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FtsW activity and lipid II synthesis are required for recruitment of MurJ to midcell during cell division in *Escherichia coli*

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Summary

Peptidoglycan (PG) is the unique cell shape-determining component of the bacterial envelope, and is a key target for antibiotics. PG synthesis requires the transmembrane movement of the precursor lipid II, and MurJ has been shown to provide this activity in *Escherichia coli*. However, how MurJ functions *in vivo* has not been reported. Here we show that MurJ localizes both in the lateral membrane and at midcell, and is recruited to midcell simultaneously with late-localizing divisome proteins and proteins MraY and MurG. MurJ septal localization is dependent on the presence of a complete and active divisome, lipid II synthesis and PBP3/FtsW activities. Inactivation of MurJ, either directly by mutation or through binding with MTSES, did not affect the midcell localization of MurJ. Our study visualizes MurJ localization *in vivo* and reveals a possible mechanism of MurJ recruitment during cell division.

Introduction

Rod-shaped bacteria such as the Gram-negative bacterium *Escherichia coli* grow by elongation and binary fission. One of the requirements for proliferation is the enlargement of their cell envelope, which contains an inner membrane

(IM) and an outer membrane (OM). The shape of the bacterium is determined by the shape of its peptidoglycan (PG) layer, which is attached to the outer membrane in the periplasmic space located between both membranes of the envelope (Vollmer *et al.*, 2008). The PG layer is a mesh-like heteropolymeric macromolecule of alternating *N*-acetylmuramyl-peptides (MurNAc-pentapeptides) and *N*-acetylglucosamine (GlcNAc) disaccharides that are connected by a β -(1,4) bond forming glycan chains that are crosslinked between *meso* diaminopimelic acid (DAP) and D-Ala at the third and fourth position of the acceptor and donor stem peptide, respectively (Bouhss *et al.*, 2008). The biosynthesis of PG is a complicated process that can be divided into three stages. In the cytoplasmic stage, the nucleotide precursors UDP-GlcNAc and UDP-MurNAc-pentapeptide are synthesized from fructose-6-phosphate by the GlmSMU and MurABCDE proteins, respectively (Barreteau *et al.*, 2008). In the IM stage, the undecaprenyl-pyrophosphate- (C₅₅) linked intermediates lipid I (C₅₅-MurNAc-pentapeptide) and lipid II (C₅₅-MurNAc(-pentapeptide)-GlcNAc) are formed by the MraY and MurG proteins, respectively (Bouhss *et al.*, 2008). During this stage, lipid II, the building unit of peptidoglycan, is translocated by a 'flippase' across the IM (Pomorski and Menon, 2006; Barreteau *et al.*, 2008; Ruiz, 2015; Scheffers and Tol, 2015; Ruiz, 2016). In the periplasmic stage, the MurNAc-pentapeptide-GlcNAc component of lipid II is inserted into the PG layer by the glycosyltransferase and transpeptidase activities of various PBPs (Macheboeuf *et al.*, 2006; Sauvage *et al.*, 2008), and the lipoyl moiety C₅₅-PP is cleaved off and recycled (Manat *et al.*, 2014).

In *E. coli*, new PG-building units are inserted into the existing PG layer during length growth by a protein complex termed the elongasome, whereas during division new poles are synthesized from completely new material (Pedro *et al.*, 1997) by proteins that collectively have been termed the divisome (Den Blaauwen *et al.*, 2008). The elongasome is organized by the actin homolog MreB, which localizes underneath the IM in patches in a helix-like distribution. MreB is bound to the membrane by its amphipathic helix, and interacts with the bitopic membrane protein RodZ, the integral membrane proteins MreD and MreC, and the PG synthesis essential proteins RodA

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and PBP2 (Den Blaauwen *et al.*, 2008; van Teeffelen *et al.*, 2011; Morgenstein *et al.*, 2015; Fenton *et al.*, 2016; Contreras-Martel *et al.*, 2017). How the elongasome is regulated is not yet very clear whereas in contrast, cell division has been well investigated. During cell division, at least 20 proteins are recruited to midcell to form the divisome. The tubulin homolog FtsZ is attached to the IM at midcell by the anchor proteins FtsA and ZipA, and polymerizes to form the Z-ring (Sun and Margolin, 1998; Blaauwen *et al.*, 1999). Simultaneously, other proteins, such as the Zap proteins and FtsEX, are recruited to the ring to form the early divisome or protoring. The protoring then recruits the later divisome proteins FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI (PBP3), PBP1b, FtsN (den Blaauwen *et al.*, 2017), and a number of hydrolases and regulatory proteins (Gray *et al.*, 2015) to form the mature divisome (Aarsman *et al.*, 2005). The arrival of FtsN completes the assembly of the core divisome, and signals to initiate septal PG synthesis (Gerding *et al.*, 2009; Lutkenhaus, 2009; Weiss, 2015). The assembled divisome functions as a scaffold to initiate septal PG synthesis, OM constriction and the cleavage of the PG layer to separate daughter cells (Gray *et al.*, 2015). Septal PG synthesis is mainly orchestrated by PBP3 and PBP1b (Bertsche *et al.*, 2006; Sauvage *et al.*, 2008), but PBP1a can substitute for PBP1b (Suzuki *et al.*, 1978; Yousif *et al.*, 1985). OM constriction is organized by the Tol/Pal system, which forms a complex connecting IM and OM (Derouichei *et al.*, 1995; Dubuisson *et al.*, 2002). Tightly controlled amidase activity is involved in the separation of the daughter cells (Priyadarshini *et al.*, 2007).

Although the stages of PG synthesis are well understood, conflicting information on the flipping of lipid II across the IM has been published over the last few years. In *E. coli*, RodA and FtsW that belong to the SEDS (shape, elongation, division, sporulation) family, and MurJ that belongs to the MOP (multidrug/oligo-saccharidyl-lipid/polysaccharide) export super family (Ruiz, 2008), are reported as candidates of lipid II flippase, either functioning in elongation or division only or in both (Ikeda *et al.*, 1989; Young, 2014; Ruiz, 2015). FtsW is an essential integral membrane cell division protein with 10 trans-membrane helices (TMHs) and is conserved in most bacterial species that have a PG cell wall (Khattar *et al.*, 1994). FtsW, PBP3 and PBP1b form a complex that is recruited to midcell at the later stage of divisome assembly (Aarsman *et al.*, 2005; Goehring *et al.*, 2005; Goehring *et al.*, 2006; Banzhaf *et al.*, 2012; Leclercq *et al.*, 2017). FtsW was shown *in vitro* to have lipid II binding and flipping activity, for which two charged residues in TMH 4, arginine 145 and lysine 153, appeared to be essential (Mohammadi *et al.*, 2011; Mohammadi *et al.*, 2014; Leclercq *et al.*, 2017). However, *in vivo* studies showed that when RodA and FtsW are both inactive, lipid II is still

continuously translocated from the inner side to the outer side of cytoplasmic membrane, which is challenging the involvement of FtsW in the lipid II flipping (Lara *et al.*, 2005; Sham *et al.*, 2014). Interestingly, the SEDS proteins RodA and FtsW are both reported as glycosyltransferases which can polymerize lipid II *in vitro* (Cho *et al.*, 2016; Meeske *et al.*, 2016; Taguchi *et al.*, 2018).

MurJ is an essential IM protein that contains 14 TMHs with both C- and N-termini in the cytoplasm (Butler *et al.*, 2013; Butler *et al.*, 2014). Depletion of MurJ will cause irregularly shaped cell, and finally result into cell lysis (Ruiz, 2008). Earlier functional and structural studies of MurJ reveal an outward-facing central cavity that is formed by TMHs 1, 2, 7 and 8, which contain several charged residues that are essential for MurJ function (Butler *et al.*, 2013, 2014), while the observation of an open inward-facing conformation in the crystal structure of MurJ from both *Thermosipho africanus* (MurJ_{TA}) and *E. coli* suggest alternative conformational changes of MurJ (Kuk *et al.*, 2017; Zheng *et al.*, 2018). *In vivo* evidence favors MurJ over FtsW as the lipid II flippase, and depletion or inhibition of MurJ caused the accumulation of lipid II in cells (Ruiz, 2008; Sham *et al.*, 2014; Young, 2014; Qiao *et al.*, 2017), and its activity is dependent on membrane potential (Rubino *et al.*, 2018). While *in vitro* flipping activity has thus far only been established for FtsW (Mohammadi *et al.*, 2011, 2014). Interestingly, the function of MurJ in *E. coli* can be replaced by other flippases that lack sequence similarity, such as the O-antigen flippase Wzk from *E. coli*, and Amj and YtgP from other species (Ruiz, 2009; Meeske *et al.*, 2015; Elhenawy *et al.*, 2016; Hong *et al.*, 2018). Two *in vitro* studies on lipid II binding of MurJ showed conflicting results, as one reported a higher lipid II binding affinity of MurJ compared with FtsW (Bolla *et al.*, 2018), while the other one showed lipid II binding only by FtsW but not by MurJ (Leclercq *et al.*, 2017). Although these data support MurJ to be involved in flipping lipid II, it is still not clear where and how MurJ functions in bacterial cells. In this study, using fluorescence microscopy combined with other biochemical approaches, we show that the cellular localization of MurJ is dependent on the availability of its substrate lipid II.

Results

Construct a functional fluorescent protein fusion to the N-terminus of MurJ

To investigate the MurJ function and localization *in vivo*, fluorescent protein (FP) fusions to either the N-terminus or the C-terminus of MurJ with different repeats of Gly-Gly-Ser (GGS) linkers were constructed (Chen *et al.*, 2013). Two low-copy-number plasmids, pTHV037 and pSAV057, were used to express the fused proteins under

Table 1. Functionality and localization of MurJ fluorescence proteins fusions with different linkers at N- or C-terminus.

Fusions	Plasmids	Complementation ^a	Localization ^b	Function ^c
No fusion	pTHV037-MurJ	≈16 h	ND	+
	pSAV057-MurJ	≈16 h	ND	+
N-terminus	pTHV037-mCh-(GGG) _n -MurJ	≈16 h	+	+
	pSAV057-mNG-(GGG) _n -MurJ	≈16 h	+	+
	pSAV057-mKO-MurJ	≈16 h	+	+
C-terminus	pTHV037-MurJ-(GGG) _n -mCh	≥3 days	–	–
	pSAV057-MurJ-(GGG) _n -mNG	≥3 days	–	–

n, Number of GGS repeats (*n* = 0, 1, 2, 3).

^aAfter transformation, the time period needed for visible white colony appearance.

^b+, FP fused MurJ is fluorescent and localizes in the membrane; –, FP fused MurJ is fluorescent and localizes in the cytosol.

^c+, functional fusion; –, loss-of-function fusion.

the control of a down-regulated $P_{trcdown}$ promoter (van der Ploeg *et al.*, 2015). Based on the physical properties of fluorescent proteins (Cranfill *et al.*, 2016; Meiresonne *et al.*, 2017), green fluorescent protein mNeonGreen (mNG) and red fluorescent protein mCherry (mCh) were chosen for MurJ fusions. The functionality of the MurJ fusions was tested by a complementation assay (Material and Methods).

