bind PG.

The importance of the PBP1b TP domain loop for its activity

In chapter 3 we identified the PBP1b TP domain loop between amino acids D535-S558 as an important structure of the TP domain for its function and interaction with other proteins. Based on an *in vivo* complementation assay, using the loop deletion mutant, we show that this loop is essential for PBP1b to be able to complement for the absence of both PBP1a and PBP1b, implicating that this mutant is not fully active. With an *in vitro* PG synthesis assays using this deletion mutant, we showed that this loop is essential for PBP1b TP activity, since absence of this loop renders PBP1b completely devoid of TP activity. This loop deletion mutant is still able to form dimers, suggesting a correct fold of the mutant protein, and the defect in TP activity not to be caused by a loss of 3D structure as a result of deletion of the loop. This is in accordance with the finding in chapter 6 that the C-terminal domain of PBP1b, even up to amino acid Y498, is not essential for dimer formation. This loop mutant is still able to bind its substrate, in the form of a donor peptide analog, suggesting that the loop is likely important for acceptor peptide binding. The inability of the PBP1b mutant having W548 replaced by the photo-crosslinker *p*Bpa to complement for the absence of both PBP1b and PBP1a suggests that this amino acid in the TP loop is essential for the acceptor peptide binding of PBP1b. Other mutants having flanking residues replaced were still fully active, and able to compensate for the absence of both PBP1b and PBP1a in the complementation assay. The importance of a single tryptophan in acceptor peptide binding by a protein with TP activity has been reported before [21]. Interestingly, the presence of a tryptophan or a tyrosine close to the active site serine or cysteine, in a loop structure covering the active site, seems to be a consistent phenomenon in TP domains of various proteins in both gram positive and gram negative bacterial species. This suggests that TP domains need an aromatic residue in close proximity to the active site, which is probably involved in acceptor peptide binding.

A different approach for PBP1b immobilization on an SPR chip surface

In chapter 4 we implemented the nonsense suppression mutagenesis technique in a new method for the immobilization of PBP1b on an surface plasmon resonance (SPR) chip. Until now, PBPs were immobilized with the use of an ampicillin coated chip surface, having a covalent interaction with the TP domain of the PBP [13], [22]–[24]. When using this method, measurements are performed with substrate bound proteins, with an inactive TP domain. Furthermore the TP domain is not exposed (since bound to the chip surface), an hence unavailable to measure interactions involving this domain of the protein. With the use of nonsense suppression mutagenesis, an azidophenylalanine was incorporated in the nonessential, cytoplasmic tail of PBP1b, to be able to couple it onto a cyclooctyne functionalized SPR chip surface. In this way PBP1b is immobilized using a nonessential part of the protein, making it possible to perform measurements with a fully active protein, which is furthermore oriented in a way more resembling the *in vivo*

orientation. This new method should make it possible to identify and characterize interactions of PBP1b with proteins that bind the TP domain, or that need an active protein for their interaction to take place.
We tested this immobilization method by analyzing the well characterized interaction of PBP1b with LpoB [13]. We observed a similar, very quick association and dissociation of the two proteins, and comparable equilibrium constants were calculated. Unfortunately we were unable to show the interaction of PBP1b with other interaction partners, like the previously identified TP domain loop binding proteins PBP5 and TolB. The PBP5 sample had a stronger association with the control surfaces, resulting in the acquirement of negative values. A component of the protein mixture apparently bound stronger to the control surfaces than to the protein immobilized surfaces. This could be avoided by changing the immobilization method, and incorporating a cyclooctyne in the protein to react with an azide on the SPR chip surface [25]. This change in chip surface could avoid the unspecific binding of a buffer component of the protein mixture to the control surfaces. Another option could be to purify the protein using a different procedure, to obtain it in a compatible solution mixture. TolB did not show any binding to the protein immobilized chip surface, nor to the control surface. It could be possible that TolB needs to be in a preformed complex with one or more partners of the Tol-Pal complex to be able to interact with PBP1b, or their interaction depends on the presence of more/other proteins.
Nevertheless, this new immobilization method does work very well, and can also be used for the investigation of other PBPs. It is actually a versatile way to immobilize any desired protein, with the added benefit of direct control of the site of immobilization and hence protein orientation by the site specific incorporation of the azide containing unnatural amino acid. This strategy will result in a homogeneous orientation of the immobilized proteins and create a possibility to study binding differences in respect to the topology of membrane proteins.

The use of different fluorescently labeled substrate variants in peptidoglycan synthase activity assays.
In chapter 5 we show that the use of Lipid II labeled with different, high quantum yield, fluorophores in the in gel PG synthesis assay highly improved the resolution and intensity of the result, compared to the use of Lipid II labeled with dansyl [26], [27]. Especially the ATTO550 and 647N labeled Lipid II variants produced high resolution results in this assay, but Megastokes dye 608 and 735 were also useful. The use of these fluorescently labeled substrates leads to a much clearer result, making it possible to observe more subtle changes in the activities of PBPs. However, to be able to observe incorporation of these new fluorescently labeled Lipid II variants into glycan chains, unlabeled Lipid II had to be added to the reaction mixture. This was not reported before, when dansylated Lipid II was

used [26], [27]. We hypothesize that this could be caused by steric hindrance of the large fluorophores (compared to dansyl) attached to Lipid II. During PG synthesis, two Lipid II substrate molecules are bound to PBP1b, one in the donor, and one in the acceptor site [28]–[30]. It could be that due to the size of the attached dyes it is not possible to have both sites occupied by these labeled Lipid II variants, and the addition of unlabeled Lipid II solved this problem.

