



Role of endopeptidases in peptidoglycan synthesis mediated by alternative cross-linking enzymes in *Escherichia coli*

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Abstract

Bacteria resist to the turgor pressure of the cytoplasm through a net-like macromolecule, the peptidoglycan, made of glycan strands connected via peptides cross-linked by penicillin-binding proteins (PBPs). We recently reported the emergence of β -lactam resistance resulting from a bypass of PBPs by the YcbB L,D-transpeptidase (LdtD), which form chemically distinct 3→3 cross-links compared to 4→3 formed by PBPs. Here we show that peptidoglycan expansion requires controlled hydrolysis of cross-links and identify among eight endopeptidase paralogues the minimum enzyme complements essential for bacterial growth with 4→3 (MepM) and 3→3 (MepM and MepK) cross-links. Purified Mep endopeptidases unexpectedly displayed a 4→3 and 3→3 dual specificity implying recognition of a common motif in the two cross-link types. Uncoupling of the polymerization of glycan chains from the 4→3 cross-linking reaction was found to facilitate the bypass of PBPs by YcbB. These results illustrate the plasticity of the peptidoglycan polymerization machinery in response to the selective pressure of β -lactams.

Keywords endopeptidase; *Escherichia coli*; L,D-transpeptidase; peptidoglycan; β -lactam

Subject Categories Membranes & Trafficking; Microbiology, Virology & Host Pathogen Interaction

DOI 10.15252/embj.2021108126 | Received 26 February 2021 | Revised 22 July 2021 | Accepted 24 July 2021 | Published online 12 August 2021

The EMBO Journal (2021) 40: e108126

Introduction

Peptidoglycan (PG) is an essential macromolecule that surrounds the bacterial cell providing resistance to the osmotic pressure of the cytoplasm and determining cell shape (Turner *et al.*, 2014). PG is

assembled from a disaccharide peptide subunit consisting of *N*-acetylglucosamine (GlcNAc), *N*-acetylmuramic acid (MurNAc), and a stem pentapeptide linked to MurNAc (L-Ala¹- γ -D-Glu²-DAP³-D-Ala⁴-D-Ala⁵ in which DAP is diaminopimelic acid) (Fig 1A). The subunit is assembled by glycosyltransferases that polymerize glycan strands and transpeptidases that form amide bonds between stem peptides linked to adjacent glycan strands. *Escherichia coli* relies on two types of transpeptidases for the latter reaction (Magnet *et al.*, 2008). The D,D-transpeptidases, also referred to as penicillin-binding proteins (PBPs), form the most abundant cross-links, which connect the fourth residue (D-Ala⁴) of an acyl donor to the third residue (DAP³) of an acyl acceptor (4→3 cross-link) (Fig 1A). The L,D-transpeptidases (LDTs) form 3→3 cross-links that connect two DAP residues (Fig 1B). The D-Ala at the 5th and 4th positions of stem peptides that do not participate in cross-link formation as donors are fully and partially trimmed by carboxypeptidases of the D,D and L,D specificities, respectively (Fig 1C). PBPs and LDTs are structurally unrelated, rely on different catalytic nucleophiles (Ser versus Cys, respectively), and use different acyl donor stems (pentapeptide versus tetrapeptide, respectively) (Mainardi *et al.*, 2008; Sauvage *et al.*, 2008). PBPs and LDTs also differ by their inhibition profiles since PBPs are potentially inhibited by all classes of β -lactams (including penams, cepheems, monobactams, and carbapenems), whereas LDTs are effectively inhibited only by carbapenems (Mainardi *et al.*, 2005). LDTs are fully dispensable for growth of *E. coli*, at least in laboratory conditions, and form a minority of the cross-links during exponential growth (6% of the total cross-links) (Glauner *et al.*, 1988; Sanders & Pavelka, 2013). The proportion of 3→3 cross-links is higher in the stationary phase (Pisabarro *et al.*, 1985) and in cells experiencing outer membrane assembly stress (Morè *et al.*, 2019). By-pass of PBPs by LDTs leads to high-level resistance to β -lactams of the penam (such as ampicillin), cephem (ceftriaxone), and monobactam (aztreonam) classes in engineered *E. coli* strains that overproduce the YcbB L,D-transpeptidase, also referred to as LdtD, and the guanosine penta- and tetra-phosphate [(p)ppGpp]

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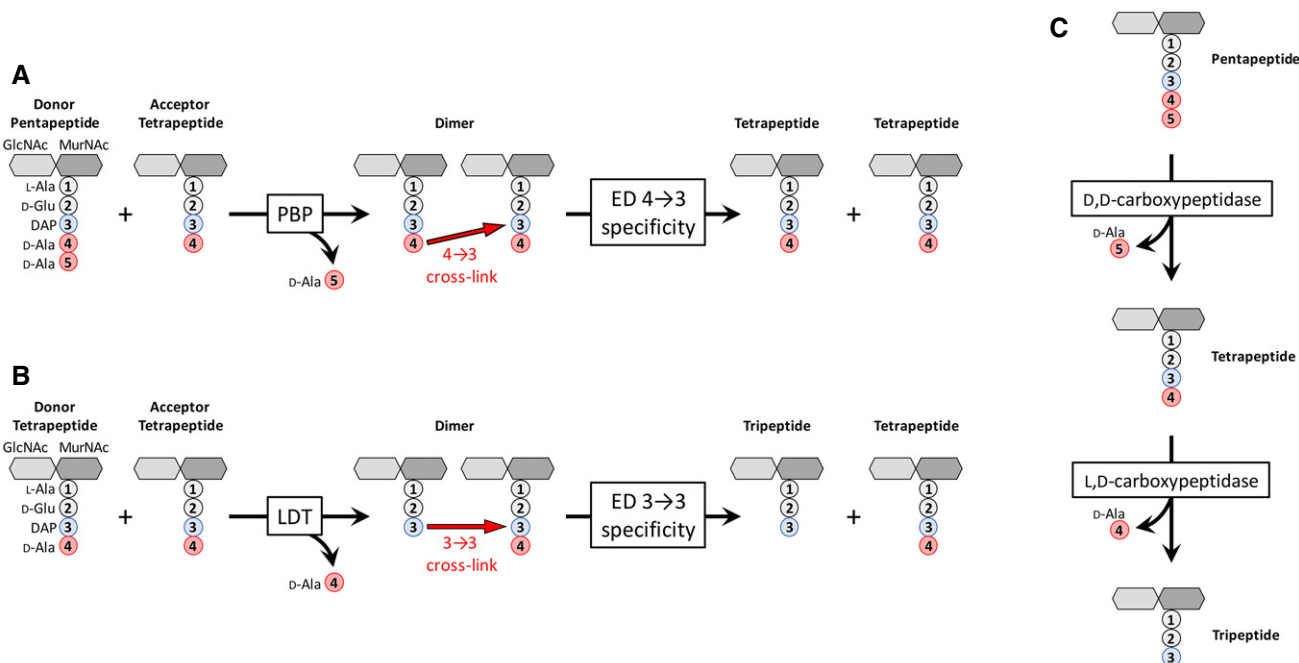


Figure 1. Metabolism of PG cross-links and maturation of free stem peptides.

A, B Formation and hydrolysis of (A) 4→3 and (B) 3→3 cross-links. The disaccharide pentapeptide unit is assembled from *N*-acetylglucosamine (GlcNAc), *N*-acetylmuramic acid (MurNAc), and five amino acids including *meso*-diaminopimelic acid (DAP), which is linked via its L (S) center to the γ -carboxyl group of D-Glu. C Hydrolysis of the D-Ala⁴-D-Ala⁵ and DAP³-D-Ala⁴ peptide bonds by carboxypeptidases of D,D and L,D specificities, respectively.

alarmones (Hugonnet *et al*, 2016). PG of such strains grown in the presence of β -lactams exclusively contains 3→3 cross-links, indicating that the D,D-transpeptidase activity of PBP is fully replaced by the L,D-transpeptidase activity of LDTs.

Expansion of PG is thought to require highly regulated hydrolytic activities that spatially control the insertion of new subunits into the growing network of cross-linked glycan strands (Singh *et al*, 2012; Vollmer, 2012). Due to the covalent net-like structure of PG, endopeptidases are predicted to be required for insertion of new subunits leading to the expansion of the PG layer (Fig EV1) (De Jonge *et al*, 1989; Höltje & Heidrich, 2001). The *E. coli* genome encodes eight endopeptidase paralogues that belong to five enzyme families (Singh *et al*, 2012; Chodiseti & Reddy, 2019; Pazos & Peters, 2019). PBP4, PBP7, and AmpH belong to the acyl-serine transferase superfamily, which also comprises D,D-transpeptidases and D,D-carboxypeptidases. Members of this superfamily are inhibited by β -lactam antibiotics. The NlpC/P60 cysteine peptidase family comprises two paralogues (MepH and MepS). Metallo-enzymes are represented by three enzyme families, LAS metallopeptidases, lysostaphin/M23 peptidases, and M15 peptidases, each contributing one paralogue (MepA, MepM, and MepK, respectively). The specificity of these eight paralogues as endopeptidases or carboxypeptidases (Fig 1) has been explored by using sacculi or purified PG fragments as substrates (Keck & Schwarz, 1979; Korat *et al*, 1991; Engel *et al*, 1992; Romeis & Holtje, 1994; Gonzalez-Leiza *et al*, 2011; Singh *et al*, 2012; Chodiseti & Reddy, 2019). PBP4, PBP7, AmpH, MepH, and MepS hydrolyze 4→3 cross-links but PG dimers containing 3→3 cross-links were not tested. In contrast, MepA, MepM, and MepK were fully characterized revealing that MepA hydrolyzes both 4→3

and 3→3 cross-links, MepM is specific to 4→3 cross-links, and MepK displays a marked preference for 3→3 cross-links. The endopeptidases of *E. coli* are redundant, and their essential roles can only be revealed by introducing multiple chromosomal deletions. One study unambiguously showed that hydrolysis of 4→3 cross-links by endopeptidases is essential as the triple deletion of genes encoding MepH, MepM, and MepS was not compatible with growth of *E. coli* in laboratory conditions (Singh *et al*, 2012; comment by Vollmer, 2012). Several endopeptidases interact genetically and physically with the outer membrane-anchored adaptor protein NlpI supporting overlapping functions during the cell cycle (Banzhaf *et al*, 2020). Various roles have been assigned to endopeptidases in bacteria other than *E. coli* such as the determination of cell shape in *Helicobacter pylori* (Bonis *et al*, 2010), release of NOD agonist in *Neisseria gonorrhoeae* (Lenz *et al*, 2017), or persistence in *Mycobacterium tuberculosis* (Healy *et al*, 2020).

The dual capacity of *E. coli* to use transpeptidases of the D,D and L,D specificities raises the possibility that polymerization of PG containing 4→3 or 3→3 cross-links involves two overlapping sets of endopeptidases. To address this question, we used an *E. coli* strain that conditionally and exclusively relies on the formation of 3→3 cross-links for growth in the presence of ampicillin or ceftriaxone (Hugonnet *et al*, 2016). By introducing serial deletions of endopeptidase genes, we showed that the 4→3 and 3→3 modes of PG polymerization both require hydrolysis of cross-links. We identified distinct sets of endopeptidases that are essential for growth involving the two modes of PG cross-linking. Strikingly, impaired digestion of nascent glycan strands by a lytic transglycosylase was found to favor PG cross-linking mediated by LDTs. These results highlight

the functional plasticity of PG polymerization complexes to accommodate various PG cross-linking enzymes and hydrolases.

Results

MepM is essential for β -lactam resistance mediated by the YcbB L,D-transpeptidase

The role of endopeptidases was assessed in *E. coli* BW25113 $\Delta relA$ pKT2(*ycbB*) pKT8(*relA'*), BW25113(*ycbB*, *relA'*) in short, which enables controlling the relative contribution of formation of 4 \rightarrow 3 and 3 \rightarrow 3 cross-links to PG polymerization (Hugonnet *et al.*, 2016). In this strain, the *ycbB* L,D-transpeptidase gene carried by plasmid pKT2 is expressed under the control of an IPTG-inducible promoter. Plasmid pKT8 carries an L-arabinose-inducible copy of the *relA'* gene encoding a truncated version of RelA (residues 1–455), which synthesizes the (p)ppGpp alarmone in an unregulated manner due to the absence of the C-terminal ribosome binding module (Schreiber *et al.*, 1991). In the presence of both inducers, production of YcbB and RelA' is sufficient for full bypass of the D,D-transpeptidase activity of PBPs by the L,D-transpeptidase activity of YcbB (Fig 2A). This enables bacterial growth in the presence of ampicillin or ceftriaxone since these drugs do not inhibit the YcbB L,D-transpeptidase. Testing for the inducible expression of β -lactam resistance in BW25113(*ycbB*, *relA'*) therefore provides a means to identify genes that are essential for growth when PG cross-linking is exclusively mediated by the YcbB L,D-transpeptidase. We used this phenotypic assay to assess the individual role of seven of the eight endopeptidases of *E. coli* following single-gene deletions in the BW25113(*ycbB*, *relA'*) strain (Fig 2B). The remaining endopeptidase MepK could not be tested by this approach since deletion of the corresponding gene was not compatible with the presence of plasmid pKT2(*ycbB*) (see below). Mutants with deletion of *mepA*, *mepH*, *dacB*, *pbpG*, or *ampH* were resistant to ceftriaxone. Deletion of *mepS* decreased plating efficiency in the presence of the drug. Deletion of *mepM* abolished the expression of β -lactam resistance. These results are surprising since MepM and MepS were not reported to hydrolyze 3 \rightarrow 3 cross-links (Singh *et al.*, 2012; Chodiseti & Reddy, 2019).

