CMLS, Cell. Mol. Life Sci. 54 (1998) 309–316
1420-682X/98/040309-08 $ 1.50 + 0.20/0
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CMLS Cellular and Molecular Life Sciences

The structure and function of *Escherichia coli* penicillin-binding protein 3

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Abstract. *Escherichia coli* penicillin-binding protein PBP3 is a key element in cell septation. It is presumed to catalyse a transpeptidation reaction during biosynthesis of the septum peptidoglycan but, in vitro, its enzymatic activity has only been demonstrated with thiolester analogues of the natural peptide substrate. It has no detectable transglycosylase activity with lipid II as substrate. This tripartite protein is constructed of an N-terminal membrane anchor-containing module that is essential for cell septation, a non-penicillin-binding (n-PB) module of unknown function and a C-terminal penicillin-binding (PB) module exhibiting all the characteristic motifs of penicilloyl serine transferases. The n-PB module, which is required for the folding and stability of the PB module, may provide recognition sites for other cell division proteins. Initiation of septum formation is not PBP3-dependent but rests on the appearance of the FtsZ ring, and is thus penicillin-insensitive. The control of PBP3 activity during the cell cycle is briefly discussed.

Key words. Penicillin-binding protein 3; division-specific peptidoglycan biosynthesis; cell division.

Introduction

Since their discovery in 1973, penicillin-binding proteins (PBPs) have attracted much attention from academic researchers as well as from pharmaceutical companies. The well-known reason is that PBPs not only play a role in peptidoglycan biosynthesis and cellular morphogenesis, but are also the targets for β-lactam antibiotics [1]. PBPs are members of the penicilloyl serine transferase family. They catalyse the transfer of the penicilloyl moiety of penicillin to their active-site serine, forming an acylenzyme which is slowly hydrolysed. There are two distinguishable groups of PBPs: the low molecular mass (LMM) PBPs and the high molecular mass (HMM) PBPs. The LMM PBPs (<60 kDa) are monofunctional enzymes acting mainly as DD-carboxypeptidases involved in the remodelling of peptidoglycan during cell growth [2, 3]. The best characterized LMM PBPs are the DD-carboxypeptidase/PBP of *Streptomyces* R61 and the DD-transpeptidase/PBP of *S.* K15 [4]. The HMM PBPs are bi(or multi)modular enzymes. They include PBPs 1a, 1b, 2 and 3 of *Escherichia coli*. These proteins are anchored to the cytoplasmic membrane by an N-terminal pseudo signal peptide, and are essentially composed of two modules localized on the outer face of the cytoplasm membrane. The C-terminal module binds penicillin and catalyses cross-linking of the peptidoglycan peptides. Depending on the primary structure and the catalytic activity of the N-terminal module, these proteins fall into classes A and B (fig. 1). PBPs 1a and 1b of class A behave as bifunc-

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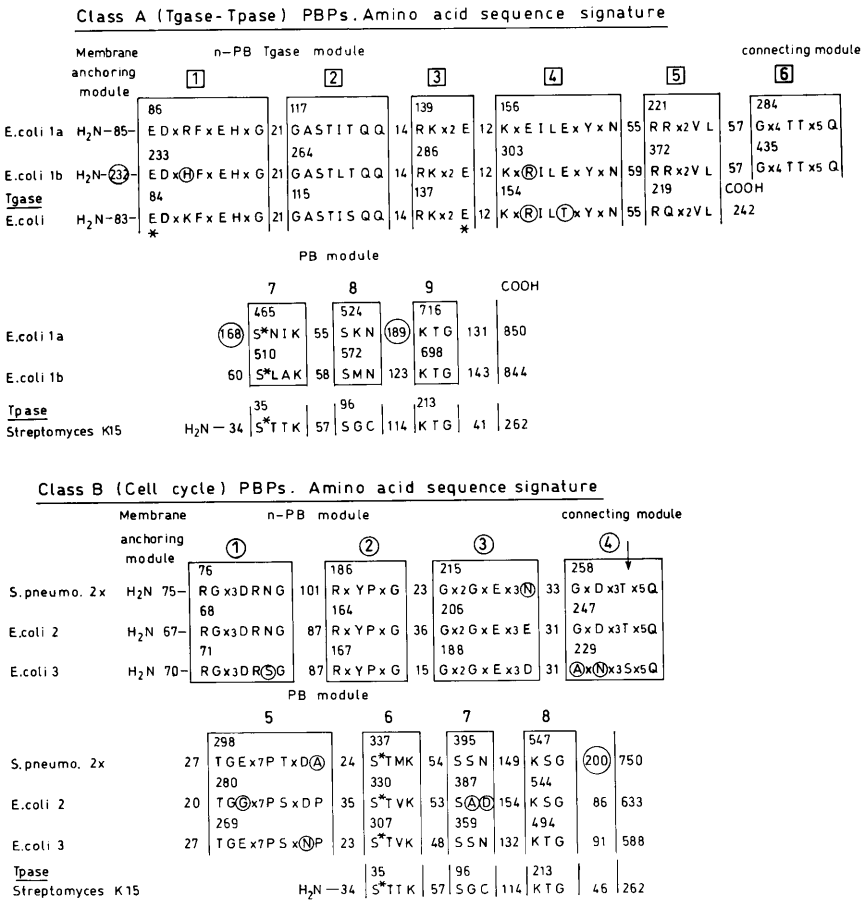