When testing the performance of these substrates in the continuous GT assay developed by Schwartz *et al.* [31], we observed that the ATTO labeled Lipid II variants were not useful in this assay, which is based on the fluorescence decrease of dansylated Lipid II as a result of environmental change. The fluorescence of dansyl is higher in a more hydrophobic (attached to the lipid carrier tail) than in a hydrophilic environment (incorporated into a glycan chain, detached from the lipid carrier tail), and therefore, the fluorescence signal will decrease as a result of glycan chain polymerization. The ATTO dyes do not have this property of changing fluorescence intensity as a result of environmental change and hence not useful in this assay. Both Megastokes dyes did have this property, but Megastokes dye 735 produced very noisy results, and therefore is not useful in this assay either. Megastokes dye 608 did show to be useful in this assay since a decrease in fluorescence as a result of glycan chain polymerization was observed. The use of this fluorophore resulted in the disappearance of a lag phase, observed at the start of the reaction when dansylated Lipid II was used, but did not result in a larger decrease in fluorescence signal (e.g. increase in resolution).

A contradiction between these two assays for PG synthesis analysis is that Megastokes dye 608 labeled Lipid II seems to be well incorporated in the continuous GT assay, though unlabeled Lipid II had to be added to the reaction mixture to obtain proper polymerization speed and chain lengths as visualized in gel. We hypothesize that this could be caused by an increased glycan chain polymerization termination, which results in the production of short glycan chains which resembles no, or barly no production when analyzed by the in gel assay, but will be interpreted as (normal) polymerization when analyzed by the continuous GT assay as a constant drop in fluorescence. Since expected long glycan chains are produced as visualized by the in gel assay, when unlabeled Lipid II is added, which does not significantly changes the result of the continuous GT assay, I would suggest to always add unlabeled Lipid II to the reaction mixture, to create a situation that is more comparable to the substrate availability *in vivo*.

Taken together, the method of fluorescent labeling on the level of Lipid II, using click chemistry showed to be very straightforward and highly efficient, since there was hardly any product loss during the procedure, and these fluorescently labeled Lipid II variants showed to be very useful for the analysis of GT and TP activities of PBP1b in these *in vitro* PG synthesis assays.

In search for the PBP1b dimer interface

In chapter 6 we show, by the production of truncated PBP1b variants, that the amino acids between F480 and Y498 are important for PBP1b dimer formation. The crystal structure shows that a large part of these amino acids are not exposed on the surface of the protein, except for a loop structure containing the amino acid E492, Q494 and F495. By the site specific incorporation of an unnatural amino acid with photo-crosslinking properties, replacing these amino acids, we show that E492 could be involved in dimer formation. A crosslink product, running slightly lower on SDS-PAGE than the dimer band observed in the unboiled sample, is produced as a result of photo-crosslinker incorporation and subsequent UV illumination at this position. It is a bit peculiar though, that especially replacement of F495, pointing in the same direction as E492, does not produce this crosslink product as well. In this respect, it is worthwhile to note that in an approach to determine the SecA dimer interface, using the same *in vivo* photo-crosslinking technique, similar results were obtained. Here, also only one amino acid position in a domain was found to render an SDS-PAGE stable dimer, though flanking residues did not [32].

Dimerization of PBP1b has been shown to highly increase both the GT and TP activity of PBP1b in an *in vitro* PG synthesis assay [23]. An interesting future experiment could comprise the creation of a PBP1b variant hampered or devoid in dimer formation ability, and expression of this variant in a PBP1b deficient strain. In this way, the importance of dimerization for the *in vivo* function of PBP1b could be investigated. Furthermore, by using this dimer formation hampered variant in an *in vitro* activity assay, the necessity of dimerization for the interaction with and effect of different regulatory proteins on its activity can be analyzed. These experiments would give a wealth of information on the role of dimerization in the regulation of PG synthesis by PBP1b *in vivo*.

Concluding remarks

Here we present new insights on the protein-protein interactions in which PBP1b is involved, which does probably still only partially cover all the existing interactions between this PG synthase, hydrolases, peptidases, regulatory proteins and cell division proteins. More evidence for the probable existence of additional interactions involving PBP1b comes from the fact that we found even more crosslink products than described in this thesis, of which the proteins involved were not identified. This reflects the high importance of the tight regulation of PG synthesis by PBP1b in space and time. The existence of interactions between proteins in the different layers of the cell envelope form a physical link between these layers, and a regulatory network between the involved proteins. The effects of different interaction partners on the activity of PG synthases can be investigated using the described and improved techniques in an effort to unravel the interplay between all these proteins and their role in PG synthesis regulation. The

knowledge on the regulation of these essential processes is highly important for the development of new antimicrobial compounds, to combat the growing amount of resistant bacterial species.

