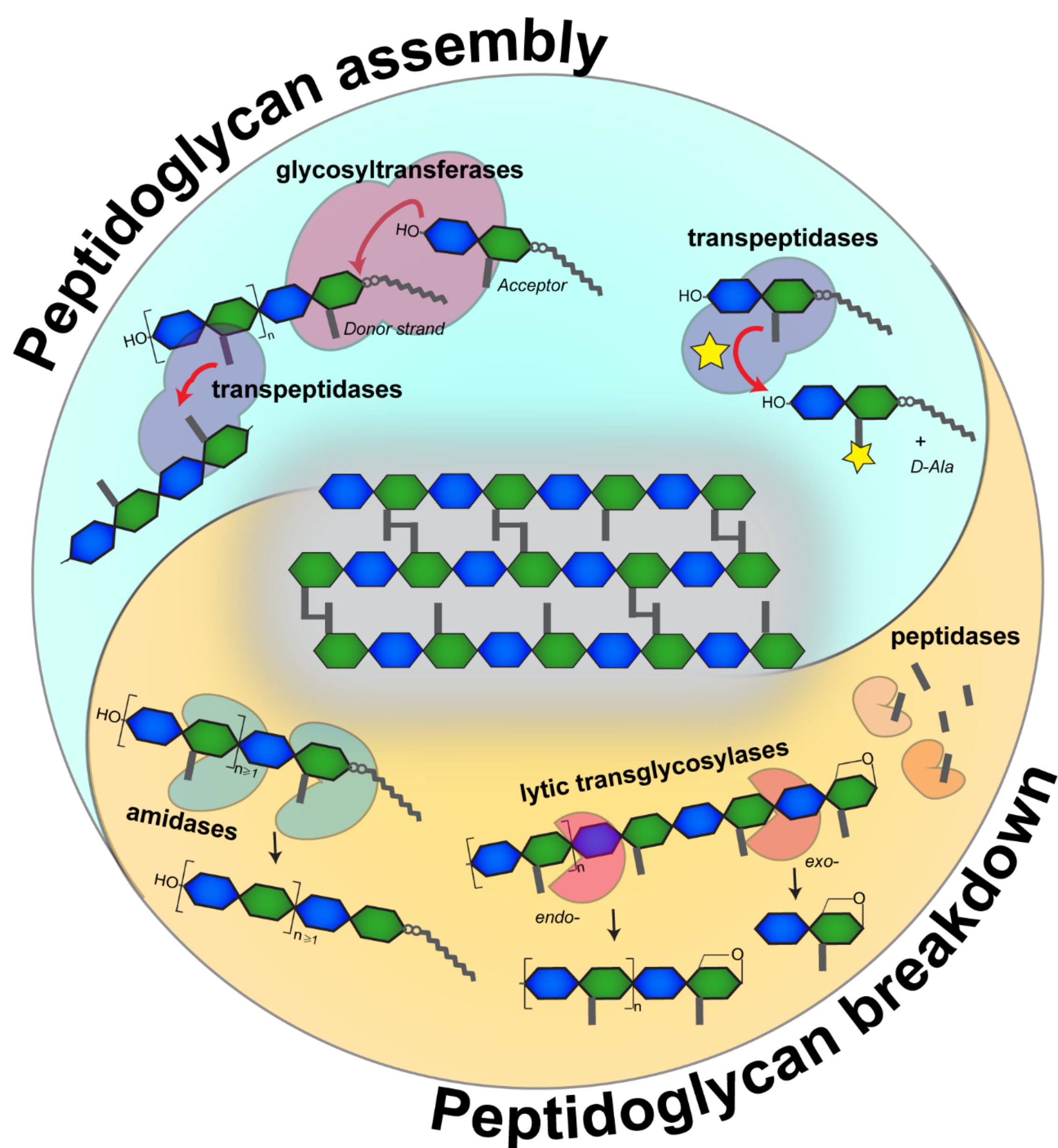
Mechanistic insights into the activities of major families of enzymes in bacterial peptidoglycan assembly and breakdown

Jeric Mun Chung Kwan[b] and Yuan Qiao*[a]



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[a] Dr, Y. Qiao
School of Chemistry, Chemical Engineering and Biotechnology (CCEB)
Nanyang Technological University (NTU), Singapore
21 Nanyang Link, Singapore 637371
E-mail: yuan.qiao@ntu.edu.sg
[b] Mr, J. M. C. Kwan
School of Chemistry, Chemical Engineering and Biotechnology (CCEB)
21 Nanyang Link, Singapore 637371
LKC School of Medicine,
11 Mandalay Road, Singapore 208232
Nanyang Technological University (NTU), Singapore

Abstract: Serving as an exoskeletal scaffold, peptidoglycan is a polymeric macromolecule that is essential and conserved across all bacteria, yet is absent in mammalian cells, which renders bacterial peptidoglycan a well-established excellent antibiotic target. In addition, soluble peptidoglycan fragments derived from bacteria are increasingly recognized as key signalling molecules in mediating diverse intra- and inter-species communications in nature, including in gut microbiota-host crosstalk. Each bacteria species encodes multiple redundant enzymes for key enzymatic activities involved in peptidoglycan assembly and breakdown. In this review, we discuss recent findings on the biochemical activities of major peptidoglycan enzymes, including peptidoglycan glycosyltransferases (PGT) and transpeptidases (TPs) in the final stage of peptidoglycan assembly, as well as peptidoglycan glycosidases, lytic transglycosylase (LTs), amidases, endopeptidases (EPs) and carboxypeptidases (CPs) in peptidoglycan turnover and metabolism. Biochemical characterisations of these enzymes provide valuable insights into their substrate specificity, regulation mechanisms and potential modes of inhibition.