As shown in Table 1, the N-terminal fusions of MurJ were fully functional, while C-terminal fusions were less functional or not functional at all. Among these, the N-terminal mNG fusion with a (GGG)₂ linker showed the most stable and strongest fluorescence signals. For mCh, the fusion without linker was the best. Thus, they were chosen for further studies. The crystal structure of MurJ from *T. africanus* reveals that TMHs 13 and 14 form a hydrophobic groove, which penetrates into the central transport cavity and is hypothesized to be important for lipid II binding (Kuk *et al.*, 2017). The loss of function of MurJ C-terminal fusions might due to the defects in folding and groove formation.

MurJ localizes in the lateral wall and also at midcell

MurJ is reported to be present at 674 and 78 molecules per cell when grown in rich and minimal medium, respectively (Li *et al.*, 2014). In order to characterize MurJ at its endogenous expression level, the MurJ fusion was introduced into the *E. coli* chromosome at its native locus. The existence of the native promoter P_{murJ} was confirmed by inserting a divergently transcribed chloramphenicol cassette between the stop codon of gene *yceM* upstream of *murJ* and the putative *murJ* promoter (Fig. S1A). However, attempts to introduce N-terminal FP-fused MurJ under the native *murJ* promoter failed, apart from a mCh fusion, which yielded a strain with defects in cell morphology and growth (Fig. S1B). Since the plasmid-expressed fusions were fully functional, a likely explanation for the morphological defect could be that the translation efficiency of

the N-terminal fused proteins was less than that of MurJ only as was observed for other N-terminal FP fusions (Veening *et al.*, 2004). Consequently, the expression of the FP-fused MurJ under the native *murJ* promoter was not enough to support growth. Therefore, we replaced the chromosomal *murJ* gene with the *fp-murJ* constructs transcribed under the control of $P_{trcdown}$ promoter to generate strains XL08 and XL09 (Material and Methods). No defect on growth or cell morphology was observed even when grown without IPTG induction (Fig. S1C and D).

Strain XL08 was grown to steady state in minimal glucose medium (Gb1) at 28°C to be able to correlate its length to its cell division cycle age (Blaauwen *et al.*, 1999; Vischer *et al.*, 2015), and the localization of MurJ without IPTG induction in living *E. coli* cells was analyzed using ImageJ and the Coli-Inspector of ObjectJ (Vischer *et al.*, 2015). The mass double time (MD) of this strain is 83 min, which is close to 80 min of wild-type *E. coli* (Vischer *et al.*, 2015). MurJ localized in the cylindrical part of the cells as well as specifically at midcell in dividing cells (Fig. 1). After normalizing and plotting the fluorescence profiles into 10 age groups, a clear midcell localization was observed starting from 50% of the cell cycle. The septal proportion of MurJ, which is indicated as the 'Ring fraction', was analyzed as function of the division cell cycle, and a maximal proportion of 8.58% was observed at 82.5% of cell cycle (Fig. S2). This number is much less compared to typical divisome proteins that are only active at midcell and similar to that of proteins involved in elongation and division (Van der Ploeg *et al.*, 2013), suggesting MurJ functions both in the lateral wall and at midcell. To show that this accumulation was not an effect of double membrane formation during cell invagination, the localization of mNG-(GGG)₂-GlpT was introduced as control. GlpT (glycerol-3-phosphate transporter) is the major transporter for the uptake of *sn*-glycerol 3-phosphate in *E. coli*. It was reported to have 12 TMHs and to function as monomer (Huang, 2003), which diffuses homogeneously in the IM (Oswald *et al.*, 2016). It was therefore expected to

Fig. 1. MurJ localizes both in the lateral wall and at midcell. Strain XL08 containing the chromosomal mNG-MurJ fusion shows a cell cycle-dependent localization in contrast to the homogeneously localizing integral membrane protein mNG-GlpT. Cells were grown in Gb1 minimal medium to steady state at 28°C. Left: MurJ profiles; Right: membrane control protein GlpT profiles.

A. Collective profiles of localization of MurJ and GlpT. For each protein, the diameter (black lines) and fluorescence (green lines) profiles along normalized cell length are shown in 10% age class bins.

B. Maps of diameter profiles (magenta) and fluorescence profiles (green). Cells are plotted by length, ascending from left to right.

C. Phase contrast image (left) and the corresponding fluorescence image (right) for each protein. Scale bar equals 5 µm. More than 1200 cells were included for both analyses.

show homogenous membrane localization. Like for MurJ, the localization of mNG-(GGG)₂-GlpT was imaged and analyzed. As expected, GlpT localized homogeneously in the membrane without midcell accumulation. After plotting into 10 age groups, GlpT showed a clear difference with MurJ, and no enhanced fluorescence was observed at midcell during constriction (Fig. 1A and B). The differences between the localization patterns of the two proteins are also obvious in images of the cells (Fig. 1C). The specific localization pattern of MurJ suggests that MurJ is likely involved in both length growth and cell division.

MurJ is recruited to midcell simultaneously with the PG synthesis proteins.

The observation of MurJ midcell localization prompted us to the next question: at which point of the cell division

cycle MurJ is recruited. To reveal this, localization of MurJ, the early and late localizing proteins FtsZ and FtsN, respectively, and the proteins MraY and MurG, which synthesize Lipid I and lipid II, respectively (Mengin-Lecreux *et al.*, 1991; Bouhss *et al.*, 1999; Aarsman *et al.*, 2005; Mohammadi *et al.*, 2007; Weiss, 2015; Bisson Filho *et al.*, 2017) was determined in steady-state grown strain XL08. Cells from the same culture and optical density were either imaged live for MurJ localization or were fixed with FGA (2.8% formaldehyde and 0.04% glutaraldehyde) and subsequently immunolabeled with antibodies specific for the above-mentioned proteins (Buddelmeijer *et al.*, 2013). The early divisome protein FtsZ was recruited at midcell at about 25% of cell cycle, and the core divisome assembly was completed by the midcell arrival of FtsN at 40–50% of the cell division cycle (Fig. 2). The measured extra fluorescence at midcell (FCplus) of these

Fig. 2. Timing of MurJ midcell localization. mNG-(GGG)₂-MurJ cellular localization was compared with the localization of divisomal and PG precursor synthesizing proteins. XL08 strain was grown to steady state in Gb1 medium. MurJ localization was determined in living cells. After fixation with FGA, the rest of cells were immunolabeled with antibodies against FtsZ, FtsN, MraY and MurG. Diameter (black lines) and Fluorescence (colored lines) profiles were plotted into 10% age class bins along the normalized cell length in %. More than 1200 cells were included.

proteins plotted as function of the cell division cycle age (Fig. S3) is in agreement with a previous study (Van der Ploeg *et al.*, 2013). Interestingly, MraY, MurG and MurJ accumulated at midcell simultaneously at approximately 50% of cell cycle at the later stage of divisome assembly.

MurJ requires a mature divisome for midcell localization

The timing of MurJ midcell localization raised the question whether its localization is dependent on particular divisome proteins. To answer this, the mNG-(GGG)₂-MurJ chromosomal fusion was introduced into a series of temperature-sensitive cell division mutant strains. FtsA is an essential early divisome protein that anchors the Z-ring to the IM (Rico *et al.*, 2004; Pichoff and Lutkenhaus, 2005). The absence of FtsA causes filamentous growth of *E. coli*, as the downstream divisome proteins are not recruited to midcell (Pichoff and Lutkenhaus, 2002). In the FtsA(*Ts*) background, MurJ showed normal midcell localization when grown at permissive temperature. However, when grown at 42°C, MurJ midcell localization was lost, and MurJ only localized in the cylindrical membrane of the filamentous cells (Figs. 3B and S4). This indicates that MurJ midcell localization is dependent on the presence of the early divisome. Like in the FtsA(*Ts*) cells, depletion of the later divisome proteins, FtsQ, FtsW, FtsI or FtsN (Taschner *et al.*, 1988; Ishino *et al.*, 1989), abolished MurJ midcell localization in the filamentous cells (Figs. 3C–F and Fig. S4). To point out, some localization bands of MurJ were observed in FtsQ(*Ts*) filaments at 42°C, which was likely caused by the relocalization of FtsQ proteins during the imaging at room temperature. As a control, MurJ still localized at midcell in a wild-type background at 42°C (Fig. 3A). These results suggest that MurJ requires a mature divisome for midcell localization. To determine to what extent MurJ is dependent on cell division, we also examined MurJ localization in strains that were deleted for PBP1a, PBP1b, Pal, TolA, or the

amidases AmiABC. The observation of a clear MurJ midcell localization in the absence of these proteins, even when cells grow as chains, indicates that MurJ midcell localization is independent of these proteins (Fig. 3G–K). All these results are consistent with the function of MurJ as the lipid II flippase during PG synthesis.

MurJ midcell localization requires PBP3 activity

After observation of MurJ midcell localization, the next obvious question would be whether this localization is dependent on septal PG synthesis. Septal PG synthesis requires the activity of PBP3 (FtsI) and PBP1b, although the latter can be replaced by PBP1a (Yousif *et al.*, 1985). The specific inhibition of PBP3 activity by aztreonam blocks septal PG synthesis without initially inhibiting the localization of the divisome, resulting in filamentous cells with divisomes localized at regular cell division distances (Den Blaauwen *et al.*, 2003; Mohammadi *et al.*, 2007). After growing the chromosomal mNG-MurJ containing strain XL08 at 28°C to steady state in Gb1 medium, MurJ localization was determined in the presence of 1 mg.L⁻¹ aztreonam. A block of MurJ midcell localization was already observed after 30 min, without much change in cell morphology (Fig. S5). After 1 and 2 MDs, MurJ midcell localization was completely abolished in the typical filamentous cells (Figs. S5 and Fig. 4). Subsequently, cells were fixed and immunolabeled with antibodies against FtsZ and FtsN to show the localization of the mature divisome, and against MraY and MurG to show the localization of the lipid II-synthesizing complex. As expected, we found that FtsZ, FtsN, MurG and MraY localized at potential division sites in the filaments (Fig. 4). Likewise, MurJ localization was determined in the presence of elongasome inhibitors A22 and mecillinam for 2 MDs, which inactivate MreB and PBP2, respectively (Den Blaauwen *et al.*, 2003; Shih *et al.*, 2005; Vollmer *et al.*, 2005; White and Gober, 2012). No influence on MurJ

Fig. 3. MurJ requires the core divisome for its midcell localization. Chromosomal mNG- (GGG)₂-MurJ fusion was introduced into strains that harbor temperature sensitive, depletable or deleted division proteins (except that the mNG-(GGG)₂-MurJ was expressed in $\Delta tolA$ and Δpal strains from plasmid pXL05, and mCherry-MurJ was expressed in $\Delta amiABC$ strain from plasmid pNM037).

A–E. MurJ localization in XL08 (WT), *ftsA* 1882 (Ts), *ftsQ1* (Ts), *ftsW* (Ts) and *ftsI2185* (Ts) backgrounds. Left: Localization at permissive temperature of 30°C. Right: Localization after growth at the non-permissive temperature of 42°C for two mass doublings (MD)s.

F. MurJ localization in FtsN depletion background at 30°C.

Left: localization in the presence of 0.2% w/v arabinose. Right: Localization of MurJ in the presence of glucose for two MDs.

(G–K) mCh-MurJ localization in $\Delta mrcA$ (PBP1A), $\Delta mrcB$ (PBP1B), Δpal , $\Delta tolA$ and $\Delta amiABC$ background at 30°C, respectively. Scale bar equals 5 μ m.

midcell localization was observed although cells started to grow spherically (Fig. S6). Together, these results suggest that MurJ midcell localization is dependent on the activity of PBP3 and septal PG synthesis.