Figure 1. Conserved motifs along the amino acid sequences of the multimodular class A PBP1a and PBP1b of *E. coli*, class B PBP2 and PBP3 of *E. coli*, and PBP2x of *Streptococcus pneumoniae*. The transglycosylase (Tgase) motifs are those of the monofunctional transglycosylase of *E. coli*. The transpeptidase (Tpase) motifs are those of *Streptomyces* K15 DD-transpeptidase/PBP.

tional proteins exhibiting both transglycosylase (N-terminal module) and transpeptidase (C-terminal module) activities. They catalyse polymerization of the peptidoglycan from undecaprenyl diphosphate-linked disaccharide peptides, presumably by producing 'primers' for PBP2 and PBP3 to act upon during cell elongation and cell division, respectively [5]. PBP2 and PBP3 of class B are likewise considered as bifunctional proteins, though the role of the N-terminal module is not clearly established (see below). PBP3 is specifically involved in polymerization of the septal peptidoglycan during cell division [5, 6].

Septum formation requires the concerted action of PBP3 (also called FtsI) and non-penicillin-binding proteins FtsA, K, L, N, Q, W, Z and ZipA [7–9]. Taken together they form a complex called divisome or septator that encompasses the cytoplasm, the inner membrane and the periplasm (fig. 2). FtsZ, a tubulin-like GTPase, forms an intracellular ring at the division site, and this cytoskeletal element is expected to focus the activity of the division proteins and to provide the force necessary for cytokinesis [10]. The FtsZ ring is required throughout septation as it directs septal growth. ZipA, a membrane-bound protein; FtsA, a cytoplasmic actin-like protein; FtsN; and PBP3 have been located at the division site by microscopic techniques [11–15]. ZipA and FtsA interact with FtsZ. PBP3 could interact with FtsA and FtsZ. So far, it is not known how the various proteins interact [9, 10, 16].

The aim of this paper is to discuss (i) the structure and enzymatic function of PBP3 and (ii) its role in the division process.

Function of the PBP3 modules

PBP3 is composed of various modules as shown in figure 3. This tripartite protein is synthesized as a 588-amino acid residue precursor which is processed by the Prc protease into a mature M1-V577 PBP3. The C-terminal D237-V577 penicillin-binding module (PB mod-