1. Overview

Peptidoglycan, the major component of the bacterial cell wall, is a polymer encasing the cytoplasmic membrane in bacteria. Its role as an exoskeletal scaffold to prevent cell lysis from internal turgor pressure makes it indispensable in bacteria; yet mammalian cells do not possess peptidoglycan, rendering bacterial peptidoglycan an attractive antibiotic target. As reflected in its name, peptidoglycan contains both '*peptide*' and '*glycan*' components. Long glycan strands make up the peptidoglycan backbone, which consists of alternating units of *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) (**Figure 1**). The termini of the glycan polymer are capped by 1,6-anhydro-MurNAc (anhMurNAc) motifs in Gram-negative bacteria.[1] In addition, a stem peptide is connected to the lactoyl group on each MurNAc residue. The sequence of the stem peptide is largely conserved with some variations across species[2]: L-Ala$_{(I)}$-γ-D-isoGln$_{(II)}$/γ-D-Glu$_{(II)}$-X$_{(III)}$-D-Ala$_{(IV)}$-D-Ala$_{(V)}$, where the subscripted Roman numeral indicates the position of the particular amino acid in the stem peptide. X$_{(III)}$ represents *meso*-diaminopimelic acid (mDAP) in most Gram-negative bacteria and L-Lys with a distinct branch peptide in different Gram-positive bacteria.[2] The stem peptides on adjacent glycan strands are inter-connected forming crosslinks that rigidify the peptidoglycan polymeric mesh. While the basic glycan composition of peptidoglycan is well-conserved across all bacteria, some bacteria are known to modify the GlcNAc-MurNAc backbone through *O*-acetylation, *N*-deacetylation, *N*-glycosylation *etc*.[1] Nevertheless, the great diversity of peptidoglycan comes from variations of the third residue (and branch peptide) in the stem peptide, with the terminal D-Ala$_{(IV)}$-D-Ala$_{(V)}$ being the only completely invariant amino acids in peptidoglycan.[2]

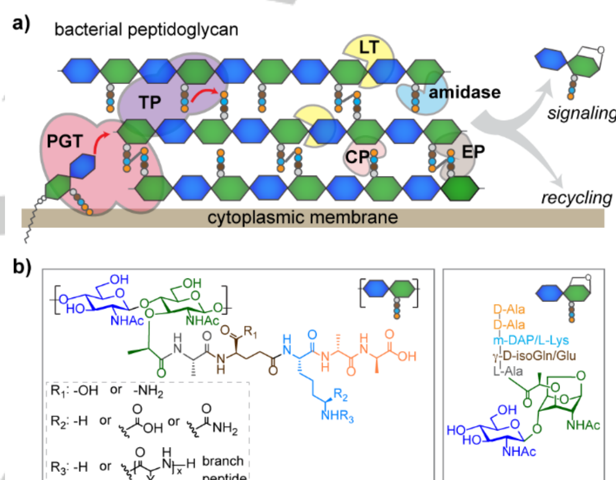

Figure 1. Schematic representation of bacterial peptidoglycan assembly and breakdown. **(a)** Peptidoglycan is a polymeric mesh on the exterior of the cytoplasmic membrane in bacteria. Gram-positive bacteria have thick layers of peptidoglycan, where Gram-negative bacteria have a thin layer of peptidoglycan in the periplasmic space. Peptidoglycan polymer is constantly assembled, re-modelled, and broken down in bacteria, during which soluble peptidoglycan fragments (*i.e.,* muropeptides) are either released into the environment or recycled back into the cytosol. The major families of peptidoglycan enzymes are shown: PGT (peptidoglycan glycosyltransferase), TP (transpeptidase), LT (lytic transglycosylase), amidase, EP (endopeptidase) and CP (carboxypeptidase). Hexagons and circles represent glycan and peptide moieties of peptidoglycan, whose chemical structures are colour-coded and shown in (**b**). **(b)** Chemical composition of the common repeating muropeptide unit of peptidoglycan polymer in bacteria (left). The structure of anhydro-muropeptide generated during peptidoglycan recycling in Gram-negative bacteria features a 1,6-anhydro-MurNAc terminus (right).

