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Bacterial cell division proteins as antibiotic targets

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ABSTRACT

Proteins involved in bacterial cell division often do not have a counterpart in eukaryotic cells and they are essential for the survival of the bacteria. The genetic accessibility of many bacterial species in combination with the Green Fluorescence Protein revolution to study localization of proteins and the availability of crystal structures has increased our knowledge on bacterial cell division considerably in this century. Consequently, bacterial cell division proteins are more and more recognized as potential new antibiotic targets. An international effort to find small molecules that inhibit the cell division initiating protein FtsZ has yielded many compounds of which some are promising as leads for preclinical use. The essential transglycosylase activity of peptidoglycan synthases has recently become accessible to inhibitor screening. Enzymatic assays for and structural information on essential integral membrane proteins such as MraY and FtsW involved in lipid II (the peptidoglycan building block precursor) biosynthesis have put these proteins on the list of potential new targets. This review summarises and discusses the results and approaches to the development of lead compounds that inhibit bacterial cell division.

1. Introduction

Cell division in bacteria is accomplished by a large and very dynamic protein complex termed the divisome of which all proteins contribute to the simultaneously synthesis of all required compounds of the cell envelope [1–3]. The envelope of Gram-negative bacteria consist from inside to outside of a cytoplasmic membrane, the single layer of peptidoglycan embedded in the periplasmic space and the outer membrane. Gram-positive bacteria lack the outer membrane and make up for this lack of protection by having a multi-layered peptidoglycan wall. Peptidoglycan is a covalently closed network of glycan strands that are interconnected by peptide side bridges. Consequently to be able to insert new material, the peptidoglycan layer has to be opened by hydrolytic enzymes [4]. During cell pole synthesis several activities can be discriminated; peptidoglycan synthesis, peptidoglycan hydrolysis and modification, synchronization of the invagination of all envelope layers and spatial and temporal organization of the process. An imbalance in these activities leads to cell death as illustrated by the lysis of bacteria at their division site due to the peptidoglycan synthesis inhibiting activity of penicillins or β-lactams. Whether inhibition of the other important protein activities would also kill bacteria is the subject of this review.

In many bacterial species investigated, cell division is initiated by the assemblage of a scaffold made of FtsZ polymers bound to the cytoplasmic membrane by proteins such as FtsA and ZipA and organized by proteins such as ZapA, B, C and D [2]. This scaffold establishes the side of division by positioning new cell envelope material (peptidoglycan) without invagination [5,6]. This activity recruits a second set of cell division proteins that are the proteins that will do the real job of synthesis of two new cell poles (Fig. 1). Depending on the species the new cell pole synthesis is either accompanied by invagination of the cell envelop (i.e. *Escherichia coli*), or the two new cell envelopes are separated in a later hydrolytic process (i.e. *Bacillus subtilis*).

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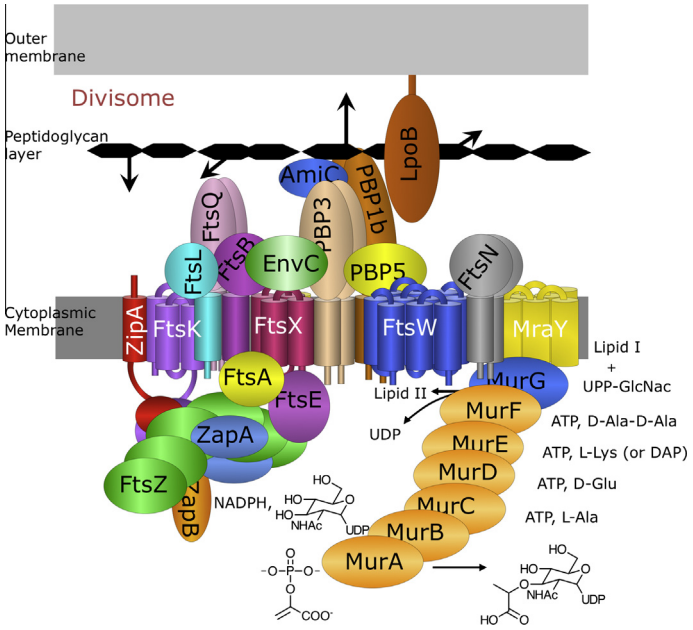

Fig. 1. Schematic overview of the three-layered cell envelop of *Escherichia coli* and the protein complex that drives new cell pole synthesis. For transparency not all known proteins that localize at mid cell during division are shown. Indicated are the Mur proteins and their substrates that might form a hyperstructure to synthesize lipid II.

2. Antibiotic inhibition of septal peptidoglycan synthesis

2.1. Transpeptidase activity

The Penicillin Binding Proteins or PBPs that synthesize peptidoglycan come in several variants. Some of the PBPs have D,D-carboxypeptidase activity, endopeptidase activity or D,D-transpeptidase (TP) activity that can all be inhibited by penicillins or β-lactams. These reactions all involve the binding of the last amino acids of the peptidoglycan peptide side chain, D-Ala-D-Ala, which has a structure similar to penicillin. To overcome the resistance to β-lactams alternative non-lactam drugs are investigated that inhibit the same reaction such as the γ-lactam Lactivicin and its derivatives ([7,8]) or compounds based on boronic acid that mimic the transition state of the enzymatic reaction, which show promising results (see for a review [9]).

2.2. Transglycosylase activity

The second reaction performed by PBPs is the polymerization of the dissacharide subunits of the peptidoglycan structure by transglycosylase activity (TG). The PBPs with TGase activity are bifunctional having TG and TP activity but enzymes with only TG activity are also present in many bacterial species. Clinically used inhibitors of the TG activity are not available. But the relatively recent publication of a number of crystal structures of bifunctional PBPs [10–12] (see for a review [13]) have given access to virtual drug screening and rational drug design. New high throughput TG assays have been described [14,15] that can replace the much more elaborate assays using fluorescently or radioactively labeled lipid II, followed by HPLC analysis of the synthesized compounds [16,17]. Lipid II (Fig. 2) is the precursor of the peptidoglycan-building unit and the substrate for the TG reaction. One of the screening assays is based on Förster Resonance Energy Transfer (FRET) to monitor the event of lipid II polymerization [14]. The lysine of the pentapeptide moiety of lipid II carries a donor fluorophore that is quenched by an acceptor fluorophore present in the lipid tail of the PG precursor. The transglycosylase reaction that attaches the dissacharide pentapeptide to the existing peptidoglycan will release the lipid moiety and increase the fluorescence of the peptide side-chain. The newly synthesized peptidoglycan is subsequently digested by N-acetylmuramidase to release the fluorescent peptidoglycan subunit in the soluble fraction of which an increase in fluorescence can be measured [14].

The only known inhibitor of the TGases is the natural product moenomycin (Fig. 2) [18] that mimics the structure of the donor substrate of the TG reaction (Fig. 2). It is a very effective antibacterial against Gram-positives that had a wide usage in the animal husbandry as growth stimulator [19]. Presently moenomycin is banned for this use, which allows its development for human treatment. A disadvantage of moenomycin is that the long lipid tail is essential for its activity but also gives the molecule poor phamacokinetics [19,20]. Therefore, attempts has been undertaken to make analogues with a shorter lipid tail [21]. Unfortunately the resulting molecules had low affinities for PBPs. However, a slightly shorter fluorescently conjugated moenomycin analogue with good PBP binding characteristics was successfully used in a replacement assay for the screening of a library of 110,000 small molecules [22]. Compound **10** (Fig. 2) of this collection replaced the fluorescent molecule in a dose depended manner and also inhibited the TG activity of several PBPs *in vitro* and bacterial growth *in vivo*, with IC_{50} and MICs in the tens of μM range, respectively. Although it has not yet been shown that these compounds also target the TG activity in the bacterial cell, this approach seems to be promising.