MurJ midcell localization requires lipid II synthesis

Next, we asked whether MurJ localization would be lipid II-dependent. The biosynthesis of lipid II requires the activity of two essential enzymes in the cytoplasmic stage, the pyridoxal 5'-phosphate (PLP) dependent alanine racemase, Alr, which converts L-alanine into D-alanine (Wood and Gunsalus, 1959; Barreteau *et al.*, 2008), and the ATP-dependent D-ala:D-ala ligase, Ddl, which catalyzes the formation of D-alanyl-D-alanine from two molecules of D-alanine (Neuhaus, 1962; Barreteau *et al.*, 2008). D-cycloserine (DCS) is a structural analog of D-alanine that inactivates Alr and Ddl, and causes rapid cell lysis (Neuhaus and Lynch, 1972; Wang *et al.*, 1978; Prosser and de Carvalho, 2013; Azam and Jayaram, 2015). A concentration of 1 μ g mL⁻¹ DCS is sufficient to deplete the UDP-MurNac-pentapeptide pool in 30 minutes when *E. coli* is grown in minimal medium (Menginlecreulx *et al.*, 1983; de Roubin *et al.*, 1992). Inhibition of the D-ala:D-ala ligase by DCS causes a 50-fold accumulation of UDP-MurNac-tripeptide (de Roubin *et al.*, 1992), suggests that *E. coli* cannot use UDP-MurNac-tripeptide to synthesize lipid II with a tripeptide side chain. Therefore, steady-state grown XL08 cells were treated with different concentrations of DCS, and MurJ localization was determined at 30 min after adding DCS. At concentrations of 0.5 μ g mL⁻¹ DCS or higher, growth inhibition and rapid cell lysis was observed after 30 min, while lower concentrations of DCS caused lysis after 1 MD or even longer incubation periods (Fig. 5A). Interestingly, DCS also showed a concentration-dependent effect on MurJ localization. Increasing DCS concentrations eventually blocked the MurJ midcell localization, and when the DCS concentration was higher than 0.5 μ g mL⁻¹, MurJ was mostly absent from midcell (Figs. 5B and S7A). In addition, localization of MraY and MurG was somewhat reduced at midcell in the presence of DCS (Fig. S7, B and C). On the contrary, the core divisome monitored by the presence of FtsN remained intact (Fig. S7D). Together, these results indicate that MurJ midcell localization is dependent on the synthesis of lipid II.

MurJ midcell localization requires FtsW activity

Our results so far showed that MurJ midcell localization is dependent on the assembly of the divisome, the activity of PBP3 and also the synthesis of lipid II. Previous investigation of divisome assembly and regulation suggests that FtsQLB normally keeps FtsW/PBP3 inactive, and once the divisome is completely assembled, the FtsW/PBP3 complex is somehow activated (Liu *et al.*, 2015; Weiss, 2015). FtsW has been shown to form a ternary

Fig. 4. Localization of cell division proteins in the presence of the PBP3 inhibitor aztreonam for two MDs. Cell division is inhibited but the localization and assemblage of the divisome continues, whereas the localization of MurJ is lost. In Gb1 at 28°C, steady-state growing XL08 was treated with 1 mg.L⁻¹ aztreonam, MurJ localization was determined in living cells, and FtsZ, FtsN, MraY and MurG were immunolabeled with antibodies after fixation. Diameter (black lines) and fluorescence (colored lines) profiles were plotted into 10% age class bins along the normalized cell length. More than 1200 cells were included.

Fig. 5. Inhibition of lipid II biogenesis blocks MurJ midcell localization. In Gb1 at 28°C, steady-state growing XL08 was treated with increasing concentrations of D-cycloserine (DCS), and mNG-MurJ localization was determined at 30 min after addition of DCS. A. Growth curves were plotted as time against the natural logarithm of the optical density of the cells. The black arrow indicates the addition of DCS. B. Fluorescence maps profiles of MurJ localization. Cells are plotted according to increasing cell length from top to bottom. From left to right, the maps correspond to 0, 0.05, 0.1, 0.5, 1 and 2 µg.L⁻¹ of DCS concentrations. More than 1200 cells were included. C. Phase contrast images (upper) and correspond fluorescence images (down) of cells treated with DCS. Scale bar equals 5 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

complex with PBP3 and PBP1b, and the lipid II binding activity of FtsW inhibits the polymerization of lipid II by

PBP1b in the absence of PBP3, while the presence of PBP3 stimulates the release of lipid II from FtsW, and activates its polymerization by PBP1b (Leclercq *et al.*, 2017). In addition, new evidence supports glycosyltransferase activity for FtsW (Taguchi *et al.*, 2018). These observations raised the possibility that FtsW activity is needed to ensure MurJ localization. To validate this hypothesis, plasmids that expressed two non-functional FtsW mutants, FtsW R145A and FtsW K153N were introduced into strain XL08 to investigate the localization of MurJ. These two mutants were shown to localize at midcell but failed to complement the FtsW(Ts) strain at non-permissive temperature, and their expression in LMC500 wild-type cells showed a dominant-negative filamentous morphology (Mohammadi *et al.*, 2014). In addition, *in vitro* data showed that these mutants lost the activity of FtsW (Mohammadi *et al.*, 2014). In our study, to eliminate the role of the wild-type FtsW, 20 µM IPTG was used to overexpress these two mutants, and thus diluted the functional wild-type FtsW expressed from the XL08 genome. When cells were grown to steady state in minimal medium, cell length increased due to the defect on cell division, especially in cells expressing mutant K153N (Fig. 6B). MurJ midcell localization was lost in the presence of non-functional FtsW mutants (Fig. 6A and B) but not wild-type FtsW (Fig. 6C) for 2 MDs, despite the overexpression of MurJ as it is also expressed under the IPTG inducible $P_{trcdown}$ promoter. In contrast, the divisome, which was monitored by immunolabeling of FtsN, was still localized at midcell in all cases (Fig. S8). These results indicate that MurJ needs a functional FtsW for its localization at midcell.

Moreover, when grown in LB medium, strains expressing these mutants showed strong defects on morphology and MurJ localization. At early exponential phase ($OD_{600} = 0.2$), filamentous morphology was observed for all cultures, even for cells grown with glucose that suppressed the expression of the FtsW mutants (Fig. S9A). Interestingly, in cultures at late log phase expressing the FtsW K153N mutant or in

Fig. 6. Expression of non-functional FtsW mutants abolishes MurJ midcell localization. XL08 expressing mutant FtsW R145A or FtsW K153N was grown in Gb1 minimal medium to steady state at 28°C. IPTG (20 μ M) was used to induce expression of the FtsW mutants from plasmid. MurJ localization was determined in living cells and the presence of the divisome was confirmed by immunolabeling of FtsN, after fixation.

A. Localization of MurJ and FtsN after expression of FtsW R145A.

B. Localization of MurJ and FtsN after expression of FtsW K153N. For each part: top, average localization profiles plotted along normalized cell length ($n \geq 1200$ cells). Black lines indicate cell diameter. Green lines and red lines indicate MurJ and FtsN localization, respectively. Middle, map profiles of cell diameter and MurJ or FtsN localization sorted by ascending cell length. Bottom, phase contrast and fluorescence microscopy images. Scale bar equals 5 μ m.

cultures at stationary phase expressing the FtsW R145A mutant, some cells started to restore the MurJ localization, especially in the cells that were induced with IPTG (Fig. S9A). Since these mutants are toxic, we wondered whether the XL08 strain lost these FtsW expressing plasmids at high frequency, restoring MurJ localization. A spot assay was carried out to test our hypothesis: cells from each culture were diluted to the same OD₆₀₀ value and spotted on LB and LB-ampicillin agar plates (these two plasmids expressing the FtsW mutants confer ampicillin resistance). For both strains, no obvious growth difference was observed on both plates when grown with glucose, indicating that most cells still contained the mutant plasmids. However, for cells that had been induced with IPTG, a large population of cells had lost the FtsW mutant plasmids, as much less growth was observed on the ampicillin plate compared with LB plate (Fig. S9B). These results strongly suggest that MurJ midcell localization requires FtsW activity, rather than its physical presence.

MurJ activity is not required for its localization

Earlier studies revealed several conserved charged residues, R18, R24, R52 and R270 in the central cavity of MurJ that are essential for its function (Butler *et al.*,

2013; Kuk *et al.*, 2017). To verify whether MurJ needed to be functional to allow midcell localization, an mNG-fused MurJ R18A mutant was expressed from plasmid in LMC500. The cells were grown in Gb1 and expression was induced with 20 μ M IPTG. As shown in Fig. 7A, the non-functional mutant R18A localized both in the lateral wall and at midcell as was observed for the wild-type MurJ fusion. Although MurJ is likely functional as a monomer based on its structure, the wild-type copy of MurJ from LMC500 genome might still contribute to the localization of this non-functional mutant. To eliminate this potential contribution, localization of the R18A mutant was also determined in strain XL30, a MurJ-depletion strain in which native MurJ production is under an arabinose-inducible promoter (Ruiz, 2008). In the presence of arabinose, no growth defect was observed, and the R18A mutant showed the same localization as in the LMC500 strain (Fig. 7B, left). When grown in the presence of glucose, native MurJ was eventually depleted as morphology defects were observed after 3 h, however, the mutant R18A was still localized in lateral wall and at midcell (Fig. 7B, right). The localization of the other loss-of-function-mutants showed the same results as R18A (see below). These results suggest that the MurJ function is not required for its localization.

Fig. 7. MurJ non-functional mutant R18A does not influence its localization.

A. The plasmid expressing the mNG-MurJ R18A fusion was introduced into LMC500. This strain was grown in Gb1 minimal medium to steady state at 28°C, and expression was induced with 20 μ M IPTG for 2 MDs. The diameter (black lines) and fluorescence (green lines) profiles along normalized cell length are shown in 10% age class bins. More than 1200 cells were included. Scale bar equals 5 μ m.

B. The plasmid expressing the mNG-MurJ R18A fusion was introduced into the MurJ depletion strain XL20. Mutant localization was determined in the presence of arabinose (wild-type MurJ expression) or glucose (wild-type MurJ depletion). Scale bar equals 1 μ m.

MTSES does not influence the localization of MurJ single-cysteine variants

The function of a number of MurJ single-cysteine mutants, A29C, N49C, S263C and E273C, was reported to be sensitive (only partially in the case of E273C) to MTSES (Butler *et al.*, 2013). MTSES covalently modifies reduced cysteine residues that are exposed to the periplasm. In the absence of MTSES, these mutant proteins are functional, but not when MTSES is bound to their cysteine residues (Sham *et al.*, 2014; Chamakura *et al.*, 2017; Qiao *et al.*, 2017). To investigate whether MTSES affects the functionality of these mutants through the disruption of their recruitment, the localization of their mNG fusions was determined in the presence of MTSES. Having optimized the induction conditions, MurJ cysteine mutants were induced with 40 μ M IPTG, and their localization was determined at 10 and 40 min (20 min for S263C variant due to the higher sensitivity) after addition of 0.5 mM MTSES (Material and Methods and Fig. S10). We found that MTSES influences only the functionality but not the localization of these mutants, as MurJ midcell localization was still observed, in spite of cell lysis after MTSES addition (Fig. 8A–D, and Fig. S11). As a control, neither the functionality nor localization of cysteine free variant (MurJ Cys⁻) was influenced by MTSES (Fig. 8E).