Production of YcbB is lethal in the absence of MepK

The gene encoding MepK, an endopeptidase with the dual 4 \rightarrow 3 and 3 \rightarrow 3 specificities, was readily deleted from the chromosome of BW25113 $\Delta relA$. The resulting strain, BW25113 $\Delta relA$ $\Delta mepK$, was transformed with pKT2(*ycbB*), pKT8(*relA'*), or both plasmids in combination (co-transformation). Tetracycline and chloramphenicol were used to select transformants that acquired pKT2(*ycbB*) and pKT8(*relA'*), respectively. Plasmid pKT8(*relA'*) was readily introduced into BW25113 $\Delta relA$ $\Delta mepK$ by transformation (10^8 transformants per μ g of DNA). Plasmid pKT2(*ycbB*) alone or in combination with pKT8(*relA'*) could not be introduced into BW25113 $\Delta relA$ $\Delta mepK$ (< 5 transformants per μ g of DNA). The same plating efficacies were observed in selective media containing IPTG, L-arabinose, or both inducers, in addition to tetracycline and chloramphenicol. These results show that production of the YcbB L,D-transpeptidase is lethal in the absence of MepK, in agreement with a recent report

(Chodiseti & Reddy, 2019). Thus, cleavage of 3 \rightarrow 3 cross-links by MepK is essential for bacterial growth when the proportion of 3 \rightarrow 3 cross-links is increased in the presence of a plasmid copy of *ycbB*. Quantitatively, the basal level of *ycbB* expression in the absence of IPTG was sufficient for the lethal phenotype associated with the *mepK* deletion. Under non-inducing conditions, the relative proportion of 4 \rightarrow 3 and 3 \rightarrow 3 cross-links in the PG extracted from exponential phase cultures of BW25113(*ycbB*, *relA'*) was in the order of 60 and 40%, respectively (data not shown). Thus, the cleavage of 3 \rightarrow 3 cross-links by MepK was essential even if these cross-links co-existed with 4 \rightarrow 3 cross-links formed by the PBPs.

The hydrolytic activity of MepM is essential for β -lactam resistance

Deletion of the *mepM* gene abolished YcbB-mediated β -lactam resistance (above, Fig 2B) even though this endopeptidase was not reported to cleave 3 \rightarrow 3 cross-links (Singh *et al.*, 2012; Chodiseti & Reddy, 2019). We therefore considered the possibility that the essential role of *mepM* in resistance could involve an as yet unknown function in addition to its 4 \rightarrow 3-endopeptidase activity. MepM (440 residues) comprises a LytM (lysostaphin/M23 peptidase) domain and a LysM PG-binding domain (Pfam: P0AFS9). Complementation analysis of the *mepM* deletion in BW25113(*ycbB*, *relA'*) was performed with plasmids encoding (i) MepM, (ii) MepM H³⁹³A harboring an Ala residue at position 393 in place of an essential catalytic His residue conserved in members of the M23 peptidase family, and (iii) MepM Δ LytM lacking the C-terminal endopeptidase catalytic domain (Fig 3). Expression of β -lactam resistance by BW25113(*ycbB*, *relA'*) $\Delta mepM$ was only restored by the plasmid harboring an intact copy of the *mepM* gene. Thus, the complementation analysis led to the conclusion that the endopeptidase activity of MepM is essential for YcbB-mediated resistance to β -lactams.

The essential role of the endopeptidase activity of MepM in β -lactam resistance is not restricted to the transition from 4 \rightarrow 3 to 3 \rightarrow 3 cross-links triggered by the induction of *ycbB* and *relA'*

The experiment reported above did not rule out the possibility that hydrolysis of 4 \rightarrow 3 cross-links by MepM might be transiently essential to enable bypass of PBPs by YcbB, *i.e.*, cleavage of 4 \rightarrow 3 cross-links by MepM could be initially essential to enable insertion of new PG subunits into the PG network by YcbB. Ultimately, this would lead to replacement of 4 \rightarrow 3 by 3 \rightarrow 3 cross-links and could then suppress the essential role of 4 \rightarrow 3 cross-link cleavage by MepM. According to this hypothesis, MepM would only be essential during the transition between the two modes of PG cross-linking. To test this possibility, we sought a plasmid construct enabling tight regulation of the *mepM* gene. In a first attempt, *mepM* was cloned under the control of the L-rhamnose-inducible promoter (P_{rhaBAD}) of the pHV30 vector. Complementation of the *mepM* deletion of BW25113(*ycbB*, *relA'*) was obtained both in the presence or absence of L-rhamnose indicating that the un-induced level of *ycbB* afforded by this plasmid construct was too high (Fig 4A). To address this issue, the level of *mepM* expression was reduced by replacing the sequence containing the translation initiation signal (TIS1) of *mepM* by a weaker translation initiation signal (TIS2). TIS1 (aAAGAGGA

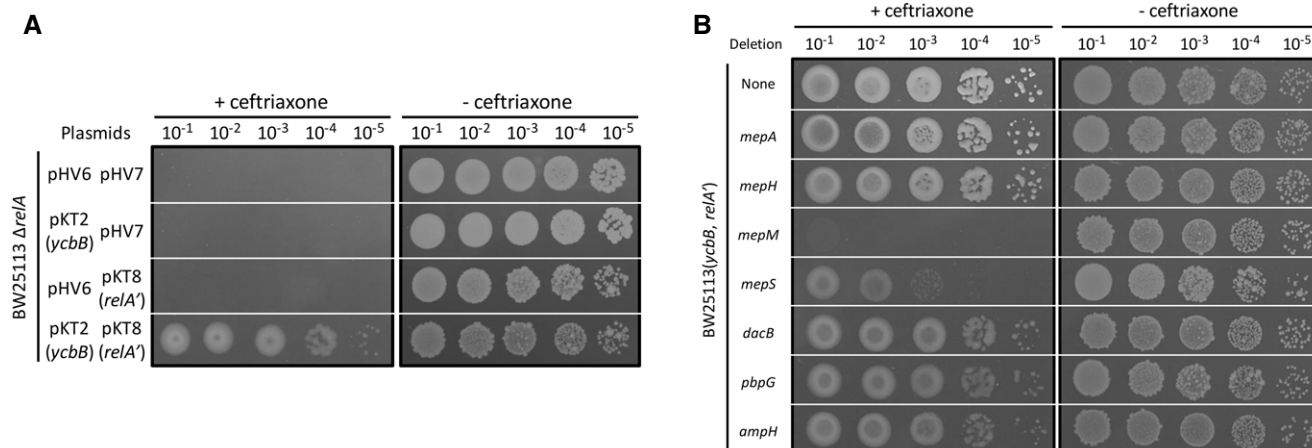


Figure 2. MepM is essential for YcbB-mediated β -lactam resistance.

Growth was tested in the presence of ceftriaxone at 8 μ g/ml (+ ceftriaxone) or in the absence of the drug (– ceftriaxone) on BHI agar plates supplemented with 40 μ M IPTG and 1% L-arabinose for induction of *ycbB* and *relA'*, respectively.

A BW25113 Δ relA derivatives harboring plasmids pKT2(*ycbB*), pKT8(*relA'*) and the vectors pHV6 and pHV7 used to construct these plasmids, respectively. Expression of β -lactam resistance requires induction of both *ycbB* and *relA'*.

B BW25113(*ycbB*, *relA'*) and its derivatives obtained by individual deletion of endopeptidase genes. BW25113(*ycbB*, *relA'*) is an abbreviated name for BW25113 Δ relA pKT2(*ycbB*) pKT8(*relA'*).

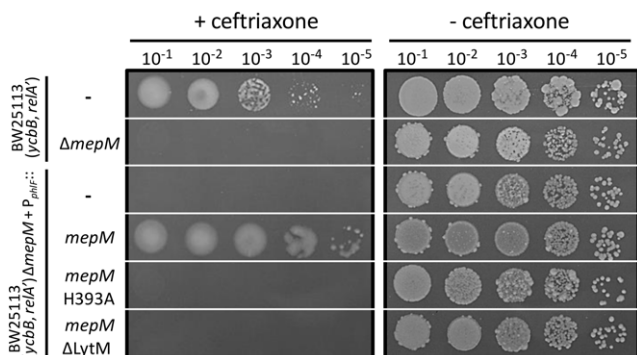


Figure 3. MepM endopeptidase activity is required for YcbB-mediated β -lactam resistance.

Growth of BW25113(*ycbB*, *relA'*), BW25113(*ycbB*, *relA'*) Δ mepM, and its derivatives harboring plasmids encoding MepM, MepM H³⁹³A, and MepM Δ LytM were tested in the presence of ceftriaxone at 8 μ g/ml (+ ceftriaxone) or in the absence of the drug (– ceftriaxone) in BHI agar plates supplemented with 40 μ M IPTG and 1% L-arabinose for induction of *ycbB* and *relA'*, respectively. The genes encoding MepM, MepM H³⁹³A, and MepM Δ LytM were inserted into the vector pHV9 under the control of the P_{phIF} promoter, which is inducible by 2,4-diacetylphloroglucinol (DAPG). Basal level of expression of *mepM* under the control of P_{phIF} was sufficient to restore ceftriaxone resistance in the absence of the inducer.

GAAAtgacataATG) combined an ATG initiation codon to a “strong” ribosome binding site (RBS) with extensive complementarity (underlined) to the 3' OH extremity of 16S rRNA (5'- AUCACCUC CUUA-3'OH) (Elowitz & Leibler, 2000). TIS2 (acacAGGAcacttaTTG) combined a TTG initiation codon to an RBS with limited complementarity to 16S rRNA (5'- AUCACCUCUUA-3'OH) (Ringquist et al., 1992; Vellanoweth & Rabinowitz, 1992; Hecht et al., 2017). In contrast to the results obtained with TIS1, the presence of L-rhamnose was required for β -lactam resistance if *mepM* was

expressed under the control of TIS2 (Fig 4A). L-rhamnose requirement for growth in the presence of ceftriaxone was not abolished by pre-exposure to the inducer (Fig 4B). These results indicate that the essential role of MepM in β -lactam resistance is not limited to the transition between the two modes of PG cross-linking, i.e., from 4 \rightarrow 3 to 3 \rightarrow 3. Since all D,D-transpeptidases are inhibited by ceftriaxone, these results also indicate that the hydrolytic activity of MepM is essential in conditions in which 4 \rightarrow 3 cross-links are not detectable (Kocaoglu & Carlson, 2015; Hugonnet et al., 2016).

MepM hydrolyzes both 4 \rightarrow 3 and 3 \rightarrow 3 cross-links in vitro

The essential role of the endopeptidase activity of MepM in the context of LDT-mediated PG cross-linking (above) led us to reconsider the specificity of the enzyme. Previous analyses were based on incubation of MepM and lysozyme with an *E. coli* PG preparation containing minute amounts of 3 \rightarrow 3 cross-links (Singh et al., 2012; Chodisetti & Reddy, 2019). Analyses of rPHPLC profiles revealed that the major dimers containing 4 \rightarrow 3 cross-links were digested by MepM but minor peaks corresponding to dimers containing 3 \rightarrow 3 cross-links remained unchanged in the presence of the enzyme leading to the conclusion that MepM was specific to 4 \rightarrow 3 cross-links (Singh et al., 2012; Chodisetti & Reddy, 2019). To improve the sensitivity of the assay, we purified MepM and reproduced this analysis with a PG preparation of *E. coli* BW25113 grown to stationary phase in minimal medium, which contained a higher proportion of 3 \rightarrow 3 cross-links (Fig 5A, upper panel). The mucopeptides corresponding to the indicated peaks are shown in Fig 5B (see Fig EV2 for determination of the structure of mucopeptides by mass spectrometry). Full digestion of all dimers was observed upon incubation of this PG preparation with MepM (5 μ M) indicating that the endopeptidase hydrolyzes both 4 \rightarrow 3 and 3 \rightarrow 3 cross-links to completion (Fig 5A, lower panel).

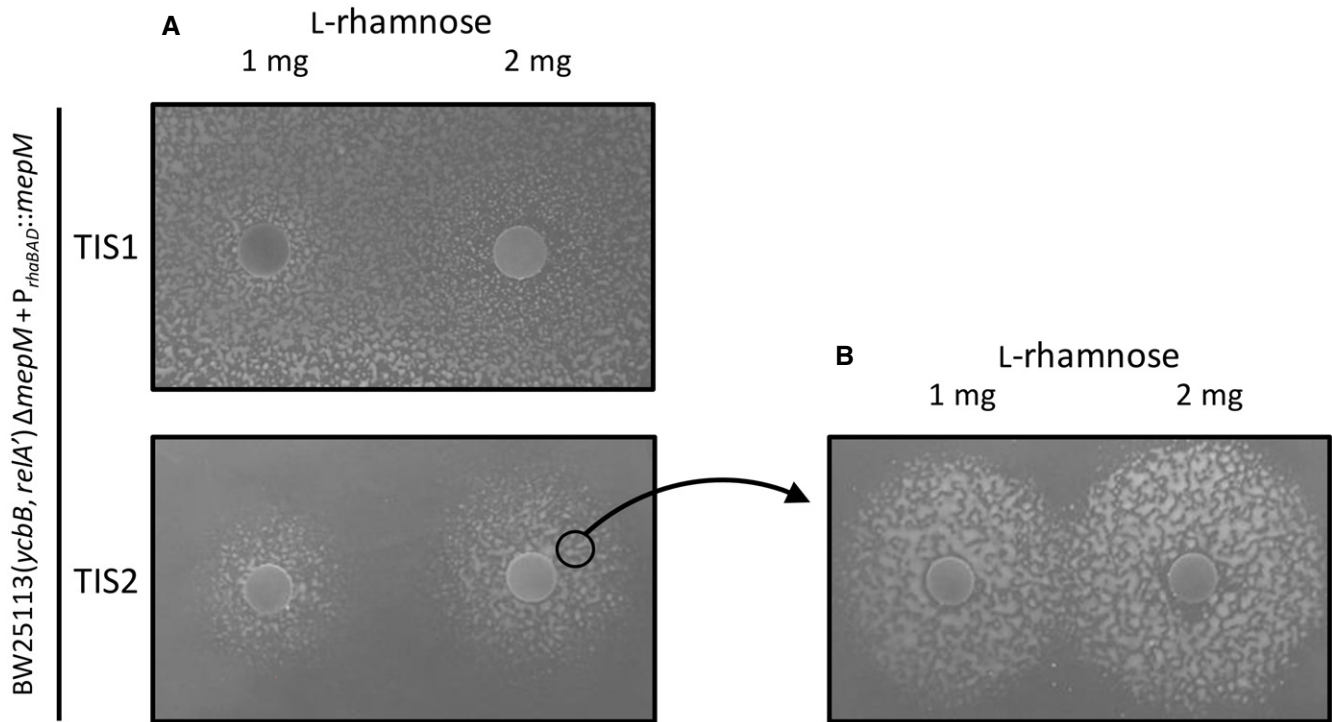


Figure 4. MepM is essential for β -lactam resistance beyond the transition from 4 \rightarrow 3 to 3 \rightarrow 3 PG cross-linking.