References

[1] P. Müller, C. Ewers, U. Bertsche, M. Anstett, T. Kallis, E. Breukink, C. Fraipont, M. Terrak, M. Nguyen-Distèche, and W. Vollmer, "The Essential Cell Division Protein FtsN Interacts with the Murein (Peptidoglycan) Synthase PBP1B in Escherichia coli," *J. Biol. Chem.*, vol. 282, no. 50, pp. 36394–36402, Dec. 2007.

[2] A. N. Gray, A. J. F. Egan, I. L. van't Veer, J. Verheul, A. Colavin, A. Koumoutsi, J. Biboy, M. A. F. Altelaar, M. J. Damen, K. C. Huang, J.-P. Simorre, E. Breukink, T. den Blaauwen, A. Typas, C. A. Gross, and W. Vollmer, "Coordination of peptidoglycan synthesis and outer membrane constriction during Escherichia coli cell division," *eLife*, p. e07118, May 2015.

[3] S. G. Addinall, C. Cao, and J. Lutkenhaus, "FtsN, a late recruit to the septum in Escherichia coli," *Mol. Microbiol.*, vol. 25, no. 2, pp. 303–309, 1997.

[4] A. I. Rico, M. García-Ovalle, P. Palacios, M. Casanova, and M. Vicente, "Role of Escherichia coli FtsN protein in the assembly and stability of the cell division ring," *Mol. Microbiol.*, vol. 76, no. 3, pp. 760–771, May 2010.

[5] T. G. Bernhardt and P. A. J. de Boer, "The Escherichia coli amidase AmiC is a periplasmic septal ring component exported via the twin-arginine transport pathway," *Mol. Microbiol.*, vol. 48, no. 5, pp. 1171–1182, Jun. 2003.

[6] M. A. Gerding, Y. Ogata, N. D. Pecora, H. Niki, and P. A. J. De Boer, "The trans-envelope Tol–Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in E. coli," *Mol. Microbiol.*, vol. 63, no. 4, pp. 1008–1025, Feb. 2007.

[7] A. Yahashiri, M. A. Jorgenson, and D. S. Weiss, "Bacterial SPOR domains are recruited to septal peptidoglycan by binding to glycan strands that lack stem peptides," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 112, no. 36, pp. 11347–11352, Sep. 2015.

[8] A. Vianney, M. M. Muller, T. Clavel, J. C. Lazzaroni, R. Portalier, and R. E. Webster, "Characterization of the tol-pal region of Escherichia coli K-12: translational control of tolR expression by TolQ and identification of a new open reading frame downstream of pal encoding a periplasmic protein.," *J. Bacteriol.*, vol. 178, no. 14, pp. 4031–4038, Jul. 1996.

[9] E. Bouveret, R. Derouiche, A. Rigal, R. Lloubes, C. Lazdunski, and H. Benedetti, "Peptidoglycan-associated lipoprotein-TolB interaction. A possible key to explaining the formation of contact sites between the inner and outer membranes of Escherichia coli," *J. Biol. Chem.*, vol. 270, no. 19, pp. 11071–11077, May 1995.

[10] R. Godlewska, K. Wiśniewska, Z. Pietras, and E. K. Jagusztyn-Krynicka, "Peptidoglycan-associated lipoprotein (Pal) of Gram-negative bacteria: function, structure, role in pathogenesis and potential application in immunoprophylaxis," *FEMS Microbiol. Lett.*, vol. 298, no. 1, pp. 1–11, Sep. 2009.

[11] E. Cascales, M. Gavioli, J. N. Sturgis, and R. Lloubès, "Proton motive force drives the interaction of the inner membrane TolA and outer membrane Pal proteins in Escherichia coli," *Mol. Microbiol.*, vol. 38, no. 4, pp. 904–915, Nov. 2000.

[12] M. A. Gerding, B. Liu, F. O. Bendezú, C. A. Hale, T. G. Bernhardt, and P. A. J. de Boer, "Self-enhanced accumulation of FtsN at Division Sites and Roles for Other Proteins with a SPOR domain (DamX, DedD, and RlpA) in Escherichia coli cell constriction," *J. Bacteriol.*, vol. 191, no. 24, pp. 7383–7401, Dec. 2009.

[13] A. J. F. Egan, N. L. Jean, A. Koumoutsi, C. M. Bougault, J. Biboy, J. Sassine, A. S. Solovyova, E. Breukink, A. Typas, W. Vollmer, and J.-P. Simorre, "Outer-membrane lipoprotein LpoB spans the periplasm to stimulate the peptidoglycan synthase PBP1B," *Proc. Natl. Acad. Sci.*, vol. 111, no. 22, pp. 8197–8202, Jun. 2014.

[14] X. Charpentier, C. Chalut, M.-H. Rémy, and J.-M. Masson, "Penicillin-binding proteins 1a and 1b form independent dimers in Escherichia coli," *J. Bacteriol.*, vol. 184, no. 13, pp. 3749–3752, Jul. 2002.