The acceptor substrate of the TG reaction is lipid-II (Fig. 2). Using the TG FRET assay [14] 120,000 natural and synthetic compounds were screened. About 25 hits were scored among which were well known inhibitors like vancomycin but also smaller molecules such as compound **24** (Fig. 2) that had a K_i of 2.3 ± 0.3 μM and a MIC of about 4 μM against several Gram-positive species. Others have synthesized Lipid II analogues to determine which parts of the precursor is essential for PBP binding, which resulted in some compounds with a K_i in the tens of μM and very weak antibacterial activity [23] and for a review see [24].

2.3. Lipid II synthesis

In *E. coli* and probably also in other bacterial species the proteins that synthesize Lipid II are shared by the elongation and the division machinery. The essential cytoplasmic proteins MurA,

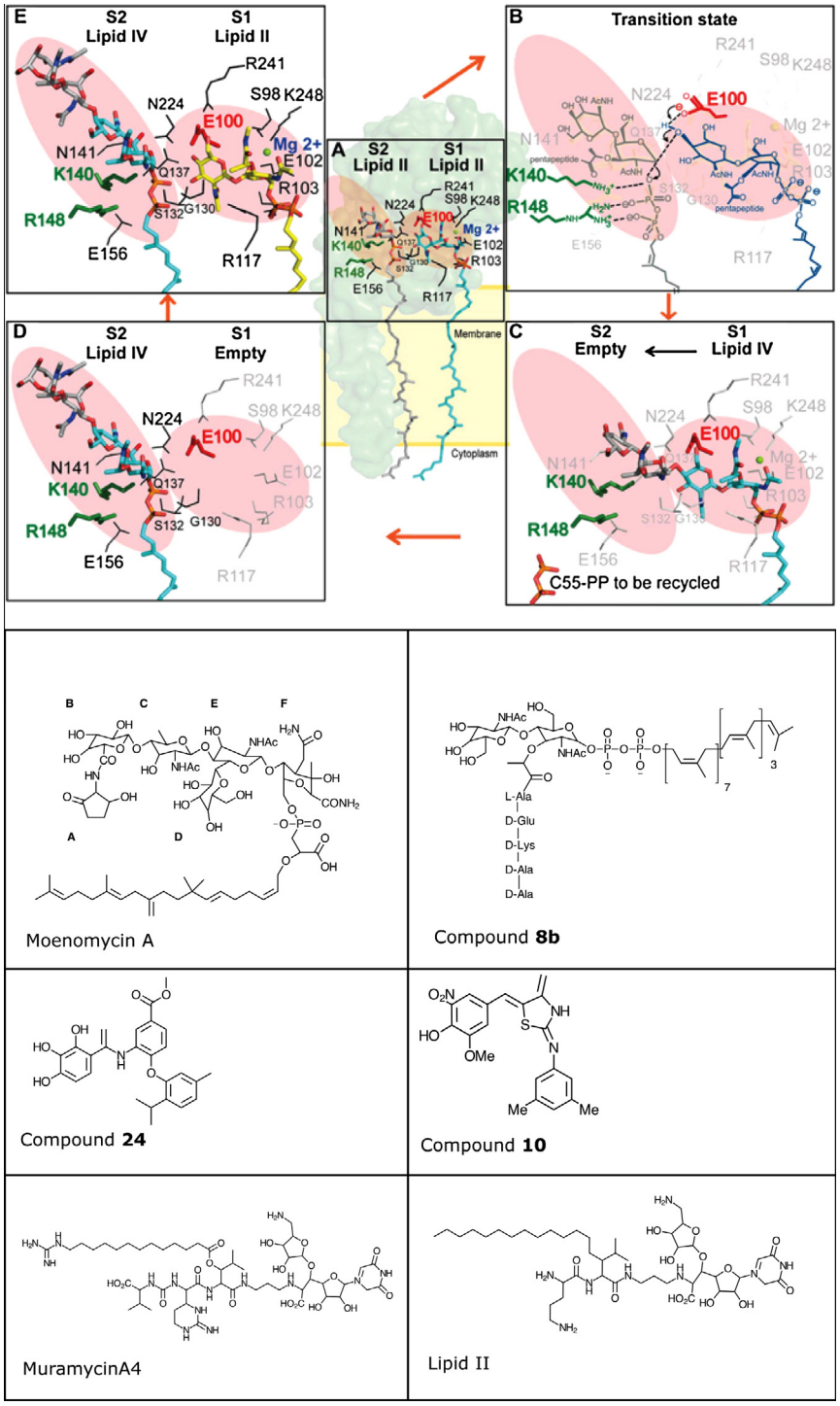

Fig. 2. Proposed mechanism for lipid II polymerization by trans glycosylases. (A) Two substrate binding sites: glycosyl acceptor (S1; shaded in red) and donor site (S2; shaded in red). The lipid II polymerization is initiated by accepting two lipid II substrates. (B) The 4-OH of GlcNAc of the lipid II (S1) is deprotonated by E100 (red stick) followed by a simultaneous reaction with the C1 of lipid II (or growing glycan chain) in S2, and the K140 and R148 (green stick) both facilitate the departure of the pyrophosphate-leaving group. (C) Lipid II (or growing glycan chain) at the glycosyl donor site (S2) reacts with the acceptor site of lipid II to form a β1–4-linked glycan chain. (D) The newly formed lipid IV is shuffled to the glycosyl donor site. (E) A new lipid II is docked at the glycosyl acceptor site (S1) again [12]. The lower panel shows the transglycosylase donor substrate analogue moenomycin A and the acceptor substrate Lipid II, the monomycin competitor compound **10** [22] and compound **24** found in the screen based on a FRET assay [14]. The last two compounds shown are the natural inhibitor of MraY muramycin4A and its derived compound **8b** [60].

MurB, MurC, MurD, MurE, MurF and the integral membrane protein MraY followed by the membrane associated protein MurG synthesize together lipid II and localize at mid cell during cell division (Fig. 1) [25–27]. The pathway has no counterpart in eukaryotic cells and is therefore deemed to contain good antibacterial targets. Crystal structures of all these proteins even for the integral membrane protein MraY [28] are available (see for a review [29] and [30]) and *in vitro* assays for their enzyme activity are well developed [31–33]. Consequently, many compounds have been synthesized that mimic the substrate molecules or transition state molecule of these proteins [30,34–39], which have high affinity for their targets. Unfortunately, the majority of these

compounds have weak or no antibacterial activity. This can be partly attributed to a failure to pass the bacterial membrane. An alternative explanation might be that although the Mur proteins function in simple one protein biochemical assays, it is possible that they form a hyperstructure [40] in bacterial cells. In such a multi-protein complex each protein passes on its reaction product to be used as substrate for the next protein. Possibly, the active sites of the proteins are not readily accessible for inhibitors in such a multi-protein complex.

