

Peptidoglycan-tethered and free forms of the Braun lipoprotein are in dynamic equilibrium in *Escherichia coli*

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Abstract

Peptidoglycan (PG) is a giant macromolecule that completely surrounds bacterial cells and prevents lysis in hypo-osmotic environments. This net-like macromolecule is made of glycan strands linked to each other by two types of transpeptidases that form either 4 → 3 (PBPs) or 3 → 3 (LDTs) cross-links. Previously, we devised a heavy isotope-based PG full labeling method coupled to mass spectrometry to determine the mode of insertion of new-subunits into the expanding PG network (Atze et al., 2022). We showed that PG polymerization operates according to different modes for the formation of the septum and of the lateral cell walls, as well as for bacterial growth in the presence or absence of β-lactams in engineered strains that can exclusively rely on LDTs for PG cross-linking when drugs are present. Here, we apply our method to the resolution of the kinetics of the reactions leading to the covalent tethering of the Braun lipoprotein (Lpp) to PG and the subsequent hydrolysis of that same covalent link. We find that Lpp and disaccharide-peptide subunits are independently incorporated into the expanding lateral cell walls, whereas tethering of Lpp to septal PG is limited. LDTs did mediate intense shuffling of Lpp between PG stems leading to a dynamic equilibrium between the PG-tethered and free forms of Lpp.

eLife assessment

This **useful** study describes a single set of label-chase mass spectrometry experiments to confirm the molecular function of YafK as a peptidoglycan hydrolase, and to describe the timing of its attachment to the peptidoglycan. Confirmation of the molecular function of YafK will be helpful in further studies to examine the function and regulation of the outer membrane-peptidoglycan link in bacteria. The evidence supporting the molecular function of YafK and that lpp molecules are shuffled on and off the peptidoglycan is **solid**, however, data supporting conclusions relating to the locations of lpp-peptidoglycan attachment are **incomplete**. The work will be of interest to microbiologists studying the bacterial cell wall.

The PG is an essential component of the bacterial cell envelope that counteracts the turgor pressure of the cytoplasm thereby preventing the cell from bursting and lysing in hypoosmotic environments (**Fig. 1A** ) (Rojas et al., 2018 ). Structural studies of the PG (**Fig. 1B** ) revealed

that this giant macromolecule consists of glycan strands linked to each other by transpeptidases belonging to the PBP and LDT families, which form 4 → 3 and 3 → 3 cross-links, respectively (Glauner et al., 1988 [\[1\]](#)). PBPs and LDTs contain different catalytic nucleophiles (Ser *versus* Cys) and use different stem peptides as the acyl donor substrate (pentapeptide *versus* tetrapeptide) (Fig. 1C [\[2\]](#)) (Mainardi et al., 2005 [\[3\]](#)). LDTs forming 3 → 3 cross-links are unessential enzymes (Magnet et al., 2008 [\[4\]](#)), which can participate in PG maintenance (Morè et al., 2019 [\[5\]](#)) and in β -lactam resistance acquired *in vitro* (Voedts et al., 2022 [\[6\]](#)). The LDT family includes additional enzymes (Fig. 1C [\[2\]](#)) that catalyze either the tethering of Lpp to PG (ErfK, Ybis, and Ycfs) (Magnet 2007) or the hydrolysis of the resulting covalent link (YafK) (Bahadur et al., 2021 [\[7\]](#)); (Winkle et al., 2021 [\[8\]](#)). Lpp is the most abundant lipoprotein (10^6 copies per cell), about one third of which is attached to PG. This dense connection between the PG and the outer membrane (OM) is thought to stabilize the bacterial envelope because mutants deficient in the tethering of Lpp to PG are viable but display increased vesiculation and lethal susceptibility to EDTA (Sanders and Pavelka, 2013 [\[9\]](#)). In addition, Lpp controls the distance between the inner (IM) and outer membranes, a structural feature that is critical for sensing stresses that affect the bacterial envelope (Asmar et al., 2017 [\[10\]](#)); (Mathelié-Guinlet et al., 2020 [\[11\]](#)).

The enzymatic activity of YafK was established by incubating PG with purified YafK and detecting the products of the hydrolysis of the PG → Lpp bonds (Bahadur et al., 2021 [\[7\]](#)); (Winkle et al., 2021 [\[8\]](#)). Intriguingly, the deletion of *yafK* was not associated with any decrease in the overall Lpp-to-PG tethering in the absence of stress (Bahadur et al., 2021 [\[7\]](#)). The absence of phenotype associated with the deletion of *yafK* implies, in the wild-type strain, either that the enzyme was not produced (or active) or that hydrolysis of PG → Lpp bonds by YafK is compensated by the formation of the same number of PG → Lpp bonds by LDTs. Here, we used a PG labeling method (Atze et al., 2022) to distinguish between these two possibilities and determine the mode of cross-linking of newly synthesized Lpp to PG.