Despite its complex structure, bacterial peptidoglycan is not a static polymer. To accommodate bacterial growth and division, the peptidoglycan scaffold requires constant remodelling and turnover, during which existing peptidoglycan polymer is broken down to generate peptidoglycan fragments (*i.e.,* muropeptides) that are either recycled back into the cytosol or released into the surroundings (**Figure 1**).[3] Most Gram-negative bacteria including *Escherichia coli* and *Pseudomonas aeruginosa* have efficient peptidoglycan recycling mechanisms to facilitate the

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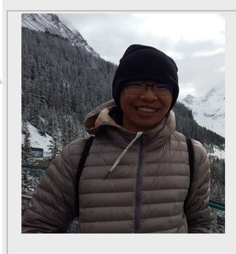
utilisation of these soluble anhydro-muropeptides as feedstocks for peptidoglycan biosynthesis.[3] On the other hand, pathogenic Gram-negative bacteria, such as *Bordetella pertussis* and *Neisseria gonorrhoeae* secrete such anhydro-muropeptides, which exhibit potent cytotoxicity against host ciliated cells.[4,5] Similarly, Gram-positive bacteria, which have much thicker peptidoglycan and lack an outer membrane, also effectively release various forms of muropeptides during normal growth.[6] Apart from the structural role of the peptidoglycan polymer, soluble peptidoglycan fragments that are derived from bacteria are increasingly recognized as key signalling molecules in diverse intra- and inter-species communications.[6,7]

Given the biological importance and clinical relevance, bacterial peptidoglycan has been an exciting topic of research over several decades, with an increasing number of key insights emerging in recent years. Remarkably, bacteria possess a redundant number of enzymes for various enzymatic activities in the peptidoglycan pathway. These include peptidoglycan glycosyltransferases (PGTs) and transpeptidases (TPs) in the final step of peptidoglycan assembly, as well as glycosidases and lytic transglycosylases (LTs), amidases, endopeptidases (EPs) and carboxypeptidases (CPs) in peptidoglycan turnover and metabolism (**Figure 1**). The functional redundancy of these major families of peptidoglycan enzymes complicates the understanding of the specific activities of individual enzymes. In many cases, deletion(s) of single or multiple genes encoding peptidoglycan enzymes of the same family may not yield significant growth defects or phenotypes,[8–12] since other members can compensate for activity, rendering it difficult to infer the function of specific gene/enzyme in the pathway. Along with genetic and structural studies of different peptidoglycan enzymes, biochemical reconstitution of enzymatic activity using defined substrates in a purified system has been valuable in elucidating specific functions of peptidoglycan enzymes. Notably, robust access to complex peptidoglycan substrates and probes, improved purification of membrane-bound proteins and complexes, and development of novel and facile assays in recent years have greatly enabled detailed characterisations of a wide range of peptidoglycan enzymes, including their enzymatic mechanisms, substrate preferences, regulations, and potential modes of inhibition.

In this review, we discuss the mechanistic insights into the activities of major families of enzymes in bacterial peptidoglycan assembly (*i.e.,* PGTs and TPs) and breakdown (*i.e.,* LTs, amidases and peptidases). Understanding of peptidoglycan enzymes is not only pertinent to the development of potential cell wall-disrupting antibiotics and the discovery of novel antibiotic targets in bacteria but also to lay important foundations and tools for unravelling peptidoglycan pathways in traditionally non-model bacteria such as the gut microbiota.



Jeric Kwan completed his B.Sc in Chemistry at the National University of Singapore where he took part in research projects in organic synthesis and catalysis. He worked as a Research Assistant at NTU-LKC before he started pursuing his PhD. Jeric joined the QIAO lab in Feb 2022 and is currently working on using computational methods to elucidate the structures of atypical peptidoglycan and characterise the peptidoglycan of gut microbiota.

Yuan Qiao is currently a Nanyang Assistant Professor in the School of Chemistry, Chemical Engineering and Biotechnology (CCEB) at Nanyang Technological University (NTU), Singapore. Her lab studies the biology of the gut microbiota-derived peptidoglycan fragments in the host, where they develop analytical chemistry platforms, organic and chemoenzymatic syntheses, genetic and biochemistry tools, as well as computational methods to elucidate the structures, production and signalling functions of natural peptidoglycan fragments in the host. Yuan obtained her PhD in Chemical Biology from Harvard University in 2016. She is a member of the early-career advisory board for *Chembiochem.*

2. Peptidoglycan glycosyltransferase (PGTs)

Peptidoglycan glycosyltransferases (PGTs) are enzymes that carry out glycan chain polymerisation of Lipid II in the final stage of bacterial peptidoglycan assembly (**Figure 1**). The specific chemical reaction catalysed by PGTs is the formation of a β-(1,4) glycosidic bond between C4-OH of GlcNAc and the C1-reducing end on MurNAc in the glycan polymer. Notably, bacterial PGTs display a considerably high degree of functional redundancy, with each bacterium encoding multiple PGTs. For instance, *E. coli* has three bifunctional Class A penicillin-binding proteins (aPBPs): PBP1a, PBP1b and PBP1c that each harbours a PGT domain (and a TP domain).[13] *Staphylococcus aureus* also possesses monofunctional PGTs (*i.e.,* MGTs), SgtA and SgtB,[14] in addition to PBP1 and PBP2 of the aPBP family. More recently, the ubiquitous Shape, Elongation, Division and Sporulation (SEDS)-family proteins, RodA and FtsW, were demonstrated as the newest family of PGTs that are widely conserved in both Gram-negative and Gram-positive bacteria.[8,15,16]

Structurally, both aPBPs and MGTs share similar catalytic glycosyltransferase domains (Pfam: PF00912) and are anchored to the cytoplasmic membrane via an *N*-terminal transmembrane helix, with the PGT domain facing outside of the membrane. On the other hand, SEDS PGTs are transmembrane proteins that do not share any homology with aPBPs and MGTs. SEDS PGTs interact with the partner bPBPs as part of the elongasome or divisome complex in bacteria,[17–20] and exhibit weak or no activity without activation from the corresponding bPBPs.[15,21–24] Below we discuss the activities of characterised PGTs from all three families.