Next, we also investigated whether MTSES influences the localization of the total-loss-of-function mutants, R18C, R24C, R52C and R270C. Similar MTSES experiments were performed on these mNG-fused mutants in the LMC500 background. To eliminate the potential influence of MTSES on wild-type MurJ from the LMC500 genome, the MurJ deletion strain expressing mNG fused wild-type MurJ was firstly checked. In agreement with the evidence that the two native cysteine residues in wild-type MurJ are not labeled by MTSES (Butler *et al.*, 2013), no defect on cell growth, morphology or MurJ localization was observed when MTSES was added (Fig. 8F and Fig. S11F). Similarly, no growth or morphology defect was observed after addition of MTSES to the non-functional single-cysteine mutants, and MurJ still localized at mid-cell (Figs. 8G–H, S11G–K and S12). To point out, when induced with 40 μ M IPTG, mutant R24C showed strongly reduced fluorescence and a cytoplasmic-like localization, compared to other mutants. However, when induced with 100 μ M IPTG, R24C showed a normal MurJ localization and comparable fluorescence, indicating that mutation R24C does not affect MurJ localization (Fig. S12C). Surprisingly, a spontaneous suppressor mutation R447H that is situated at the cytoplasmic end of TMH13 restored fluorescence and showed a typical MurJ localization when induced with 40 μ M IPTG (Fig. S7D–F). Since the R24C

Fig. 8. Localization of MurJ cysteine mutants in the presence of MTSES. Strains expressing MurJ single-cysteine mutants from plasmid were grown to steady state in Gb1 medium at 28°C, and expression was induced with IPTG for 2 MDs. MurJ localization was determined at 10 and 40 min after the addition of MTSES (for mutant S263C, localization was determined at 10 and 20 min because of its higher sensitivity to MTSES). Average of MurJ localization profiles were plotted along normalized cell axis. Black lines indicate the MurJ localization in the absence of MTSES, green and red lines indicate the MurJ localization at 10 and 40 min in the presence of MTSES, respectively. More than 1200 cells were included for each experiment.

A–D. Only growth but not localization of functional mutants A29C, S263C, N49C and E273C is affected by MTSES.

E. Neither growth nor localization of cysteine free mutants is influenced by MTSES.

F. Neither growth nor localization of wild-type MurJ is influenced by MTSES.

G and H. Localization of non-functional MurJ mutants R18C and R52C is not affected by MTSES.

mutant was reported not to affect protein level (Butler *et al.*, 2014), R24C might slightly change the structure of MurJ, and affect mNG folding and fluorescence, while mutation R447H could somehow suppress this defect.

Together, the investigation of MTSES on MurJ cysteine variants supports the notion that MurJ activity is not essential for its localization.

Discussion

Recruitment of MurJ to midcell and the coordination of cell division and septal PG synthesis

The synthesis of PG has been studied for decades, and FtsW and RodA have been always considered as the lipid II flippases in elongasome and divisome,

respectively (Matsuzawa *et al.*, 1973; Ikeda *et al.*, 1989; Dai and Lutkenhaus, 1991; Den Blaauwen *et al.*, 2008; Mohammadi *et al.*, 2011, 2014; Sieger *et al.*, 2013). Only recently, MurJ was identified as the lipid II flippase (Ruiz, 2008; Sham *et al.*, 2014; Meeske *et al.*, 2015; Elhenawy *et al.*, 2016; Qiao *et al.*, 2017). FtsW has been shown to bind and flip lipid II and other lipids *in vitro* but not *in vivo* (Mohammadi *et al.*, 2011; 2014), whereas evidence has been provided that MurJ has the lipid II flipping activity *in vivo*, but only lipid II binding activity so far *in vitro* (Sham *et al.*, 2014; Leclercq *et al.*, 2017; Qiao *et al.*, 2017; Bolla *et al.*, 2018).

Our results show for the first time the specific MurJ cellular localization in *E. coli*. We show that MurJ localizes both in the lateral wall and also at midcell during cell division, and its midcell localization is dependent on the

presence of its substrate lipid II since inhibition of lipid II synthesis by DCS delocalizes MurJ from midcell. This substrate driven localization is also in agreement with evidence that: (i) The essential function of MurJ can be smoothly replaced with other flippases that lack sequence and/or structure similarity (Ruiz, 2009; Meeske *et al.*, 2015; Elhenawy *et al.*, 2016; To Sham *et al.*, 2018), (ii) Our attempts to probe the protein–protein interaction between MurJ and the potential interacting proteins FtsW and RodA using a cytoplasmic FRET assay (Alexeeva *et al.*, 2010) failed (data not shown), (iii) The addition of DCS to inhibit lipid II synthesis or aztreonam to inhibit specifically the cell division PG transpeptidase PBP3 only caused the delocalization of MurJ from midcell, but neither of the divisome components nor of PG precursor synthesis proteins (Fig. 5 and Fig. S7) and (iv) Non-functional MurJ proteins that are stable but have defects in sites within the functional cavity, which are not accessible for protein–protein interactions, localize to the septum but are not dominant negative. These proteins would be expected to have a dominant-negative effect if MurJ was recruited to the divisome through protein–protein interactions with essential proteins like MurG and FtsW (Fig. 7 and Fig. S11).

Moreover, we found that FtsW/PBP3 activities are also required for MurJ midcell localization, and the inactivation of either protein caused the delocalization of MurJ from midcell. It is well known that the inactivation of PG polymerization and crosslinking will cause the accumulation of unused lipid II at the periplasmic side of the IM, and likely result into the depletion the undecaprenyl carrier on its cytoplasmic side since this carrier is limiting in cells (Mengin-Lecreux *et al.*, 1991; Hug and Feldman, 2011; Manat *et al.*, 2014). The beta-lactam antibiotics have been shown to induce a futile cycle that depletes lipid II precursors in cell (Cho *et al.*, 2014). Both inhibitions lead therefore to the absence of substrate for MurJ and consequently its delocalization from midcell. Interestingly, the SEDS proteins RodA and FtsW are reported as glycosyltransferases that polymerize lipid II, and this activity of FtsW is dependent on its cognate class B PBP (Meeske *et al.*, 2016; Taguchi *et al.*, 2018), PBP3 in *E. coli* (Fraipont *et al.*, 2011). Although MurJ needs an active FtsW for midcell localization, its activity is not dependent on FtsW or RodA (Sham *et al.*, 2014). Together all these observation indicate that the cellular recruitment of MurJ is driven by the localization of its substrate lipid II.

Unexpected, we found that the activity of MurJ is not required for MurJ localization, as the inactivation of MurJ, either by mutation or MTSES treatment, did not affect its cellular localization. Given the above evidence that its localization is substrate-dependent, these inactivated proteins should still be able to recognize lipid II but not be able translocate it across the IM. This is in conflict with the recent *in vitro* evidence suggesting that binding of MTSES

at A29C reduced the lipid II binding affinity of MurJ protein (Bolla *et al.*, 2018). This would predict delocalization for the MTSES bound A29C MurJ mutant, whereas we observe the typical midcell localization *in vivo*. Since *in vitro* data in detergents are not always completely comparable to the *in vivo* situation and given the conflicting lipid II binding evidences *in vitro* (Leclercq *et al.*, 2017; Bolla *et al.*, 2018), we assume that after MTSES binding, the MurJ A29C mutant could still recognize lipid II sufficiently to localize MurJ.

Model of MurJ function in septal PG synthesis

Based on our evidence and that of others, we arrived at a model for the mechanism of MurJ recruitment and lipid II flipping during septal PG synthesis (Fig. 9). After maturation of the core divisome complex, MurJ is recruited to midcell through the recognition of its substrate lipid II (Fig. 9A), which ensures the precise positioning of septal PG synthesis. Interruption of lipid II synthesis by D-cycloserine, or inactivation of PBP3 by aztreonam, or inactivation of FtsW would deplete undecaprenyl phosphate and block the recruitment of MurJ to midcell. Once recruited, MurJ flips lipid II by changing from the inward-facing state to an outward-facing conformation, and the flipped lipid II can be used for new PG synthesis (Fig. 9B and C). Subsequently, the unloaded outward facing MurJ switches back to the inward-facing state, and can be recruited for new cycles (Fig. 9D). Inactivation of MurJ, either by direct mutation at the charged residues R18, R24, R52, R270, or by binding of MTSES to residues A29C, N49C, S263C and E273C, will disrupt the translocation of lipid II across the membrane (Fig. 9B). It should be noted that although we mainly focused on the septal MurJ, the D-cycloserine experiments, MurJ depletion experiment and MTSES experiments, showed a global effect on *E. coli*, indicating that MurJ very likely will be positioned close to the elongasome and likely be dependent on RodA activity for this localization. Unless a specific localization of MurJ is not a requirement because the diffusion rate of lipid II might be sufficient to access the elongasome after being randomly flipped by MurJ.

Experimental Procedures

Strains and plasmids construction

Strains and plasmids used in this study are listed in Table. S1. To construct the MurJ N-terminal fluorescent protein (FP) fusion plasmids, mCherry (mCh) (Cranfill *et al.*, 2016) or *E. coli* codon optimized mNeonGreen mNG (Meiresonne *et al.*, 2017), were firstly cloned into plasmids pTHV037 (Den Blaauwen *et al.*, 2003) and pSAV057 (Alexeeva *et al.*, 2010) by restriction and ligation (*Nco*I

Fig. 9. Model of MurJ recruitment and function in septal peptidoglycan synthesis.

A. Recruitment of MurJ to midcell when FtsW is activated.

B. MurJ binds substrate.

C. MurJ flips lipid II across the IM by changing its structure from the inward-facing conformation into the outward-facing state.

D. Unloaded MurJ changes back into the default inward-facing state to participate in a new cycle. (i) Loss-of-function MurJ mutants that localize but are not able to flip lipid II. (ii) Functional MurJ mutants that are inactivated by MTSES still localize but are not able to flip lipid II.

and *EcoRI*), to generate plasmid pSAV047 and pSAV057-mNG, respectively. Subsequently, amplified MurJ fragments with different linkers were cloned into these two plasmids by restriction and ligation (*EcoRI* and *HindIII*), to generate the derived N-terminal fusion plasmids. To construct the MurJ C-terminal FP fusion plasmids, MurJ gene was firstly cloned into plasmids pTHV037 and pSAV057 with *NcoI* and *BamHI*, to generate plasmids pXL09 and pXL10. Subsequently, FP genes with different linkers were cloned into these two plasmids with *BamHI*

and *HindIII*, to generate the derived C-terminal fusion plasmids. GlpT fusion plasmid pXL28 was constructed in the same way. To construct the MurJ mutant plasmids, QuickChange site directed mutagenesis (Agilent technologies, Santa Clara, CA) and Gibson assembly (Gibson *et al.*, 2009) approaches were applied. Plasmid pXL74 was firstly generated from pXL05 by replacing the wild-type MurJ with the cysteine free mutant that was amplified from plasmid pFLAGMurJΔCys (Butler *et al.*, 2013). Single-cysteine mutant plasmids were constructed afterward by

replacing the original residues with cysteine. The double mutation plasmid pXL93 was constructed by introducing the additional R52C mutation into plasmid pXL82.