The *mepM* gene was expressed under the control of TIS1 or TIS2 (translation initiation) and of P_{rhaBAD} (the L-rhamnose-inducible promoter of vector pHV30). Growth of BW25113(*ycbB*, *relA'*) Δ *mepM* harboring the pHV30 derivatives was tested on BHI agar supplemented with 8 μ g/ml ceftriaxone, 40 μ M IPTG (induction of *ycbB*) and 1% L-arabinose (induction of *relA'*).

- A For TIS2, growth around paper disks containing 1 or 2 mg of L-rhamnose indicated that induction of the expression of *mepM* was required for ceftriaxone resistance. In contrast, a higher level of translation from TIS1 was sufficient for ceftriaxone resistance in the absence of the inducer.
- B The experiment was repeated with bacteria pre-exposed to L-rhamnose that were harvested at the vicinity of the disk containing 2 mg of L-rhamnose. Expression of ceftriaxone resistance remained dependent on the presence of L-rhamnose indicating that the requirement for MepM is not transient. The diameter of the growth zones is larger in panel (B) than in panel (A) as expected from the fact that bacteria in the inoculum used in (B) had been grown in the presence of the inducer and already contained MepM. In (A) growth is only possible after diffusion of L-rhamnose in the medium prior to the action of ceftriaxone. At a distance from the disk, diffusion was not sufficiently rapid to observe resistance.

Incubation of the PG preparation with lower concentrations of MepM led to partial hydrolysis of the dimers (Fig 5C). Comparison of the relative abundance of mucopeptides based on the integration of peak areas in the chromatograms showed the expected increase in monomers upon digestion of dimers (Fig 5D). The concentrations of MepM required for hydrolysis of half of the mucopeptides containing 4 \rightarrow 3 and 3 \rightarrow 3 cross-links were 0.4 and 0.7 μ M, respectively, revealing similar apparent hydrolysis efficacies for the two types of cross-links under the assay conditions (Fig 5D). These results indicate that hydrolysis of 3 \rightarrow 3 cross-links may account for the essential role of MepM in conditions in which the L,D-transpeptidase activity of YcbB fully replaces the D,D-transpeptidase activity of the PBPs, as inferred from expression of ceftriaxone resistance by BW25113(*ycbB*, *relA'*) but not by its Δ *mepM* derivative (Fig 2B).

The sum of the relative proportion of tripeptide and tetrapeptide stems in all monomers and dimers did not vary upon addition of MepM (Fig 5E). This observation indicates that MepM did not hydrolyze D-Ala⁴ from tetrapeptide monomers or from tetrapeptide stems located in the acceptor position of dimers. Thus, MepM did not display any L,D-carboxypeptidase activity.

Design of an assay to investigate the redundancy of endopeptidases required for YcbB-mediated β -lactam resistance

Single deletion of endopeptidase genes revealed four phenotypes (above, Fig 2B). (i) Deletion of *mepK* was not compatible with production of YcbB. (ii) Deletion of *mepM* abolished YcbB-mediated ceftriaxone resistance. (iii) Deletion of *mepS* impaired growth in the presence of ceftriaxone. (iv) Deletion of *mepA*, *mepH*, *dacB*, *pbpG*, or *ampH* had no impact on growth in the presence of ceftriaxone. The absence of any phenotypic alteration associated with the individual deletion of the latter genes does not necessarily imply that the corresponding endopeptidases are unable to participate in the hydrolysis of 3 \rightarrow 3 cross-links. Indeed, the function of these enzymes may be redundant. Alternatively, their level of production may be insufficient under the tested growth conditions. To investigate these possibilities, each of the eight endopeptidase genes was independently cloned under the control of the “strong” TIS1 translation initiation signal downstream from the P_{rhaBAD} promoter of the vector pHV30 in order to modulate the level of endopeptidase production based on induction by L-rhamnose. The plasmids were introduced into BW25113(*ycbB*, *relA'*) Δ *mepM*, and growth of the

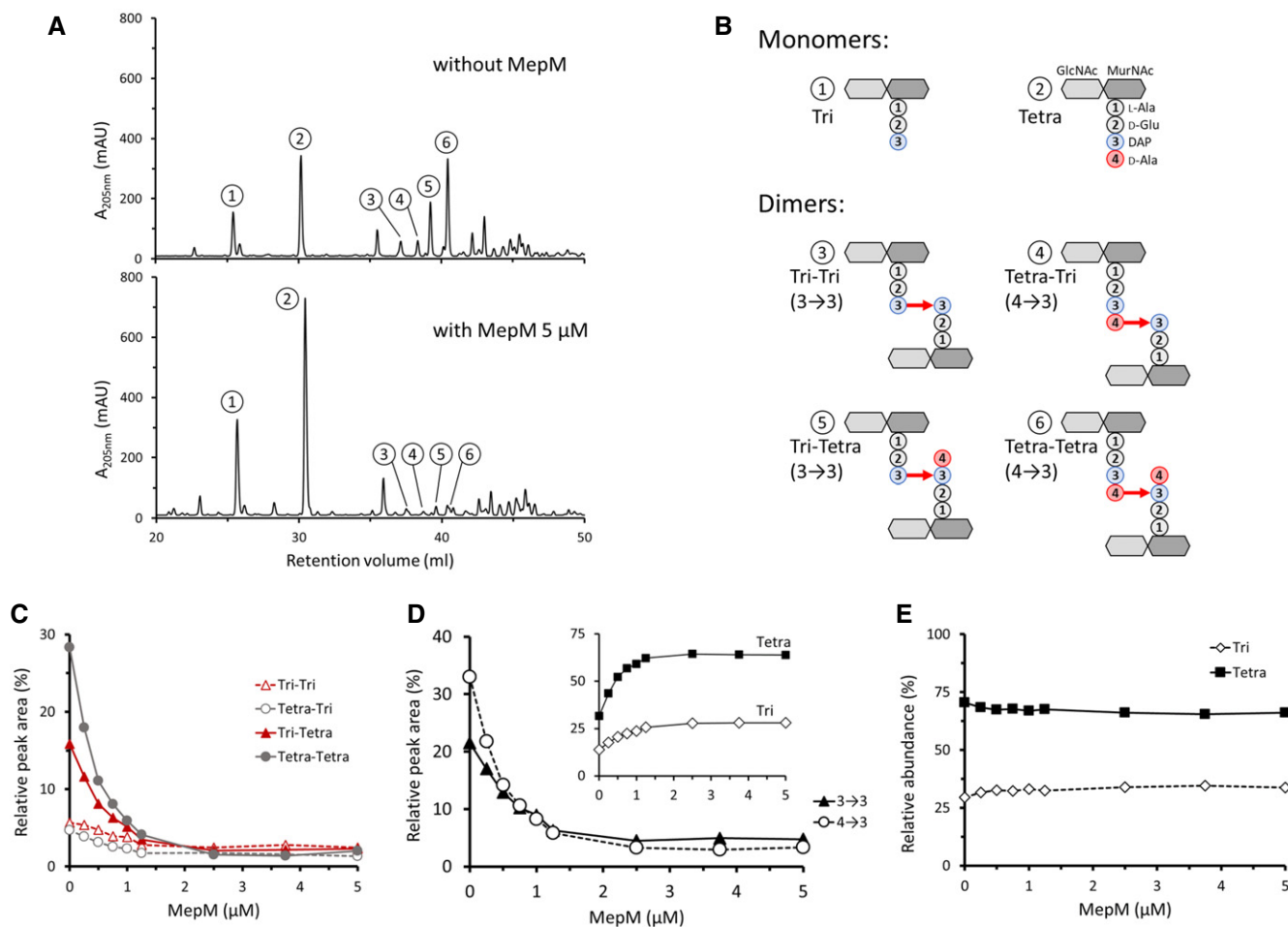


Figure 5. Hydrolysis of 4→3 and 3→3 cross-links by purified MepM.

- A *r*PHPLC chromatograms of sacculi isolated from BW25113 grown in minimal medium to stationary phase and digested by lysozyme (upper panel) or by lysozyme and 5 μM MepM (lower panel). Absorbance was monitored at 205 nm (mAU, milli-absorbance unit).
- B Structure of the muropeptides as determined by mass spectrometry (Fig EV2).
- C Hydrolysis of the four types of dimers by MepM. Sacculi were incubated with lysozyme and MepM at various concentrations. The relative abundance of the muropeptides was estimated by calculating the relative peak areas.
- D Hydrolysis of dimers containing 4→3 and 3→3 cross-links by MepM. The relative peak areas of Tri-Tri and Tri-Tetra containing 3→3 cross-links and that of Tetra-Tri and Tetra-Tetra containing 4→3 cross-links were combined. The inset shows variations in the relative peak areas of the Tri and Tetra monomers.
- E Relative abundance of Tri and Tetra stems in all muropeptides.

resulting strains was tested in the presence or absence of L-rhamnose and in the presence or absence of ceftriaxone in all combinations (Fig 6).

Overexpression of *mepM* is toxic in the presence of ceftriaxone

The assay described above revealed that the basal level of expression of the plasmid copy of *mepM* in the absence of the inducer was sufficient to restore growth of BW25113(*ycbB*, *relA'*) Δ *mepM* in the presence of ceftriaxone (Fig 6). Induction of the *mepM* gene by L-rhamnose prevented growth in the presence of ceftriaxone but not in the absence of the drug. Importantly, MepM toxicity was only observed when its production was under the control of the effective TIS1 translation initiation signal, but not of the weaker TIS2 signal

used for the experiment described in Fig 4. These results suggest that overproduction of MepM inhibits growth by cleavage of 3→3 cross-links if 4→3 cross-links are absent due to the inactivation of the PBPs by ceftriaxone.

Overexpression of *mepS* complements the *mepM* deletion for expression of YcbB-mediated β -lactam resistance

MepS restored growth of BW25113(*ycbB*, *relA'*) Δ *mepM* only in the presence of the inducer (Fig 6). Thus, MepM and MepS have overlapping functions although overproduction of MepS was required to compensate for the absence of MepM. As mentioned above (Fig 2B), deletion of *mepS* impaired but did not abolish ceftriaxone resistance in BW25113(*ycbB*, *relA'*). Together, these results indicate that

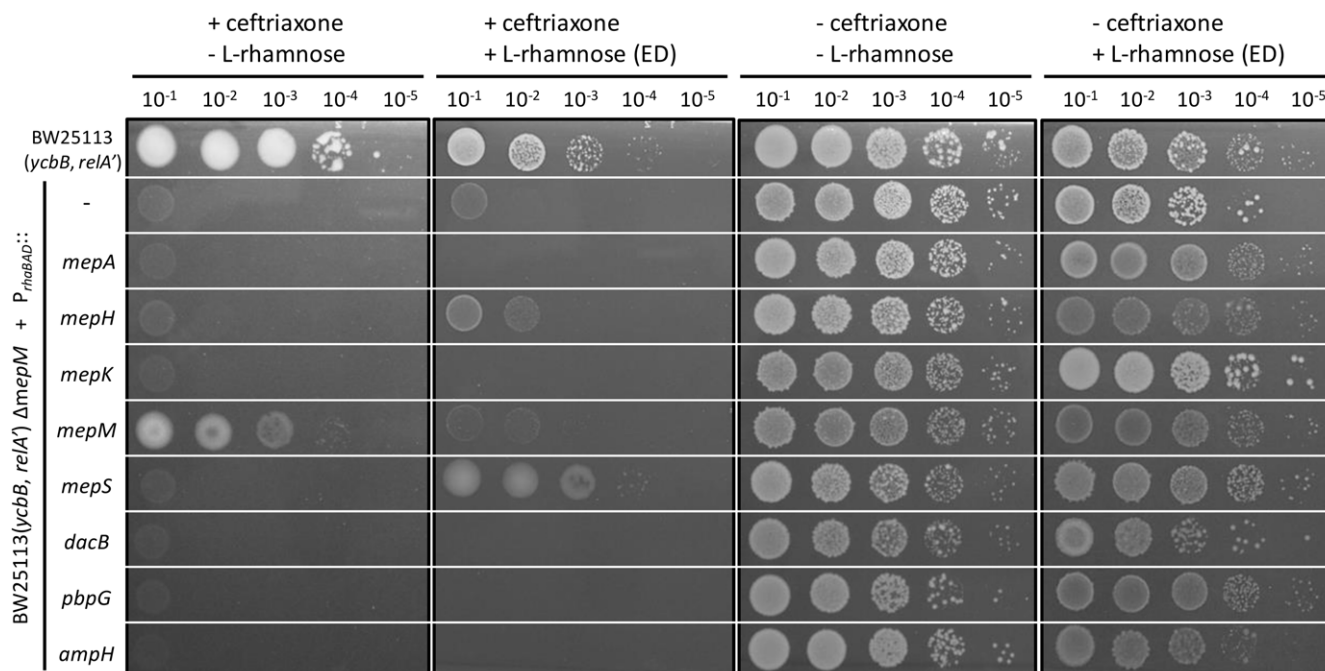


Figure 6. Complementation of the *mepM* deletion by plasmids encoding L-rhamnose-inducible copies of the eight endopeptidase genes.