[15] R. van der Ploeg, J. Verheul, N. O. E. Vischer, S. Alexeeva, E. Hoogendoorn, M. Postma, M. Banzhaf, W. Vollmer, and T. den Blaauwen, "Colocalization and interaction between elongasome and divisome during a preparative cell division phase in Escherichia coli," *Mol. Microbiol.*, vol. 87, no. 5, pp. 1074–1087, Mar. 2013.

[16] A. K. Fenton and K. Gerdes, "Direct interaction of FtsZ and MreB is required for septum synthesis and cell division in Escherichia coli," *EMBO J.*, vol. 32, no. 13, pp. 1953–1965, Jul. 2013.

[17] F. van den Ent, M. Leaver, F. Bendezu, J. Errington, P. de Boer, and J. Löwe, “Dimeric structure of the cell shape protein MreC and its functional implications,” *Mol. Microbiol.*, vol. 62, no. 6, pp. 1631–1642, Dec. 2006.

[18] A. V. Divakaruni, R. R. O. Loo, Y. Xie, J. A. Loo, and J. W. Gober, “The cell-shape protein MreC interacts with extracytoplasmic proteins including cell wall assembly complexes in Caulobacter crescentus,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 51, pp. 18602–18607, Dec. 2005.

[19] C. L. White, A. Kitich, and J. W. Gober, “Positioning cell wall synthetic complexes by the bacterial morphogenetic proteins MreB and MreD,” *Mol. Microbiol.*, vol. 76, no. 3, pp. 616–633, May 2010.

[20] L. Potluri, A. Karczmarek, J. Verheul, A. Piette, J.-M. Wilkin, N. Werth, M. Banzhaf, W. Vollmer, K. D. Young, M. Nguyen-Distèche, and T. den Blaauwen, “Septal and lateral wall localization of PBP5, the major D,D-carboxypeptidase of Escherichia coli, requires substrate recognition and membrane attachment,” *Mol. Microbiol.*, vol. 77, no. 2, pp. 300–323, Jul. 2010.

[21] S. Triboulet, C. M. Bougault, C. Laguri, J.-E. Hugonnet, M. Arthur, and J.-P. Simorre, “Acyl acceptor recognition by Enterococcus faeciuml,d-transpeptidase Ldtfm,” *Mol. Microbiol.*, vol. 98, no. 1, pp. 90–100, Oct. 2015.

[22] W. Vollmer, M. von Rechenberg, and J.-V. Höltje, “Demonstration of Molecular Interactions between the Murein Polymerase PBP1B, the Lytic Transglycosylase MltA, and the Scaffolding Protein MipA of Escherichia coli,” *J. Biol. Chem.*, vol. 274, no. 10, pp. 6726–6734, Mar. 1999.

[23] U. Bertsche, E. Breukink, T. Kast, and W. Vollmer, “In Vitro Murein (Peptidoglycan) Synthesis by Dimers of the Bifunctional Transglycosylase-Transpeptidase PBP1B from Escherichia coli,” *J. Biol. Chem.*, vol. 280, no. 45, pp. 38096–38101, Nov. 2005.

[24] U. Bertsche, T. Kast, B. Wolf, C. Fraipont, M. E. G. Aarsman, K. Kannenberg, M. Von Rechenberg, M. Nguyen-Distèche, T. Den Blaauwen, J.-V. Höltje, and W. Vollmer, “Interaction between two murein (peptidoglycan) synthases, PBP3 and PBP1B, in Escherichia coli,” *Mol. Microbiol.*, vol. 61, no. 3, pp. 675–690, Aug. 2006.

[25] T. Plass, S. Milles, C. Koehler, C. Schultz, and E. A. Lemke, “Genetically Encoded Copper-Free Click Chemistry,” *Angew. Chem. Int. Ed Engl.*, vol. 50, no. 17, pp. 3878–3881, Apr. 2011.

[26] N. Helassa, W. Vollmer, E. Breukink, T. Vernet, and A. Zapun, “The membrane anchor of penicillin-binding protein PBP2a from Streptococcus pneumoniae influences peptidoglycan chain length,” *FEBS J.*, vol. 279, no. 11, pp. 2071–2081, Jun. 2012.

[27] A. Zapun, J. Philippe, K. A. Abrahams, L. Signor, D. I. Roper, E. Breukink, and T. Vernet, “In vitro Reconstitution of Peptidoglycan Assembly from the Gram-Positive Pathogen Streptococcus pneumoniae,” *ACS Chem. Biol.*, vol. 8, no. 12, pp. 2688–2696, Dec. 2013.

[28] M. Terrak, T. K. Ghosh, J. van Heijenoort, J. Van Beeumen, M. Lampilas, J. Aszodi, J. A. Ayala, J. M. Ghuysen, and M. Nguyen-Distèche, “The catalytic, glycosyl transferase and acyl transferase modules of the cell wall peptidoglycan-polymerizing penicillin-binding protein 1b of Escherichia coli,” *Mol. Microbiol.*, vol. 34, no. 2, pp. 350–364, Oct. 1999.

[29] D. L. Perlstein, Y. Zhang, T.-S. Wang, D. E. Kahne, and S. Walker, “The Direction of Glycan Chain Elongation by Peptidoglycan Glycosyltransferases,” *J. Am. Chem. Soc.*, vol. 129, no. 42, pp. 12674–12675, Oct. 2007.