2.4. MraY

MraY is maybe a slightly different case as it is an integral membrane protein that is also accessible from the periplasmic side of the cytoplasmic membrane. It catalyzes the transfer of muramylpentapeptide from UDP-muramylpentapeptide to the lipid carrier undecaprenyl phosphate to form Lipid I the precursor of lipid II (Fig. 1). Since its isolation and detailed biochemical and enzymatic characterization [41–46] and the development of high throughput screening assays [47,48], MraY has been investigated as a potential target for new antibiotics. The recently published crystal structure of the protein will give this research without doubt an extra boost [28]. Several natural inhibitors of MraY have been identified. The bacterolytic E peptide of the bacteriophage ΦX174 [49,50] does not interact with the active site of MraY but associates with the protein in the membrane [51] but does not inhibit the enzymatic activity of soluble MraY. The authors [51] suggest that the E-peptide inhibits a higher active state of Mray by preventing its association with another membrane protein (i.e. MurG or/and FtsW). Other natural compounds that inhibit MraY are tunicamycin, mureidomycin A and liposidomycin B [52,53], which inhibitory mechanisms are not well described. In search of the pharmacophore of these natural inhibitors a number of variants have been synthesized that show moderate activity [54–57] for liposidomycin B, and [58,59] for mureidomycin A. In a recent structure–activity relationship study of the muramycins (Fig. 2) [60] several molecules were synthesized that inhibited MraY in the nM range and also inhibited the growth of Gram-positive bacteria such as *Staphylococcus aureus* (see for an example compound **8b** in Fig. 2). Interestingly, the compounds were competitive inhibitors of UDP-MurNac pentapeptide but not of undecaprenyl phosphate despite the requirement for the lipophilic side chain of the compounds. As solubilized MraY was used for the assays, the inhibitory activity of the side chain cannot be explained by the inhibition of protein–protein interactions as in the case of the E-peptide.

Lipid II itself is also a target for antibiotics such as vancomycin and nisin (see for reviews [61,62]). Because this review deals with protein targets, lipid II targeting is beyond the scope of this review.

3. Inhibition of the cell division initiating protein FtsZ

3.1. FtsZ polymerization

Bacteria contain several cytoskeletal-like polymerizing proteins that are essential and that have been recognized as potential new targets for the development of novel antimicrobials [63]. The tubulin homologue FtsZ is essential in all bacteria. FtsZ is a 45-kDa protein that polymerizes into protofilaments using a GTP molecule to couple the FtsZ monomers head to tail. The GTP binding site is shared by the two FtsZ subunits. The major part of the nucleotide is bound by the T2, T3 and T4 loops of the FtsZ monomer, whereas the γ-phosphate and the magnesium-ion are also bound by the T7 loop of the incoming FtsZ molecule (see Fig. 3). Multiple protofilaments form a ring-like structure at mid cell underneath the cytoplasmic membrane (see for a review on the structure and

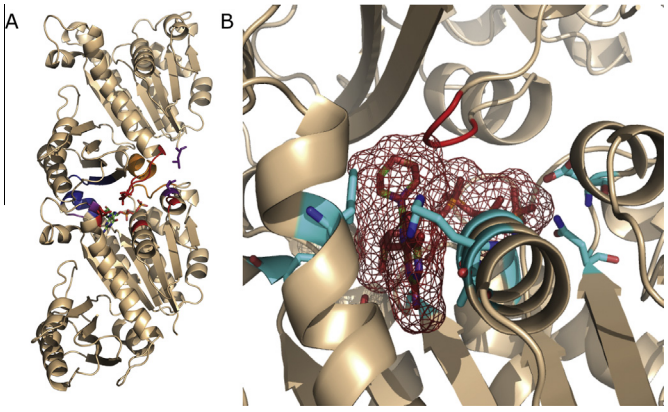

Fig. 3. (A) A dimer of *Bacillus subtillis* FtsZ (PDB 2vxy) modeled by homology onto the *Staphylococcus aureus* FtsZ filament crystal structure (PDB 3vo8) with GDP extended to GTP. The loops of both subunits that interact between them are colored. B. The same structure but with GTP replaced by 8-morpholino-GTP in its NMR-determined anti-conformation [81]. The T7-loop (in red) of the incoming FtsZ molecule clashes with the electron density of the nucleotide analogue.

function of FtsZ [64]). Hydrolysis of GTP causes dissociation of the polymers. The half-life of the Z-ring in the bacterial cell is about 10 s [65]. This dynamic nature of the ring is essential, as the diameter of the ring has to become continuously smaller during the division process. Although the precise structure and function in the cell is still debated, inhibition of the Z-ring formation invariably leads to inhibition of cell division, filamentation and eventually to cell death. Crystal structures of FtsZ from a number of bacterial species are available [66–70] and *in vitro* assays to detect its polymerization state by light scattering [Mukherjee:1999wa 71] or to determine its GTPase activity by fluorescence [66] or colorimetry [72] are well established. Also a medium throughput assay to detect its polymerization is available [73]. In addition, the localization of FtsZ can easily be monitored in the bacterial cell by FtsZ-fluorescent protein fusions [74,75] and details of the filaments formed *in vitro* can be assessed by electron microscopy [76]. The possibility to screen for FtsZ inhibitor *in vivo* and *in vitro* makes it an attractive new target for antibiotics. The fact that a small mouse macrophage derived peptide CRAMP appears to inhibit the Z-ring when expressed in *Salmonella typhimurium* [77] and that *B. subtilis* uses a small peptide MciZ to prevent Z-ring formation [78], indicates that natures also uses FtsZ as an inhibitable target.

The precise regulation of the FtsZ polymerization in the bacterial cell to ensure that the Z-ring is not prematurely produced or misplaced or persists too long during the division process makes that different types of FtsZ inhibitors can be envisioned. These are: type 1; molecules that prevent the association between two FtsZ subunits by blocking the GTP binding site or other interfacial site, type 2; molecules that stabilize the protofilaments or promote protofilament bundling, which also inhibits the GTPase activity, type 3; molecules that interfere with one of the many interactions of FtsZ with other essential cells division proteins.

In addition, some of the FtsZ inhibitors might also inhibit Tubulin or *vice versa* given their similarities in the structure of the GTP binding site. Because the amino acid sequence of the two proteins does not have a high degree of identity, the chance of finding an inhibitor that affects both proteins equally is in general limited. However, the possibility should be taken into account in particularly with inhibitors binding to the GTP binding site.

3.2. Rational drug design

Of type 1 are the majority of the compound that are based on rational drug design. Addition of GTP analogues with bulky

substitutions such as a methoxy- or a morpholino-group at the C8 position (Fig. 4) [66,79–81] inhibit FtsZ polymerization. The GTP analogues are still able to bind FtsZ, but since the C8 group protrudes from the surface of the FtsZ molecule at the interface of the subunits in a protofilament, binding of the GTP analogues prevent the association of a second FtsZ subunit. This also inhibits the GTPase activity because the second molecule is essential for the hydrolysis of the gamma phosphate of GTP (Fig. 3). Interestingly despite the strong homology between the FtsZ genes of the various species and the almost identical active sites, variations in the inhibitory mode of the GTP analogues were still observed [81]. Despite the many similarities between the polymerization behavior and structure of Tubulin and FtsZ, the GTP analogues with a relative small group at the C8 position did not inhibit tubulin polymerization [66]. Although GTP analogue as such are not very suitable as antibiotics, the results of these studies are very informative for further design of FtsZ inhibitors and are promising for the selectivity of FtsZ interface association inhibitors (see Section 3.3).