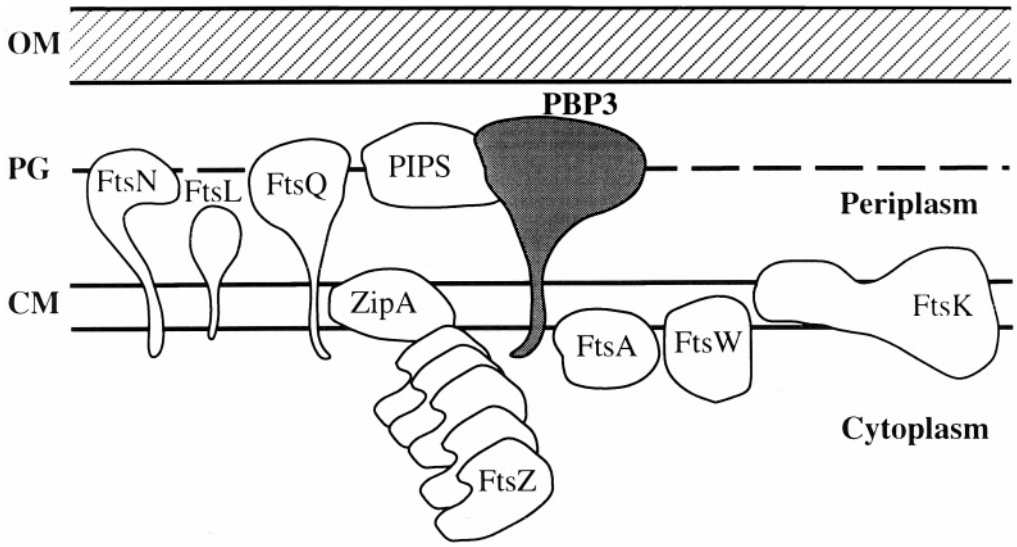

Figure 2. Schematic representation of the proteins forming the divisome. See text for description of the proteins. OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane.

ule) possesses the three conserved motifs of the penicilloyl serine transferases and the N-terminal G57-I236 non-penicillin-binding module (n-PB module), the four motifs characteristic of the class B PBPs (fig. 4; [17]). The role of the different modules has been investigated by dissecting the protein into its component parts.

The M1-D56 membrane anchor-containing module

The replacement of the membrane anchor-containing module by the cleavable OmpA signal peptide results in the production of a soluble active G57-V577 PBP3 in the periplasmic space of *E. coli* [18]. The thermostability and the specificity profile for β-lactams of this periplasmic PBP3 are similar to those of the membrane-bound enzyme. On the basis of these results, one can assume that the periplasmic protein adopts the native folded conformation. However, production of the soluble PBP3 in *E. coli* RP41, a thermosensitive *fts*I mutant, does not restore cell division at 42 °C. The membrane anchor-containing module, although not essential for the folding of the protein, is required for cell septation activity [19]. The transmembrane segment of PBP3 cannot be exchanged for another one, indicating that it is not simply a sequence that contributes to the anchoring of the protein into the membrane [20, 21].

The replacement of the M1-R23 intracellular peptide by that of FtsL or FtsQ results in the loss of ability to support the division of a null *fts*I mutant, suggesting that the cytoplasmic domain performs an important role perhaps to signal localization to the cell septum by interacting with some other components of the cell division machinery such as FtsZ [20]. However, in some conditions, the FtsL (or FtsQ) PBP3 hybrid is able to complement the thermosensitive *fts*I23 mutation. In addition, the replacement of residues 3 to 22 by an Arg is tolerated at a nonpermissive temperature in *E. coli* RP41, a *fts*I *ts* mutant (H. Matsuzawa, unpublished results). One explanation of these results is that the thermosensitive PBP3 would form a dimer with the modified PBP3, generating a partially functional dimer. Dimerization of PBP3 has been shown in vitro by two-dimensional (2D) electrophoresis and by using the surface plasma resonance technique (J. Ayala, J. Höltje and N. Nanninga, unpublished results).

The penicillin-binding module

Indirect experimental evidence suggests that the penicillin-binding (PB) module of PBP3 is involved in peptide cross-linking during the biosynthesis of the septal peptidoglycan [22]. PBP3 has no detectable activity on the natural pentapeptide, but it catalyses in vitro the hydrolysis of thiolester substrates of general structure C_6H_5-CONH-CHR′-COS-CHR′-COOH. Table 1 compares the k_{cat}/K_m values obtained for the periplasmic PBP3 with those obtained with the class B PBP2x of *Streptococcus pneumoniae*, the monofunctional DD-transpeptidase of *Streptomyces* K15 and the monofunctional DD-carboxypeptidase of *Streptomyces* R61. The activity of the periplasmic PBP3 on this limited series of substrates is similar to that of the K15 enzyme.