Functional complementation of the *mepM* deletion in BW25113(*ycbB*, *relA'*) Δ *mepM* was performed with the pHV30 vector or recombinant plasmids encoding each of the eight endopeptidases under the control of the P_{rhoBAD} promoter and of the TIS1 translation initiation signal. Induction of endopeptidase (ED) genes was performed with 0.2% L-rhamnose in the presence or absence of 8 μ g/ml ceftriaxone. BHI agar plates contained 40 μ M IPTG and 1% L-arabinose for induction of *ycbB* and *relA'*, respectively.

expression of *mepS* in its native chromosomal environment contributes to resistance but the level of its expression is not sufficient to compensate for the absence of MepM.

Partial complementation of the *mepM* deletion by *mepH*

The plasmid encoding MepH partially restored growth of BW25113(*ycbB*, *relA'*) Δ *mepM* on ceftriaxone only in the presence of L-rhamnose (Fig 6). This result indicates that MepH, like MepS, replaces MepM for the expression of ceftriaxone resistance if MepH is overproduced.

Purified MepS and MepH hydrolyze 4→3 and 3→3 cross-links (endopeptidase activity) and the DAP-D-Ala bond of tetrapeptide stems (L,D-carboxypeptidase activity)

Complementation of Δ *mepM* by overproduction of MepS and MepH prompted us to evaluate the specificity of these enzymes, as described above for MepM. MepH and MepS both hydrolyzed 4→3 and 3→3 cross-links (Fig 7A and B). MepS showed no preference for 4→3 or 3→3 cross-links, while MepH displayed a strong preference for 4→3 cross-links. The weak hydrolytic activity of MepH on 3→3 cross-links may account for the fact that the overproduction of MepH can only partially compensate for the absence of MepM (Fig 6, above). Both MepS and MepH displayed L,D-carboxypeptidase activity leading to rapid conversion of tetrapeptide stems into tripeptide stems (Fig 7A and B).

MepA and MepK do not compensate the absence of MepM

In spite of the fact that MepA and MepK were previously shown to cleave 3→3 cross-links (Engel *et al*, 1992; Chodisetti & Reddy, 2019), growth of BW25113(*ycbB*, *relA'*) Δ *mepM* on ceftriaxone was not restored by overproduction of these enzymes (Fig 6). Thus, there was not a strict correlation between the ability of the endopeptidases to hydrolyze 3→3 cross-links *in vitro* and their ability to restore growth of BW25113(*ycbB*, *relA'*) Δ *mepM*. This absence of correlation was particularly striking for MepK since this endopeptidase, which preferentially hydrolyze 3→3 cross-links *in vitro* (Chodisetti & Reddy, 2019), did not complement the *mepM* deletion although it was required for growth of BW25113(*ycbB*, *relA'*) expressing the *ycbB* L,D-transpeptidase gene.

These results indicate that functional properties of the endopeptidases, beyond their mere hydrolytic specificity, are relevant to the bypass of the D,D-transpeptidase activity of PBPs by the L,D-transpeptidase activity of YcbB. These properties may include the interaction of the endopeptidases with other proteins that regulate their spatiotemporal activity (see Discussion section).

Endopeptidases of the PBP family do not compensate the absence of MepM

Complementation of the *mepM* deletion was not observed for PBP4, PBP7, or AmpH in the presence or absence of induction of the corresponding genes by L-rhamnose (Fig 6). There is a caveat for these

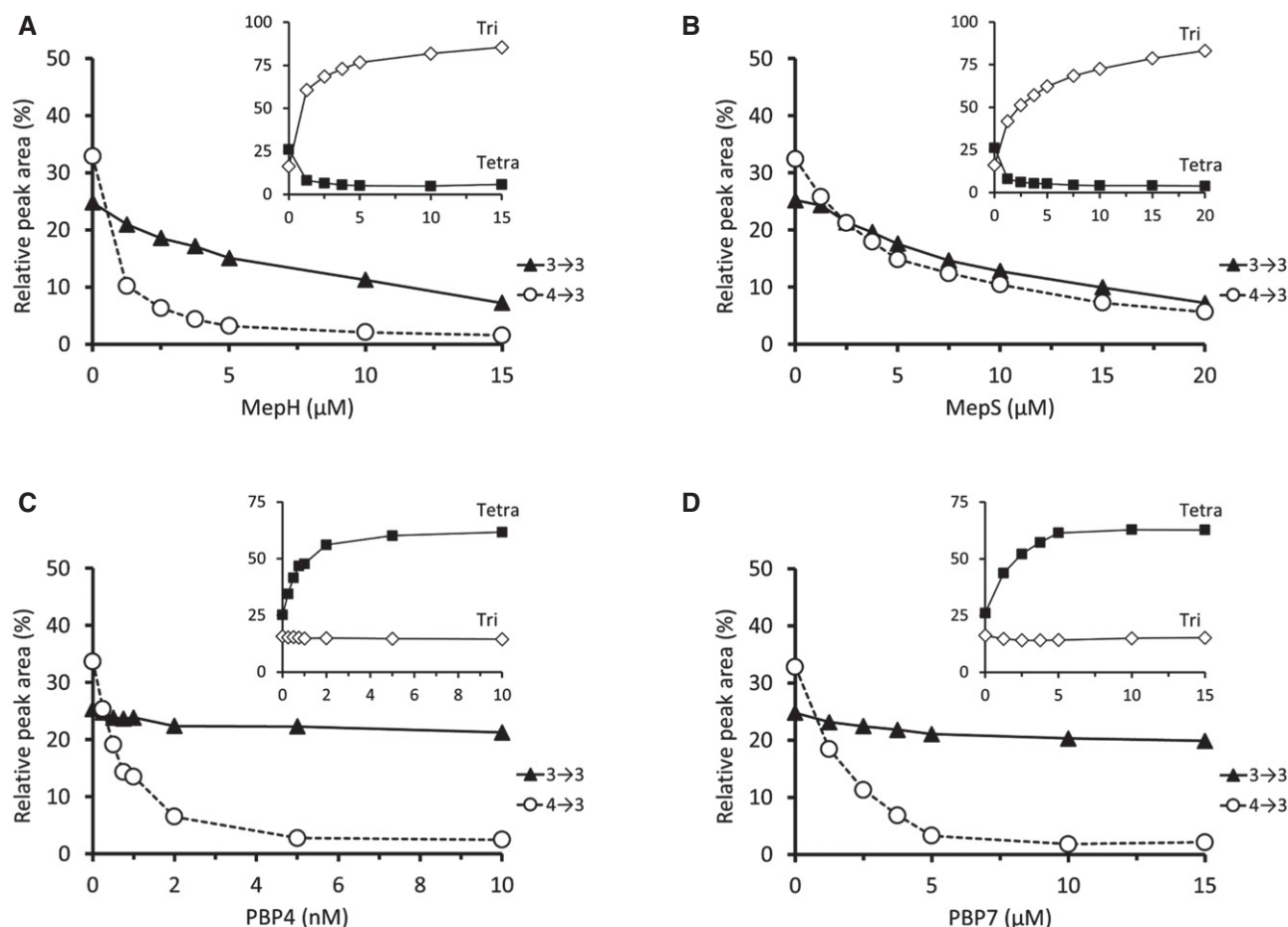


Figure 7. Hydrolysis of 4→3 and 3→3 cross-links by purified endopeptidases.

A–D Sacculi were incubated with lysozyme and purified MepH (A), MepS (B), PBP4 (C), and PBP7 (D). The relative peak areas of Tri-Tri and Tri-Tetra containing 3→3 cross-links and that of Tetra-Tri and Tetra-Tetra containing 4→3 cross-links were combined. MepH preferentially hydrolyzed dimers containing 4→3 cross-links. MepS hydrolyzed dimers containing 4→3 and 3→3 cross-links with similar efficacies. PBP4 and PBP7 only hydrolyzed dimers containing 4→3 cross-links. The insets show variations in the relative peak areas of the Tri and Tetra monomers. MepH and MepS displayed L,D-carboxypeptidase activity, but not PBP4 and PBP7.

endopeptidases since they are potentially inhibited by ceftriaxone. To address this issue, the complementation test was repeated with ampicillin, cefsulodin, and aztreonam, which were reported to exhibit different selectivities for inhibition of the PBPs (Henderson *et al*, 1997; Kocaoglu & Carlson, 2015). Plasmids encoding PBP4, PBP7, and AmpH did not restore growth of BW25113 (*ycbB*, *relA'*) Δ *mepM* in the presence of ampicillin, cefsulodin, and aztreonam (Fig EV3) confirming that these endopeptidases are unable to compensate for the absence of MepM. PBP4 and PBP7 were purified and shown to only cleave 4→3 cross-links (Fig 7C and D). Thus, the absence of complementation of the *mepM* deletion by the plasmids encoding these PBPs can be accounted for by their lack of hydrolytic activities on 3→3 cross-linked dimers. PBP4 and PBP7 did not display L,D-carboxypeptidase activity.

Minimal complement of endopeptidases required for growth in the context of the formation of 4→3 cross-links by PBPs

Previous analyses based on multiple deletions showed that genes encoding endopeptidases belonging to the PBP family (PBP4, PBP7,

and AmpH) are collectively dispensable (Denome *et al*, 1999). Independently, deletion of the genes encoding MepH, MepM, and MepS in various combinations revealed that at least one of these endopeptidases was essential (Singh *et al*, 2012). Here, we extend these analyses to the full complement of the eight endopeptidase genes.

Serial deletions of endopeptidase genes were introduced into the chromosome of *E. coli* BW25113 Δ *relA* generating the lineages depicted in Appendix Fig S1. This approach culminated in the construction of a viable derivative of BW25113 Δ *relA*, designated Δ 7EDs (lineage 5 in Appendix Fig S1), which retained only one of the eight endopeptidase genes (*mepM*). Thus, MepM alone was necessary and sufficient to support bacterial growth in the context of the 4→3 mode of cross-linking.

Our next objective was to determine whether deletion of *mepM* could be complemented by overproduction of other endopeptidases. To address this question, the *mepM* gene was cloned under the L-rhamnose-inducible promoter of vector pHV30 and introduced into the Δ 7EDs strain. The chromosomal copy of *mepM* was deleted from the resulting strain leading to strain Δ 8EDs pHV53(*mepM*), which

was dependent upon the presence of L-rhamnose for growth (Fig 8A). The plasmids enabling L-arabinose-inducible expression of the eight endopeptidase genes (above) were introduced in the Δ 8EDs pHV53(*mepM*) strain to determine which endopeptidase could functionally replace MepM (Fig 8B). Induction by L-arabinose of the genes encoding MepM, MepH, MepS, and PBP7 suppressed the requirement for L-rhamnose for growth. These results indicate that a single endopeptidase, MepM, MepH, MepS, or PBP7 is potentially sufficient for growth in the context of the 4 \rightarrow 3 mode of cross-linking. Except for MepM, this required overproduction of the enzymes following induction of the P_{araBAD} promoter of the recombinant plasmids.

MepA and MepS compensate for the absence of MepK when *ycbB* is induced

The basal production of the YcbB L,D-transpeptidase encoded by plasmid pKT2(*ycbB*) in the absence of induction was found to be lethal in a derivative of BW25113 lacking *mepK* (above). To investigate the possibility that MepK might be replaced by another endopeptidase, the *ycbB* gene was cloned under the control of the P_{araBAD} promoter of vector pHV7 to obtain a lower level of expression of the L,D-transpeptidase gene. The resulting plasmid, pHV63 (*ycbB*), was successfully introduced into the BW25113 Δ *mepK* strain indicating that the basal level of expression of *ycbB* in the absence of induction was compatible with the absence of *mepK*. The disk diffusion assay revealed a clear zone around the disk containing L-arabinose indicating that induction of *ycbB* in the Δ *mepK* background prevented bacterial growth (Fig 9A). Plasmids for expression of each of the eight endopeptidases under the control of the P_{rhaBAD} promoter (above) were introduced in this strain (Fig 9B). Bacterial growth was observed in conditions of induction of *ycbB* by L-arabinose and of genes encoding MepA or MepS by L-rhamnose. This result indicates that the essential role of MepK for PG polymerization mediated by the YcbB L,D-transpeptidase was bypassed by overproduction of MepA or MepS.

Complementation of the *mepK* deletion by catalytically inactivated endopeptidases

Since overproduction of MepK, MepS, and MepA was found to complement the chromosomal deletion of the *mepK* gene (above, Fig 9), we focused on these three endopeptidases. Plasmids encoding catalytically inactive MepK H¹³³A, MepS C⁹⁴A, and MepA H¹¹³A were used to determine whether the endopeptidase activity of MepK, MepA, and MepS was required to compensate for the chromosomal deletion of *mepK*. Overproduction of the endopeptidases was tested in the Δ *mepK* background (single-deletion mutant retaining all chromosomal endopeptidase genes except *mepK*, Fig 10A) and in the Δ 7EDs background (seven-deletion mutant retaining only *mepM*, Fig 10B). Overproduction of MepK but not MepK H¹³³A was essential for growth in both backgrounds indicating that the catalytic activity of the endopeptidase was essential. Overproduction of MepS restored growth in both backgrounds but complementation by MepS C⁹⁴A was only observed in the Δ *mepK* single-deletion background. Since the periplasmic protease Prc hydrolyzes MepS (Singh et al, 2015), overproduction of MepS C⁹⁴A may saturate the protease enabling sufficient chromosomally-encoded MepS to

escape hydrolysis and support growth. Likewise, saturation of the Prc protease is likely to be responsible for the apparent complementation mediated by overproduction of MepA and MepA H¹¹³A since overproduction of these enzymes restored growth in the Δ *mepK* single-deletion background but not in the Δ 7EDs background. Together, these results indicate that MepS is the only endopeptidase that can compensate for the absence of MepK. This required overproduction of MepS. Alternatively, saturation of the Prc protease by overproduction of MepA, MepA H¹¹³A, or MepS C⁹⁴A prevented hydrolysis of MepS produced at a lower level from the native chromosomal *mepS* gene.