Results and discussion

Time-resolved characterization of the isotopic composition of the Tri → KR muropeptide by mass spectrometry

To investigate the mode of tethering of Lpp to PG, bacteria were grown in minimal medium containing [^{13}C]glucose and [^{15}N]ammonium chloride, thus affording fully labeled PG. At the late log phase, bacteria were transferred to a light isotope medium and the PG macromolecule was extracted from culture samples collected at various times after the medium switch. Digestion of PG with proteinases and muramidases released soluble disaccharide-peptide fragments (muropeptides) and eliminated the bulk of Lpp except for its C-terminal Arg-Lys dipeptide that remained bound to tripeptide stems (muropeptide Tri → KR in Fig. 1B [\[2\]](#)). The isotopic composition of the Tri → KR isotopologues was determined by mass spectrometry (Figure 2 [\[12\]](#)) and tandem mass spectrometry (Table S1 [\[13\]](#)). This analysis revealed that tethering of Lpp to PG did generate Tri → KR isotopologues containing light and heavy moieties at both the donor (tripeptide) and acceptor (Lpp) positions.

Newly synthesized Lpp and stem peptides are independently incorporated into PG

The PG is polymerized from lipid II consisting of the disaccharide-pentapeptide subunit linked to the undecaprenyl lipid carrier by a pyrophosphate bond. In Gram-positive bacteria, proteins are tethered to PG by sortases that use lipid II as the acceptor of the transpeptidation reaction (Drams et al., 2008 [\[14\]](#)). Kinetic analysis of the appearance of isotopologues of the Tri → KR muropeptide ruled out this mode of tethering for Lpp. Indeed, this would have resulted in the linking of newly

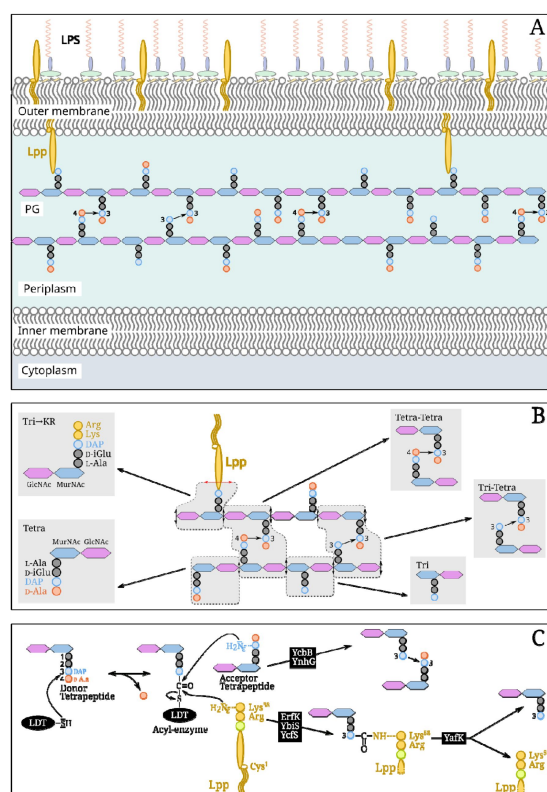


Figure 1.

Structure and synthesis of the cell envelope of *Escherichia coli*.

(A) Structure of the main polymers of the envelope. The envelope of Gram-negative bacteria contains two membranes. The inner membrane (IM) is made of phospholipids. The outer membrane (OM) is asymmetric, with phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet. The PG is located in the periplasmic space delimited by the IM and the OM. The PG consists of glycan chains linked to each other by short peptides. The glycan chains consist of alternating $\beta 1 \rightarrow 4$ -linked *N*-acetyl-glucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc) residues. The sequence of the stem peptide linked to the d-lactoyl group of MurNAc, as assembled in the cytoplasm, is l-Ala¹-d-iGlu²-DAP³-d-Ala⁴-d-Ala⁵, in which DAP is a diaminopimelyl residue. Polymerization of this disaccharide-pentapeptide subunit by glycosyltransferases results in linear glycan strands that are inserted into the expanding PG network by the combined actions of space-making hydrolases and transpeptidases. The majority of the cross-links connecting d-Ala⁴ of a donor stem peptide to DAP³ of an acceptor stem peptide (4 → 3 cross-links) is made by d,d-transpeptidases belonging to the penicillin-binding protein (PBP) family that are the essential targets of β -lactam antibiotics. L,D-transpeptidases (LDTs) catalyze the formation of 3 → 3 cross-links connecting two DAP residues (see panel C). The Braun lipoprotein (Lpp) provides a link between the OM and the PG since its C-terminus is covalently bound to DAP residues (panel C), whereas its N-terminal Cys residue carries three alkyl chains, one of which is carried by the amino group of the C-terminal Cys residue and the other two by a glycerol moiety linked to the sulfhydryl of that residue by a thioether bond. These alkyl chains are inserted into the inner leaflet of the OM (PG-tethered form of Lpp) or to the outer leaflet of that membrane (free form of Lpp facing the bacterial cell surface).