2.1. Direction of glycan polymerisation

Since the Lipid II monomer can potentially act both as a glycosyl donor and acceptor substrate in PGT glycosylation (**Figure 2a**), the direction of peptidoglycan polymerisation was difficult to determine without appropriate substrate analogues. To conclusively address this point, Perlstein *et al.* beautifully designed and chemoenzymatically synthesized an array of galactose (Gal)-blocked radioactive Lipid II and short oligomer analogues, in which the C4-OH on GlcNAc at the non-reducing

end was blocked such that they could only act as glycosyl donors for peptidoglycan elongation at the reducing end.[25] Incubation of the radioactive Gal-blocked oligomers with non-labelled Lipid II monomers in the presence of an aPBP (*i.e., E. coli* PBP1a, PBP1b, *Aquifex aeolicus* PBP1a, or *S. aureus* PBP2) led to glycan chain extension products as observed in gel electrophoresis assays, which are indicative of successful PGT activity.[25] This seminal study provides direct biochemical evidence for the direction of glycan polymerisation in the PGT reaction, which occurs with the addition of incoming Lipid II (*i.e.,* glycosyl acceptor) to the reducing end of the growing peptidoglycan polymer (*i.e.,* glycosyl donor).[25] (**Figure 2a**).

Using these radioactive Gal-blocked Lipid II analogues, Welsh *et al.* recently established that both SEDS-family proteins RodA and FtsW also polymerise peptidoglycan in the same direction as aPBPs.[26] Although the direction of glycan polymerisation has not been directly demonstrated for bacterial MGTs, it is conceivable that they catalyse polymerization in the same direction, as their catalytic domains are highly similar to that of the aPBPs. Thus, the reducing end addition appears to be a common mechanism for all bacterial PGTs. In the case of aPBPs, whose PGT activity is closely coupled with the TP-catalysed crosslinking (see section 3), this mechanism may ensure the nascent peptidoglycan strands are efficiently processed for crosslinking and other modifications.

2.2. Rate, processivity and chain length

Thanks to synthetic radiolabelled Lipid II analogues and gel electrophoresis assays for convenient visualisation and quantification of polymeric peptidoglycan products, it is possible to perform in-depth kinetic studies for PGT catalysis (**Figure 2b**). Taking *E. coli* and *S. aureus* PBPs as examples (*i.e., E. coli* PBP1a, *S. aureus* PBP2 and SgtB), Wang *et al.* found that the initial round of PGT catalysis, during which two Lipid II monomers are coupled to form a tetra-saccharide lipid-linked product (*i.e.,* Lipid IV), is the rate-limiting step since the addition of the appropriate 'donor-only' synthetic Lipid IV analogues bypassed the slow step and significantly accelerated the polymerisation of PBP1b.[27] Consistently, Lipid IV and longer Lipid II oligomers are readily accepted by PGT's donor site, undergoing elongation in the presence of Lipid II.[28,29] However, it remains to be determined if the initial rate-limiting step is a common mechanism among all bacterial PGTs, as such detailed kinetic experiments are yet to be performed for the newly discovered SEDS PGTs.

Moreover, biochemical reconstitutions of several PGTs (*e.g. A. aeolicus* PBP1a and *E. coli* PBP1a and 1b) with radiolabelled Lipid II have established that peptidoglycan polymerisation occurs in a processive manner, in which the PGT holds onto the growing peptidoglycan strand without releasing the product after each round of coupling.[27,28,30–32] In contrast, certain PGTs such as *A. aeolicus* PBP1a and *E. coli* PBP1a, when utilizing Lipid IV as the sole substrate, proceed in a slow distributive manner, where the polymeric products are released after each round of coupling thus giving rise to initial products with shorter chains (**Figure 2b**).[28,29] Interestingly, the mutant $PBP2^{Y196D}$ and $SgtB^{Y181D}$ enzymes in *S. aureus* that confer moenomycin resistance also display a distributive mechanism and produce short peptidoglycan oligomers instead.[33]

Furthermore, aPBPs from different bacteria were found to produce peptidoglycan of different lengths *in vitro* independent of the enzyme/substrate ratio,[31] suggesting the possibility of different intrinsic termination determinants in PGTs despite a common polymerisation mechanism. Curiously, *E. coli* PBP1a appears to produce peptidoglycan strands of reduced lengths *in vitro* when coupled with ongoing transpeptidation activities from either itself or the interacting bPBP, PBP2[34] (section 3), both of which represent possible *in vivo* factors that could influence peptidoglycan chain lengths in bacteria.