For both enzymes, the presence of D-Ala in the reaction mixture results in a simple partitioning between hydrolysis of the carbomyl donor into benzoyl-D-Ala and its aminolysis into benzoyl-D-Ala-D-Ala with the concomi-

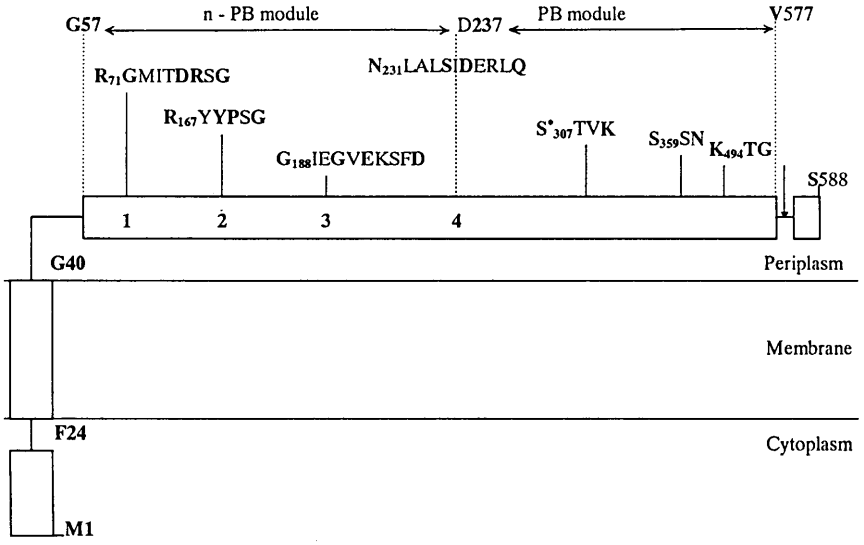

Figure 3. Modular organization of *E. coli* PBP3. The vertical arrow indicates the cleavage site of the Prc protease. The conserved motifs of the n-PB module and the PB module are shown.

tant release of thiolglycolate. PBP3 also uses as acceptor the L-Ala-γ-D-Glu-(NH_2)-*meso*-A_2pm-D-Ala tetrapeptide which is the tetrapeptide unit of the *E. coli* peptidoglycan containing an α-amidated D-Glu residue. These results show that the PB module of PBP3 can catalyse acyl transfer reactions [23].

The n-PB module of PBP3

The function of the n-PB module is not clear. Matsuhashi and his group have suggested that PBP3 could perform a transglycosylase reaction [24]. With lipid II intermediate as substrate, however, the soluble PBP3 and the membrane-bound enzyme do not catalyse the polymerization of the glycan chains under conditions where the class A PBP1b functions as a transglycosylase [23–25]. That the n-PB module of class B PBP3 is probably not a transglycosylase is consistent with the fact that the primary structure of the n-PB module of class B PBP3 greatly differs from those of class A PBPs and of the monofunctional transglycosylases.

It is reasonable to ask whether the PB and n-PB modules can act independently of each other, so the role of the n-PB module in the folding of the PB module was investigated by mutagenesis. All the attempts to produce a functional PB module independently of the n-PB module failed. This shows that in marked contrast to the low-molecular-mass PBPs, which are autonomous folding entities, the PB module of PBP3 cannot fold by itself. It requires the assistance of the complete n-PB module, which might function as an intramolecular chaperone. Key elements to the folding information are found in motif 1 and motif 3 [19].

The three-dimensional (3D) structure of PBP3 is not known, but that of a soluble form of *S. pneumoniae* PBP2x has been solved at 3.5 Å [26]. The soluble PBP2x is composed of three modules (fig. 4). The C-terminal extension is not present in PBP3. The PB module has the characteristic topology of the penicilloyl serine transferases. The n-PB module is shaped as a pair of sugar tongs the head of which fits in a groove of the PB module (fig. 4). The four conserved motifs of the n-PB module are located in the head of the sugar tongs and are expected to be important structural elements involved in intermodular interactions. Motif 4 is at the end of the n-PB module and is part of helix α1 of the PB module.

One of the roles of the n-PB module could be to provide specific interaction regions for other proteins of the divisome. One indication that the n-PB module is involved in complex formation arises from the lethal effects of its overproduction [27]. It has also been proposed that the transpeptidase activity of PBP3 is coupled to the transglycosylase activity of PBP1b. Höltje and his group showed an interaction between the lytic transglycosylase, MltB, PBP7, PBP1b and PBP3 using affinity chromatography and surface plasma resonance methods. This suggests the occurrence of a multienzyme complex involving lytic and synthetic activities related to divisome-specific peptidoglycan synthesis [28].