Minimal complement of endopeptidases required for growth in the presence of β -lactams in the context of the exclusive formation of 3 \rightarrow 3 cross-links by the YcbB L,D-transpeptidase

As previously described (Hugonnet et al, 2016), induction of *relA'* led to mecillinam resistance in BW25113(*ycbB*, *relA'*), whereas induction of both *ycbB* and *relA'* was required for ampicillin and ceftriaxone resistance (Table 1 and Fig EV4). Strain BW25113(*ycbB*, *relA'*) Δ 6EDs (harboring only *mepM* and *mepK*) was also resistant to the three β -lactams upon induction of *ycbB* and *relA'* indicating that 6 of the 8 endopeptidase genes were dispensable for expression of β -lactam resistance. In contrast to BW25113(*ycbB*, *relA'*), the Δ 6EDs derivative was resistant to ampicillin and ceftriaxone in the absence of induction of *ycbB* by IPTG. The basal level of *ycbB* expression in the absence of induction was required for resistance since susceptibility to ampicillin and ceftriaxone was observed in the absence of pKT2(*ycbB*). These observations indicate that deletion of 6 of the 8 endopeptidase genes was associated with a decrease in the level of expression of *ycbB* required for β -lactam resistance. In combination with the analysis based on single-gene deletions (Fig 2B), these results show that MepM and MepK are necessary and sufficient for bacterial growth in conditions in which YcbB is the only functional transpeptidase.

Mutations impairing the lytic transglycosylase activity of Slt70 favors YcbB-mediated PG synthesis

Although BW25113(*ycbB*, *relA'*) displays high β -lactam resistance on BHI agar supplemented with IPTG and L-arabinose, the strain was found to remain susceptible to β -lactams in BHI broth supplemented with the same inducers. Mutations leading to expression of β -lactam resistance in liquid medium were sought by selecting mutants derived from *E. coli* BW25113 M1 (Hugonnet et al, 2016). The latter strain overexpresses *ycbB* from plasmid pJEH12(*ycbB*) in response to induction by IPTG and overproduces the (p)ppGpp alarmone due to impaired expression of the isoleucine tRNA synthetase gene *ileS* (Hugonnet et al, 2016). Derivatives of BW25113 M1 were selected in BHI broth containing 16 μ g/ml ampicillin and 50 μ M IPTG. Four independent mutants derived from BW25113 M1 (M1.1 to M1.4) were isolated and shown to grow in liquid medium supplemented with ampicillin and IPTG (Fig 11A). Whole genome sequencing revealed single mutations all located in the *sltY* gene encoding the lytic transglycosylase Slt70 (Table 2). One of the mutants (M1.3) most probably harbored a null allele of *sltY* since a 7-bp deletion introduced a frameshift at the 9th codon of the gene. To confirm this conclusion, the *sltY* gene

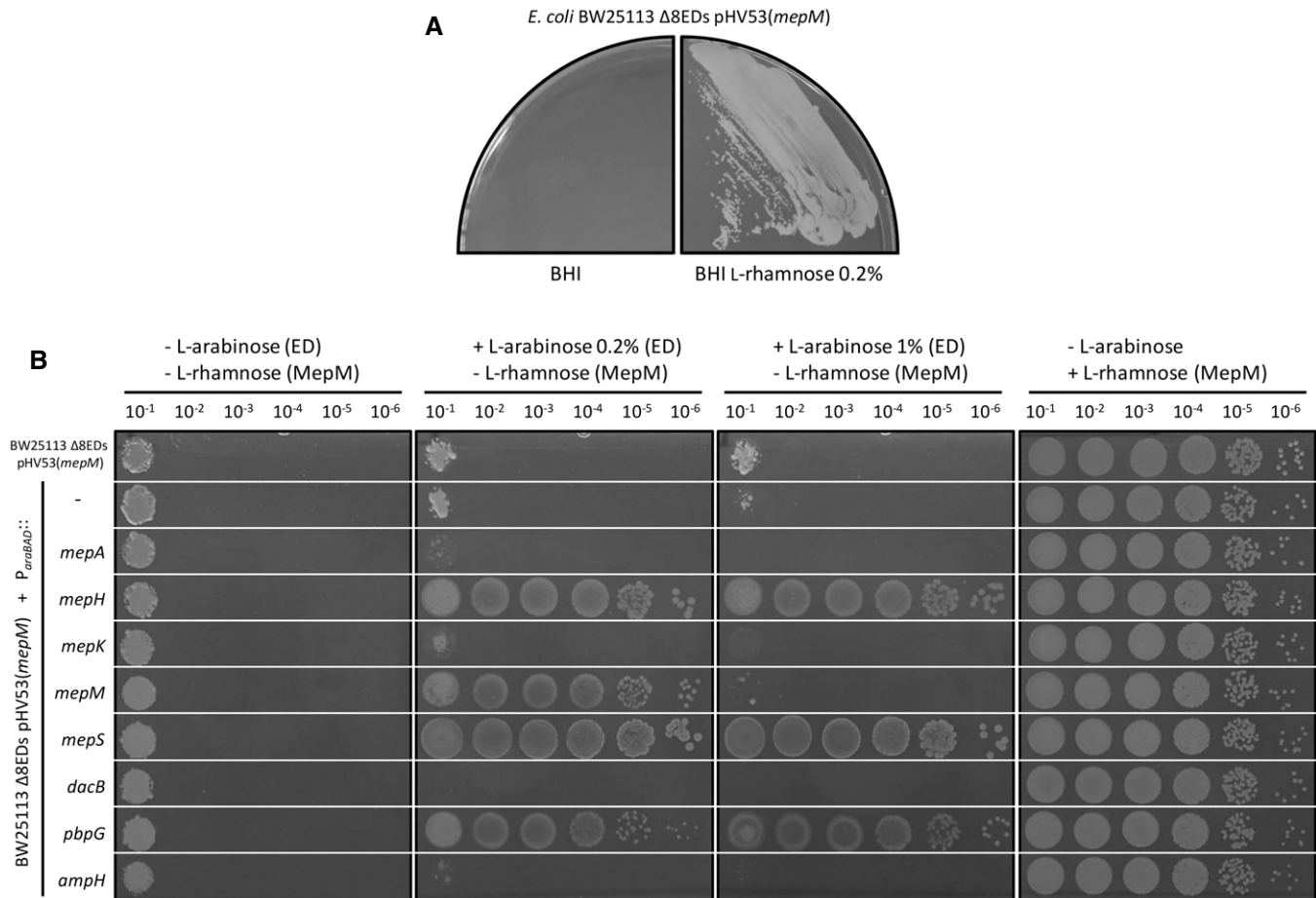


Figure 8. Minimal complement of endopeptidases required for growth in the context of the formation of 4→3 cross-links by PBPs.

- A** BW25113 Δ 8EDs harboring a plasmid carrying the *mepM* gene under the control of the P_{rhaBAD} L-rhamnose-inducible promoter with the “weak” TIS2 translation initiation signal (plasmid pHV53) was grown on BHI agar in the absence or presence of 0.2% L-rhamnose. Growth was dependent upon induction of the *mepM* copy carried by pHV53.
- B** The plating efficiency assay was performed with derivatives of BW25113 Δ 8EDs pHV53(*mepM*) harboring the vector pHV7 or recombinant plasmids carrying each of the eight endopeptidase genes under the control of the P_{araBAD} promoter. In this assay, functional replacement of MepM is detected based on growth in media containing 0.2% or 1% L-arabinose for expression of the endopeptidase gene carried by vector pHV7, thereby by-passing the requirement for induction of the *mepM* copy of pHV53 by L-rhamnose. Complementation was observed with both concentrations of inducer for *mepH*, *mepS*, and *pbpG*. Overproduction of *mepM* encoded by the pHV7 derivative in the presence of the high dose of L-arabinose (1%) was lethal. The right panel presents the growth control performed in the presence of 0.2% L-rhamnose for induction of the *mepM* copy carried by pHV53.

was deleted from the chromosome of BW25113(*ycbB*, *relA'*) strain. The resulting strain, BW25113(*ycbB*, *relA'*) Δ *slyT*, was also resistant to ceftriaxone in liquid medium in the presence of IPTG and L-arabinose (Fig 11A).

Comparison of the resistance phenotype of BW25113(*ycbB*, *relA'*) and its Δ *slyT* derivative on BHI agar revealed that induction of *ycbB* by IPTG is required for ampicillin and ceftriaxone resistance in the former but not in the latter strain (Fig 11B for representative antibiograms and Table 3 for the full set of data). Expression of resistance to ampicillin and ceftriaxone on BHI agar remained dependent upon induction of *relA'* since BW25113 Δ *relA* Δ *slyT* pKT2(*ycbB*) pKT8 (*relA'*) remained susceptible to these drugs in the absence of L-arabinose. Expression of resistance to ampicillin and ceftriaxone also remained dependent upon the presence of pKT2(*ycbB*) since

replacement of this plasmid by the pHV6 vector abolished resistance [strain BW25113 Δ *relA* Δ *slyT* pHV6 pKT8(*relA'*); Table 3]. These results indicate that loss of Slt70 enabled expression of YcbB-mediated β -lactam resistance in liquid medium and reduced the level of production of the YcbB L,D-transpeptidase required for expression of resistance to ampicillin and ceftriaxone on solid medium. This observation suggests that accumulation of uncross-linked glycan chains in the absence of Slt70 may improve the capacity of YcbB to catalyze PG cross-linking accounting for the lower level of expression of *ycbB* required for resistance.

For the sake on completeness, we also noticed that BW25113 Δ *relA* Δ *slyT* pKT2(*ycbB*) pKT8(*relA'*) expressed intermediate resistance to ceftriaxone in the presence of IPTG (induction of *ycbB*) and in the absence of L-arabinose (no induction of *relA'*) because the

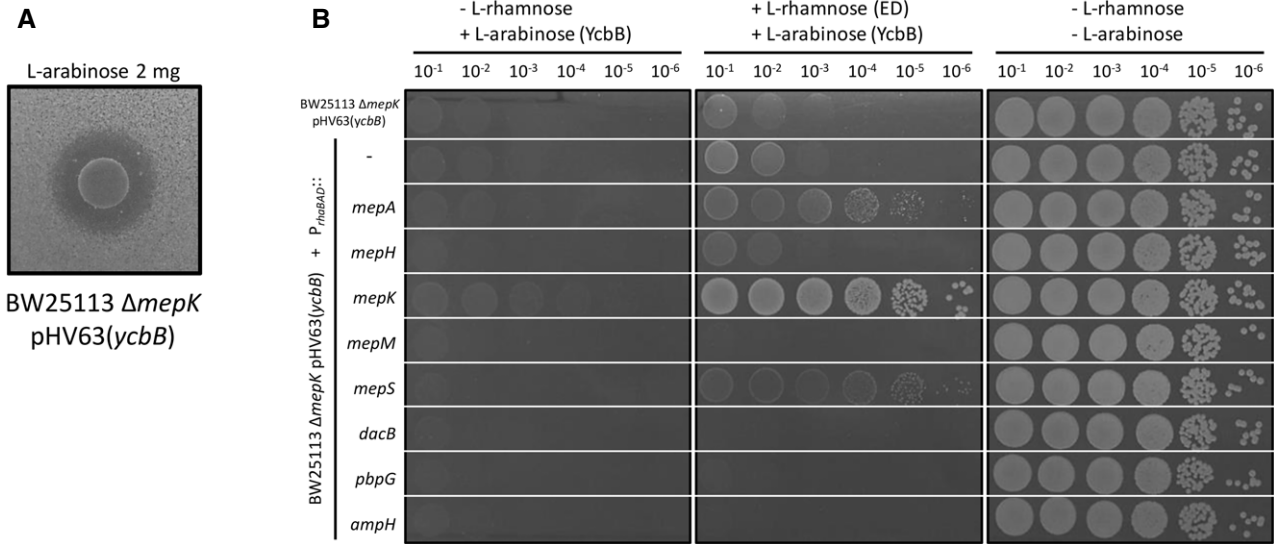


Figure 9. Complementation of the *mepK* deletion by plasmids encoding L-rhamnose-inducible copies of the eight endopeptidase genes.

A Induction of *ycbB* under the control of the P_{araBAD} promoter in BW25113 $\Delta mepK$ pHV63(*ycbB*) was studied by the disk diffusion assay. The clear zone around the disk containing L-arabinose indicates that production of YcbB inhibited growth.
 B Functional complementation of the *mepK* deletion of BW25113 $\Delta mepK$ pHV63(*ycbB*) was performed with the pHV30 vector or recombinant plasmids encoding each of the eight endopeptidases under the control of the P_{rhaBAD} promoter. Induction of *ycbB* and of endopeptidase (ED) genes was performed with 0.2% L-arabinose and 1% L-rhamnose, respectively. BHI agar plates contained chloramphenicol (20 μ g/ml) to counter-select loss of pHV63(*ycbB*).