An alternative approach is again imitated from nature. Under stress conditions in which the bacteria have to postpone cell division, the FtsZ polymerization inhibiting protein SulA is synthesized. This protein binds to the T7 loop of FtsZ and reduces so the number of FtsZ molecules that can participate in the Z-ring formation [82,83]. Molecules that bind the T7 loop but also molecules that mimic the T7-loop [84] would inhibit the addition of new FtsZ molecules to the existing protofilaments. Because of the very dynamic nature of the Z-ring, such molecules would effectively inhibit cell division.

3.3. Bioinformatics tools

In structure based drug design small molecules are docked onto the crystal structure of the protein target in order to investigate possible chemical modifications of the molecule that could improve the specificity. X-ray crystallography together with bioinformatics has been very successful for drug design as have been demonstrated in different areas [12,85–87]. One of the most relevant drugs creating studies was the design of the antiviral Tamiflu [88]. An analysis of the FtsZ inhibitors shows that several of them are aromatics compounds with more than one ring, like, polyphenols, zantrin and those derived from indol groups [89]. These inhibitors can be used as templates when they are bound to the protein binding site, whose tridimensional structure can be obtained from the crystal or the NMR structure of the protein–inhibitor complex or predicted from a reliable model of the complex obtained by molecular docking and molecular dynamics. Unfortunately, crystal structures of FtsZ in complex with only two inhibitors (morpholino-GTP and PC190723; [66,67,90,91]) are known at the time of writing this review. Therefore, this strategy has been used to search for synthetic compounds that replace GTP and inhibit FtsZ polymerization and cell division [92,93]. Based on the docking study, a number of small inhibitors and their simplified derivatives were tested in a mant-GTP competition assay on FtsZ molecules of *B. subtilis* [92]. Compound UCM44 and a chlorinated analog UCM53 (Fig. 4) had a Kb $\sim$ 1–1.5 μM for FtsZ and inhibited the growth of *B. subtilis* with a MIC of $\sim$13–25 μM. Subsequently, UCM-44 and −05 were tested on FtsZ of three very different species; the Gram-positive bacterium *B. subtilis*, the Gram-negative bacterium *E. coli* and the archaea *Methanococcus jannaschii* using a variety of assays to monitor FtsZ assembly and GTPase activity as well as investigating the polymers by electron microscopy. Interestingly, the compounds caused an aberrant assembly of the *B. subtilis* FtsZ polymers *in vitro* and dissociation of the Z-ring and aggregation *in vivo* but only weakly inhibited *E. coli* FtsZ. They acted as non-hydrolyzable GTP analogues on the archaeal FtsZ and also affected the structure of its protofilaments. The compounds were relatively non-toxic to eukaryotic cells and lacked effect on microtubules at much higher concentrations than the MIC found for the inhibition of bacterial growth. In conclusion these are the first promising and species-specific hit compounds that have been obtained starting from an *in silico* approach.

3.4. Screening for FtsZ inhibitors

Screening of small compound libraries or of molecules of natural sources have resulted in a large number of FtsZ inhibitors including 4-aminofurazan derivative-A189 [94], OBTA [95], trisubstituted benzimidazoles [96], Zantrins [97], 3-methoxybenzamine derivatives [98–100], rhodanine [101], dibenzo[a,g]quinolizin-7-ium and 5-methylbenzo[c]phenanthridinium derivatives [102–104], guanidinomethyl biaryl [105], anti-Mycobacterium tuberculosis screens [96,106–108], natural compounds; berberine [109,110] and derivatives [111], Chrysophaentins [112], cinnamaldehyde [113], curcumin [114,115], sanguinarine [116] and derivatives [117], sulfoalkylresorcinol [118], SA-05 [119], viriditoxin [120], Plumbagin [121] and dichamanetin [122], phenylpropanoids such as cafeic acid [123], anti-tubulin compounds [5,5-bis-8-anilino-1-naphthalenesulfonate] [124], totarol [125], albendazole sulfone [126] and taxanes [127]. Although many of these compounds have been shown to inhibit FtsZ polymerization *in vitro* and *in vivo* (e.g. sanguinarine), their primary target is not always FtsZ and the IC50s are often more than 50 μM. The association to the cytoplasmic membrane of many proteins is sensitive to the presence of the transmembrane potential or $\Delta\Psi$, which is the difference in charge between the cytoplasm side and the periplasmic or outside of the cytoplasmic membrane. The loss of $\Delta\Psi$ causes the dissociation of FtsA and consequently the release of FtsZ from the membrane in *B. subtilis* and inhibition of cell division [128]. Many of the compounds that are weak lipophilic acids with electron withdrawing groups that have been shown to cause dislocation of FtsZ, appear to affect the $\Delta\Psi$ as their first mode of action [129]. All cytoplasmic membranes have a net charge difference and the putative FtsZ inhibitors that cause a loss of $\Delta\Psi$ in bacteria, will also depolarize eukaryotic mitochondrial membranes. This is illustrated by the depolarization of mitochondria of mouse melanoma cells by for instance sanguinarine [130]. Interestingly, some membrane depolarization compounds seem to be able to discriminated bacterial and eukaryotic membranes [131]. Therefore, the FtsZ inhibitors that affect the polarity of the membranes should not by default be disregarded.

As many reviews have been written on FtsZ inhibitors, only the more recent and most effective compounds will be discussed in this review. In a structure activity relationship (SAR) study on trisubstituted benzimidazoles, several small variants were characterized that inhibited *Mycobacterium tuberculosis* growth at about the same concentration as they inhibited FtsZ polymerization *in vitro* (e.g. compound **5f** in Fig. 4). The compounds were not cytotoxic in an epithelium cell assay, suggesting that these molecules do not affect membranes and might be indeed specific for FtsZ [108].

Screening of a bank of 151 rhodanine compounds revealed a molecule CCR-11 (Fig. 4) that had a high affinity for FtsZ (Kd 1.5 ± 0.3 μM), inhibited its GTPase activity *in vitro*, FtsZ-ring formation *in vivo* and inhibited *B. subtilis* growth with a MIC of 3 μM. Growth of HeLa cells was inhibited but at a 15-fold higher concentration. CCR-11 did not compete for the GTP binding site of FtsZ and based on *in silico* docking it might bind in a cavity close to the T7 loop and inhibit the interaction of the loop with the GTP in the binding site of the other FtsZ monomer [101].