(B) Determination of peptidoglycan structure. The sacculi are extracted by the hot SDS procedure, treated with pronase and trypsin (red double arrows), and digested by muramidases that cleave the MurNAc-GlcNAc bonds (black double arrows). The structure of the resulting fragments (enclosed in rounded polygons) is determined by mass spectrometry following reduction of MurNAc residues and separation by *rp*HPLC.

(C) Reactions catalyzed by L,D-transpeptidases. In *E. coli*, the LDT protein family includes six paralogues that catalyze (i) the formation of 3 → 3 cross-links (YcbB and YnhG), (ii) the covalent anchoring of Lpp to PG (YfbB, YfbC, and ErfK), and (iii) the hydrolysis of the resulting PG → Lpp bond (YafK). Formation of the 3 → 3 and PG → Lpp bonds involves a common catalytic intermediate resulting from the nucleophilic attack of the DAP³-D-Ala⁴ bond of an acyl-donor tetrapeptide stem by the active Cys residue of the LDTs, the release of D-Ala⁴, and the formation of a d-Ala⁴-Cys thioester bond. For the formation of a 3 → 3 cross-link, the side-chain amino group of DAP at the third position of an acyl acceptor stem reacts with the acyl enzyme. For the formation of a PG → Lpp bond, the side-chain of the C-terminal Lys residue of Lpp (at position 58) reacts with the acyl enzyme.