[30] M. Terrak, E. Sauvage, A. Derouaux, D. Dehareng, A. Bouhss, E. Breukink, S. Jeanjean, and M. Nguyen-Distèche, “Importance of the Conserved Residues in the Peptidoglycan Glycosyltransferase Module of the Class A Penicillin-binding Protein 1b of Escherichia coli,” *J. Biol. Chem.*, vol. 283, no. 42, pp. 28464–28470, Oct. 2008.

[31] B. Schwartz, J. A. Markwalder, S. P. Seitz, Y. Wang, and R. L. Stein, “A Kinetic Characterization of the Glycosyltransferase Activity of Eschericia coli PBP1b and Development of a Continuous Fluorescence Assay,” *Biochemistry (Mosc.)*, vol. 41, no. 41, pp. 12552–12561, Oct. 2002.

[32] D. Yu, A. J. Wowor, J. L. Cole, and D. A. Kendall, “Defining the Escherichia coli SecA Dimer Interface Residues through In Vivo Site-Specific Photo-Cross-Linking,” *J. Bacteriol.*, vol. 195, no. 12, pp. 2817–2825, Jun. 2013.

Nederlandse samenvatting

Een essentieel en uniek onderdeel van de bacteriële cel enveloppe is de peptidoglycaan laag. Deze laag, gevormd uit suikerketens, die door eiwit peptide bruggen verbonden worden tot een netwerk, is verantwoordelijk voor het vormbehoud van de bacterie, evenals het bieden van weerstand tegen het grote verschil in osmotische druk tussen de binnen en buitenkant van de cel. Omdat dit netwerk zo belangrijk is voor het overleven van bacteriën is er een enorme hoeveelheid eiwitten betrokken bij de synthese hiervan, en even zoveel bij de regulatie en coördinatie van de synthese met betrekking tot waar en wanneer dit plaatsvindt. Om het voor bacteriële cellen mogelijk te maken om te groeien, moet het peptidoglycaan netwerk vergroot worden, en tegelijkertijd in stand gehouden worden, om lekken te voorkomen, wat een nog grotere uitdaging is tijdens de cel deling.
Omdat dit peptidoglycaan netwerk zo essentieel is voor het overleven van de bacterie, het redelijk makkelijk bereikbaar is aan de buitenkant van de cel, en wij mensen deze structuur en daardoor ook de eiwitten verantwoordelijk voor de synthese niet bezitten, is dit van oudsher een aantrekkelijk doelwit voor antibacteriële middelen. Door het ontstaan van steeds meer resistente bacteriën tegen de nu beschikbare antibiotica, is het van essentieel belang om nieuwe te ontwikkelen. Om nieuwe doelwitten aan te kunnen wijzen voor het ontwikkelen van antibiotica is het van belang om het essentiële en complexe proces van peptidoglycaan synthese tot in detail te begrijpen. In de afgelopen tientallen jaren is er al veel bekend geworden over de eiwitten die betrokken zijn bij de synthese van het peptidoglycaan netwerk, en de regulatie hiervan. Echter, er blijven nieuwe dingen ontdekt worden en de details van het exacte mechanisme zijn nog niet bekend. Daarom wordt er veel onderzoek gedaan om meer inzicht te krijgen in de interacties tussen alle verschillende eiwitten die betrokken zijn bij de peptidoglycaan synthese en de regulatie hiervan. Dit alles met als doel om mogelijke nieuwe aangrijpingspunten voor de ontwikkeling van nieuwe antibiotica te vinden. In dit perspectief hebben wij ons gericht op een van de twee essentiële bifunctionele peptidoglycaan synthases van *Escherichia coli,* Penicillin Binding Protein 1b (PBP1b), die tevens bij de cel deling betrokken is. Dit eiwit kan zowel de suikergroepen van het substraat Lipid II polymeriseren tot ketens (glycosyltransferase GT reactie), en de peptide bruggen vormen, die deze suikerketens tot een netwerk verbinden (transpeptidase TP reactie).

Door het vervangen van specifieke aminozuren in PBP1b door een onnatuurlijk aminozuur met foto cross-linkende eigenschappen hebben wij nieuwe eiwitten geïdentificeerd die een interactie aan gaan met PBP1b, en tevens hebben wij het interactie oppervlakte van PBP1b met deze nieuwe en al bekende interactie partners vast gesteld. Met deze techniek hebben wij in hoofdstuk 2 aangetoond dat PBP1b een interactie aan gaat met CpoB en