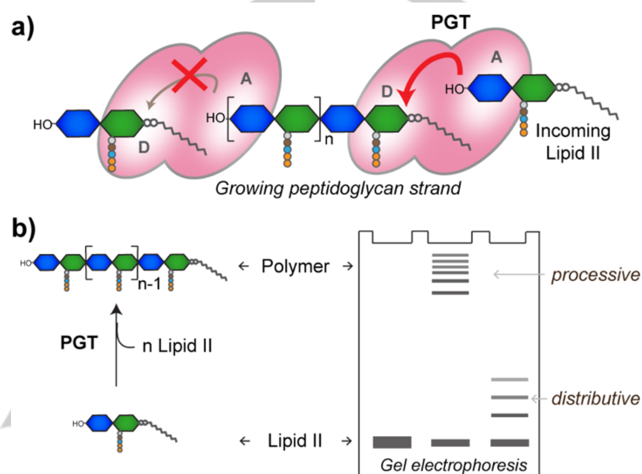


Figure 2. (a) Schematic representation shows the direction of bacterial PGT glycosylation. Incoming monomeric Lipid II acts as the glycosyl acceptor but not the glycosyl donor in the PGT reaction. The growing peptidoglycan strand is extended at its reducing terminus.[25,26] D indicates the donor site and A indicates the acceptor sites on PGT **(b)** PGT reaction can be analysed by an *in vitro* gel electrophoresis assay, which separates Lipid II substrates and polymeric products based on size and electrophoretic mobility. The monomeric Lipid II molecules migrate to the bottom of the gel, whereas the polymeric peptidoglycan products retain on top. Processive PGTs result in long peptidoglycan strands with minimal accumulation of short chains, whereas distributive polymerases produce shorter chains for the initial rounds of coupling. Radiolabelled, fluorescent and bio-orthogonal Lipid II substrates are commonly used to enable visualization.

2.3. Substrate selectivity of PGTs

Understanding the structure-activity relationships of bacterial PGTs with various Lipid II analogues is essential for the design and development of small molecule PGT inhibitors. Below we discuss characterisations of PGT activity concerning changes in three key moieties on the Lipid II substrate: pyrophosphate-linked lipid tail, glycan, and stem peptide, respectively (**Figure 3**).

2.3.1. Lipid tail

The natural undecaprenyl pyrophosphate (UndPP) lipid tail in Lipid II consists of a C55 chain with Z_8, E_2, ω stereochemistry (**Figure 3a**). However, due to the scarcity of this lipid carrier in bacteria, earlier chemical syntheses of Lipid II had utilized (Z_7, E_3, ω)-undecaprenol isolated from plants instead, which is much more abundant in nature.[35] Since these Lipid II analogues are well tolerated by PGTs in biochemical assays, many researchers have conveniently annotated the lipid tail of natural bacterial Lipid II as Z_7, E_3, ω, which is a misnomer.[35]

Natural bacterial Lipid II molecules are complex and hydrophobic, which are considerably difficult to work with. In efforts to search for shorter lipid surrogates, Ye *et al.* evaluated a panel of Lipid II analogues with varying lipid tails for PGT polymerisation *in vitro*.[36] Interestingly, the Z_4, E_2, ω–heptaprenyl (C35) lipid tail analogue undergoes polymerisation with crude *E. coli* membranes more readily compared to the natural C55 substrate.[36] This work was later expanded upon by Perlstein *et al.* with synthetic blocked oligomers to specifically tease out the lipid requirements of the glycosyl acceptor and donor sites of the PGT domain in *A. aeolicus* PBP1a, respectively.[37] In general, its acceptor site demonstrates broad tolerance to substrate lipid tail – accepting analogues as short as C10, containing unnatural double bond geometry and even fully saturated (**Figure 3b**).[36,37] In contrast, the donor site is more stringent: it requires a C35 lipid with the natural double bond geometry (Z_4, E_2, ω) for processive polymerisation, while it also recognises shorter C20 lipid for distributive polymerisation.[36,37] This indicates that the length of the lipid tail on the glycosyl donor plays a major role in determining PGT processivity. Interestingly, the SEDS polymerase, *S. aureus* FtsW was recently shown to exhibit similar lipid selectivity of glycosyl donors and acceptors as the previously characterised *E. coli* and *A. aeolicus* aPBPs.[26]

Moreover, synthetic Lipid II analogues containing a fluorescent tag in the lipid tail have been developed. For instance, Liu *et al.* studied *Clostridium difficile* PBP1b and *E. coli* PBP1b using a dansylated Lipid II analogue with a C20 lipid tail (while it is important to note that the presence of the terminal dansyl group makes this lipid effectively C30), where the release of the fluorescent lipid was taken as a readout of PGT activity.[38] However, caution should be taken as the increase in fluorescent signals could be due to incidental hydrolysis instead of polymerisation. To circumvent this potential issue, Huang *et al.* extended the idea by synthesising the dual labelled Lipid II probes, which bear a coumarin fluorophore in the peptide chain and a dabsyl-quencher in the lipid tail.[39] Upon PGT-catalysed polymerisation, the quencher-bearing lipid is released and the fluorescent signals (on individual muropeptides) are detected upon muramidase digestion of the polymeric peptidoglycan product.[39] This fluorometric study also confirmed that a minimum of four *cis* isoprene units on the lipid tail is critical for *E. coli* PBP1b PGT activity.[39] However, *C. difficile* PBP1b, unlike other known PGTs, readily uses a shorter lipid analogue with a C15 tail *in vitro*.[39] The shorter transmembrane region of *C. difficile* PBP1b may allow it to accept substrates with shorter lipid tails.[38,39] Thus, PBPs of different bacteria species may exhibit different selectivity for the substrate lipid tail.