The MurJ deletion strain XL03 (LMC500 $\Delta murJ::FRT/$ pRC7MurJKan) was constructed by λ -Red recombination (Datsenko and Wanner, 2000) in the presence of the complementing plasmid pRC7MurJ (Butler *et al.*, 2013). Primers priXL17 and priXL18 were used to amplify the MurJ upstream homologous sequence region ahead of MurJ start codon from the *E. coli* genome, primers priXL19 and priXL20 were used to amplify the chloramphenicol (*cat*) cassette from plasmid pKD3 (Datsenko and Wanner, 2000), and primers priXL21 and priXL22 were used to amplify the downstream homologous sequence that comes after MurJ stop codon from the *E. coli* genome. An overlap PCR was performed using the obtained homologous sequences and *cat* gene with primers priXL17 and priXL21 to generate the final recombination fragment. This PCR product was used to generate the MurJ depletion strain sNM01 (LMC500 $\Delta murJ::cat$ pRC7MurJKan). Subsequently, the chloramphenicol resistance cassette was removed by plasmid pCP20 (Datsenko and Wanner, 2000), which resulted in the final *murJ* deletion strain XL03/pRC7MurJ Δ Kan (The kanamycin cassette on this plasmid is also removable).

To prove the existence of the native *murJ* promoter, a chloramphenicol cassette was inserted after the stop codon of *yceM* upstream of *murJ* with a similar strategy as above. Primers priXL49 and priXL50 were used to amplify the upstream homologous sequence from the *E. coli* genome, priXL54 and priXL52 were used to amplify the *cat* cassette from plasmid pKD3, and priXL45 and priXL48 were used to amplify the downstream homologous sequence from the *E. coli* genome. The amplified overlap PCR product with primers priXL49 and priXL48 was used to construct the recombinant strain XL04 that interrupted MurJ expression from the upstream gene cluster. To construct MurJ chromosomal fusion strains that are expressed under its native promoter, plasmids pXL36 and pXL37 were firstly constructed with Gibson assembly: primers priXL51 and priXL52 were used to amplify the pKD3 backbone, primers priXL45 and priXL46 were used to amplify the P_{murJ} sequence from the *E. coli* genome, primers priXL53 and priXL47 were used to amplify FPs-MurJ sequence from plasmid pXL05 and pNM037. The final recombination fragments were generated by overlap PCR of 2 fragments with primers priXL17 and priXL48 from fragment *yceM-cat* amplified from the XL04 genome with primers priXL17 and priXL50, and fragment *cat-P_{murJ}-FPs-murJ* amplified from these assembled plasmids with primers priXL54 and priXL48. To construct the MurJ chromosomal FP fusion strains XL06 and XL07 expressing MurJ under control of the P_{trcdwn} promoter, fragment *yceM-cat* was amplified from the XL04 genome with primers priXL17 and priXL52, and fragment P_{trcdwn} -FPs-MurJ was amplified from plasmids pXL06 or pNM037

with primers priXL77 and priXL48. The two fragments were used for an overlap PCR with primers priXL17 and priXL48 and the resulting sequence was used to construct strains XL06 and XL07 from LMC500 by λ -Red recombination. To insert the mNG-MurJ fusion in the chromosomal of temperature sensitive strains and deletion strains, PCR fragment *yceM-cat-P_{trcdwn}-NG-(GGS)₂-MurJ* was firstly amplified from the XL06 genome with primer priXL17 and priXL48, and subsequently introduced into other strains by λ -Red recombination.

To construct an FtsN depletion strain, primers priXL140 and priXL141 were used to amplify the upstream sequence of FtsN from the *E. coli* genome, priXL54 and priXL52 were used to amplify the *cat* cassette from plasmid pKD3, and priXL138 and priXL139 were used to amplify the *araC-P_{BAD}* region from plasmid pJC83 (Chen and Beckwith, 2008). An overlap PCR with priXL140 and priXL138 was performed subsequently to generate the final product for recombination. The MurJ depletion strain was constructed in a similar way, primers priXL17 and priXL52 were used to amplify the *YceM-cat* region from the XL06 genome, priXL126 and priXL157 were used to amplify *araC-P_{BAD}* region from plasmid pKD46, priXL156 and priXL48 were used to amplify the MurJ region from the *E. coli* genome. The final PCR product for MurJ depletion was generated by an overlap PCR with primers priXL17 and priXL48.

All PCR amplifications were performed using the DNA polymerase pfuX7 prepared in our lab as described (Nørholm, 2010), and all restriction enzymes used were purchased from New England Biolabs Inc (NEB, Ipswich, MA).

Medium and growth conditions

LB medium (10 g of tryptone (Bacto laboratories, Australia), 10 g of NaCl (Merck, Kenilworth, NJ), 5 g of yeast extract (Duchefa, Amsterdam, The Netherlands) per liter) was used for growth in rich medium at 30, 37 and 42°C. The FtsW(*Ts*) strain was grown in LB Δ NaCl medium (LB without NaCl and containing 0.1% glucose and 20 mg thymine per liter) as described previously (Pastoret *et al.*, 2004). Gb1 minimal medium (6.33 g of K₂HPO₄ (Merck), 2.95 g of KH₂PO₄ (Riedel de Haen, Seelze, Germany), 1.05 g of (NH₄)₂SO₄ (Sigma, St. Louis, MO), 0.10 g of MgSO₄·7H₂O (Roth, Karlsruhe, Germany), 0.28 mg of FeSO₄·7H₂O (Sigma), 7.1 mg of Ca(NO₃)₂·4H₂O (Sigma), 4 mg of thiamine (Sigma), 2 mg of uracil (Sigma), 2 mg of lysine (Sigma), 2 mg of thymine (Sigma), and 0.5% glucose (Merck) per liter, pH 7.0) was prepared for steady-state growth at 28°C as described (Van der Ploeg *et al.*, 2013). Protein expression was induced with isopropyl β -D-1-thiogalactopyranoside (IPTG, Promega, Madison WI) or L-(+)-arabinose (Sigma) at described concentrations. All antibiotics concentrations were: 100 μ g mL⁻¹ ampicillin (10 μ g mL⁻¹

for $\Delta tolA$ and Δpal strains), 25 $\mu\text{g mL}^{-1}$ chloramphenicol, 50 $\mu\text{g mL}^{-1}$ kanamycin, 10 $\mu\text{g mL}^{-1}$ tetracycline (for chromosomal recombinant strains, half of the concentrations were used). Optical density (OD) was measured at 450 and 600 nm when grown in minimal medium and rich medium, respectively.

Testing functionality of MurJ fusions

Functionality experiments were performed as described previously (Butler *et al.*, 2013, 2014) using the MurJ depletion strain XL03 that expresses a functional MurJ copy from plasmid pRC7MurJ Δ Kan. This plasmid has partitioning defects causing some of the daughter cells to lose this plasmid and die after division, unless they are complemented with a functional copy of MurJ since MurJ is essential for *E. coli*. In addition, pRC7MurJ Δ Kan contains the *lacZ* operon and thus cells that lost the plasmid will also lose their ability to produce the blue color on 20 $\mu\text{g.L}^{-1}$ X-gal (Sigma) agar plates, as our pTHV and pSAV-based plasmids do not. The functional FPs fusions to MurJ were obtained by selecting the white colonies on Ampicillin or chloramphenicol X-gal agar dish after transformation. The growth and fluorescence intensity of strains containing a fusion expressing plasmid were investigated with Synergy Mx BioTek plate reader and microscopy.

Immunolocalization experiments

E. coli cells were fixed with FGA (2.8% formaldehyde and 0.04% glutaraldehyde final concentration) for 15–20 min in a shaking waterbath after steady-state growth in Gb1 minimal medium at 28°C (Den Blaauwen *et al.*, 2003). Subsequently, cells were permeabilized and immunolabeled with antibodies as described (Buddelmeijer *et al.*, 2013). The antibodies against FtsZ, FtsN, MurG used in this study were purified as described (Aarsman *et al.*, 2005; Mohammadi *et al.*, 2007). Polyclonal antibodies against MraY were obtained from Rabbits that were injected with an antigenic peptide that was designed with the Epiros program (Biosiris). Based on the predicted topology by Bouhss *et al.* (Bouhss *et al.*, 1999; Al-Dabbagh *et al.*, 2008) the peptide localizes in the cytoplasmic loop between TMH 1 and 2. The sequence was H2N-G54QVVRNDGPESHFS67C-COOH. The cysteine was added at the C-terminal to perform the coupling with the KLH carrier protein.

Depletion or inactivation of divisome proteins

Strain XL17, XL15, XL13 and XL14 that contain a MurJ FP fusion and the temperature sensitive divisome mutant proteins FtsW, FtsI, FtsQ and FtsA, respectively, were diluted 1:1000 from overnight cultures (grown at 30°C) into fresh

LB medium (LB Δ NaCl medium for XL13) with 12.5 $\mu\text{g mL}^{-1}$ chloramphenicol, and grown at 30°C to OD₆₀₀ of approximately 0.2. Cells were diluted 1:5 into the same fresh medium that was pre-warmed at 30 and 42°C, respectively, and kept growing for 2 mass doublings. To deplete FtsN, strain XL23 was diluted 1:1000 from an overnight culture (grown at 30°C) into fresh LB medium with 12.5 $\mu\text{g mL}^{-1}$ chloramphenicol and 0.5% w/v glucose, and kept growing at 30°C to OD₆₀₀ of approximately 0.2. Induced with 0.2% w/v arabinose was set as control. After growth, cells were immobilized on 1% agarose slides, and imaged live by phase contrast and epifluorescence microscopy.

MTSES assay

Sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES) was purchased from Santa Cruz Biotechnology Company. A gradient assay on MTSES concentrations (0, 0.01, 0.05, 0.1 or 0.5 mg.mL^{-1}) was firstly applied on Gb1 steady-state grown strain XL03 that expressed the MurJ A29C mutant. MurJ localization was determined by microscopy at 10 and 40 min after addition of MTSES. Upon induction of expression with 20 μM IPTG for 2 MDs rapid cell lysis was observed after adding MTSES for 10 min at concentrations 0.1 or 0.5 mM. A pattern of MurJ midcell localization was observed despite the increase in MTSES concentrations (Fig. S7A). However, we noticed that lower concentration of MTSES (0.01 mM) was not sufficient to influence the cell growth, and cells lysed too fast at higher MTSES concentrations (above 0.05 mM) to determine MurJ localization at a longer time scale. Since MTSES targets MurJ A29C by covalent binding, we suspected that the higher expression of MurJ might help to increase the resistance to MTSES. Thus, 40 μM IPTG was used for MurJ induction (2 MDs). Indeed, higher expression of A29C slightly improved the survival of cells, with a yield of better fluorescent signal, and MurJ midcell localization was able to be determined at both 10 and 40 min after MTSES addition. In addition, 0.5 mM MTSES was sufficient to show the influence on cell growth. Thus, 0.5 mM MTSES and 40 μM IPTG inductions were used for the investigation of MurJ localization in the presence of MTSES.

Microscope and image analysis

For localization imaging, cells were immobilized on 1.0% agarose (w/v in Gb1) pads and imaged immediately. Fluorescence microscopy was carried out either with an Olympus BX-60 fluorescence microscope equipped with a CoolSnap fx (Photometrics) CCD camera, a 100 $\times$ /N.A. 1.35 oil objective, and software 'ImageJ-MicroManager', or with a Nikon Eclipse Ti microscope equipped with a C11440-22CU

Hamamatsu ORCA camera, a CFI Plan Apochromat DM 100 × oil objective, an Intensilight HG 130W lamp and the NIS elements software (version 4.20.01).