FtsN via een opening tussen het UB2H domein en het TP domein. Deze eiwitten hebben een regulerende functie met betrekking tot de activiteit van PBP1b. CpoB verlaagt de activerende werking van een ander eiwit (LpoB) op het TP domein, en FtsN verhoogt de activiteit van zowel het TP als het GT domein. In hoofdstuk 3 laten wij met deze techniek tevens zien dat een lus van het TP domein tussen de aminozuren D535-S558 betrokken is bij vele verschillende interacties, met FtsN, PBP1a, PBP5, MreC en wellicht ook met TolB en PBP6. Al deze interacties vormen een fysieke link tussen de drie lagen van de bacteriële cel enveloppe, die (in gram negatieve bacteriën zoals *E. coli*) bestaat uit een binnen en een buiten membraan, met daartussenin het peptidoglycaan netwerk. Deze link wordt gevormd door FtsN en MreC die zich in de binnen membraan bevinden, en een interactie aangaan met PBP1b, die het peptidoglycaan netwerk bind door zijn synthetiserende functie. Hiernaast bind FtsN zelf ook peptidogylcaan met zijn SPOR domein. Dit alles staat in verbinding met de buiten membraan door de interactie van PBP1b met het Tol-Pal (buiten membraan constrictie) complex via CpoB en wellicht ook via TolB. Deze interacties vormen een ingewikkeld peptidoglycaan synthese en regulatie eiwit-netwerk, die de synthese in overeenstemming met de constrictie van de drie cel enveloppe lagen coördineert, op de juiste plaats en het juiste moment gedurende het cel delings proces.

In hoofdstuk 3 hebben we tevens laten zien dat deze lus van het TP domein van PBP1b, die betrokken is bij verscheidene eiwit-eiwit interacties die hierboven beschreven worden, essentieel is voor de functie van PBP1b. Cellen die een eiwit hebben dat deze lus mist, in plaats van het wild type eiwit, groeien significant minder snel dan cellen die het wild type eiwit produceren. Een *in vitro* peptidoglycaan synthese experiment laat zien dat dit wordt veroorzaakt doordat dit eiwit de TP reactie niet kan uitvoeren. Voor het plaatsvinden van deze reactie, bind het eiwit een donor peptide, wat in een chemische reactie gekoppeld wordt aan de acceptor peptide. Het eiwit dat de lus mist is echter nog steeds in staat het donor peptide te binden, wat suggereert dat deze lus een functie in acceptor peptide binding heeft. Het feit dat een PBP1b variant waarin het aminozuur W548 is vervangen door een onnatuurlijk aminozuur niet meer functioneel is, en eiwitten waarin omliggende aminozuren vervangen zijn nog wel, impliceert dat dit aminozuur essentieel is voor deze acceptor peptide bindende functie. Interessant is dat voor een ander eiwit wat ook TP activiteit bezit, aangetoond is dat een tryptofaan in een vergelijkbare lus structuur verantwoordelijk is voor het binden van de acceptor peptide. De analyse van TP domeinen van verschillende eiwitten van diverse bacteriesoorten (zowel gram positief als gram negatief) laat zelfs zien dat ze allemaal een lus structuur bevatten die het actieve centrum bedekt, met een tryptofaan of een tyrosine dicht bij het actieve centrum. Dit terugkerende fenomeen suggereert dat TP domeinen een aromatisch aminozuur dichtbij de actieve kern nodig hebben, die waarschijnlijk betrokken is bij acceptor peptide binding.

In hoofdstuk 4 wordt een nieuwe methode beschreven voor het immobiliseren van PBP1b op een SPR chip oppervlak. Met SPR kan de kinetiek van eiwit-eiwit interacties geanalyseerd worden. Hiervoor wordt een eiwit van het interactie koppel geïmmobiliseerd op het chip oppervlak, het andere eiwit stoomt hier, opgelost in een vloeistof overheen. Tot nu toe werden PBPs geïmmobiliseerd met behulp van een ampicilline gecoate chip, wat een covalente binding aangaat met het TP domein van de PBP. Echter, op deze manier worden metingen verricht met een eiwit dat substraat gebonden is, waardoor dit domein inactief is. Verder is hierdoor het TP domein niet beschikbaar (omdat het gebonden is aan het chip oppervlak) voor interacties die via dit domein plaatsvinden. Door het inbouwen van een onnatuurlijk aminozuur met een reactieve groep in de vorm van een azide, in de niet essentiële cytoplasmatische staart van PBP1b, is het mogelijk om deze te koppelen aan een met cyclooctyne gecoat SPR chip oppervlak. Op deze manier wordt PBP1b geïmmobiliseerd via een niet essentieel deel van het eiwit, wat het mogelijk maakt om metingen te doen met een geheel actief eiwit. Daarnaast resulteert deze immobilisatie methode in een oriëntatie die meer lijkt op de *in vivo* oriëntatie van PBP1b. Deze nieuwe methode maakt het mogelijk om interacties te identificeren met eiwitten die het TP domein van PBP1b binden, of die een actief eiwit nodig hebben om de interactie te doen plaatsvinden. We hebben deze nieuwe methode getest met behulp van de goed gekarakteriseerde interactie tussen PBP1b en LpoB. Een vergelijkbaar interactie patroon werd verkregen met behulp van deze nieuwe immobilisatie methode. Tevens werd een vergelijkbare dissociatie constante in chemisch evenwicht (equilibrium constanten) berekend voor deze interactie. Helaas zijn wij niet in staat geweest om de interactie van PBP1b met nieuwe interactie partners te laten zien, zoals de hiervoor beschreven TP domein bindende eiwitten PBP5 en TolB. Desalniettemin heeft deze nieuwe immobilisatie methode wel gewerkt voor het analyseren van de interactie tussen PBP1b en LpoB, en kan ook gebruikt worden voor andere PBPs. Het is eigenlijk een hele makkelijke manier om ieder gewenst eiwit te immobiliseren, met het voordeel dat door de vervanging van een specifiek aminozuur door het azide bevattende aminozuur, ook de oriëntatie van het eiwit bepaald kan worden. Dit zal resulteren in een homogene oriëntatie van de geïmmobiliseerde eiwitten en schept de mogelijkheid om bindings verschillen in relatie tot de topologie van (membraan) eiwitten te kunnen meten.