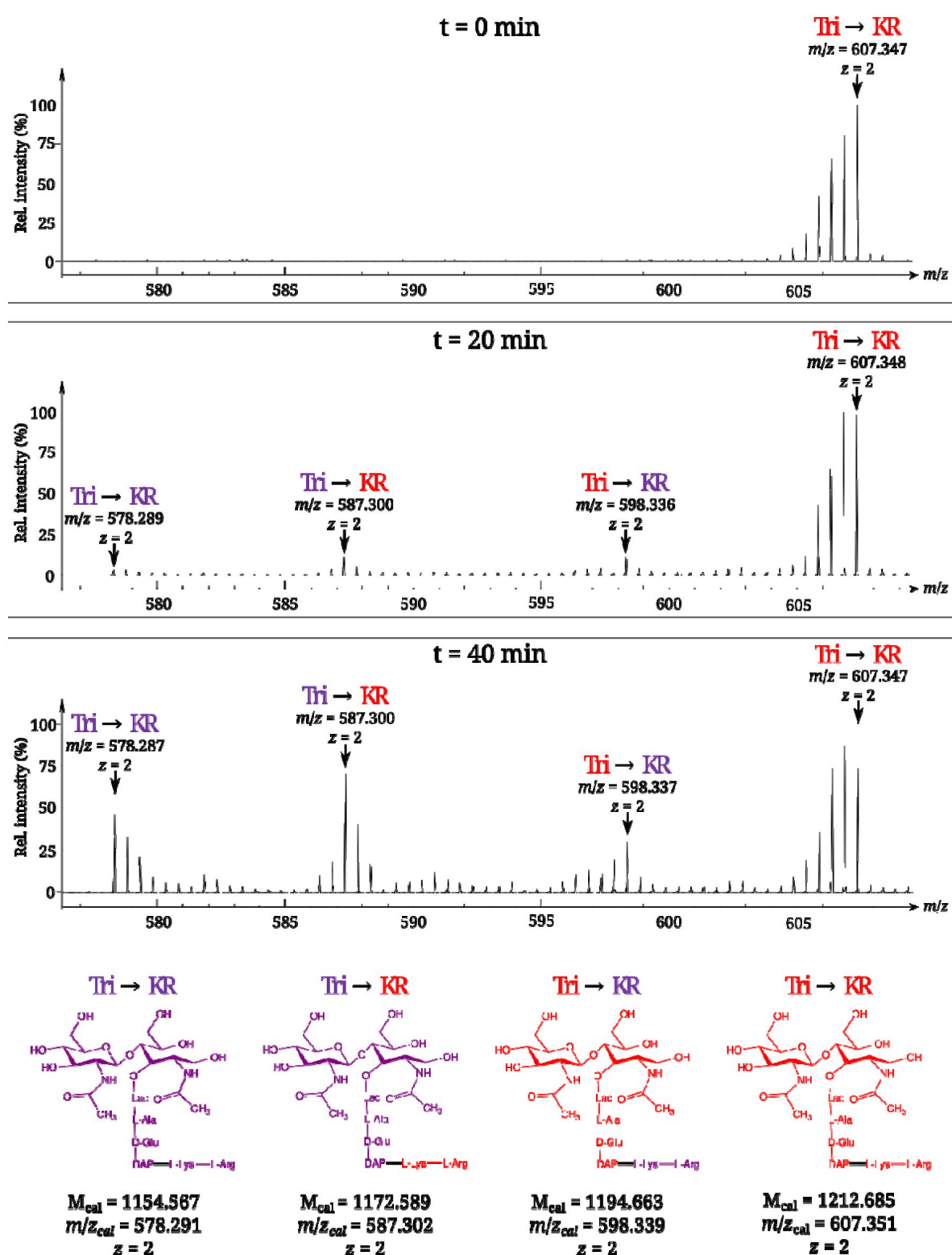


Figure 2.

Time-resolved appearance of differentially labeled Tri → KR mucopeptide isotopologues containing new (light) and old (heavy) Lpp and PG moieties.

Light and heavy residues are indicated in purple and in red, respectively. M_{cal} , calculated monoisotopic mass (see Atze et al., 2022) for the detailed description of the method used to determine M_{cal} values).

synthesized Lpp to newly synthesized stem peptide in lipid II, thus leading to the formation of Tri → KR isotopologues of the new → new type, which were only detected at late times of the kinetics, when light tetrapeptide stems became abundant in the PG (**Fig. 3A** [↗](#)). The low abundance of Tri → KR isotopologues of the new → new type at early times of the kinetics also indicates that Lpp is not preferentially incorporated at locations in which new disaccharide-peptide subunits are incorporated into the expanding PG. Thus, the tethering of Lpp to PG and the incorporation of newly synthesized subunits are spatially independent.

Tethering of Lpp to septal PG might be limited

The mode of insertion of PG subunits into the expanding PG is different in the side walls and in the septum (de Jonge et al., 1989 [↗](#)). One-at-a time insertion of newly synthesized glycan strands into the side-walls leads to a mosaic of new and old subunits gradually enriched in new subunits. In contrast, the septum mainly consists of newly synthesized PG subunits. The low abundance of new → new Tri → KR isotopologues at early time points (**Fig. 3A** [↗](#)) thus suggests that Lpp might be incorporated in very limited amounts into nascent septal PG.

YafK hydrolyzes tripeptide → Lpp bonds

After the medium switch, the relative abundance of old → old isotopologues is expected to decrease as a result of (i) the synthesis of new Lpp and PG stem peptides leading to a twofold decrease in one generation (*ca.* 60 min) and (ii) the hydrolysis of the tripeptide → Lpp bond by YafK. Indeed, the latter reaction releases heavy Lpp, which may remain unbound (free Lpp) or subsequently cross-linked to newly synthesized peptide stems. The decrease in the relative abundance of old → old isotopologues was slower in the absence of YafK (**Fig. 3B** [↗](#)), thus establishing that YafK is actively hydrolyzing tripeptide → Lpp bonds.

Hydrolysis of tripeptide → Lpp bonds by YafK contributes to the shuffling of Lpp between old and new peptidoglycan stems

At the beginning of the kinetics (0 to 25 min), the absence of YafK had little impact on the relative abundance of old → new isotopologues (**Fig. 3C** [↗](#)). This is expected since the bulk of the PG contains old tetrapeptide stems that act as donors in the transpeptidation reaction, thus leading to the tethering of newly synthesized Lpp to old PG. At later times, the proportion of old → new isotopologues levels off earlier in the BW25113 strain than in the $\Delta yafK$ mutant. This may be accounted for by the hydrolysis of old → new isotopologues by YafK, thus generating light Lpp molecules that can be cross-linked to light tetrapeptide donor stems, the abundance of which increases after the medium switch. Thus, the larger relative abundance of new → old isotopologues in the $\Delta yafK$ mutant provides a first indication that hydrolysis of tripeptide → Lpp bonds by YafK results in the shuffling of Lpp between PG stem peptides.

The relative abundance of new → old isotopologues was higher in the BW25113 strain than in the $\Delta yafK$ mutant at all incubation times (**Fig. 3D** [↗](#)). The heavy Lpp molecules used for the formation of these isotopologues are expected to originate both from the old pool of free Lpp and from the YafK-mediated hydrolysis of old → old isotopologues. Thus, the reduction in the relative abundance of new → old isotopologues upon deletion of *yafK* additionally indicates that hydrolysis of tripeptide → Lpp bonds by YafK results in the shuffling of Lpp between PG stem peptides.