The natural products Chrysophaentins A-H (Fig. 4) are a family of bisdiarylbutene macrocycles isolated from the marine chrysophyte alga *Chrysophaeum taylori* that inhibit the growth of several Gram-positive bacterial species including the methillicin resistant *S. aureus* (MRSA) and the vancomycin resistant *Enterococcus*

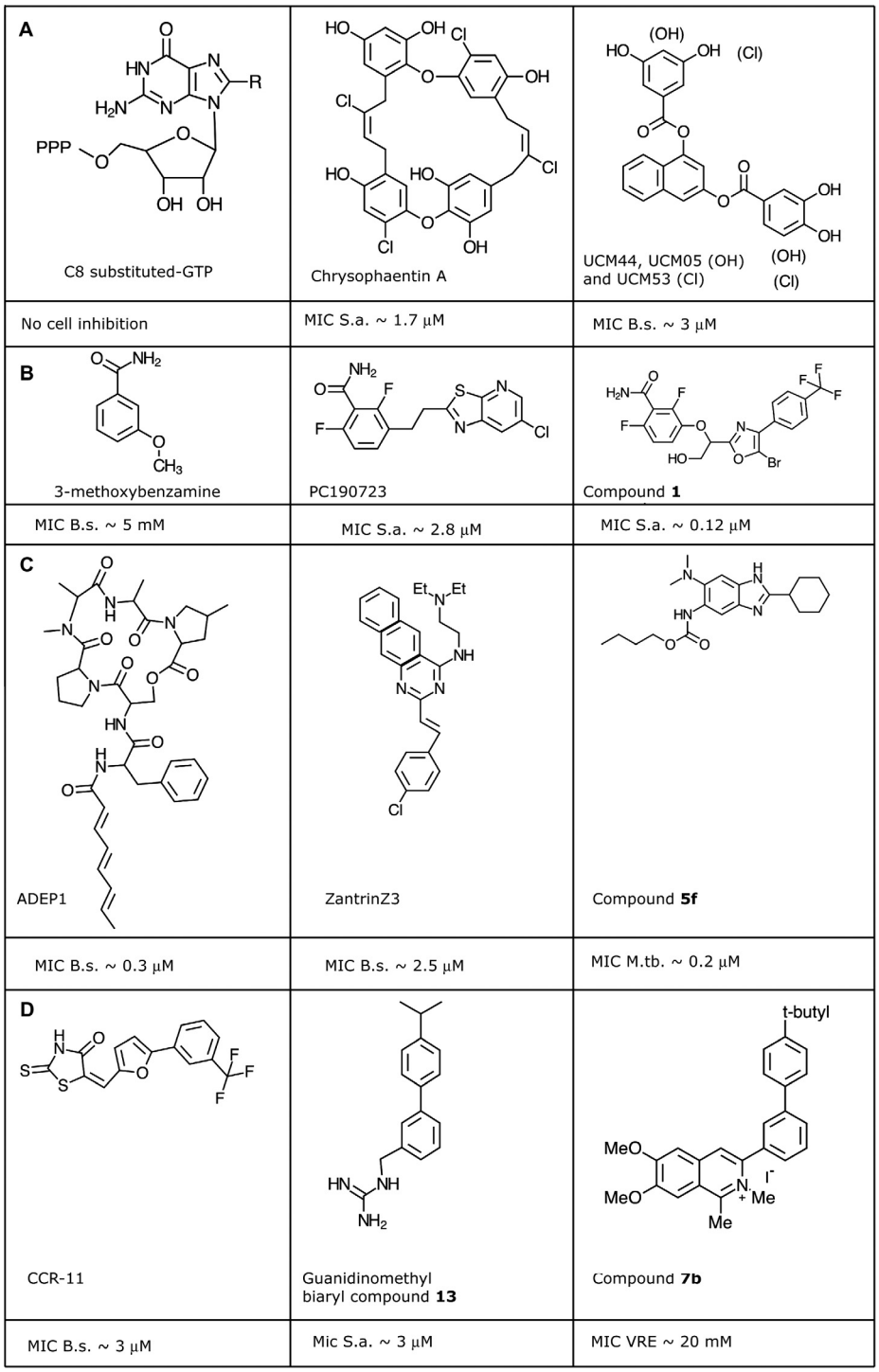

Fig. 4. Examples of FtsZ inhibitors. (A) Inhibitors that compete with GTP for binding. C8-substituted GTP analogues (R could be -H, -Cl,-Br, -I, Me, OMe, Pyrr, -tBu,-Ph, -Morph, -NMePip)) have been useful for the *in silico* search for inhibitors [66]. Chrystopheantin A [112] and UCM44 [92] are both promising hits for further development. (B) Inhibitors that do not bind close or in the GTP binding pocket. The inhibitor 3-methoxy benzamine [136] and its improved derivatives PC190723 [98] and compound **1** [135]. (C) Indirect FtsZ inhibitors such as ADEP1 [186], inhibitors with unknown binding site such as the zantrins of which zantrin 3 is one of the few inhibitors that inhibits *E. coli* cell growth with a MIC of 5 μM provided that the multidrug efflux pump AcrAB has been deleted [97] and trisubstituted benzamidazoles such as compound **5f** [108]. (D) Rhodanine-like compound CCR-11 [101], Guanidiomethyl biaryl compound **13** [105] and the beberberine-like compound **7b** [111] that acts on vancomycin resistant *Enterococcus feacalis* (VRE) seem to be non-competitive inhibitors of FtsZ. B.s. is *Bacillus subtilis*, S.a. is *Staphylococcus aureus*, MRSA is Methicillin resistant S.a. and M.tb. is *Mycobacterium tuberculosis.*

faecium. [112]. The compounds were shown to be competitive inhibitors of the GTPase activity of FtsZ *in vitro* and *in vivo* by occupying a large part of the GTP-binding site of the protein [112,132]. Algae isolated from different sources produced a different spectrum of chysophaentins including linear molecules that still have bactericidal properties [133]. A number of Chrysophaentin fragments was synthesized and analyzed in a SAR study [133], which showed that although the cyclic variants are more potent, the linear fragments have a good efficiency for their size [132,133] and are a good starting point for further synthesis.

SAR studies on synthetic analogues of sanguinarine and berberine [111,117] produced several compounds with improved antibacterial activity. Compound **7b** (Fig. 4) inhibited the GTPase activity of *S. aureus* FtsZ, did not inhibit tubulin polymerization and had good antibacterial activity against this species as well as activity against vancomycin resistant *Enterococcus faecalis*, which is not inhibited by the parental compound.

3.5. One molecule that cures infection models

PC190723 [98,134,135] and its further optimized derivative compound **1** [135] are based on the weak inhibitor 3-methoxybenzamide [136] (Fig. 4). The heterocyclic compound **1** FtsZ inhibitor has a very good activity against gram-positive bacteria (MIC of 0.12 μM) and was shown to cure mice from a *S. aureus* infection. The PC190723 can be easily synthesized [137] and has no effect on the $\Delta\Psi$ or the permeability of the cytoplasmic membrane [129]. Based on mutants of *S. aureus* FtsZ that are not able to bind PC19073 and on a co-crystal structure of GDP-FtsZ and PC19073, the compound binds in between the C-terminal domain and the central helix 7 close to the T7 loop of FtsZ [67,90,91]. This position might also explain the specificity for *S. aureus* FtsZ as the amino acids in this region and the residues that bind the compound are not completely conserved between various bacterial species.

A N-Mannich base derivative of PC190723, TXY436, has a 100 times higher solubility than its precursors and acts as a prodrug, which is converted to PC190723 with a half-live of ~18 min. TXY436, was shown to inhibit FtsZ-ring formation *in vivo* and to be efficacious in a mouse model against both MRSA oral and systemic infections [138]. Similar results were found for another prodrug TXY541 of PC190723 [139].