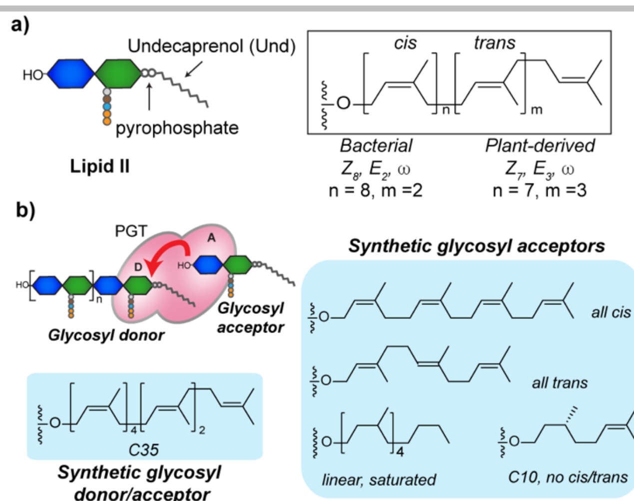


Figure 3. Substrate specificity of bacterial PGTs with respect to the lipid tail in Lipid II. **(a)** Schematic representation of bacterial Lipid II. Lipid II consists of a muropeptide linked to the pyrophosphate undecaprenol lipid. The structure of plant-derived undecaprenol differs from bacterial undecaprenol.[35] **(b)** The canonical C55 chain in Lipid II can be substituted with a shorter synthetic lipid tail. For most characterised bacterial PGTs, the glycosyl donor site has a more stringent requirement compared to the acceptor site, and only accepts lipids of at least C30 in length with natural double bond geometry for processive polymerisation.[26,36,37] The left box shows a synthetic C35 lipid that can be utilized both as a glycosyl donor and acceptor; the right box shows synthetic lipids that only act as glycosyl acceptors.

2.3.2. Glycan

The GlcNAc-MurNAc disaccharide moiety is an essential component of Lipid II that is recognised by PGT for polymerisation since the Lipid I substrate that lacks the GlcNAc moiety is completely excluded by PGTs.[25] PGTs' substrate selectivity to modifications on GlcNAc has been explored in detail (**Figure 4**).[40] For instance, the absence of the C2 *N*-acetyl group on GlcNAc was found to greatly reduce PGT activity,[40] suggesting a possible interaction between this acetyl group with PGT for substrate recognition. In addition, the C4-epimer of Lipid II, GalNAc-MurNAc analogue, which features an improper orientation of C4-OH for glycosylation, inhibits PGT reaction with a 14-fold increase in the binding affinity to *C. difficile* PBP1b.[40] Specifically, the K_D of the epimer was 33 μM in contrast with 445 μM of the original Lipid II analogue.[40] Interestingly, the binding affinity of the GalNAc-MurNAc epimeric analogue to PGT was greatly attenuated in the absence of the lipid moiety (with a K_D of 778 μM) and non-existent without both lipid and stem peptide.[40] Such observation is consistent with previous structural studies that the pyrophosphate-lipid motif on Lipid II participates in PGT binding.[36,37,41] Furthermore, Lipid II analogues with an azido moiety on C6 of GlcNAc were well-tolerated by PGTs *in vitro*.[40] Recently, C2 *N*-acetyl-modified MurNAc and GlcNAc probes have been used for metabolic labelling of peptidoglycan in live bacteria,[42,43] which implies that bacterial PGTs can tolerate these particular modifications for glycan polymerisation.

2.3.3. Stem peptide

The stem pentapeptide connected to the D-lactoyl group on MurNAc is an invariant component of Lipid II (although the exact amino acid composition does vary across bacterial species).[2]

Lipid II analogues that lack the stem peptide are not suitable substrates for PGT polymerisation, indicating the essentiality of the stem peptide for reaction.[25,40] Interestingly, analogues bearing MurNAc-L-Ala$_{(I)}$ were found to represent the minimally active substrate for PGT binding and activity *in vitro* (**Figure 4**).[44] In particular, the methyl groups on both the D-lactoyl group and L-Ala$_{(I)}$ likely participate in critical hydrophobic interactions with PGT, since the removal of either methyl substituent significantly weakens PGT binding with a considerably high K_D of at least 420 μM.[44] Consistently, DeMeester *et al.* established that MurNAc probes with an azido group attached to the D-lactoyl moiety were well tolerated by PGTs for *in situ* peptidoglycan labelling in bacteria.[42]