Images were analyzed with Coli-Inspector supported by the ObjectJ plugin for ImageJ (version 1.49v) (Vischer *et al.*, 2015). Briefly, the length and diameter of more than 1200 individual cells were marked and analysis in the phase contrast images. Fluorescence and phase contrast images were aligned and fluorescence background was subtracted as described (Vischer *et al.*, 2015). The fluorescence of each cell was collected in a one pixel wide bar with the length of the cell. A map of the diameter or the fluorescence localization and intensity was generated with the cells sorted according to increasing cell from left to right. Because cells were grown to steady state, the length of the cells can be directly correlated to the cell division cycle age. A collective profile is created from all cell profiles in a map. They are first resampled to a normalized cell length of 100 data points, and then averaged to a single plot, in either 1 group or more age bins. The FCplus (the extra fluorescence at midcell in comparison to the fluorescence in the rest of the cell) and Ringfraction (FCplus/Total cellular fluorescence) profiles were generated with the 'graph assistant' macro in ObjectJ (18).

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X.L. and T.B. designed most experiments. X.L. performed most experiments and N.Y.M. designed and performed some of the experiments. X.L. and TdB analyzed the data and wrote the manuscript. A.B. isolated MraY and made antibodies against the protein. All authors critically read the manuscript and gave feedback.

Conflict of interests

The authors declare no conflicting interests.

References

- Aarsman, M.E.G., Piette, A., Fraipont, C., Vinkenvleugel, T.M.F., Nguyen-Distèche, M., and den Blaauwen, T. (2005) Maturation of the *Escherichia coli* divisome occurs in two steps. *Molecular Microbiology*, **55**, 1631–1645.
- Al-Dabbagh, B., Henry, X., El Ghachi, M., Auger, G., Blanot, D., Parquet, C., *et al.* (2008) Active site mapping of MraY, a member of the Polyprenyl-phosphate N-Acetylhexosamine 1-Phosphate transferase superfamily, catalyzing the first membrane step of peptidoglycan biosynthesis †. *Biochemistry*, **47**, 8919–8928.
- Alexeeva, S., Gadella, T.W.J., Verheul, J., Verhoeven, G.S. and den Blaauwen, T. (2010) Direct interactions of early and late assembling division proteins in *Escherichia coli* cells resolved by FRET. *Molecular Microbiology*, **77**, 384–398.
- Azam, M.A. and Jayaram, U. (2015) Inhibitors of alanine racemase enzyme: a review. *Journal of Enzyme Inhibition and Medicinal Chemistry*, **6366**, 1–10.
- Banzhaf, M. and van den Berg van Saparoea, B., Terrak, M., Fraipont, C., Egan, A., Philippe, J., *et al.* (2012) Cooperativity of peptidoglycan synthases active in bacterial cell elongation. *Molecular Microbiology*, **85**, 179–194.
- Barreteau, H., Kovač, A., Boniface, A., Sova, M., Gobec, S. and Blanot, D. (2008) Cytoplasmic steps of peptidoglycan biosynthesis. *FEMS Microbiology Reviews*, **32**, 168–207.
- Bertsche, U., Kast, T., Wolf, B., Fraipont, C., Aarsman, M.E.G., Kannenberg, K., *et al.* (2006) Interaction between two murein (peptidoglycan) synthases, PBP3 and PBP1B, in *Escherichia coli*. *Molecular Microbiology*, **61**, 675–690.
- Bisson Filho, A.W., Hsu, Y.-P., Squyres, G., Kuru, E., Wu, F. and Jukes, C. *et al.* (2017) Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. *Science* (80-) **355**: 739–743.
- Blaauwen, T.D., Aarsman, M.E.G., Vischer, N.O.E. and Nanninga, N. (2003) Penicillin-binding protein PBP2 of *Escherichia coli* localizes preferentially in the lateral wall and at mid-cell in comparison with the old cell pole. *Molecular Microbiology*, **47**, 539–547.
- den Blaauwen, T., Buddelmeijer, N., Aarsman, M.E.G., Hameete, C.M. and Nanninga, N. (1999) Timing of FtsZ assembly in *Escherichia coli*. *Journal of Bacteriology*, **181**, 5167–5175.
- den Blaauwen, T., Hamoen, L.W. and Levin, P.A. (2017) The divisome at 25: the road ahead. *Current Opinion in Microbiology*, **36**, 85–94.
- den Blaauwen, T., de Pedro, M.A., Nguyen-Distèche, M. and Ayala, J.A. (2008) Morphogenesis of rod-shaped sacculi. *FEMS Microbiology Reviews*, **32**, 321–344.
- Bolla, J.R., Sauer, J.B., Wu, D., Mehmood, S., Allison, T.M. and Robinson, C.V. (2018) Direct observation of the influence of cardiolipin and antibiotics on lipid II binding to MurJ. *Nature Chemistry*, **10**, 363–371.
- Bouhss, A., Mengin-Lecreulx, D., le Beller, D. and van Heijenoort, J. (1999) Topological analysis of the MraY protein catalysing the first membrane step of peptidoglycan synthesis. *Molecular Microbiology*, **34**, 576–585.
- Bouhss, A., Trunkfield, A.E., Bugg, T.D.H. and Mengin-Lecreulx, D. (2008) The biosynthesis of peptidoglycan lipid-linked intermediates. *FEMS Microbiology Reviews*, **32**, 208–233.
- Buddelmeijer, N., Aarsman, M. and den Blaauwen, T. (2013) Immunolabeling of proteins in situ in *Escherichia coli* K12 strains. *Bio-Protocol*, **3**, 1–5.
- Butler, E.K., Davis, R.M., Bari, V., Nicholson, P.A. and Ruiz, N. (2013) Structure-function analysis of MurJ reveals a solvent-exposed cavity containing residues essential for peptidoglycan biogenesis in *Escherichia coli*. *Journal of Bacteriology*, **195**, 4639–4649.
- Butler, E.K., Tan, W.B., Joseph, H. and Ruiz, N. (2014) Charge requirements of lipid II flippase activity in *Escherichia coli*. *Journal of Bacteriology*, **196**, 4111–4119.

- Chamakura, K.R., Sham, L.-T., Davis, R.M., Min, L., Cho, H., Ruiz, N., et al. (2017) A viral protein antibiotic inhibits lipid II flippase activity. *Nature Microbiology*, **2**(11), 1480–1484.
- Chen, J.C. and Beckwith, J. (2008) FtsQ, FtsL and FtsI require FtsK, but not FtsN, for co-localization with FtsZ during *Escherichia coli* cell division. *Molecular Microbiology*, **42**, 395–413.
- Chen, X., Zaro, J.L. and Shen, W.C. (2013) Fusion protein linkers: property, design and functionality. *Advanced Drug Delivery Reviews*, **65**, 1357–1369.
- Cho, H., Uehara, T. and Bernhardt, T.G. (2014) Beta-lactam antibiotics induce a lethal malfunctioning of the bacterial cell wall synthesis machinery. *Cell*, **159**, 1310–1311.
- Cho, H., Wivagg, C.N., Kapoor, M., Barry, Z., Rohs, P.D.A., Suh, H. et al. (2016) Bacterial cell wall biogenesis is mediated by SEDS and PBP polymerase families functioning semi-autonomously. *Nature Microbiology*, **1**, 16172.
- Contreras-Martel, C., Martins, A., Ecobichon, C., Trindade, D.M., Mattei, P.-J., Hicham, S., et al. (2017) Molecular architecture of the PBP2–MreC core bacterial cell wall synthesis complex. *Nature Communications*, **8**, 776.
- Cranfill, P.J., Sell, B.R., Baird, M.A., Allen, J.R., Lavagnino, Z., de Gruiter, H.M., et al. (2016) Quantitative assessment of fluorescent proteins. *Nature Methods*, **13**, 557–562.
- Dai, K. and Lutkenhaus, J. (1991) *ftsZ* is an essential cell division gene in *Escherichia coli*. *Journal of Bacteriology*, **173**, 3500–3506.
- Datsenko, K. and Wanner, B. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences*, **97**(12), 6640–6645.
- Derouichei, R., Bénédicti, H., Lazzaroni, J.C., Lazdunski, C. and Lloubès, R. (1995) Protein complex within *Escherichia coli* inner membrane: TolA N-terminal domain interacts with TolQ and TolR proteins. *Journal of Biological Chemistry*, **270**, 11078–11084.
- Du, S., Pichoff, S. and Lutkenhaus, J. (2016) FtsEX acts on FtsA to regulate divisome assembly and activity. *Proceedings of the National Academy of Sciences* 201606656.
- Dubuisson, J.F., Vianney, A. and Lazzaroni, J.C. (2002) Mutational analysis of the TolA C-Terminal domain of *Escherichia coli* and genetic evidence for an interaction between TolA and TolB. *Journal of Bacteriology*, **184**, 4620–4625.
- Elhenawy, W., Davis, R.M., Fero, J., Salama, N.R., Felman, M.F. and Ruiz, N. (2016) The O-Antigen flippase wzk can substitute for MurJ in peptidoglycan synthesis in *Helicobacter pylori* and *Escherichia coli*. *PLoS One*, **11**, 1–16.
- Fenton, A.K., el Mortaji, L., Lau, D.T.C., Rudner, D.Z. and Bernhardt, T.G. (2016) CozE is a member of the MreCD complex that directs cell elongation in *Streptococcus pneumoniae*. *Nature Microbiology*, **2**, 16237.
- Fraipont, C., Alexeeva, S., Wolf, B., derPloeg, R., Schloesser, M. and den Blaauwen, T. et al. (2011) The integral membrane FtsW protein and peptidoglycan synthase PBP3 form a subcomplex in *Escherichia coli*. *Microbiology*, **157**, 251–259.
- Gerding, M.A., Liu, B., Bendezi, F.O., Hale, C.A., Bernhardt, T.G. and de Boer, P.A.J. (2009) 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. *Journal of Bacteriology*, **191**, 7383–7401.
- Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A. and Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, **6**, 343–345.
- Goehring, N.W., Gonzalez, M.D. and Beckwith, J. (2006) Premature targeting of cell division proteins to midcell reveals hierarchies of protein interactions involved in divisome assembly. *Molecular Microbiology*, **61**, 33–45.
- Goehring, N.W., Gueiros-filho, F. and Beckwith, J. (2005) Premature targeting of a cell division protein to midcell allows dissection of divisome assembly in *Escherichia coli*. *Genes & Development*, **19**, 127–137.
- Gray, A.N., Egan, A.J.F., van't Veer, I.L., Verheul, J., Colavin, A., Koumoutsis, A. et al. (2015) Coordination of peptidoglycan synthesis and outer membrane constriction during *Escherichia coli* cell division. *eLife*, **4**, 1–29.
- Hong, Y., Liu, M.A. and Reeves, P.R. (2018) Progress in our understanding of Wzx flippase for translocation of bacterial membrane lipid-linked oligosaccharide. *Journal of Bacteriology*, **200**, 1–14.
- Huang, Y. (2003) Structure and mechanism of the Glycerol-3-Phosphate transporter from *Escherichia coli*. *Science* (80-), **301**, 616–620.
- Hug, I. and Feldman, M.F. (2011) Analogies and homologies in lipopolysaccharide and glycoprotein biosynthesis in bacteria. *Glycobiology*, **21**, 138–151.
- Ikeda, M., Sato, T., Wachi, M., Jung, H.K., Ishino, F., Kobayashi, Y. et al. (1989) Structural similarity among *Escherichia coli* FtsW and RodA proteins and *Bacillus subtilis* SpoVE protein, which function in cell division, cell elongation, and spore formation, respectively. *Journal of Bacteriology*, **171**, 6375–6378.
- Ishino, F., Jung, H.K., Ikeda, M., Doi, M., Wachi, M. and Matsubashi, M. (1989) New mutations *fts-36*, *fts-33*, and *ftsW* clustered in the *mra* region of the *Escherichia coli* chromosome induce thermosensitive cell growth and division. *Journal of Bacteriology*, **171**, 5523–5530.
- Khattar, M.M., Begg, K.J. and Donachie, W.D. (1994) Identification of FtsW and characterization of a new *ftsW* division mutant of *Escherichia coli*. *Journal of Bacteriology*, **176**, 7140–7147.
- Kuk, A.C.Y., Mashalidis, E.H. and Lee, S.Y. (2017) Crystal structure of the MOP flippase MurJ in an inward-facing conformation. *Nature Structural & Molecular Biology*, **24**, 171–176.
- Lara, B., Mengin-Lecreulx, D., Ayala, J. A. and van Heijenoort, J. (2005) Peptidoglycan precursor pools associated with MraY and FtsW deficiencies or antibiotic treatments. *FEMS Microbiology Letters*, **250**, 195–200.
- Leclercq, S., Derouaux, A., Olatunji, S., Fraipont, C., Egan, A.J.F., Vollmer, W., et al. (2017) Interplay between penicillin-binding proteins and SEDS proteins promotes bacterial cell wall synthesis. *Scientific Reports*, **7**(1), 43306.
- Li, G.W., Burkhardt, D., Gross, C. and Weissman, J.S. (2014) Quantifying absolute protein synthesis rates reveals principles underlying allocation of cellular resources. *Cell*, **157**, 624–635.
- Liu, B., Persons, L., Lee, L. and deBoer, P.A.J. (2015) Roles for both FtsA and the FtsBLQ subcomplex in