3.6. On in vitro assays

FtsZ polymerization has proven to be fairly complicated given the massive number of papers that contradict each other on the subject. FtsZ polymerizes cooperatively and needs a critical concentration to be able to polymerize [140–144]. The reaction is sensitive to pH and salt concentration [71,145] and the protofilaments can easily be made to aggregate and bundle in a variety of structures [146–149]. It is therefore very easy to misinterpret the effects the addition of compounds have. For instance, if the GTPase activity of the protein, which is a consequence of its polymerization, would be determine without analysis of the structure of the polymers by a light scattering assay, sedimentation, or by electron microscopy, one could easily think that the GTPase activity is inhibited whereas in reality the compound stimulated bundling of FtsZ, which automatically reduces the GTPase activity. Because of this type of misinterpretations, it is useful to do the FtsZ activity assays in the presence of not more than 5 mM $MgCl_2$ in case of *E. coli* FtsZ. Under these conditions all polymers are virtually protofilaments [147,150]. For FtsZ of other bacterial species different assay conditions can apply [151]. High concentrations (more than 10 mM) of cat-ions cause aggregation of FtsZ and discrimination between aggregation due to addition of the compound or due to the cat-ions becomes difficult. Many drugs absorb light or are fluorescent. The absorption of the compound can cause an inner-filter effect in the light-scattering assay and in the GTPase assays that are based on fluorescence spectroscopy. The standard conditions for the light scattering assay are excitation and emission at 350 nm. The inner filter effect can be avoided by excitation and emission at 450 or 550 nm although the signal will become weaker. Aggregation of the added compounds, could also lead to an under estimation of the extent of FtsZ polymerization by light scattering. Aggregates can easily be discriminated by light scattering or by centrifugation and filtration of the compounds. A good positive control is the FtsZ inhibitor zantrin 3 (Fig. 4), which inhibits the polymerization of a number of FtsZ proteins form various species and does not aggregate [132,152]. A SAR study of zantrin 3 showed that most groups are essential for efficient inhibition of FtsZ [152].

GTPase activity can be measured through several different methods (see Table 1 for an overview of specific methods for FtsZ GTPase activity). It is of paramount importance to measure the effect of inhibitors on the FtsZ polymerization and GTPase activity in an assay that resemble the physiological conditions of the cell. To keep the concentration of GTP constant during the polymerization reaction at least two enzymatic GTP regenerating systems have been used. The most commonly applied has been acetate kinase that uses acetyl phosphate to phosphorylate GDP [153]. The extension of the polymerization steady state can be controlled through the concentration of acetyl phosphate. A similar coupled system uses pyruvate kinase to convert phosphoenol pyruvate to pyruvate coupled to the phosphorylation of GDP [154]. The GTPase activity can be measure continuously adding the enzyme pyruvate dehydrogenase plus NADH to produce lactate and NAD^+. The conversion of NADH to NAD^+ is monitored by the decrease in absorbance at 340 nm. To quantify the amount of GTP hydrolyzed an extinction coefficient of 6220 $M^{-1}cm^{-1}$ is used for NADH [155]. It should be noted that to determine the phosphate content at different times during the course of the reaction the malachite green dye method can be used only in combination with the pyruvate/pyruvate kinase coupled system because acetyl phosphate is hydrolyzed in acid conditions.

For historical reasons the majority of the polymerization assays for FtsZ are performed at pH 6.5 whereas the physiological pH for

Table 1
Specific methods to determine the GPase activity of FtsZ.

GTP version	Enzymes or FtsZ mutants employed	Treatment	Compound measured	Measurement method	Source
GTP-γ^{32}P		Perchloric acid and $KHCO_3$	Radioactive phosphate	Scintillation	[207]
GTP		Perchloric acid and $KHCO_3$	GTP and GDP	HPLC	[208]
Mant-GTP			Mant-GTP release upon competition	Fluorescence anisotropy	[209]
GTP		Malachite green and molybdate	Green molybdophosphoric acid	Absorbance at 630 nm	[210]
GTP	Nucleoside phosphorylase	Fluorescent 7-methylguanosine	Non-fluorescent 7-methylguanine (7MG) and ribose 1-phosphate	Decrease in fluorescence emission at 390 nm	[66,79,140]
GTP	FtsZF268C-tetramethylrhodamine (acceptor) and FtsZL68W (donor)		FRET	Fluorescence	[211]
GTP	FtsZY22W-S151C-ATTO-655		Trp quenching decrease	Fluorescence	[212]

most bacterial species is pH 7.0–7.5. The GTPase activity of FtsZ is higher and the polymers less stable at pH 7.5 [71,150]. Although working at physiological pH *in vitro* makes the experiments less easy, it would probably enhance the chance to find an inhibitor that has some *in vivo* use. Many drugs aggregate [156] and because of that sequester the protein target instead of inhibiting it or bind without specificity to proteins *in vitro* and bind for that reason to non-related targets *in vivo*. Addition of TritonX-100 and BSA to drug screening assays abolishes many of those promiscuous interactions, which reduces the number of false positives. Although Tx100 has been used at low concentrations to test FtsZ inhibiting drugs (den Blaauwen unpublished results and [152]), unfortunately it also inhibits the polymerization reaction of FtsZ [92]. Therefore, this aspect of FtsZ drug screening needs further optimization. Another possibility is to include crowding agents to mimic the cytoplasmic conditions more closely and prevent non-specific binding. Notably, crowding agents such as high concentrations of BSA or ficoll in general [157,158] will push the FtsZ polymer/bundle equilibrium towards bundling. In the absence of the other divisome proteins, this might lead to over interpretation of the importance of bundling.

Several methods based on nuclear magnetic resonance (NMR) have been applied to drug discovery [159], especially for the screening of new drugs [160]. Among them, saturation transfer difference NMR (STD NMR) has been used to characterize the binding of ligands to macromolecules at atomic resolution [161]. In a STD NMR experiment the sample should contain both the macromolecule and the ligand in a molar ratio 1/100, which allows the quantification of the macromolecule-bound and free ligand. This method has been primarily used for the characterization of protein-carbohydrate interactions, after its introduction by the Meyer group [162]. Applications to FtsZ ligand interactions have been rare, however, the binding of two natural plant alkaloids, cinnamaldehyde [113], berberine [110], and from algae isolated Chrystopaentins [112] to FtsZ has been characterized with this method. These STD NMR studies in combination with *in silico* molecular docking suggested that cinnamaldehyde binds to a pocket at the C-terminal region involving the T7 loop of FtsZ. STD NMR experiments clearly show that H2 and H3 protons of the conjugated alkene of cinnamaldehyde are in close contact with FtsZ protons in its binding pocket [113] and berberine binds to a hydrophobic pocket of FtsZ [110]. More recently the binding of C8-substituted guanosine nucleotides to *M. jannaschii* and *B. subtilis* FtsZ was studied at molecular level using transfer NOESY and STD NMR experiments. The results showed that the binding of the C8-adducts produced changes in size, shape and electrostatic surface at the interface of FtsZ monomers that explained the polymerization inhibition by these guanosine nucleotide analogs [81].