Since FtsQ seems to occur specifically in peptidoglycan-containing organisms, the possibility should be considered that FtsQ is involved in peptidoglycan

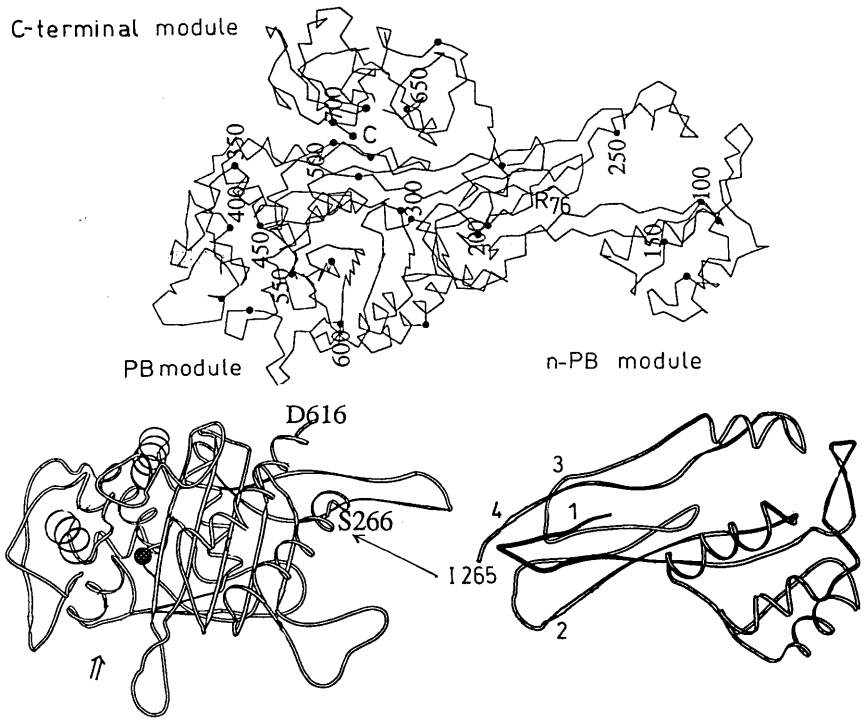

Figure 4. Schematic representation of the three-module structure of *S. pneumoniae* PBP2x. The PB module and the n-PB module are presented as disconnected entities in the lower part (adapted from ref. 26). Open arrow, catalytic site of the PB module; black ball, active-site serine. Numbers 1 to 4 in the n-PB module indicate the four conserved motifs.

assembly. If so, interaction with PBP3 might occur. This has been studied by two different in vitro binding assays. First, affinity blotting was carried out with purified histidine-tagged FtsQ proteins and a purified soluble form of G57-V577 PBP3. Second, both proteins were incubated in solution and immunoprecipitated by their respective antibodies. The two binding assays failed to show an interaction between PBP3 and FtsQ (our unpublished observations, and see ref. 29). Alternatively, the interaction could be mediated by one or several other division proteins.

Functional specificity of PBP3

The n-PB module and the PB module of PBP3 are probably in close interaction and form interdependent folding entities. PBPs of class B do not fulfill identical functions. PBP2 is involved in cell elongation; PBP3 is involved in cell septation. The PB module of PBP3 cannot be replaced in vitro or in vivo by that of PBP2 and vice versa [30]. The unique combination of the n-PB and PB modules appears, therefore, to determine the functional specificity of the proteins. The modular design is also class-specific. Attempts have been made to find out whether the transpeptidase modules of PBP3 and PBP1b could be exchanged. A similar exchange has been tried for the n-PB module of PBP3 and that of PBP1b. In no case could replacement of function be demonstrated, suggesting that each domain needs the other one to carry out its function [31]. In the case of the PBP1b-PBP3 transglycosylase-transpeptidase hybrid encoded by pCM13 [31], [^{3}H]benzylpenicillin does not bind to the PB module (our unpublished observations), indicating that transpeptidase activity has not been retained.