Bacterial PGTs are promiscuous to the identity of the third amino acid in Lipid II, (**Figure 4**) since N^{ε}-decorated mDAP-Lipid II,[45] Lys-Lipid II,[46–48] and N^{ε}-decorated Lys-Lipid II[32,38,39,44,45,48] are all suitable substrates for PGTs. Taking advantage of the substrate tolerance, Hernández-Rocamora *et al.* designed a novel continuous Föster resonance energy transfer (FRET) assay to monitor peptidoglycan synthesis by PBP1b homologues from various bacteria including *E. coli*, *P. aeruginosa* and *Acinetobacter baumannii in vitro*.[45] Two versions of Lipid II probes, which feature either Atto647n or Atto550 fluorophore onto L-Lys$_{(III)}$ on Lipid II, are incorporated into the peptidoglycan product in the PBP1b reaction.[45] FRET signals arise due to the proximity of both fluorophores in the peptidoglycan products. The PGT activity of PBP1b was investigated with the addition of beta-lactam to prevent crosslinking, allowing analysis of probes in the same peptidoglycan strands only (*i.e.,* intra-chain FRET signals).[45] On the other hand, without inhibition of neither domain in PBP1b, FRET occurs between probes in the same strand as well as from different strands that are crosslinked (*i.e.,* intra-chain and inter-chain) as a result of both PGT and TP activities.[45] Interestingly, it was determined that inter-chain FRET contributes stronger signals.[45] This assay was performed in solution, liposomes and supported lipid bilayers, providing information on PGT activity in the natural lipid environment.[45] The robustness of this assay makes it an attractive platform for the high-throughput screening of potential PGT inhibitors.

Furthermore, the absence of the D-Ala$_{(IV)}$-D-Ala$_{(V)}$ terminus of the stem peptide in Lipid II does not affect PGT activity (**Figure 4**).[44] Based on the crystal structures of bacterial PGTs in complex with substrate analogues, the stem peptide of Lipid II is found to orient away and has minimal interactions with the PGT active site.[49,50] Hence, it is not surprising that the distal portion of the stem peptide is non-essential for the PGT reaction. Consistently, native Lipid II molecules in vancomycin-resistant strains of *Enterococcus* and *Staphylococcus* showcase modified depsi-peptide D-Ala$_{(IV)}$-D-Lac$_{(V)}$ termini, which evade vancomycin binding and yet are still substrates of both *Enterococcus* and *Staphylococcus* PGTs.[51–54] Lipid II analogues with a D-Ala$_{(IV)}$-L-Ala$_{(V)}$ terminus are likewise polymerised by *E. coli* PBP1b *in vitro*.[48]

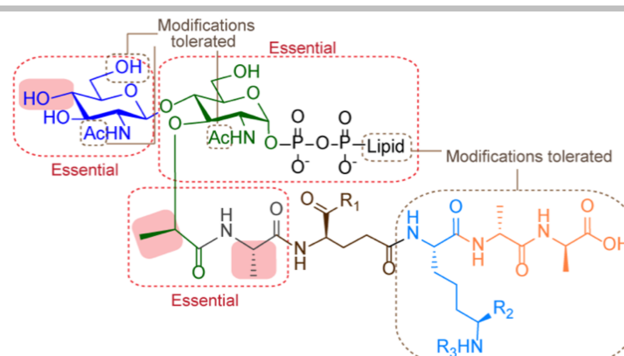


Figure 4. Summary of bacterial PGT's substrate specificity to various modifications on glycan and peptide of Lipid II. *E. coli* PBP1b and *C. difficile* PBP1b were used as model PGTs in most cited studies. Facile Lipid II probes can be created by modifying Lipid II on one or more of the following sites: *N*-acetyl groups on either sugar,[42,43] C6 on GlcNAc,[40] the terminus of the lipid tail,[38,39] and the third-fifth amino acids of the stem peptide.[32,38,39,44,45,48]

2.4. Moenomycin A, a natural product inhibitor of PGTs

Moenomycin, which is a natural product phosphoglycolipid, represents the only known class of PGT (*i.e.,* aPBPs and MGTs) inhibitors with excellent *in vitro* activities.[55] Moenomycin A (MoeA) is the founding member of the family. It contains a 3-phosphoglyceric acid (3-PG) core, with an isoprenoid chain attached to the C2-OH of 3-PG and a complex penta-saccharide unit at the phosphate group of 3-PG.[55] The structural resemblance of MoeA with the growing peptidoglycan strand substrate (Lipid IV) has led to the proposal that MoeA is an active site inhibitor. Correspondingly, several crystal and CryoEM structures of MoeA-bound PGTs (*i.e.,* aPBPs and MGTs) revealed that MoeA resides in the glycosyl donor site and its carbohydrate and 3-PG moieties are extensively hydrogen bonded to a number of residues in the active site, locking the PGT into an inactive conformation.[49,50,56–60] Given the strict substrate lipid requirement of the PGTs' donor sites, it remains unclear how the C25 lipid of MoeA with unnatural double bond geometry interacts with PGTs, as the molecular details are not resolved in current structural studies. The natural geometry of the lipid might only be essential for PGT polymerisation but not binding to PGT, as in the case of MoeA. On the other hand, the presence of the lipid tail and hence the lipophilic nature of MoeA renders it undesirable pharmacokinetic properties for clinical applications.[55] Thus, many efforts have been devoted to the discovery of alternative scaffolds for PGT inhibitors.[61,62] For instance, based on the minimal pharmacophore of MoeA, Gampe *et al.* developed a fluorescent probe using a truncated MoeA analogue for high-throughput screening of potential PGT inhibitors.[61] Hopefully, new classes of PGT inhibitors will emerge from both rational designs and screening assays for further development. Intriguingly, SEDS PGTs are insensitive to MoeA, which led to the initial discovery of this family of PGTs.[15,21,23] Structural details of the SEDS PGTs' active sites will be needed to address the differences.