- FtsN-stimulated cell constriction in *Escherichia coli*. *Molecular Microbiology*, **95**, 945–970.
- Lutkenhaus, J. (2009) FtsN - trigger for septation. *Journal of Bacteriology*, **191**, 7381–7382.
- Macheboeuf, P., Contreras-Martel, C., Job, V., Dideberg, O. and Dessen, A. (2006) Penicillin binding proteins: key players in bacterial cell cycle and drug resistance processes. *FEMS Microbiology Reviews*, **30**, 673–691.
- Manat, G., Roure, S., Auger, R., Bouhss, A., Barreteau, H., Mengin-Lecreulx, D. et al. (2014) Deciphering the metabolism of undecaprenyl-phosphate: the bacterial cell-wall unit carrier at the membrane frontier. *Microbial Drug Resistance*, **20**, 199–214.
- Matsuzawa, H., Hayakawa, K., Sato, T. and Imahori, K. (1973) Characterization and genetic analysis of a mutant of *Escherichia coli* K-12 with rounded morphology. *Journal of Bacteriology*, **115**, 436–442.
- Meeske, A.J., Riley, E.P., Robins, W.P., Uehara, T., Mekalanos, J.J., Kahne, D., et al. (2016) SEDS proteins are a widespread family of bacterial cell wall polymerases. *Nature*, **537**, 1–15.
- Meeske, A.J., Sham, L.-T., Kimsey, H., Koo, B.-M., Gross, C.A., Bernhardt, T.G. et al. (2015) MurJ and a novel lipid II flippase are required for cell wall biogenesis in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the USA*, **112**, 6437–42.
- Meiresonne, N.Y., van der Ploeg, R., Hink, M.A. and den Blaauwen, T. (2017) Activity-related conformational changes in d,d-Carboxypeptidases revealed by in vivo periplasmic Förster resonance energy transfer assay in *Escherichia coli*. *MBio*, **8** (5), e01089-17.
- Mengin-lecreulx, D., Flouret, B., Heijenoort, J. V. A. N., S. E.R.C.N.R., de Biochimie, I. and Paris-sud, U., (1983) Pool levels of UDP N-acetylglucosamine and UDP N-acetylglucosamine-enolpyruvate in *Escherichia coli* and correlation with peptidoglycan synthesis. *Journal of Bacteriology*, **154**, 1284–1290.
- Mengin-Lecreulx, D., Texier, L., Rousseau, M. and van Heijenoort, J. (1991) The *murG* gene of *Escherichia coli* codes for the UDP-N-acetylglucosamine: N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase involved in the membrane steps of peptidoglycan synthesis. *Journal of Bacteriology*, **173**, 4625–4636.
- Mohammadi, T., van Dam, V., Sijbrandi, R., Vernet, T., Zapun, A., Bouhss, A., et al. (2011) Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane. *The EMBO Journal*, **30**, 1425–1432.
- Mohammadi, T., Karczarek, A., Crouvoisier, M., Bouhss, A., Mengin-Lecreulx, D. and den Blaauwen, T. (2007) The essential peptidoglycan glycosyltransferase MurG forms a complex with proteins involved in lateral envelope growth as well as with proteins involved in cell division in *Escherichia coli*. *Molecular Microbiology*, **65**, 1106–1121.
- Mohammadi, T., Sijbrandi, R., Lutters, M., Verheul, J., Martin, N.I., den Blaauwen, T. et al. (2014) Specificity of the transport of lipid II by FtsW in *Escherichia coli*. *Journal of Biological Chemistry*, **289**, 14707–14718.
- Morgenstein, R.M., Bratton, B.P., Nguyen, J.P., Ouzounov, N., Shaevitz, J.W. and Gitai, Z. (2015) RodZ links MreB to cell wall synthesis to mediate MreB rotation and robust morphogenesis. *Proceedings of the National Academy of Sciences USA*, **112**, 12510–12515.
- Neuhaus, F.C. (1962) The enzymatic synthesis of D-alanyl-D-alanine. I. Purification and properties of D-alanyl-D-alanine synthetase. *Journal of Biological Chemistry*, **237**, 778–786.
- Neuhaus, F.C. and Lynch, J.L. (1972) Studies on the inhibition of D-alanyl-D-alanine synthetase by the antibiotic D-cycloserine. *Biochemical and Biophysical Research Communications*, **8**, 377–382.
- Nørholm, M.H.H. (2010) A mutant Pfu DNA polymerase designed for advanced uracil-excision DNA engineering. *BMC Biotechnology*, **10**, 21.
- Oswald, F., Varadarajan, A., Lill, H., Peterman, E.J.G. and Bollen, Y.J.M. (2016) MreB-dependent organization of the *E. coli* cytoplasmic membrane controls membrane protein diffusion. *Biophysical Journal*, **110**, 1139–1149.
- Pastoret, S., Fraipont, C., den Blaauwen, T., Aarsman, M.E.G., Thomas, A. and Brasseur, R. et al. (2004) Functional analysis of the cell division protein FtsW of *Escherichia coli*. *Journal of Bacteriology*, **186**, 8370–8379.
- de Pedro, M.A., Quintela, J.C., Höltje, J.V. and Schwarz, H. (1997) Murein segregation in *Escherichia coli*. *Microbiology*, **179**, 2823–2834.
- Pichoff, S. and Lutkenhaus, J. (2002) Unique and overlapping roles for ZipA and FtsA in septal ring assembly in *Escherichia coli*. *The EMBO Journal*, **21**, 685–693.
- Pichoff, S. and Lutkenhaus, J. (2005) Tethering the Z ring to the membrane through a conserved membrane targeting sequence in FtsA. *Molecular Microbiology*, **55**, 1722–1734.
- van der Ploeg, R., Goudelis, S.T. and den Blaauwen, T. (2015) Validation of FRET assay for the screening of growth inhibitors of *Escherichia coli* reveals elongasome assembly dynamics. *International Journal of Molecular Sciences*, **16**, 17637–17654.
- van der Ploeg, R., Verheul, J., Vischer, N. O. E., Alexeeva, S., Hoogendoorn, E. and Postma, M. et al. (2013) Colocalization and interaction between elongasome and divisome during a preparative cell division phase in *Escherichia coli*. *Molecular Microbiology*, **87**, 1074–1087.
- Pomorski, T. and Menon, A.K. (2006) Lipid flippases and their biological functions. *Cellular and Molecular Life Sciences*, **63**, 2908–2921.
- Priyadarshini, R., De Pedro, M.A. and Young, K.D. (2007) Role of peptidoglycan amidases in the development and morphology of the division septum in *Escherichia coli*. *Journal of Bacteriology*, **189**, 5334–5347.
- Prosser, G.A. and de Carvalho, L.P.S. (2013) Kinetic mechanism and inhibition of *Mycobacterium tuberculosis* D-alanine: D-alanine ligase by the antibiotic D-cycloserine. *The FEBS Journal*, **280**, 1150–1166.
- Qiao, Y., Srisuknimit, V., Rubino, F., Schaefer, K., Ruiz, N., Walker, S. et al. (2017) Lipid II overproduction allows direct assay of transpeptidase inhibition by [beta]-lactams. *Nature Chemical Biology*, **13**, 793–798.
- Rico, A.I., García-Ovalle, M., Mingorance, J. and Vicente, M. (2004) Role of two essential domains of *Escherichia coli* FtsA in localization and progression of the division ring. *Molecular Microbiology*, **53**, 1359–1371.
- de Roubin, M.R., Mengin-Lecreulx, D. and van Heijenoort, J. (1992) Peptidoglycan biosynthesis in *Escherichia coli*.