Role of PBP3 during the cell cycle

Temperature-sensitive mutants, when grown at the non-permissive temperature or the presence of specific antibiotics such as furazlocillin or cephalexin, produce filamentous cells indicating that PBP3 is involved in the division process [6, 32]. Because such filaments still reveal indentations, it has been assumed that PBP3 was not involved in the initiation of division, but rather in its continuation and completion [33]. This notion has been corroborated by electron microscopic autoradiography in the sense that incorporation of radioactive diaminopimelic acid (A_2pm) can still occur at the developing division site when PBP3 is impaired [33]. Incorporation does not occur in the above-mentioned indentations [33, 34]. Very recently it was found that

Table 1. Interaction of the G57-V577 PBP3 with thiolester substrates C_6H_5-CO-NH-CH(R)-CO-S-CH(R′)-COOH → C_6H5-CO-NH-CH(R)-COOH + HS-CH(R′)-COOH.

Thiolester	R	R′	k_{cat}/K_m (M^{-1} s^{-1})			
			PBP3p	PBP2x	K15 PBP	R61 PBP
1	CH_3	H	100⇑20	5000	75	700,000
2	H	H	20⇑5	600	8	100,000
3	CH_3	CH_3	40⇑10	4900	3	125,000
4	H	CH_3	30⇑7	3200	75	100,000
5	$(CH_3)_2$-CH	H	NA	ND	NA	8000

Thiolesters (1) and (3): the asymmetric carbon atoms have the D-configuration. Thiolesters (4) and (5), racemic mixtures. ND, not determined. NA, no detectable activity.

FtsZ rings can form and initiate septation in *ftsI*, *ftsA* and *ftsQ* mutants [12] or after treatment of the cell with cephalexin [35] or aztreonam (fig. 5). This suggests that peptidoglycan synthesis during initiation of the septum and FtsZ ring formation are coordinated and independent of PBP3. The enzymes responsible for the initial peptidoglycan synthesizing activity during division have been proposed to be penicillin-insensitive ([36], PIPS [penicillin-insensitive peptidoglycan synthesis]), but this still remains an open question. Though penicillin-insensitive enzymes involved in peptidoglycan synthesis have been described, division-specific ones have not yet been found [36].

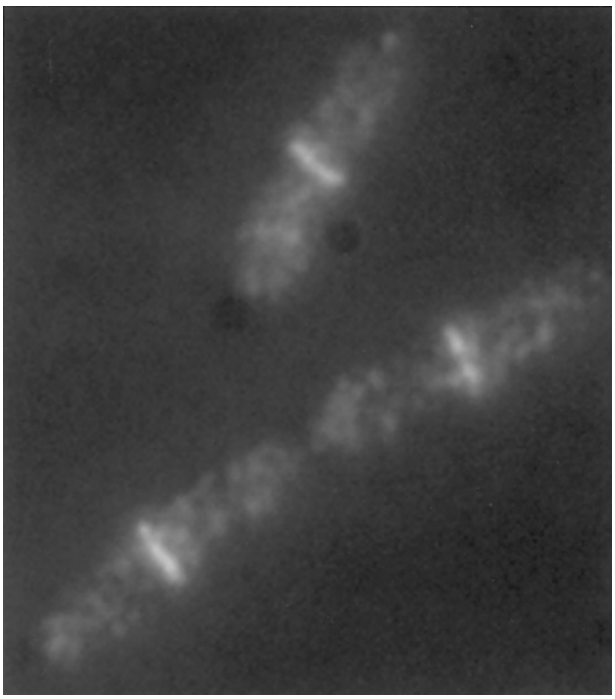

Figure 5. Localization of FtsZ rings in *ftsI* 2158 *E. coli* cells treated with aztreonam at 30 °C by immunofluorescence microscopy. Labelling was performed with monoclonal antibodies MAB212 directed against FtsZ [43].