3.7. In cell screening assays

The multitudes of FtsZ *in vitro* inhibitors that have been published illustrate how difficult it is to find or design a chemical compound that inhibits FtsZ in cells. Therefore, it is instrumental to design very specific and efficient screening assays that reveal simultaneously whether a compound is killing bacteria as well as reveal the target of the compound. Such an assay has been described and used for the screening of 105,000 synthetic compounds in *B. subtilis* [163]. The assay combined two reporter-constructs that produced different colors, one that expressed β-galactosidase constitutively in the cytoplasm till the cells start to sporulate and one that expressed β-glucuronidase at the onset of sporulation. The assay is performed in a multi-well plate with in each well a mixture of sporulating and exponentially growing bacteria, which gives in a colorimetric measurement a particular color ratio. Inhibition of cell division will inhibit sporulation and will cause a shift in the color ratio. The screen yielded a cell division inhibiting compound that was shown to inhibit FtsZ *in vitro*. Unfortunately this screen did not yield a clinically suitable inhibitor and has not been further used for other screenings as far as publications reveal.

An elegant cell-based screening was based on with *Wolbachia* infected *Drosophilla* cell lines that constitutively produced a Jupiter-GFP fusion that labeled microtubules [126]. *Wolbachia* is an obligate symbiont of nematodes that are the causative agent of African river blindness. Killing *Wolbachia* also kills the nematodes, therefore an assay was developed to screen for anti *Wolbachia* compounds. The bacteria interact with interphase microtubules, which allows high throughput automated microscopy to look for a reduction in the *Wolbachia* titer. Of 5000 compound about 40 were found to have an effect among which was the FtsZ inhibitor totarol. Three compounds showed structural similarity to albendazole a compound approved to treat nematode infections. Surprisingly, these compounds did not show toxicity against eukaryotic cells. The effective molecule was albendazole sulfone a breakdown product of albendazole that does not affect microtubule assembly. Therefore it was suggested that the compounds inhibit *Wolbachia* FtsZ.

An alternative approach could be to use Förster Resonance Energy Transfer (FRET) to screen in living bacteria for the loss of FtsZ interactions (i.e. polymerization). If FtsZ is expressed as two different fluorescent reporter proteins within one cell of which the donor protein can transfer its fluorescence to an acceptor fluorescent protein in polymerized FtsZ, it is possible to measure the loss of energy transfer upon addition of an FtsZ polymerization inhibiting drug. The advantage of such an assay is that one would simultaneously screen against promiscuous binding, for membrane impermeability and for target specificity. This would be much more efficient than to screen inhibitors with an FtsZ polymerization or GTPase activity *in vitro* assay to discover that the selected inhibitor with nM affinity cannot pass the bacterial envelop. Alternatively, and potentially equally inefficient is doing an *in vivo* screen to find a nice growth inhibitor without the faintest idea what its target is and therefore having a high probability that a similar target will exist in eukaryotic cells. Although these FRET assays do exist [5,164,165], they have at this moment not yet been adapted to high throughput plate reader modes.

4. Other proteins related to cell division as potential antimicrobial target

4.1. FtsA, ZipA and ClpP

The *divisome* formation can be separated in 2 stages. In the first stage the assembly of the Z-ring occurs and a ternary complex called proteo-ring is the first macrostructure formed by the interaction of the Z-ring with ZipA and FtsA (and other regulatory proteins) to anchor this ring to the inner surface of the cytoplasmic membrane and to recruit later the other proteins of the *divisome* [3,166–168]. Recently, it has been proposed that FtsA has an active dynamic role in the contraction of the ring, while ZipA plays a more passive structural role [169]. The interaction of FtsZ with ZipA and FtsA is essential [170,171] and it is produced through its C-terminal region [172,173]. The amino acids residues of this region that are involved in the interaction with ZipA and FtsA are different [174] and not all bacteria have both proteins. This will allow the use of small compounds and mimetic peptides to inhibit selectively these interactions. Five small peptides that inhibit the ATPase activity of FtsA were selected from a large pool of random peptide permutations, using the phage-display technique [175]. By competition with FtsZ at least one peptide was found to affect the

interaction of FtsZ with FtsA. Potentially, small molecules that bind with high affinity to the ATP binging site of FtsA will be a means to find future antibiotics due to the low affinity of this protein for ATP [176]. ZipA contains a hydrophobic cleft in which a peptide of 17 residues of the C-terminus of FtsZ was co-crystalized [172]. The Kd of 7 μM for this interaction was determined using a fluorescence polarization based assay [177]. With the same assay in a high-throughput screen of 250,000 compounds several small aromatic derivatives were selected and the best of them inhibited the FtsZ–ZipA interaction with a K_i of 12 μM and was shown to bind to the same hydrophobic pocket on ZipA as the FtsZ peptide [177]. Unfortunately, this pyridylpyrimidine is toxic for eukaryotic cells. Therefore, its structure determinants were used in an *in silico* approach to screen for additional small molecules [178], which yielded three new scaffolds as leads for the inhibition of this interaction. Combinatorial chemistry of indol based compounds have yielded derivatives that inhibit this interaction in the μM range: 3-(2-indoyl) piperidines and 2-phenyl indoles [179]; indolo[2,3-a]quinolizin-7-one [180] and carboxybiphenylindole [181]. NMR-based fragment screening of an 825 fragment library and subsequent combinatorial chemistry of the most promising molecule did not yield a molecule with improved binding parameters [182]. The absence of recent papers on the subject, suggests that ZipA has been abandoned for the moment as suitable target or is continued in a pharmaceutical context.

Part of the regulation of FtsZ comes from its degradation at the end of the cell cycle by the ClpXP ATP-dependent protease. ClpX recognizes the C-terminal flexible domain of FtsZ [183] and presents it unfolded for proteolysis to ClpP. ClpX forms an hexamer attached to the tetradecameric ClpP and stimulates the degradation of unfolded proteins in general by ClpP [184]. A new class of antibiotics, Cyclic acyldepsipeptides or ADEP, activates ClpP independent of ClpX [185] and uncontrolled FtsZ degradation causing filamentation and cell death in *B. subtilis* [186].

4.2. FtsEX

In the second stage the rest of the proteins are recruited sequentially to the septal ring. The first complex recruited in the second step is FtsEX, which poses structural and sequence similarity to an ABC transporter [187], where FtsE corresponds to the ATP binding subunit and FtsX to the integral membrane subunit (Fig. 5). ATP binding by FtsE is critical for division, but not in the formation of the divisome. Both proteins FtsE and FtsX have to be present in the septal site for the recruitment of the later proteins [188]. FtsE and FtsX are essential in the pathogenic bacterium *Streptococcus pneumoniae* [189] but not in *E. coli*, where they are only essential at low ionic strength growth conditions [187]. It has been proposed that the PG hydrolytic activity at the division site in both bacteria is controlled by the ATPase activity of FtsEX through the protein PcsB in *S. pneumoniae* [190] and EnvC in *E. coli* [191,192] (Fig. 5). The inhibition of the ATPase activity of FtsE or interference in the formation of these regulatory complexes could be a good strategy for the development of new antibiotics.