- variations in the metabolism of alanine and D-alanyl-D-alanine. *Journal of General Microbiology*, **138**(Pt 8), 1751–1757.
- Rubino, F.A., Kumar, S., Ruiz, N., Walker, S. and Kahne, D. (2018) Membrane potential is required for MurJ function. *Journal of the American Chemical Society*, **140**(13), 4481–4484. <https://doi.org/10.1021/jacs.8b00942>
- Ruiz, N. (2008) Bioinformatics identification of MurJ (MviN) as the peptidoglycan lipid II flippase in *Escherichia coli*. *Proceedings of the National Academy of Sciences USA*, **105**, 15553–15557.
- Ruiz, N. (2009) *Streptococcus pyogenes* YtgP (Spy-0390) complements *Escherichia coli* strains depleted of the putative peptidoglycan flippase MurJ. *Antimicrobial Agents and Chemotherapy*, **53**, 3604–3605.
- Ruiz, N. (2015) Lipid flippases for bacterial peptidoglycan biosynthesis. *Lipid Insights*, **8**, 21–31.
- Ruiz, N. (2016) Filling holes in peptidoglycan biogenesis of *Escherichia coli*. *Current Opinion in Microbiology*, **34**, 1–6.
- Sauvage, E., Kerff, F., Terrak, M., Ayala, J.A. and Charlier, P. (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiology Reviews*, **32**, 234–258.
- Scheffers, D.J. and Tol, M.B. (2015) LipidII: just another brick in the wall? *PLOS Pathogens*, **11**, 1–12.
- Sham, L.-T., Butler, E. K., Lebar, M. D., Kahne, D., Bernhardt, T. G. and Ruiz, N. (2014) MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis. *Science* (80-) **345**: 220–222.
- Shih, Y.L., Kawagishi, I. and Rothfield, L. (2005) The MreB and Min cytoskeletal-like systems play independent roles in prokaryotic polar differentiation. *Molecular microbiology*, **58**, 917–928.
- Sieger, B., Schubert, K., Donovan, C. and Bramkamp, M. (2013) The lipid II flippase RodA determines morphology and growth in *Corynebacterium glutamicum*. *Molecular Microbiology*, **90**, 966–982.
- Sun, Q. and Margolin, W. (1998) FtsZ dynamics during the division cycle of live. *Escherichia coli Cells*, **180**, 2050–2056.
- Suzuki, H., Nishimura, Y. and Hirota, Y. (1978) On the process of cellular division in *Escherichia coli*: a series of mutants of *E. coli* altered in the penicillin-binding proteins. *Proceedings of the National Academy of Sciences USA*, **75**, 664–668.
- Taguchi, A., Welsh, M.A., Marmont, L.S., Lee, W., Kahne, D., Bernhardt, T. G. et al. (2018) FtsW is a peptidoglycan polymerase that is activated by its cognate penicillin-binding protein. *bioRxiv*. Available at: <https://biorxiv.org/content/early/2018/06/29/358663.abstract>.
- Taschner, P.E., Huls, P.G., Pas, E. and Woldringh, C.L. (1988) Division behavior and shape changes in isogenic *ftsZ*, *ftsQ*, *ftsA*, *pbpB*, and *ftsE* cell division mutants of *Escherichia coli* during temperature shift experiments. *Journal of Bacteriology*, **170**, 1533–1540.
- van Teeffelen, S., Wang, S., Furchtgott, L., Huang, K.C., Wingreen, N.S., Shaevitz, J.W. et al. (2011) The bacterial actin MreB rotates, and rotation depends on cell-wall assembly. *Proceedings of the National Academy of Sciences*, **108**, 15822–15827.
- To Sham, L., Zheng, S., Yakhnina, A.A., Kruse, A.C. and Bernhardt, T.G. (2018) Loss of specificity variants of Wzx suggest that substrate recognition is coupled with transporter opening in MOP-family flippases. *Molecular Microbiology*. 2018. doi: 10.1111/mmi.14002.
- Tsang, M.J. and Bernhardt, T.G. (2015) Guiding divisome assembly and controlling its activity. *Current Opinion in Microbiology*, **24**, 60–65.
- Veening, J., Smits, W.K., Hamoen, L.W., Jongbloed, J.D.H. and Kuipers, O.P. (2004) Visualization of Differential gene expression by improved cyan fluorescent protein and yellow fluorescent protein production in *Bacillus subtilis*. *Applied and Environmental Microbiology*, **70**, 6809–6815.
- Vischer, N.O.E., Verheul, J., Postma, M., Berg van Saparoea, B., van den Galli, E. and Natale, P. et al. (2015) Cell age dependent concentration of *Escherichia coli* divisome proteins analyzed with ImageJ and ObjectJ. *Frontiers in Microbiology*, **6**, 1–18.
- Vollmer, W., Blanot, D. and de Pedro, M.A. (2008) Peptidoglycan structure and architecture. *FEMS Microbiology Reviews*, **32**, 149–167.
- Vollmer, W., Blanot, D., de Pedro, M.A., Scheffers, D., Pinho, M., Nikolaidis, I. et al. (2005) Structural and mechanistic basis of penicillin-binding protein inhibition by lactvicins. *FEMS Microbiology Reviews*, **30**, 565–569.
- Wang, E., Walsh, C. and Walsh, C. (1978) Suicide substrates for the alanine racemase of *Escherichia coli*. *Biochemistry*, **17**, 1313–1321.
- Weiss, D.S. (2015) Last but not least: new insights into how FtsN triggers constriction during *Escherichia coli* cell division. *Molecular Microbiology*, **95**, 903–909.
- White, C.L. and Gober, J.W. (2012) MreB: pilot or passenger of cell wall synthesis? *Trends in Microbiology*, **20**, 74–79.
- Wood, B.W.A. and Gunsalus, I.C. (1959) D-Alanine formation; a racemase in *Streptococcus faecalis*. *Journal of Biological Chemistry*, **190**, 403–416.
- Young, K.D. (2014) A flipping cell wall ferry. *Microbiology*, **345**, 139–140.
- Yousif, S.Y., Broome-Smith, J.K. and Spratt, B.G. (1985) Lysis of *Escherichia coli* by beta-lactam antibiotics: deletion analysis of the role of penicillin-binding proteins 1A and 1B. *Journal of General Microbiology*, **131**, 2839–2845.
- Zheng, S., Sham, L.-T., Rubino, F.A., Brock, K.P., Robins, W.P., Mekalanos, J.J., et al. (2018) Structure and mutagenic analysis of the lipid II flippase MurJ from *Escherichia coli*. *Proceedings of the National Academy of Sciences*, **115**: 6709, LP-6714.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. 1. MurJ localizes both in the lateral wall and at mid-cell. Strain XL08 containing the chromosomal mNG-MurJ fusion shows a cell-cycle-dependent localization in contrast to the homogenously localizing integral membrane protein mNG-GlpT. Cells were grown in Gb1 minimal

medium to steady state at 28°C. Left: MurJ profiles; Right: membrane control protein GlpT profiles. (A) Collective profiles of localization of MurJ and GlpT. For each protein, the diameter (black lines) and fluorescence (green lines) profiles along normalized cell length are shown in 10 % age class bins. (B) Maps of diameter profiles (magenta) and fluorescence profiles (green). Cells are plotted by length, ascending from left to right. (C) Phase contrast image (left) and the corresponding fluorescence image (right) for each protein. Scale bar equals 5 µm. More than 1200 cells were included for both analyses.

Fig. 2. Timing of MurJ midcell localization. mNG-(GGS)2-MurJ cellular localization was compared with the localization of divisomal and PG precursor synthesizing proteins. XL08 strain was grown to steady state in Gb1 medium. MurJ localization was determined in living cells. After fixation with FGA, the rest of cells were immunolabeled with antibodies against FtsZ, FtsN, MraY and MurG. Diameter (black lines) and Fluorescence (colored lines) profiles were plotted into 10 % age class bins along the normalized cell length in %. More than 1200 cells were included.

Fig. 3. MurJ requires the core divisome for its midcell localization. Chromosomal mNG-(GGS)2-MurJ fusion was introduced into strains that harbor temperature sensitive, depletable, or deleted division proteins (except that the mNG-(GGS)2-MurJ was expressed in Δ tolA and Δ pal strains from plasmid pXL05, and mCherry-MurJ was expressed in Δ amiABC strain from plasmid pNM037). (A-E) MurJ localization in XL08 (WT), ftsA 1882 (Ts), ftsQ1 (Ts), ftsW (Ts) and ftsI2185 (Ts) backgrounds. Left: Localization at permissive temperature of 30 °C. Right: Localization after growth at the non-permissive temperature of 42 °C for 2 mass doublings (MD)s. (F) MurJ localization in FtsN depletion background at 30 °C. Left: localization in the presence of 0.2% w/v arabinose. Right: Localization of MurJ in the presence of glucose for 2 MDs. (G-H) mCh-MurJ localization in Δ mrcA (PBP1A), Δ mrcB (PBP1B), Δ pal, Δ tolA and Δ amiABC background at 30 °C, respectively. Scale bar equals 5 µm.

Fig. 4. Localization of cell division proteins in the presence of the PBP3 inhibitor aztreonam for 2 MDs. Cell division is inhibited but the localization and assemblage of the divisome continues, whereas the localization of MurJ is lost. In Gb1 at 28 °C, steady state growing XL08 was treated with 1 mg.L⁻¹ aztreonam, MurJ localization was determined in living cells, and FtsZ, FtsN, MraY and MurG were immunolabeled with antibodies after fixation. Diameter (black lines) and fluorescence (colored lines) profiles were plotted into 10 % age class bins along the normalized cell length. More than 1200 cells were included.

Fig. 5. Inhibition of lipid II biogenesis blocks MurJ mid-cell localization. In Gb1 at 28 °C, steady state growing

XL08 was treated with increasing concentrations of D-cycloserine (DCS), and mNG-MurJ localization was determined at 30 minutes after addition of DCS. (A) Growth curves were plotted as time against the natural logarithm of the optical density of the cells. The black arrow indicates the addition of DCS. (B) Fluorescence maps profiles of MurJ localization. Cells are plotted according to increasing cell length from top to bottom. From left to right, the maps correspond to 0, 0.05, 0.1, 0.5, 1 and 2 µg.L⁻¹ of DCS concentrations. More than 1200 cells were included. (C) Phase contrast images (upper) and correspond fluorescence images (down) of cells treated with DCS. Scale bar equals 5 µm.

Fig. 6. Expression of non-functional FtsW mutants abolishes MurJ midcell localization. XL08 expressing mutant FtsW R145A or FtsW K153N was grown in Gb1 minimal medium to steady state at 28 °C. IPTG (20 µM) was used to induce expression of the FtsW mutants from plasmid. MurJ localization was determined in living cells and the presence of the divisome was confirmed by immunolabeling of FtsN, after fixation. (A) Localization of MurJ and FtsN after expression of FtsW R145A. (B) Localization of MurJ and FtsN after expression of FtsW K153N. For each part: top, average localization profiles plotted along normalized cell length (n 1200 cells). Black lines indicate cell diameter. Green lines and red lines indicate MurJ and FtsN localization, respectively. Middle, map profiles of cell diameter and MurJ or FtsN localization sorted by ascending cell length. Bottom, phase contrast and fluorescence microscopy images. Scale bar equals 5 µm.

Fig. 7. MurJ non-functional mutant R18A does not influence its localization. (A) The plasmid expressing the mNG-MurJ R18A fusion was introduced into LMC500. This strain was grown in Gb1 minimal medium to steady state at 28 °C, and expression was induced with 20 µM IPTG for 2 MDs. The diameter (black lines) and fluorescence (green lines) profiles along normalized cell length are shown in 10 % age class bins. More than 1200 cells were included. Scale bar equals 5 µm. (B) The plasmid expressing the mNG-MurJ R18A fusion was introduced into the MurJ depletion strain XL20. Mutant localization was determined in the presence of arabinose (wild type MurJ expression) or glucose (wild type MurJ depletion). Scale bar equals 1 µm.

Fig. 8. Localization of MurJ cysteine mutants in the presence of MTSES. Strains expressing MurJ single-cysteine-mutants from plasmid were grown to steady state in Gb1 medium at 28 °C, and expression was induced with IPTG for 2 MDs. MurJ localization was determined at 10 min and 40 min after the addition of MTSES (for mutant S263C, localization was determined at 10 min and 20 min because of its higher sensitivity to MTSES). Average

of MurJ localization profiles were plotted along normalized cell axis. Black lines indicate the MurJ localization in the absence of MTSES, green and red lines indicate the MurJ localization at 10 and 40 min in the presence of MTSES, respectively. More than 1200 cells were included for each experiment. (A-D) Only growth but not localization of functional mutants A29C, S263C, N49C and E273C is affected by MTSES. (E) Neither growth nor localization of cysteine free mutants is influenced by MTSES. (F) Neither growth nor localization of wild type MurJ is influenced by MTSES. (G and H) Localization of non-functional MurJ mutants R18C and R52C is not affected by MTSES.

Fig. 9. Model of MurJ recruitment and function in septal peptidoglycan synthesis. (A) Recruitment of MurJ to midcell when FtsW is activated. (B) MurJ binds substrate. (C) MurJ flips lipid II across the IM by changing its structure from the inward-facing conformation into the outward-facing state. (D) Unloaded MurJ changes back into the default inward-facing state to participate in a new cycle. (i) Loss-of-function MurJ mutants that localize but are not able to flip lipid II. (ii) Functional MurJ mutants that are inactivated by MTSES still localize but are not able to flip lipid II.