Despite the continuous presence of PBP3 during the cell cycle (see below), inhibition of PBP3 with cephalexin occurs specifically during the division process. This could be deduced by measuring A_2pm incorporation in synchronized cells [5]. Thus, presumably PBP3 is specifically activated during the division process. When the cell constricts, the diameter of the FtsZ ring decreases as it maintains its position at the leading edge of the septum. During this stage, the FtsZ ring is joined by FtsN and probably the other cell division proteins such as PBP3. PBP3 might be activated through its interaction with the FtsZ ring and/or the other cell division proteins to synthesize the septal peptidoglycan. It has been suggested that the switch from cell elongation to septation depends on changes in the availability of tripeptide side chains of peptidoglycan which are used preferentially by PBP3 as acceptors in the transpeptidation reaction [37]. This tripeptide substrate could be the result of the relative activities of LMM PBPs 5 and 6 and of the LD-carboxypeptidase which remove the ultimate and penultimate D-Ala of the pentapeptide side chains respectively. In addition, it could arise from the specific production of tripeptide-containing lipid II resulting from the recycling of peptidoglycan [38, 39].

The number of PBP3 molecules in fast-growing cells has recently been estimated to be of the order of 100 molecules per cell [14, 40]. By contrast, thousands of FtsZ molecules are considered to take part in the so-called FtsZ ring [7]. As a consequence it seems unlikely that PBP3 (like other low-copy-number division proteins such as, for instance, FtsQ) can form an intact ring. Presumably, the FtsZ ring contains subassemblies where the low-copy-number division proteins are grouped together (fig. 6). The low number of PBP3 molecules also explains why it has taken such a long time to really establish their location near the cell centre during the later stages of cell growth [14].

Since PBP3 is already present before division starts [41], this leads to the question of how its positioning in the

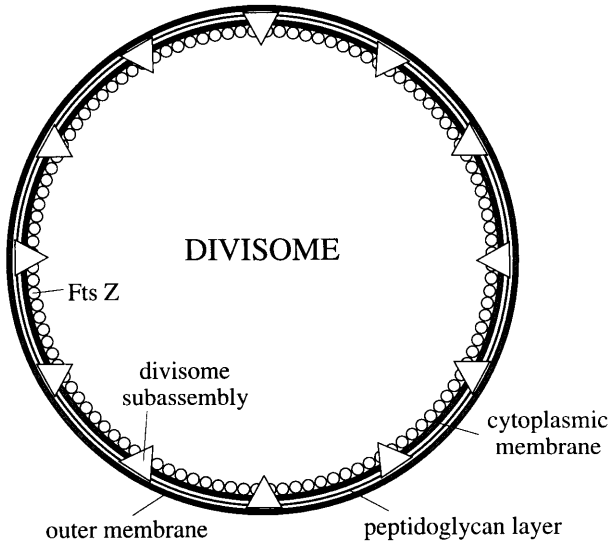

Figure 6. Schematic representation of the FtsZ ring and subassemblies of cell division proteins.

cell centre is regulated. Two possibilities can be envisaged. PBP3 could either be present at the future division site or might be transported to the division site. In both cases one can imagine that PBP3 molecules become inserted in the membrane at a limited number of centres. If these centres are not present at the future division site, they might find their location in a manner similar to the way in which receptor-bound low-density-lipid particles are 'trapped' by coated pits in the plasma membrane of an animal cell [42].

Concluding remarks

Though it is clear that the PB domain of PBP3 plays a role in peptidoglycan synthesis during the division process, many questions remain to be answered. (i) The function of the n-PB module remains to be established. It is clearly essential for the function of the PB module. Though the n-PB module most probably does not function as a transglycosylase, it could provide specific interaction regions for other proteins of the divisome. (ii) More research will be needed to find out with which proteins PBP3 is associated in the divisome. It clearly takes part in a division-specific multienzyme complex, which also contains lytic enzymes [28]. (iii) Since PBP3 is continuously present, it will be important to establish how it becomes activated or inhibited during division and cell elongation, respectively. (iv) Related to this point is the question of where PBP3 becomes inserted in the cytoplasmic membrane and of how it finds its location at midcell.

Acknowledgements. This work was supported in part by (i) the Belgian programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Services fédéraux des affaires scientifiques, techniques et culturelles (PAI P4/03) and the Fonds de la Recherche Fondamentale Collective (contract no. 2.4534.95), and by (ii) Life Sciences Foundation (S.L.W.), which is subsidized by the Netherlands Organization for Scientific Research (N.W.O.) (contract no. 33.220-P).

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