In hoofdstuk 5 laten we zien dat het gebruik van Lipid II gelabeld met andere fluoroforen, met een hogere relatieve fluorescentie, de resolutie en intensiteit van de in gel peptidoglycaan synthese assay sterk verbeterd, vergeleken met het gebruik van Dansyl gelabeld Lipid II. In deze assay wordt *in vitro* peptidoglycaan gesynthetiseerd van fluorescent gelabeld substraat, waarbij op verschillende tijdspunten een monster genomen wordt. De inhoud van deze monsters wordt vervolgens gescheiden met behulp van een Tris/Tricine SDS-PAGE gel, waarop de gepolymeriseerde suikerketens worden

gescheiden op grootte. Korte ketens lopen lager en zijn zichtbaar als gescheiden banden, langere ketens lopen hoger, die een smeer op de gel vormen. Een door peptide bruggen gevormd netwerk van peptidoglycaan is te groot om de gel in te migreren, en is zichtbaar als een band in de onderkant van het slotje. Omdat het substraat fluorescent gelabeld is, kan dit zichtbaar gemaakt worden door middel van een fluorescentie scanner. De hogere resolutie en intensiteit behaald door het gebruik van Lipid II gelabeld met nieuwe fluoroforen maakt het mogelijk om kleinere veranderingen in de activiteit van PBPs te detecteren. Vooral de met de fluoroforen ATTO 550 en 647N gelabelde Lipid II varianten genereerden een resultaat met hoge resolutie, hoewel de Megastokes dye 608 en 735 gelabelde Lipid II varianten ook bruikbaar zijn in deze assay. Het was echter wel nodig om niet gelabeld substraat toe te voegen aan deze reacties om peptidoglycaan synthese te observeren, wat niet beschreven is voor het gebruik van Dansyl gelabeld Lipid II. Een andere assay om PBP activiteit te meten is de continue GT assay. Deze assay is gebaseerd op een dalende fluorescentie, veroorzaakt door verandering van de chemische omgeving van de fluorofoor. Het fluorescente signaal is hoger in een meer hydrofoob milieu (verbonden aan de dragende lipide staart) dan in een meer hydrofiel milieu (ingebouwd in de suikerketen, losgemaakt van de lipide staart), en daardoor daalt het fluorescente signaal als resultaat van suikerketen polymerisatie. De ATTO gelabelde Lipid II varianten waren niet bruikbaar in deze assay, omdat zij deze eigenschap van veranderend fluorescent signaal als resultaat van een veranderd chemisch milieu niet bezitten. Beide Megastokes fluoroforen hebben deze eigenschap wel, maar gebruik van Megastokes dye 735 gelabeld Lipid II resulteerde in een signaal met veel ruis, wat deze Lipid II variant niet erg bruikbaar maakt voor deze assay. Megastokes dye 608 gelabeld Lipid II kan zeer goed gebruikt worden in deze assay, wat resulteerde in het verdwijnen van een lag fase aan het begin van de meting die gezien wordt wanneer dansyl gelabeld Lipid II gebruikt wordt, maar niet in een grotere fluorescente daling (verhoogde resolutie). Een tegenstrijdigheid tussen de twee beschreven assays voor het meten van peptidoglycaan synthese is dat Megastokes dye 608 gelabeld Lipid II goed ingebouwd lijkt te worden in de continue GT assay, maar dat er niet gelabeld Lipid II toegevoegd moet worden om een redelijke inbouw snelheid en suikerketen lengte te behalen als het geanalyseerd wordt op gel. Onze hypothese is dat dit wordt veroorzaakt doordat de polymerisatie vroegtijdig wordt beëindigd. Dit resulteert in de productie van korte suikerketens, wat lijkt op geen, of bijna geen productie als het geanalyseerd wordt op gel, maar geïnterpreteerd zal worden als normale polymerisatie als het geanalyseerd wordt met behulp van de continue GT assay, door een constante daling van het signaal. Verder is de manier van fluorescent labelen op het niveau van Lipid II met gebruik van click chemie een makkelijke recht toe recht aan methode gebleken die zeer efficiënt is. De op deze wijze geproduceerde gelabelde Lipid II varianten bleken erg bruikbaar te zijn in het analyseren van de GT en TP activiteit van PBP1b in deze *in vitro* peptidoglycaan synthese assays.