At least 50% of the tripeptide → Lpp bonds are hydrolyzed in one generation

The twofold decrease in the relative abundance of old → old isotopologues over the course of one generation, as observed in the absence of YafK (**Fig. 3B** [↗](#)), can be fully accounted for by the synthesis of new PG stem peptides and Lpp molecules. Thus, there was no detectable loss of

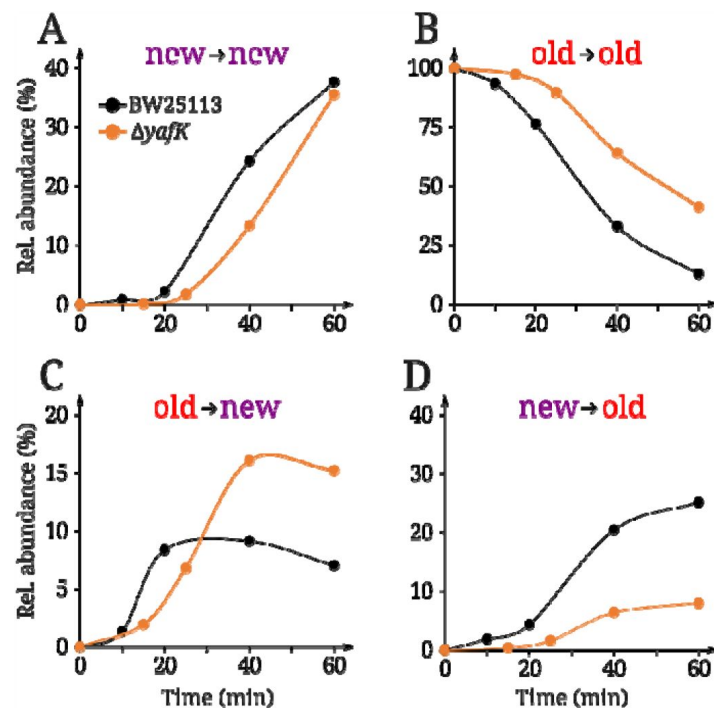


Figure 3.

Kinetics of variations in the relative abundance of Tri → KR isotopologues.

(A) Unlabeled isotopologue containing newly synthesized muropeptide and Lpp moieties (new → new). (B) Fully labeled isotopologue containing existing muropeptide and Lpp moieties (old → old). (C) Hybrid isotopologue containing old and new muropeptide and Lpp moieties, respectively (old → new). (D) Hybrid isotopologue containing new and old muropeptide and Lpp moieties, respectively (new → old). Light and heavy moieties are indicated in purple and in red, respectively. Muropeptide analysis was performed for the BW25113 strain (black) and the $\Delta yafK$ mutant (orange). Data are the average of two biological replicates. Rel. abundance, relative abundance, defined as the ratio (%) between the intensity of the indicated isotopologue and the sum of the intensities of all four isotopologues. This implies that an isotopologue that is present at the medium switch and is neither synthesized nor degraded during the following incubation of 60 min (one generation) will display a 50% reduction in its relative abundance because the total number of stem peptides doubles in one generation.

old → old isotopologues in the *ΔyafK* strain.

Furthermore, the relative abundance of old Lpp present in both the old → old and new → old isotopologues decreased twofold in 60 min both in the BW25113 strain and in the *ΔyafK* mutant (Fig. 4A and 4B). This implies that hydrolysis of tripeptide → Lpp bonds by YafK was not associated with any proteolytic degradation of the released Lpp. In contrast, the relative abundance of old tripeptide stems present in both the old → old and old → new isotopologues decreased more rapidly in the BW25113 strain than in the *ΔyafK* mutant (four versus twofold in 60 min; Fig. 4C and 4D, respectively). Thus, hydrolysis of the Tri → KR bond by YafK resulted in a twofold decrease in the relative abundance of old tripeptide stems in these isotopologues, providing an estimate of 50% for the proportion of tripeptide → Lpp bonds hydrolyzed in one generation. This is a low estimate since old Lpp released by YafK can be cross-linked to a donor containing an old tetrapeptide stem, a reaction that regenerates old → old and old → new isotopologues.

Conclusions

Here we show that Lpp is not tethered to PG precursors prior to their polymerization, as is the case for the reaction catalyzed by sortases in Gram-positive bacteria (Dramsi et al., 2008). Instead, Lpp is incorporated into the expanding lateral cell walls independently from their sites of expansion. The free form of Lpp faces the bacterial surfaces raising the possibility that pools of free and PG-tethered Lpp might not be in dynamic equilibrium (Asmar and Collet, 2018). This is not the case because new → old isotopologues are formed in the absence of YafK (Fig. 3), showing that existing (old) Lpp is translocated from the outer leaflet of the OM to the periplasm and tethered to neo-synthesized PG subunits. Since Lpp tethered to PG is expected to prevent OM budding, it has been proposed that vesicles are released from OM domains lacking PG-tethered Lpp (Schwechheimer et al., 2014) (McMillan and Kuehn, 2021). The dynamic equilibrium between free and PG-tethered Lpp may therefore be physiologically relevant to spatially control the formation of OM vesicles, and thereby, their periplasmic content.