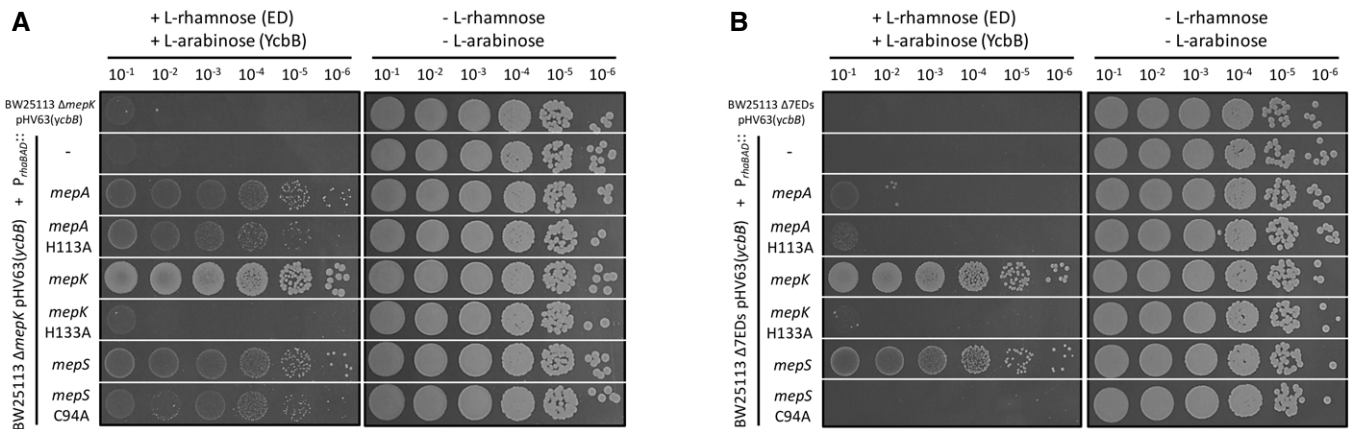


Figure 10. Complementation of *mepK* deletion with catalytically inactivated endopeptidases.

A Functional complementation of the *mepK* deletion of BW25113 $\Delta mepK$ pHV63(*ycbB*) was performed with the pHV30 vector or recombinant plasmids encoding *mepA*, *mepK*, *mepS*, or derivatives encoding catalytically inactive endopeptidases under the control of the P_{rhaBAD} promoter.
 B The complementation assay was repeated for BW25113 $\Delta 7EDs$ pHV63(*ycbB*), which was obtained by deletion of all chromosomal endopeptidase genes except *mepM*. Induction of *ycbB* and of endopeptidase (ED) genes was performed with 0.2% L-arabinose and 1% L-rhamnose, respectively. BHI agar plates contained chloramphenicol (20 μ g/ml) to counter-select loss of pHV63(*ycbB*).

size of the inhibition zone (26 mm) was intermediate between those of susceptible and resistant strains (range of 32–36 mm versus 9–15 mm) (Table 3). This phenotype was independent from *relA'* since replacement of plasmid pKT8(*relA'*) of BW25113 $\Delta relA$ $\Delta sltY$ pKT2(*ycbB*) pKT8(*relA'*) by the vector pHV7 also led to intermediate resistance to ceftriaxone in the presence of IPTG for induction of *ycbB* (25 mm).

Discussion

Endopeptidases are essential for expansion of sacculi

PG polymerization requires a combination of synthetases, the transpeptidases and the glycosyltransferases, in addition to hydrolases that fulfill two essential roles. Since PG is a net-like

Table 1. YcbB-mediated β-lactam resistance in BW25113 derivatives harboring all endopeptidase genes or only *mepM* and *mepK* (Δ6EDs).

Host	Plasmid	Inducer ^b	Inhibition zones (mm) ^a		
			Mec	Amp	Cro
BW25113 Δ <i>relA</i>					
	pKT2(<i>ycbB</i>) pKT8(<i>relA'</i>)	None	20	18	34
		IPTG (<i>ycbB</i>)	21	16	32
		Ara (<i>relA'</i>)	9	18	35
		IPTG + Ara	< 8	< 8	15
BW25113 Δ <i>relA</i> Δ6EDs					
	pKT2(<i>ycbB</i>) pKT8(<i>relA'</i>)	None	19	22	37
		IPTG (<i>ycbB</i>)	20	19	35
		Ara (<i>relA'</i>)	< 8	10	18
		IPTG + Ara	< 8	< 8	16
BW25113 Δ <i>relA</i> Δ6EDs					
	pHV6 ^c pKT8(<i>relA'</i>)	None	22	18	35
		IPTG	22	18	35
		Ara (<i>relA'</i>)	10	18	37
		IPTG + Ara	9	17	37

^aThe diameter of inhibition zones was determined by the disk diffusion assay around disks containing 10 μg mecillinam (Mec), 10 μg ampicillin (Amp), or 30 μg ceftriaxone (Cro). Data are the medians from three experiments. Examples of the original results are presented in Fig EV4.

^bThe *ycbB* and *relA'* genes carried by plasmid pKT2 and pKT8 were induced with 40 μM IPTG and 1% L-arabinose (Ara), respectively.

^cpHV6 is the vector used for construction of pKT2(*ycbB*).

Table 2. Mutations detected in the *sltY* gene of mutants BW25113 M1.1 to M1.4.

Mutant	Position	Mutation	Impact ^a
M1.1	4,621,672	C→T	Gln ³⁷⁵ stop
M1.2	4,622,297	Duplication of AGG: CAGGG→CAGGAGGG	Insertion of Gly position 584 (codon GGN)
M1.3	4,620,572	7-bp deletion: CCTGGCGGC→CC	Trp ⁹ frameshift
M1.4	4,621,507	C→T	Arg ³²⁰ stop

^aSlT70 comprises 645 amino acid residues.

macromolecule completely surrounding the cytoplasmic membrane, it seems clear that insertion of new disaccharide peptide subunits into the growing cell wall requires cleavage of covalent bonds (Höltje & Heidrich, 2001; Vollmer, 2012). The stress-bearing PG being present during the entire cell cycle, it is also obvious that PG hydrolases are required to split daughter cells following completion of septum synthesis (Heidrich *et al*, 2002). Some of these hydrolases generate PG fragments that are imported into the cell and recycled, a complex pathway that is not essential for growth in laboratory conditions but bears important roles in (i) minimizing energy costs, (ii) sensing the appropriate balance between synthetic and hydrolytic activities, which may be altered by β-lactam antibiotics and other toxic agents, and (iii) avoiding the release of proinflammatory molecules recognized by the host immune system (Johnson *et al*, 2013; Bastos *et al*, 2021). PG hydrolases specifically acting on each of the ten amide, ether, and glycosidic bonds present in the PG poly-

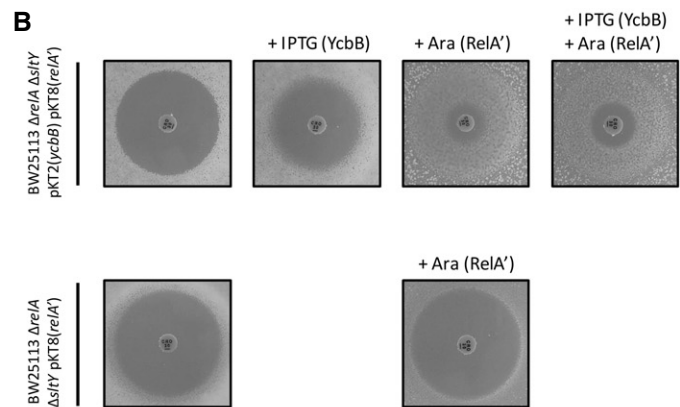
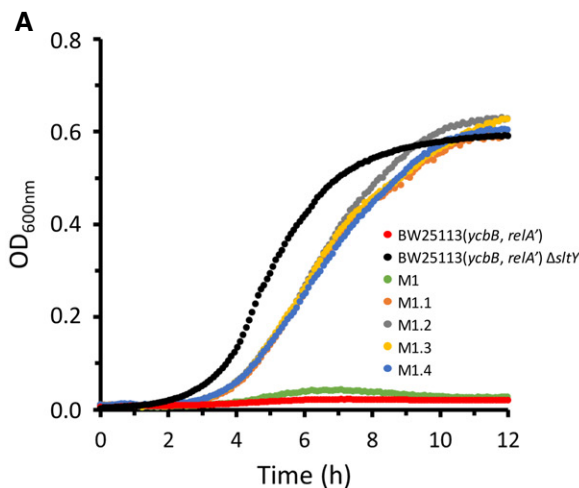


Figure 11. Growth phenotype of *sltY* mutants.

A Growth curves of (i) BW25113(*ycbB*, *relA'*) and its Δ*sltY* derivative; and (ii) strain M1 and its derivatives (M1.1 to 1.4) harboring mutations in *sltY*. The growth medium contained (i) 40 μM IPTG, 1% L-arabinose, and 8 μg/ml ceftriaxone for BW25113(*ycbB*, *relA'*) and its Δ*sltY* derivative or (ii) 50 μM IPTG and 16 μg/ml ampicillin for M1 and its derivatives.

B Ceftriaxone-resistance depends upon *ycbB* and *relA'* expression. The disk diffusion assay was performed with BW25113 Δ*relA* Δ*sltY* harboring pKT2(*ycbB*) and pKT8 (*relA'*) or pKT8(*relA'*) only. Induction was performed with 40 μM IPTG and 1% L-arabinose for *ycbB* and *relA'*, respectively. Disks were loaded with 30 μg of ceftriaxone.

Table 3. YcbB-mediated β -lactam resistance in BW25113(*ycbB*, *relA'*) and its derivative obtained by deletion of *sltY*.

Host	Plasmid	Inducer ^b	Inhibition zones (mm) ^a		
			Mec	Amp	Cro
BW25113 Δ <i>relA</i>					
	pKT2(<i>ycbB</i>) pKT8(<i>relA'</i>)	None	20	18	34
		IPTG (<i>ycbB</i>)	21	16	32
		Ara (<i>relA'</i>)	9	18	35
		IPTG + Ara	< 8	< 8	15
BW25113 Δ <i>relA</i> Δ <i>sltY</i>					
	pKT2(<i>ycbB</i>) pKT8(<i>relA'</i>)	None	21	21	33
		IPTG (<i>ycbB</i>)	22	18	26
		Ara (<i>relA'</i>)	< 8	< 8	9
		IPTG + Ara	< 8	< 8	12
BW25113 Δ <i>relA</i> Δ <i>sltY</i>					
	pHV6 ^c pKT8(<i>relA'</i>)	None	20	22	34
		IPTG	22	22	34
		Ara (<i>relA'</i>)	< 8	21	36
		IPTG + Ara	< 8	20	35
BW25113 Δ <i>relA</i> Δ <i>sltY</i>					
	pKT2(<i>ycbB</i>) pHV7 ^d	None	23	21	34
		IPTG (<i>ycbB</i>)	25	18	25
		Ara	25	21	34
		IPTG + Ara	24	17	26

^aThe diameter of inhibition zones was determined by the disk diffusion assay around disks containing 10 μ g mecillinam (Mec), 10 μ g ampicillin (Amp), or 30 μ g ceftriaxone (Cro). Data are the medians from three experiments.

^bThe *ycbB* and *relA'* genes carried by plasmid pKT2 and pKT8 were induced with 40 μ M IPTG and 1% L-arabinose (Ara), respectively.

^cpHV6 is the vector used for construction of pKT2(*ycbB*).

^dpHV7 is the vector used for construction of pKT8(*relA'*).

mer have been described and most cleavage specificities involve multiple enzymes (Fig EV5A). Enzymes of different specificities can at least in part compensate for each other, e.g., lytic glycosyltransferases and amidases both contribute to the separation of daughter cells (Heidrich *et al*, 2002; van Heijenoort, 2011). In this study, we show that endopeptidases are specifically required for bacterial growth not only in the context of the formation of 4 \rightarrow 3 cross-links by PBPs but also in the context of the formation of 3 \rightarrow 3 cross-links by YcbB (Table 4). We also identify for the first time the minimum sets of endopeptidases for each mode of PG cross-linking, namely MepM for 4 \rightarrow 3 cross-links and MepM plus MepK for 3 \rightarrow 3 cross-links. Endopeptidase overproduction resulting from expression of the genes under the control of heterologous promoters revealed potential functional redundancies in the endopeptidase families. In particular, overproduction of MepH, MepS, or PBP7 compensated for the absence of MepM in the context of a 4 \rightarrow 3 cross-linked PG (Fig 12A). Overproduction of MepS compensated for the absence of MepM or MepK for growth with a 3 \rightarrow 3 cross-linked PG. Overproduction of MepM prevented growth probably due to unbalanced synthesis and hydrolysis of PG cross-links (observed for both 4 \rightarrow 3

and 3 \rightarrow 3 cross-linked PG). Production of catalytically inactive endopeptidases suggested that MepA and MepS are negatively regulated by Prc-mediated proteolysis, as previously established for MepS (Singh *et al*, 2015; Lai *et al*, 2017). In conclusion, MepK is specifically required for growth if the peptidoglycan contains a large proportion of 3 \rightarrow 3 cross-links, while MepM is additionally required for β -lactam resistance, unless it is the only endopeptidase remaining, in which case it is also required for growth in the absence of the drug.

Specificity of purified endopeptidases for 4 \rightarrow 3 and 3 \rightarrow 3 cross-links

The cleavage specificity of the endopeptidases was determined by mass spectrometry (Figs 5 and 7, Appendix Fig S2). Endopeptidases of the PBP family were specific to 4 \rightarrow 3 cross-links. Endopeptidases belonging to other families cleaved both 4 \rightarrow 3 and 3 \rightarrow 3 cross-links with similar efficacies (MepM, MepS, MepA) or with a preference for 4 \rightarrow 3 (MepH) or 3 \rightarrow 3 (MepK) cross-links (Figs 5 and 7) (Engel *et al*, 1992; Chodisetti & Reddy, 2019). This is unexpected since 4 \rightarrow 3 and 3 \rightarrow 3 cross-links contain amide bonds connecting two R stereocenters (D-Ala⁴ \rightarrow DAP³) or an S to an R stereocenter (DAP³ \rightarrow DAP³), respectively (Fig EV5A). This could imply that endopeptidases of the Mep families mainly interact with the acceptor stems of cross-linked muropeptides, which are the same for both types of cross-links, whereas endopeptidases of the PBP family interact with a donor tetrapeptide stem only present in 4 \rightarrow 3 cross-linked muropeptides (Fig EV5B).