4.3. FtsW

The essential cell division integral membrane protein FtsW interacts with PBP3 essential for septal peptidoglycan synthesis in *E. coli* [165]. The protein is able to translocate lipid II *in vitro* [193] and specific residues in the first four transmembrane helices were shown to be essential for this activity. Expression of the mutants resulted in a block in cell division and even a dominant negative lethal phenotype ([213]). These characteristics suggest that FtsW is a suitable target for new antibiotics. The availability of an *in vitro* activity assay [193] and an *in vivo* FtsW-PBP3 interaction assay [165] will have to be developed into high throughput screening to be useful for efficient screening. Although the topology of the protein is known [194], the structure of the protein urgently needs to be resolved for *in silico* screening of chemical compounds and to determine the binding site of its substrate lipid II. It would be interesting to test the recently developed lipid II analogues as competitors in the existing *in vitro* FtsW activity assay.

4.4. FtsQLB

The trimeric FtsQ, FtsL, and FtsB complex is essential for cell division and thus far has escaped its functional elucidation. All three proteins are bitopic membrane proteins with the largest domain in the periplasm. FtsQ, -L and -B have been shown to interact with almost all cell division proteins by bacterial two hybrid assays [195–200] and with ZapA and FtsN using an in cell FRET assay [164]. Possibly, the heterotrimer functions as the structural core for the assembly of late division proteins. Because FtsQ is present in only 25–50 copies per cells, it might also be the

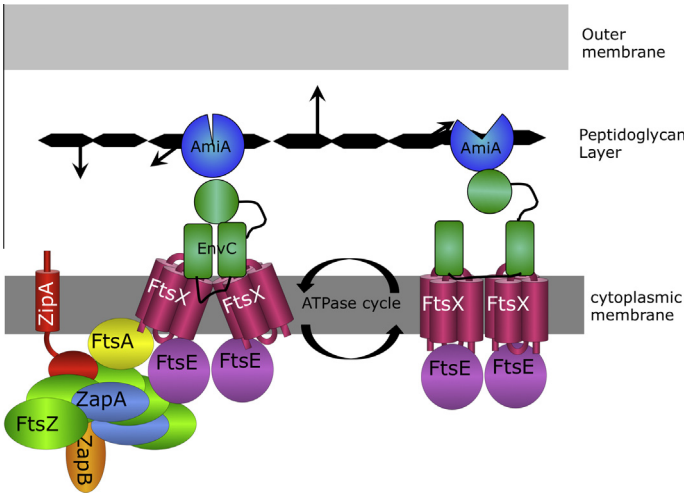

Fig. 5. Model for FtsEX function in regulating PG hydrolase activity at the division site. Shown is a schematic diagram of a putative FtsEX-EnvC-amidase complex at the Z-ring. It is proposed that conformational changes in FtsEX induced by FtsE-mediated ATP hydrolysis are transmitted to EnvC to control its ability to activate the amidases so that they can cleave the septal PG [191,192]. The model is not meant to reflect actual interaction stoichiometries, because they have yet to be determined. In addition, it is not yet clear if the amidases remain in complex with EnvC as drawn or if this interaction is also regulated (figure adapted from [191]).

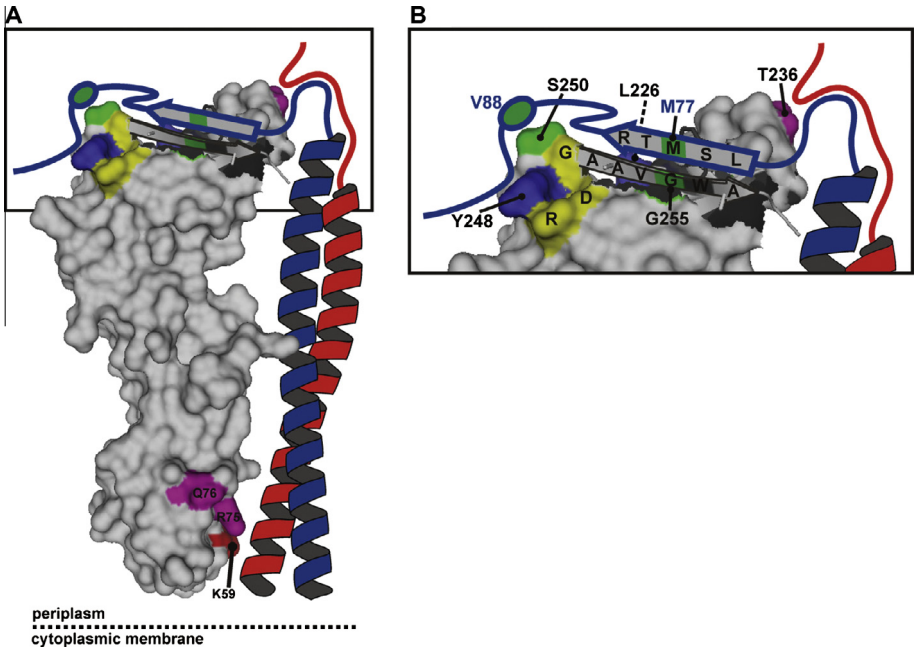

Fig. 6. Model of the periplasmic part of the *E. coli* FtsQBL complex. (A) A surface plot of the periplasmic domain of FtsQ exposing the C-terminal β-strand in graphic style was created in PyMOL using PDB code 2VH1. The surface plot of the structure of FtsQ amino acids 58–260 is in dark gray with the following exceptions. Red, position that showed cross-linking biased toward FtsL; blue, position that showed cross-linking biased toward FtsB; purple, position that showed apparently equal cross-linking to both FtsB and FtsL and green the cysteine cross-linking positions FtsQ 250 and 255 and FtsB 77 and 88. FtsB (blue) and FtsL (red) are drawn schematically forming a coiled-coil that contacts FtsQ in the α-domain around residue 59. (B) A close-up of the distal end of the model. The C-terminal regions of FtsB and FtsL are both in close proximity to residue Thr-236 of FtsQ. The C-terminal region of FtsB around residue 77 further engages in a β-sheet-like interaction with the C-terminal β-strand of FtsQ, whereas the region around residue 88 interacts with the hot spot on FtsQ including residues Leu-226, Tyr-248, and Ser-250. Surrounded by these extensive interactions are residues Asp-245, Arg-247, and Gly-251 (yellow) that are part of a strong consensus motif, DLRY(d/e)(s/t)G. This figure was originally published in [204].

determinant for the number of septum synthesizing complexes along the Z-ring. A combination of *in vitro* reconstitution [201], small-angle neutron and X-ray scattering [202], modeling [203], cross-linking [204] and crystallography [205] has provided information on the interaction surfaces and the stoichiometry of the three proteins (Fig. 6). The binding sites of FtsB and FtsL on FtsQ are sufficiently identified to start an *in silico* docking experiment with small molecule libraries to identify competitive inhibitors of the interaction between the three proteins. It has been proposed that the FtsL instability could be a control point in the divisome formation, given by the proteolysis of FtsL by the metalloprotease YulC [206]. This will be also a point for the design of new inhibitors.

5. Concluding remarks

Many bacterial cell division proteins are essential. The inhibition of their activity or their ability to assembly as part of the division machinery will result in loss of viability in many bacterial species. Although the core of the cell division proteins is similar in most bacterial species investigated thus far [3], many additional proteins are present that are unique to their genus and their inhibition might provide species specific antibiotics. Due to availability of crystal structures, enzyme assays, high throughput screening assays and in cell assays, these proteins have become accessible to drug design and screening. This review shows that many activities in this direction are undertaken, but given the challenge to develop new antibiotics it may take time before the first cell division inhibitor will be approved for the medical market.

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