Materials and methods

To investigate the mode of tethering of the Braun lipoprotein to PG, the *E. coli* BW25113 strain, which produces the full complement of six L,d-transpeptidases, and its derivative obtained by deletion of the *yafK* gene, were grown in minimal medium containing [¹³C]glucose and [¹⁵N]ammonium chloride to obtain fully labeled PG (all procedures are described in details in (Atze et al., 2022)). At an optical density of 0.4 at 600 nm, bacteria were centrifuged, resuspended in a minimal medium with the same chemical composition but containing the light isotopes of carbon and nitrogen ([¹²C] and [¹⁴N]) for a continued incubation of 60 min corresponding to one generation. Culture samples were collected immediately before the medium switch (time = 0) and at four times after the medium switch in order to characterize the kinetics of the replacement of heavy (existing) by light (newly synthesized) isotopes. The PG macromolecule was extracted from these culture samples by the hot SDS procedure, digested with pronase and trypsin, and treated with muramidases, which cleave the β-glycosidic bond connecting MurNAc to GlcNAc residues. This procedure led to the release of soluble disaccharide-peptide fragments (muropeptides) that were reduced with sodium borohydride (conversion of MurNAc to muramitol residues) and purified by *rp*HPLC. The isotopic compositions of the Tri → KR isotopologues was determined by mass spectrometry and tandem mass spectrometry.

Supplementary File 1. Time-resolved appearance of differentially labeled Tri → KR muropeptide isotopologues containing new (light) and old (heavy) Lpp and PG moieties. This file contains mass spectral data obtained for the kinetics analyses for the BW25113 strain and the

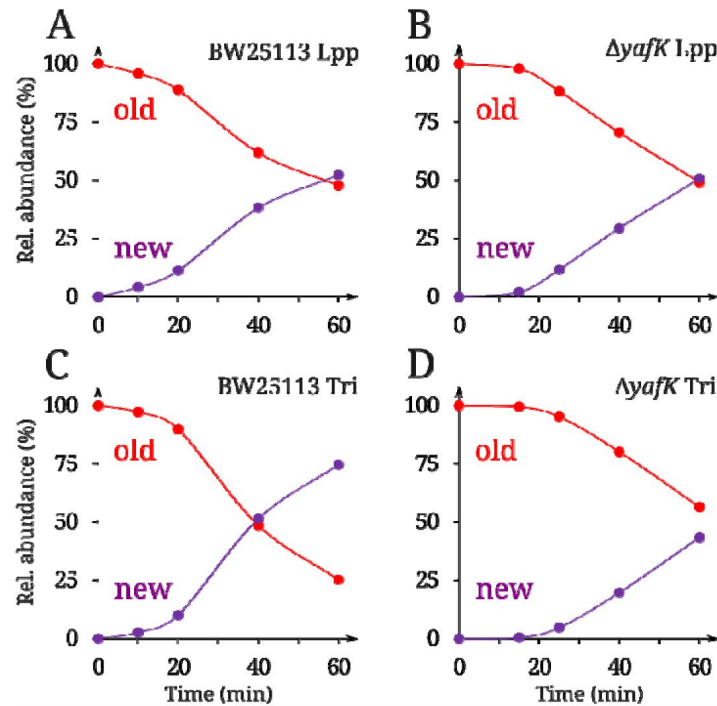


Figure 4.

Kinetics of variations in the proportions of old and new moieties in the Tri → KR muropeptide.

(A) and (B), relative abundance of old (red) and new (purple) Lpp moieties in the isotopologues from the BW25113 strain and the $\Delta yafK$ mutant, respectively. The relative abundances were calculated from data appearing in Fig. 3 by adding the relative abundances of the new → old and old → old isotopologues (red curves; old Lpp) or of the new → new and old → new isotopologues (purple curves; new Lpp). (C) and (D), relative abundance of old (red) and new (purple) tripeptide moieties in the isotopologues from the BW25113 strain and the $\Delta yafK$ mutant, respectively. The relative abundances were calculated from data appearing in Fig. 3 by adding the relative abundances of the old → old and old → new isotopologues (red curves; old tripeptide) or of the new → new and new → old isotopologues (purple curves; new tripeptide).