In hoofdstuk 6 laten we zien, door de productie van verkortte PBP1b varianten, dat de aminozuren tussen F480 en Y498 belangrijk zijn voor PBP1b dimeer formatie. De kristal structuur van PBP1b laat zien dat een groot deel van deze aminozuren zich aan de binnenkant van het eiwit bevinden, behalve een lus die de aminozuren E492, Q494 en F495 bevat. Door het vervangen van deze specifieke aminozuren in PBP1b door een onnatuurlijk aminozuur met foto cross-linkende eigenschappen, hebben we laten zien dat E492 betrokken zou kunnen zijn bij dimeer formatie van PBP1b. Het inbouwen van het onnatuurlijke aminozuur op deze positie resulteert in het verschijnen van een cross-link product, wat net iets hoger loopt op SDS-PAGE dan de dimeer band. Dimeer formatie heeft een hogere GT en TP activiteit van PBP1b tot gevolg in *in vitro* peptidoglycaan synthese assays. Het zou interessant zijn om het effect van dimeer formatie op de *in vivo* functie van PBP1b te onderzoeken. Daarnaast zou ook de noodzakelijkheid van dimeer formatie voor de interactie met andere eiwitten, en het effect van regulerende eiwitten op de activiteit van PBP1b, in relatie tot dimeer formatie, interessant zijn om te onderzoeken.

In deze thesis zijn nieuwe eiwit-eiwit interacties, waarin PBP1b betrokken is beschreven. De nu beschikbare informatie weerspiegeld waarschijnlijk nog maar deels alle bestaande interacties tussen deze peptidoglycaan synthase, hydrolases, peptidases, cel delings en regulerende eiwitten. Bewijs dat er waarschijnlijk nog meer interacties zijn waarin PBP1b betrokken is komt uit het feit dat er nog meer cross-link producten gevonden zijn dan degenen die in deze thesis beschreven worden, waarvan de betrokken eiwitten niet geïdentificeerd konden worden. Deze hoeveelheid aan interacties weerspiegeld het belang van een strikte regulatie van peptidoglycaan synthese door PBP1b, vooral tijdens het cel delings proces. De kinetiek van deze interacties, en het effect van de interacties op de activiteit van peptidoglycaan synthases kan onderzocht worden met behulp van de beschreven en verbeterde technieken in een streven naar het ontrafelen van het interactienetwerk tussen al deze eiwitten, en hun rol in de regulatie van peptidoglycaan synthese. Kennis over de regulatie van dit essentiële proces voor het overleven van bacteriën is erg belangrijk voor het ontwikkelen van nieuwe antibiotica, in het gevecht tegen het groeiende aantal resistente bacterie soorten.

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Curriculum Vitae

Inge van ’t Veer was born on October 26th 1986 in Maassluis, The Netherlands. She graduated from secondary school at the Maascollege in Maassluis in 2005. She continued her education at the University of Amsterdam, studying Biomedical sciences. She obtained her bachelor degree in 2008, and continued with the master Life Sciences. As part of her master program she performed two internships. The first internship was performed at the Swammerdam Institute for Life Sciences (SILS) at the group of Molecular cytology, under supervision of Dr. T.W.J. Gadella, where the interaction between the receptor like kinases LYK3 and NFP was investigated by FLIM/FRET microscopy. The second internship was performed at the Hubrecht institute, at the mechanics of cell adhesion group, under supervision of Dr. J. de Rooij, where the role of polarity proteins in endothelial cell-cell adhesion was investigated. After obtaining her master’s degree in 2010, she started her PhD research at the University Utrecht, at the Membrane Biochemistry and Biophysics group, under supervision of Dr. E.J. Breukink and Prof. dr. J.A. Killian. The results of the research are described in this thesis.

List of publications

Andrew N Gray, Alexander J F Egan, **Inge L van 't Veer**, Jolanda Verheul, Alexandre Colavin, Alexandra Koumoutsi, Jacob Biboy, Maarten A F Altelaar, Mirjam J Damen, Kerwyn Casey Huang, Jean-Pierre Simorre, Eefjan Breukink, Tanneke den Blaauwen, Athanassios Typas, Carol A Gross, Waldemar Vollmer.
Coordination of peptidoglycan synthesis and outer membrane constriction during *Escherichia coli* cell division, *eLife*, p. e07118, May 2015.

Alexander J. F. Egan, Jacob Biboy, **Inge van 't Veer**, Eefjan Breukink, Waldemar Vollmer
Activities and regulation of peptidoglycan synthases, *Phil Trans R Soc B*, vol. 370, no. 1679, p. 20150031, Oct. 2015

Diana Stork, **Inge van 't Veer**, Monique P. C. Mulder, Reinout Raijmakers, Jacob Biboy, Rob M. J. Liskamp, Waldemar Vollmer, Eefjan Breukink
Identification of an essential interaction site in the transpeptidase domain of PBP1b from *Escherichia coli*.
Manuscript in preparation

Inge van 't Veer, Nadia Leloup, Bert Janssen, Nathaniel Martin and Eefjan Breukink
Site specific immobilization of PBP1b on an SPR chip surface
Manuscript in preparation

Inge van 't Veer, Alexander Egan, Jacob Biboy, Waldemar Vollmer and Eefjan Breukink
The use of different fluorescently labeled Lipid II variants for the improvement of glycosyltransferase assays
Manuscript in preparation