Integration of endopeptidases into the global regulation of 4 \rightarrow 3 and 3 \rightarrow 3 PG cross-linking

D,D-carboxypeptidases, which cleave off the terminal residue (D-Ala⁵) of pentapeptide stems, are thought to negatively control the transpeptidase activity of PBPs since these enzymes require a pentapeptide donor (Fig 1). A less studied impact of D,D-carboxypeptidases is the formation of the essential tetrapeptide donor substrate of the LDTs, except for two publications reporting that PBP5 and PBP6a are essential for YcbB-mediated β -lactam resistance and for rescue of a defect in lipopolysaccharide synthesis, respectively (Hugonnet *et al*, 2016; Morè *et al*, 2019). Thus, D,D-carboxypeptidases have crucial roles in controlling the relative contributions of transpeptidases of the D,D and L,D specificities to PG cross-linking by both decreasing access of PBPs to pentapeptide stems and increasing access of LDTs to tetrapeptide stems.

Endopeptidases participate in the metabolism of PG cross-links in several ways. MepH and MepS display L,D-carboxypeptidase activity leaving tripeptides as the main (> 80%) end product of *in vitro* PG hydrolysis (Fig 7). This activity may negatively control the L,D-transpeptidase activity of YcbB by hydrolysis of D-Ala⁴, thereby preventing access to its tetrapeptide donor. In addition, hydrolysis of 4 \rightarrow 3 cross-linked Tetra \rightarrow Tetra and Tetra \rightarrow Tri dimers by the endopeptidases generates free tetrapeptide stems (Figs 5 and 7). These tetrapeptide stems can be used as donor by YcbB for formation of 3 \rightarrow 3 cross-links, as demonstrated for the LDTs of *Mycobacterium smegmatis* (Baranowski *et al*, 2018). In contrast, the D,D-transpeptidase activity of PBPs exclusively requires pentapeptide-containing donors. Analyses of peptidoglycan structure indicate that

Table 4. Characteristics of the endopeptidases.

Endopeptidase	<i>In vitro</i> hydrolysis of 4→3 and 3→3 cross-links ^c	Role in the context of the two modes of cross-linking (revealed by endopeptidase overproduction ^f)	
		4→3 cross-links	3→3 cross-links
Acyl-serine transferase			
PBP4 ^a	4→3	Not essential (None)	Not essential (None)
PBP7 ^a	4→3	Not essential (Compensates for the absence of MepM)	Not essential (None)
AmpH ^b	4→3 ^d	Not essential (None)	Not essential (None)
NlpC/P60 peptidase			
MepH ^a	4→3 > 3→3 L,D-carboxypeptidase	Not essential (Compensates for the absence of MepM)	Not essential (None)
MepS ^a	4→3 = 3→3 L,D-carboxypeptidase	Not essential (Compensates for the absence of MepM)	Not essential (Compensates for the absence of MepM or MepK) ^e
Lysostaphin/M23 peptidase			
MepM ^a	4→3 = 3→3	Sufficient (Prevents growth)	Essential (Prevents growth)
LAS metallopeptidase			
MepA ^b	4→3 = 3→3	Not essential (None)	Not essential (None)
M15 peptidase			
MepK ^b	4→3 < 3→3	Not essential (None)	Essential (None)

^aCharacterized in this study.

^bData from the literature (Engel *et al*, 1992; Gonzalez-Leiza *et al*, 2011; Chodiseti & Reddy, 2019).

^cMepM, MepS, and MepA cleaved 4→3 and 3→3 cross-links with similar efficacies (4→3 = 3→3). MepH and MepK displayed a preference for 4→3 cross-links (4→3 > 3→3) or for 3→3 cross-links (4→3 < 3→3), respectively. L, D-carboxypeptidase, hydrolysis of the DAP³-D-Ala⁴ amide bond of tetrapeptide stems.

^dHydrolysis of 3→3 cross-links was not tested.

^eReplacement of both MepM and MepK by MepS was not tested.

^fNone, no phenotype associated with endopeptidase overproduction.

D-Ala⁵ is rapidly cleaved off from pentapeptide stems by D,D-carboxypeptidases if they are not used for formation of 4→3 cross-links (Glauner *et al*, 1988). These observations suggest that the transpeptidase activity of PBPs exclusively relies on *de novo* synthesis and translocation of pentapeptide-containing subunits. Thus, YcbB is expected to function as a rescue enzyme to restore cross-linking in regions of the PG that are compromised by 4→3 or 3→3 endopeptidases. This mechanism was proposed for PG repair

following disassembly of the lipopolysaccharide export machinery that crosses the PG layer (Morè *et al*, 2019). The combined activities of endopeptidases cleaving 4→3 cross-links and of L,D-transpeptidases could contribute to the enrichment in 3→3 cross-links in stationary phase cultures (Pisabarro *et al*, 1985). In turn, this enrichment may protect cells from hydrolases active on 4→3 cross-linked PG. Previous analyses proposed that two L,D-transpeptidases may contribute to the enrichment of PG in 3→3 cross-links, namely YcbB (LdtD), which is induced by the cell envelope Cpx stress system, and YnhG (LdtE), which is expressed under the control of sigma S and induced in stationary phase (Weber *et al*, 2005; Delhaye *et al*, 2016).

Participation of YcbB to PG polymerization complexes

PG polymerization is generally thought to be performed by two multiprotein complexes involved in the expansion of the lateral cell wall (elongasome) and in the formation of the septum (divisome) (Pazos *et al*, 2017). Replacement of the D,D-transpeptidase activity of all PBPs by the L,D-transpeptidase activity of YcbB raises several questions regarding the identity of the partners of YcbB for the assembly of lateral wall and septum PG, and whether YcbB physically replaces PBPs in the PG polymerization complexes. Our data support a model in which YcbB functions with two different sets of partners for lateral wall and septum PG assembly as follows.

For the assembly of lateral wall PG, inactivation of the transpeptidase domain of PBP2 by β -lactams leads to uncoupling of the glycosyltransfer and transpeptidation reactions, the former being most probably catalyzed by RodA (Uehara & Park, 2008; Cho *et al*, 2014) (Fig 12B). Uncross-linked glycan chains accumulate in the periplasm and are eventually cleaved by the Slt70 lytic transglycosylase and recycled. According to the model presented in Fig 12, and in agreement with a previous study (Cho *et al*, 2014), the reactions catalyzed by Slt70 and YcbB occur in competition implying that YcbB-mediated cross-linking is not coupled to glycan chain polymerization by RodA. This also implies that YcbB could function in the PG layer in combination with the MepM endopeptidase known to participate in cell elongation (Uehara *et al*, 2009; Singh *et al*, 2012; Banzhaf *et al*, 2020; Truong *et al*, 2020). In agreement with this model, impaired Slt70 activity had a positive impact on β -lactam resistance mediated by YcbB (Fig 12C). This was established both by the selection of mutations enabling expression of β -lactam resistance in liquid medium, which mapped in the *sltY* gene encoding Slt70 (Table 2) and by the deletion of *sltY*, which lowered the level of *ycbB* expression required for resistance (Fig 11).

For the assembly of septum PG, YcbB was proposed to cooperate with the glycosyltransferase activity of Class A PBP1b (Hugonnet *et al*, 2016; Caveney *et al*, 2019). In support of this hypothesis, microscale thermophoresis experiment revealed that purified YcbB interacts with PBP1b and PBP5 (D,D-carboxypeptidase). Furthermore, the glycosyltransferase activity of PBP1b is essential for YcbB-mediated β -lactam resistance, whereas the combined deletion of class A PBP1a and PBP1c had no impact. We cannot rule out the possibility that the glycosyltransferase activity of FtsW also contributes to septum PG polymerization in the presence of β -lactams but this would require that the inactivation of PBP3 by β -lactams lead to the uncoupling of glycan chain polymerization and PG cross-linking, as proposed for the RodA-PBP2 complex (see above). This

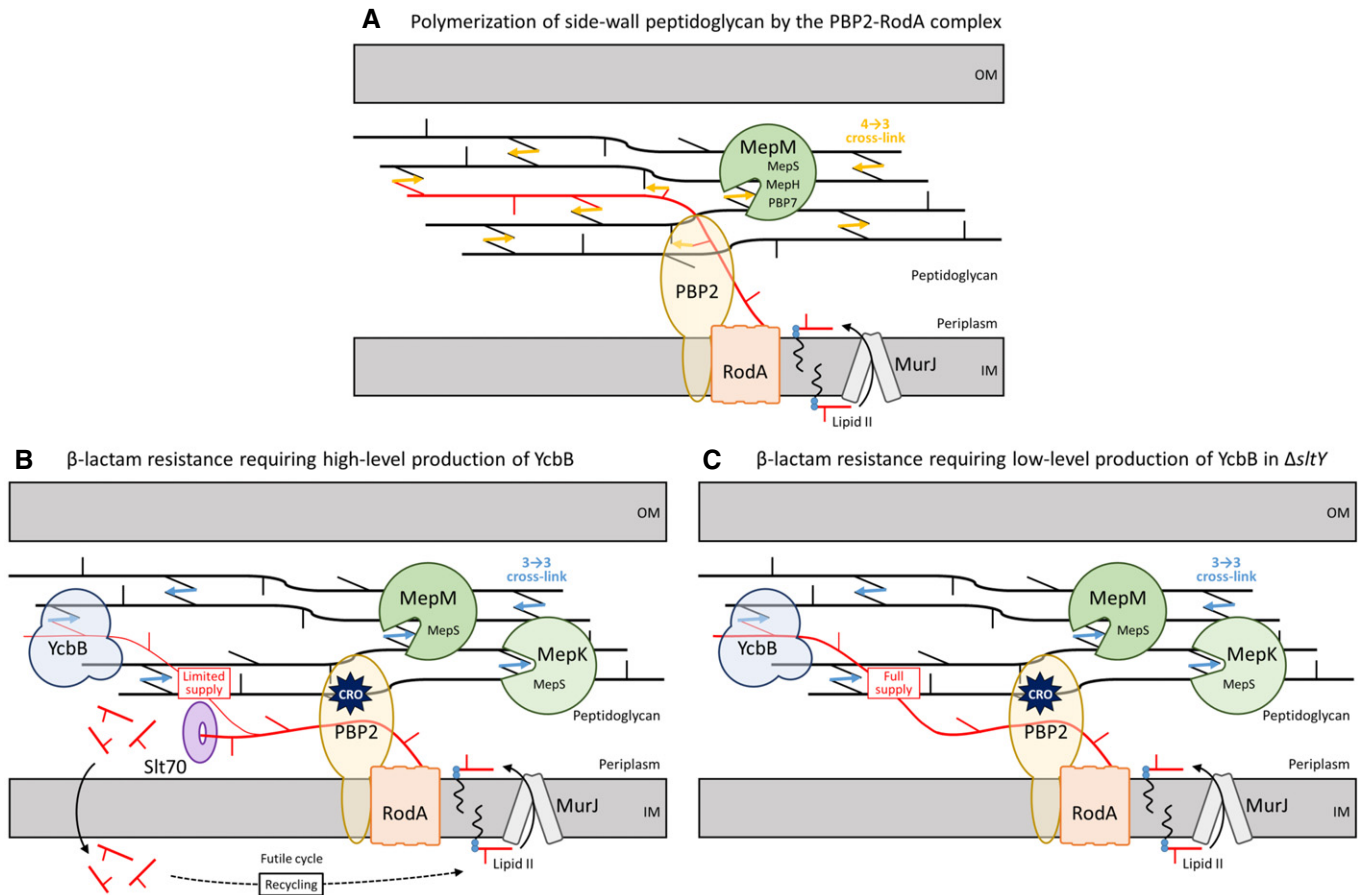


Figure 12. Proposed model for polymerization of side wall PG by transpeptidases of the D,D or L,D specificity.

- A In wild-type cells, the disaccharide pentapeptide subunit linked to the undecaprenyl lipid transporter (Lipid II) is translocated to the outer leaflet of the cytoplasmic membrane by MurJ and polymerized by the glycosyltransferase and D,D-transpeptidase activities of the RodA-PBP2 complex. MepM is essential and sufficient for insertion of new material in the PG net, although this endopeptidase can be replaced by overproduction of MepH, MepS, or PBP7.
- B Inhibition of PBP2 by β -lactams leads to the accumulation of uncross-linked glycan chains that are cleaved by the Slt70 lytic transglycosylase. This limits the supply of glycan chains for cross-linking by the YcbB L,D-transpeptidase. Under this condition, MepM or overproduction of MepS is required for insertion of new glycan strands. Production of YcbB leads to a requirement for an additional endopeptidase, MepK. This function of MepK can be bypassed by overproduction of MepS.
- C Deletion of the *sltY* gene encoding lytic transglycosylase Slt70 prevents digestion of uncross-linked glycan chains leading to a full supply of neo-synthesized glycan chains to YcbB and improved expression of β -lactam resistance. IM, inner membrane; OM, outer membrane; CRO, ceftriaxone.

is not supported by the analyses of PG recycling in conditions of selective inhibition of PBP3 by aztreonam (Uehara & Park, 2008). The endopeptidases involved in septum formation have not been identified, except for a contribution of PBP4, which has an effect on the timing of septation (preprint: Verheul *et al*, 2020). MepK is a candidate for this function in 3→3 cross-linked PG although this is currently not supported by experimental evidence, and it remains to be seen if endopeptidases are needed for septum PG synthesis.

Materials and Methods

Strains, plasmids, and growth conditions

All strains were derived from *E. coli* BW25113 (Baba *et al*, 2006). The origin and characteristics of plasmids are listed in

Appendix Table S1. The origin and genotype of strains are listed in Appendix Table S2. Bacteria were grown in brain heart infusion (BHI; Difco) broth or agar at 37°C unless otherwise specified. Liquid cultures were performed with aeration (180 rpm). The growth media were systemically supplemented with drugs to counter-select plasmid loss (Appendix Table S1). The same drugs at the same concentrations were used to select transformants. Kanamycin at 50 μ g/ml was used for the Km^R cassette. Induction of the *lacZYA*, *araBAD*, and *rhaBAD* promoters was performed with isopropyl β -D-1-thiogalactopyranoside (IPTG, 40 or 50 μ M), L-arabinose (0.2 or 1%), and L-rhamnose (0.2 or 1%), respectively. Plasmids constructed in this study were obtained by using NEBuilder HiFi DNA assembly (New England Biolabs) method, unless otherwise specified.