Deduced structure of fragments	Mass of ions generated by fragmentation of indicated isotopologues ^a							
	Tri→KR		Tri→KR		Tri→KR		Tri→KR	
	M _{cal}	M _{obs}	M _{cal}	M _{obs}	M _{cal}	M _{obs}	M _{cal}	M _{obs}
GlcNAc-MurNAc ^R -L-Ala-D-iGlu-DAP→L-Lys-L-Arg ^b	1,154.57	1,154.56	1,172.59	1,172.58	1,194.66	1,194.65	1,212.69	1,212.68
MurNAc ^R -L-Ala-D-iGlu-DAP→L-Lys-L-Arg	951.49	951.49	969.51	969.52	982.56	982.56	1,000.58	1,000.58
L-Ala-D-iGlu-DAP→L-Lys-L-Arg	674.37	674.38	692.39	692.39	693.41	693.41	711.43	711.43
D-iGlu-DAP→L-Lys-L-Arg	603.33	603.34	621.36	621.37	618.37	618.36	636.39	636.39
DAP→L-Lys-L-Arg	474.29	474.29	492.31	492.31	483.31	483.31	501.33	501.33
L-Lys-L-Arg	302.21	302.21	320.23	320.23	302.21	302.21	320.23	320.23
L-Arg	174.11	174.11	184.12	184.12	174.11	174.11	184.12	184.12

^aNewly synthesized and existing moieties are indicated in purple and red, respectively. M_{cal} and M_{obs}, calculated and observed monoisotopic masses (Da), respectively.

^bParental ion

Table S1.

MS-MS analysis of the four isotopologues of the Tri → KR muropeptide

ΔyafK mutant.

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Reviewer #1 (Public Review):

Summary:

Liang et. al., uses a previously devised full isotope labeling of peptidoglycan followed by mass spec to study the kinetics of Lpp tethering to PG and the hydrolysis of this bond by YafK.

Strengths:

-The labeling and mass spec analysis technique works very well to discern differentially labelled Tri-KR mucopeptide containing new and old Lpp and PG.

Weaknesses:

-Only one line of experimentation using mass spec based analysis of labeled PG-Lpp is used to make all conclusions in the paper. The evidence is also not enough to fully delineate the role of YafK.

-Only one mutant (YafK) is used to make the conclusion.

-The paper makes a lot of 'implications' with minimal proof to support their hypothesis. Other lines of experimentations must be added to fully delineate their claims.

-Time points to analyse Tri-KR isotopologues in Wt (0,10,20,40,60 min) and yafK mutant (0,15, 25, 40, 60 min) are not the same.

-Experiments to define physiological role of YafK are also missing.

Reviewer #2 (Public Review):

Summary:

The authors of this study have sought to better understand the timing and location of the attachment of the lpp lipoprotein to the peptidoglycan in *E. coli*, and to determine whether YafK is the hydrolase that cleaves lpp from the peptidoglycan.

Strengths:

The method is relatively straightforward. The authors are able to draw some clear conclusions from their results, that lpp molecules get cleaved from the peptidoglycan and then re-attached, and that YafK is important for that cleavage.

Weaknesses:

However, the authors make a few other conclusions from their data which are harder to understand the logic of, or to feel confident in based on the existing data. They claim that their 5-time point kinetic data indicates that new lpp is not substantially added to lipidII before it is added to the peptidoglycan, and that instead lpp is attached primarily to old peptidoglycan. I believe that this conclusion comes from the comparison of Figs 3A and 3C, where it appears that new lpp is added to old peptidoglycan a few minutes before new lpp is

added to new peptidoglycan. However, the very small difference in the timing of this result, the minimal number of time points and the complete lack of any presentation of calculated error in any of the data make this conclusion very tenuous. In addition, the authors conclude that lpp is not significantly attached to septal peptidoglycan. The logic behind this conclusion appears to be based on the same data, but the authors do not provide a quantitative model to support this idea.

This work will have a moderate impact on the field of research in which the connections between the OM and peptidoglycan are being studied in *E. coli*. Since lpp is not widely conserved in gram negatives, the impact across species is not clear. The authors do not discuss the impact of their work in depth.

Author Response

We decided to address the comments of the reviewers with additional experiments and modification of the text with the aim of submitting a new version of the report.

We would like to underline that the current study is an extension of the work published in eLife (Atze et al., 2021). For this reason, and in agreement with eLife guidelines, we did not repeat all the background information on the method used to identify PG subunit isotopologues using mass spectrometry.

Reviewer #1 (Public Review):

Summary:

Liang et. al., uses a previously devised full isotope labeling of peptidoglycan followed by mass spec to study the kinetics of Lpp tethering to PG and the hydrolysis of this bond by YafK.

Strengths:

-The labeling and mass spec analysis technique works very well to discern differentially labelled Tri-KR muropeptide containing new and old Lpp and PG.

Weaknesses:

-Only one line of experimentation using mass spec based analysis of labeled PG-Lpp is used to make all conclusions in the paper. The evidence is also not enough to fully deleanate the role of YafK.

Our approach based on heavy isotope labelling and mass spectrometry has the power to identify and kinetically characterize the specific products of the reaction leading to the tethering of Lpp to PG and the hydrolysis of the corresponding bond. We therefore advocate that our experimentation is sufficient to obtain meaningful results without combining other lines of experimentation.

-Only one mutant (YafK) is used to make the conclusion.

The aim of the study is to determine the effect of the hydrolysis of the PG → Lpp bond on the dynamics of the tethering of Lpp to PG. Since YafK is the only enzyme catalyzing this reaction, it is appropriate to compare the wild-type strain to an isogenic yafK deletion mutant. Nonetheless, we carefully consider this comment and will investigate the dynamics of the tethering of Lpp to PG in mutants deficient in the production of the L,D-transpeptidases responsible for tethering Lpp to PG.

-The paper makes a lot of 'implications' with minimal proof to support their hypothesis. Other lines of experimentations must be added to fully delineate their claims.

See our answer to the first comment.

-Time points to analyse Tri-KR isotopologues in Wt (0,10,20,40,60 min) and yafK mutant (0,15, 25, 40, 60 min) are not the same.

The purpose of the experiments is to compare the kinetics of formation and hydrolysis of the PG → Lpp bond in the WT versus $\Delta yafK$ strains. Comparison of the kinetics is therefore possible even though the kinetics are not based on the exact same time points. Nonetheless, we will reproduce the kinetics experiment (see also answers to Reviewer 2) and use the same time points in these additional experiments.

-Experiments to define physiological role of YafK are also missing

We will investigate the effect of the yafK deletion on the formation of outer membrane vesicles.

Reviewer #2 (Public Review):

Summary:

The authors of this study have sought to better understand the timing and location of the attachment of the lpp lipoprotein to the peptidoglycan in E. coli, and to determine whether YafK is the hydrolase that cleaves lpp from the peptidoglycan.

Strengths:

The method is relatively straightforward. The authors are able to draw some clear conclusions from their results, that lpp molecules get cleaved from the peptidoglycan and then re-attached, and that YafK is important for that cleavage.

Weaknesses:

However, the authors make a few other conclusions from their data which are harder to understand the logic of, or to feel confident in based on the existing data. They claim that their 5-time point kinetic data indicates that new lpp is not substantially added to lipid II before it is added to the peptidoglycan, and that instead lpp is attached primarily to old peptidoglycan. I believe that this conclusion comes from the comparison of Fig.s 3A and 3C, where it appears that new lpp is added to old peptidoglycan a few minutes before new lpp is added to new peptidoglycan. However, the very small difference in the timing of this result, the minimal number of time points and the complete lack of any presentation of calculated error in any of the data make this conclusion very tenuous. In addition, the authors conclude that lpp is not significantly attached to septal peptidoglycan. The logic behind this conclusion appears to be based on the same data, but the authors do not provide a quantitative model to support this idea.

The reviewer is correct in stating that we claim that Lpp is not substantially added to lipid II before incorporation of the disaccharide-pentapeptide subunit into the expanding PG network. This conclusion is based on the paucity of PG-Lpp covalent adducts containing light PG and Lpp moieties at the earliest time points. To substantiate more thoroughly this finding, we will reproduce the kinetic experiments with more early time points. The paucity of the new → new PG-Lpp isotopologues also implies that Lpp might not be extensively tethered to septal peptidoglycan since the latter is assembled from newly synthesized PG (see our

previous publication Atze et al. 2021 and references therein). Quantitatively, septal synthesis roughly accounts for one third of the total PG synthesis. It is therefore expected that tethering of Lpp to septal PG would represent one third of the total number of newly synthesized Lpp molecules tethered to PG. We therefore proposed that the paucity of new → new PG- Lpp isotopologues at early time points of the kinetics implies that Lpp is preferentially tethered to the side wall. This is only one of several conclusions that we reach in the present study and we were very careful in the wording of our results.

-This work will have a moderate impact on the field of research in which the connections between the OM and are being studied in E. coli. Since lpp is not widely conserved in gram negatives, the impact across species is not clear. The authors do not discuss the impact of their work in depth.

We respectfully disagree with this reviewer's comment. The work reported in this article for E. coli opens the way to the analysis and comparison of the mechanisms of the tethering of proteins to PG in various bacteria. In addition, we would like to stress that the Gram-negative bacteria that produce Lpp-related proteins and tether them to the PG include other major pathogens such as Pseudomonas aeruginosa (DOI: 10.1128/spectrum.05217-22).