Growth curves were obtained in a 96-well plate using an Infinite 200 PRO microplate reader (TECAN). Briefly, bacteria were grown

to the late exponential phase, i.e., to an optical density at 600 nm (OD_{600}) greater than 1.0 (ca. 6 h at 37°C under agitation). The OD_{600} was adjusted to 1.0, and 5 μ l were inoculated in 195 μ l of BHI broth supplemented with drugs and inducers, as specified in the legends to figures. Growth was monitored at 600 nm every 5 min for 12 h at 37°C with vigorous shaking.

Construction of *E. coli* strains carrying gene deletions

The Keio collection comprises 3,985 mutants obtained by replacement of non-essential genes by a kanamycin resistance (Km^R) cassette (Baba *et al.*, 2006). P1 transduction of the Km^R cassette from selected mutants was used to introduce deletions of specific genes involved in PG synthesis (Datsenko & Wanner, 2000). For multiple gene deletions, the Km^R cassette was removed by the FLT recombinase encoded by plasmid pCP20. The presence of the expected deletions was confirmed by PCR amplification at each deletion step. Appendix Fig S1 shows lineages that have been obtained by parallel serial deletions.

To study the 3 \rightarrow 3 mode of cross-linking, plasmid pKT2(*ycbB*) and pKT8(*relA'*) were introduced into the derivatives of *E. coli* BW25113 Δ *relA* obtained by deletion of various combinations of endopeptidase genes. For the sake of simplicity, the latter strains were referred to as BW25113(*ycbB*, *relA'*) derivatives even though gene deletions preceded the introduction of pKT2 (*ycbB*) and pKT8(*relA'*).

Construction of *E. coli* BW25113 Δ *relA* Δ 8EDs

The plasmid pHV53(P_{thaBAD^-} -TIS2-*mepM*) was introduced into BW25113 Δ *relA* Δ 7EDs harboring *mepM* as the only chromosomal endopeptidase-encoding gene. The *mepM* deletion was introduced into BW25113 Δ *relA* Δ 7EDs pHV53(P_{thaBAD^-} -TIS2-*mepM*) in the presence of 0.2% L-rhamnose by P1 transduction as described above. Growth of the resulting BW25113 Δ *relA* Δ 8EDs pHV53(P_{thaBAD^-} -TIS2-*mepM*) strain was dependent on the induction of the plasmid copy of *mepM* mediated by L-rhamnose.

Mutant selection and whole-genome sequencing

E. coli BW25113 M1 was streaked for isolated colonies on agar plates containing 10 μ g/ml tetracycline to counter-select loss of plasmid pJEH12(*ycbB*). The selection procedure was independently carried out starting with four independent colonies. Briefly, 5 ml of BHI broth supplemented with 10 μ g/ml tetracycline and 50 μ M IPTG were inoculated with a colony. Bacteria were grown overnight with shaking (180 rpm). A fraction of 1 ml of the culture was inoculated in 250 ml of BHI broth supplemented with 16 μ g/ml ampicillin and 50 μ M IPTG. Bacteria were grown overnight with shaking and streaked on BHI agar containing 16 μ g/ml ampicillin and 50 μ M IPTG. A colony was inoculated in 250 ml of BHI broth supplemented with 16 μ g/ml ampicillin and 50 μ M IPTG. Bacteria were grown overnight with shaking and streaked on BHI agar containing 16 μ g/ml ampicillin and 50 μ M IPTG. Five ml of BHI broth containing 10 μ g/ml tetracycline was inoculated with a single colony and genomic DNA was extracted (Wizard DNA extraction kit, Promega). Genomic DNA was sequenced by paired-end joining Illumina (Biomics Platform of the Institut Pasteur, Paris, France).

Identification of the mutations was performed with the *breseq* pipeline (Deatherage & Barrick, 2014).

Plating efficiency assay

Bacteria were grown to the late exponential phase, i.e., to an optical density at 600 nm (OD_{600}) greater than 1.0 (ca. 6 h at 37°C under agitation). The OD_{600} was adjusted to 1.0- and 10-fold dilutions (10^{-1} to 10^{-6}) were prepared in BHI broth. Ten μ l of the resulting bacterial suspensions was spotted on BHI agar supplemented with inducers and drugs as indicated in the legend to figures. For the disk diffusion assay, 5 μ l of the bacterial suspension adjusted to an OD_{600} of 1.0 was inoculated in 5 ml of water. BHI agar plates were flooded with the latter suspension, excess liquid was removed, and the plates were kept at room temperature for 15 min prior to the addition of paper disks containing antibiotics or inducers. Plates were imaged after 16 h (or 24 h for plates containing ceftriaxone) of incubation at 37°C.

Detection of the production of PBP4 and AmpH with a fluorescent penicillin

Derivatives of BW25113(*ycbB*, *relA'*) Δ *mepM* harboring plasmids pHV48(*dacB*) or pHV50(*ampH*) were grown in the presence of L-rhamnose (0, 0.01, 0.1, and 1%), 10 μ g/ml tetracycline, 20 μ g/ml chloramphenicol, and 25 μ g/ml zeocin to the early stationary phase ($OD_{600} = 3.0$). The antibiotics were used to counter-select loss of plasmids pKT2, pKT8, and pHV48 or pHV50, respectively. Bacteria (1.5 ml) were washed in PBS, concentrated 10-fold (150 μ l), and incubated with 200 μ M BOCILLIN® FL Penicillin (Invitrogen) for 10 min at 20°C. Bacteria were washed with PBS, resuspended in 100 μ l of PBS, and 100 μ l of 2 \times Laemmli loading buffer was added. The mixture was incubated at 92°C for 3 min, and 30 μ l was analyzed by 12% SDS-PAGE. Images were acquired using the IBright FL 1500 (Thermo Fisher) imager in the protein fluorescence mode.

Purification of endopeptidases

The *mepM* gene was amplified by PCR and cloned into pET-TEV between the NdeI and XhoI restriction sites. The fusion protein comprised a 6 \times histidine tag, a TEV protease cleavage site, and residues 41–440 of MepM. The *mepH* and *mepS* genes were independently amplified by PCR and cloned in frame with *dsbC* into pETMM82 using NEBuilder HiFi DNA assembly (New England Biolabs). The fusion proteins comprised the DsbC chaperone (Firczuk & Bochtler, 2007), a 6 \times histidine tag, a TEV protease cleavage site, and residues 28–271 of MepH or 25–188 of MepS. The enzymes were produced in *E. coli* BL21(DE3) following induction by 0.5 mM IPTG for 18 h at 16°C. The endopeptidases were purified in 50 mM Tris-HCl pH 8.0 from a clarified lysate by nickel affinity chromatography (elution with 0.5 mM imidazole). The endopeptidases were dialyzed overnight at 4°C against 50 mM Tris-HCl pH 8.0, 0.5 mM EDTA. N-terminal tags were cleaved overnight at room temperature following addition of 10 μ g of TEV protease for every mg of protein and DTT at a final concentration of 0.5 mM. MepM, MepH, and MepS were further purified by size-exclusion chromatography (Superdex 75 HiLoad 26/60, GE Healthcare) in 50 mM Tris-HCl pH 7.5, 200 mM NaCl.

PBP4 was purified from strain BL21(DE3) pET21b Ω PBP4 Δ 1-60 as previously reported (Banzhaf *et al*, 2020). Briefly, cells were grown in the presence of 1 mM IPTG for 8 h at 20°C and then harvested by centrifugation at 7,500 \times g, 4°C, 15 min. Cell pellets were resuspended in 50 mM Tris-HCl pH 8.0, 300 mM NaCl and lysed by sonication. Following centrifugation at 14,000 \times g, 1 h, 4°C, the NaCl concentration was reduced by stepwise dialysis in a Spectra/Por dialysis membrane (MWCO 12–14 kDa) against 50 mM Tris-HCl pH 8.5 containing (i) 200 mM NaCl, (ii) 100 mM NaCl, and (iii) 30 mM NaCl. The sample was centrifuged at 7,500 \times g, 4°C, 10 min, and the supernatant was applied to a 5 ml HiTrap Q HP IEX column in 25 mM Tris-HCl pH 8.5, 30 mM NaCl. Protein was eluted from the column with a linear gradient from 50 mM Tris-HCl pH 8.5, 100 mM NaCl, to 25 mM Tris-HCl pH 8.0, 1 M NaCl, over a 100 ml volume. Fractions containing PBP4 were combined and dialyzed against 10 mM potassium phosphate pH 6.8, 300 mM NaCl. Protein was applied at 1 ml/min to a 5 ml ceramic hydroxyapatite column (Bio-Rad BioscaleTM) in the dialysis buffer, and a 50 ml linear gradient to 500 mM potassium phosphate pH 6.8, 300 mM NaCl was applied. Fractions with PBP4 were dialyzed overnight against 25 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 10% glycerol, and concentrated to *ca.* 5 ml using a Vivaspin concentrator spin column (Sartorius). The protein sample was applied to a HiLoad 16/600 Superdex 200 column (GE healthcare) at 1 ml/min and eluted in a linear gradient to 25 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 10% glycerol. The collected fractions containing PBP4 were combined.

PBP7 was purified from strain BL21(DE3) pET28a Ω pbpG Δ 1-75 as previously reported (Banzhaf *et al*, 2020). Briefly, cells were grown in the presence of 1 mM IPTG for 3 h at 30°C before being harvested by centrifugation and resuspended in 25 mM Tris-HCl pH 7.5, 500 mM NaCl, 20 mM imidazole. Following sonication and subsequent centrifugation, the lysate was applied to a 5 ml HisTrap HP column (GE healthcare) and washed with 4 column volumes of 25 mM Tris-HCl pH 7.5, 500 mM NaCl, 20 mM imidazole. Bound protein was eluted with 25 mM Tris-HCl pH 7.5, 300 mM NaCl, 400 mM Imidazole. Elution fractions containing PBP7 were combined and dialyzed overnight against 25 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 10% glycerol, in the presence of 1 unit/ml of restriction grade thrombin (Novagen) to remove the oligohistidine tag. The sample was then concentrated to *ca.* 5 ml using a Vivaspin concentrator spin column (Sartorius) at 4,500 \times g, 4°C. The protein sample was applied to a HiLoad 16/600 Superdex 200 column (GE healthcare) at 1 ml/min and eluted in 25 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 10% glycerol. Elution fractions containing PBP7 were combined.

Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin as a standard. Endopeptidases were stored at –80°C.

Preparation of sacculi

Bacteria were grown in M9 minimal medium supplemented with 0.1% glucose at 37°C for 48 h. Bacteria were harvested by centrifugation and boiled in 4% sodium dodecyl sulfate (SDS) for 1 h. Sacculi were harvested by centrifugation (20,000 \times g for 20 min at 20°C), washed five times with water, and incubated with 100 μ g/ml pronase overnight at 37°C in 20 mM Tris-HCl pH 7.5. Sacculi were washed five times with water and incubated overnight at 37°C with 100 μ g/ml trypsin in 20 mM sodium phosphate pH 8.0. Sacculi

were washed five times with water, boiled for 5 min, collected by centrifugation, resuspended in water, and stored at –20°C.

Digestion of sacculi

Sacculi were digested overnight at 37°C with 120 μ M lysozyme alone or in association with an endopeptidase in 40 mM Tris-HCl pH 8.0. Insoluble material was removed by centrifugation, and the soluble fraction containing mucopeptides was reduced with sodium borohydride for 1 h in 125 mM borate buffer pH 9.0. The pH of the solution containing the reduced mucopeptides was adjusted to 4.0 with phosphoric acid. Mucopeptides were separated by *rp*HPLC in a C18 column (Hypersil GOLD aQ; 250 \times 4.6 mm; 3 μ m, Thermo Scientific) at a flow rate of 1 ml/min with a linear gradient (0–20%) applied between 10 and 60 min (buffer A, TFA 0.1%; buffer B, acetonitrile 20% TFA 0.1%). Absorbance was monitored at 205 nm and fractions were collected, lyophilized, and analyzed by mass spectrometry. Mass spectra were obtained on a Bruker Daltonics maXis high-resolution mass spectrometer (Bremen, Germany) operating in the positive mode (Analytical Platform of the Muséum National d'Histoire Naturelle, Paris, France). Theoretical masses were predicted using massXpert, and mass spectral data were explored using mineXpert2 (Rusconi, 2009; Langella & Rusconi, 2021).

Data availability

Whole genome sequencing raw data of *sltY* mutants (M1.1 to M1.4) are available at the Sequence Read Archive database (<https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA748867>).

Expanded View for this article is available online.

Acknowledgements

This work was supported by the French National Research Agency ANR “RegO-Peps” (grant ANR-19-CE44-0007 to JEH). HV is the recipient of a doctoral fellowship from Sorbonne-Université (ED 515, complexité du vivant). We thank L. Dubost and A. Marie for technical assistance in the collection of mass spectra at the Bio-organic Mass Spectrometry Platform. We also thank L. Ma and R. Legendre for technical assistance in genome sequencing. The Biomics Platform is a member of the “France Génomique” consortium supported by the French National Research Agency ANR (grant ANR-10-INBS-0009) and IBISA. We also thank Z. Edoou for proofreading the manuscript.

Author contributions

HV: conception and design, acquisition, analysis, and interpretation of data, drafting, and revising the article. DD and AL: acquisition of data. WV: interpretation of data, drafting and revising the article. MA and JEH: conception and design, analysis and interpretation of data, drafting and revising the article.

Conflict of interest

The authors declare that they have no conflict of interest.

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