3. Transpeptidases (TPs)

In the last step of bacterial peptidoglycan assembly, the stem peptides on adjacent peptidoglycan strands are crosslinked by bacterial transpeptidases (TPs), strengthening the stress-

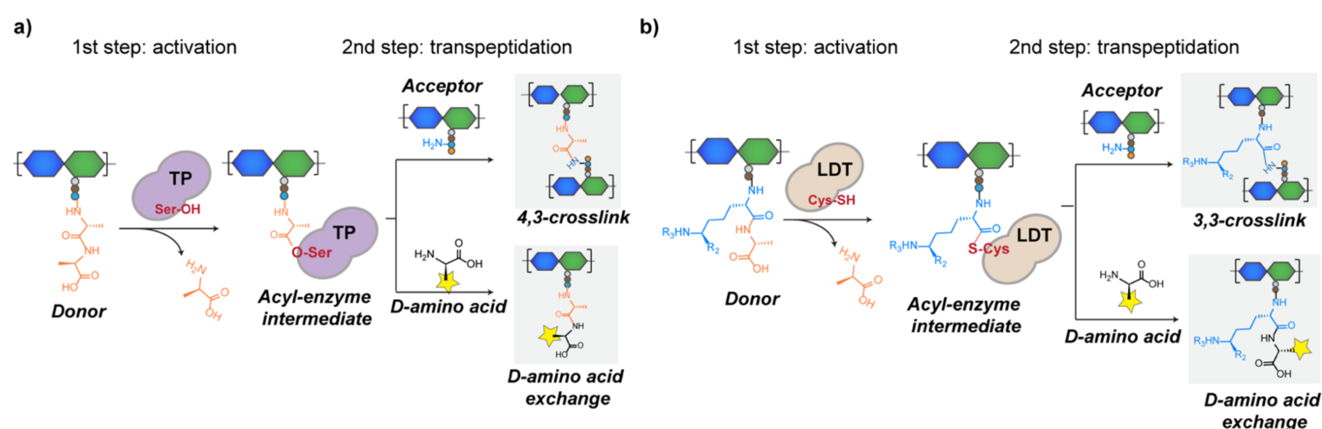


Figure 5. Both TP **(a)** and LDT **(b)** follow a similar two-step reaction mechanism. In the first activation step, the active site Ser in TP attacks the D-Ala-D-Ala terminus on a donor pentapeptide substrate, whereas the active site Cys residue in LDT attacks the L,D-configuration bond in a tetrapeptide stem, forming covalent acyl-enzyme intermediates. In the second transpeptidation step for both TP and LDT, the intermediate is attacked by an acceptor peptidoglycan strand to form either 4-3 crosslink and 3-3 crosslink, respectively. Both TP and LDT catalyse D-amino acid exchange reaction, in which an exogenous D-amino acid is incorporated into the stem peptide terminus.

bearing polymeric mesh (**Figure 1**). The world's most widely used class of antibiotics, the beta-lactams, inhibit bacterial TP activity to impair peptidoglycan assembly and function. Bacterial TPs, which can be either part of the bifunctional aPBP (*i.e.,* both PGT and TP domains) or monofunctional bPBPs and low-molecular-weight PBPs (*i.e.,* only TP domain), are D,D-transpeptidases that form 4-3 crosslinks. Most bacteria possess predominantly 4-3 crosslinked peptidoglycan formed by TPs. However, certain bacteria such as mycobacteria[63,64] and *C. difficile*[65] showcase extensive 3-3 crosslinking, which are products of L,D-transpeptidases (LDTs) belonging to the peptidoglycan remodelling enzymes. The importance of LDTs was first realised by Gutmann and co-workers who discovered that beta-lactam-resistant *E. faecium* maintained the integrity of its peptidoglycan by gradually replacing 4-3 crosslinks with 3-3 crosslinks when challenged with beta-lactams.[66–68] Later studies determined that *E. coli* remodels its peptidoglycan crosslinking to increase 3-3 crosslinks as a stress response.[69,70] Herein, we discuss biochemical studies of both TPs and LDTs involved in the formation of peptidoglycan crosslinks.

Bacterial TPs catalyse transpeptidation in a two-step manner (**Figure 5, top**). In the first activation step, the catalytic Ser residue of TP attacks the D-Ala-D-Ala terminus of the stem peptide on a donor peptidoglycan strand to form a covalent intermediate between Ser and the penultimate D-Ala, releasing the terminal D-Ala. In the second step, the acceptor strand, either the *N*-terminus of the L-$Lys_{(III)}$-connected branch peptide or the NH_2 on $mDAP_{(III)}$ residue, acts as the acceptor nucleophile to attack the acyl-enzyme intermediate and forms a new peptide bond. 4-3 crosslinking indicates the bond formed is between the 4th residue (in the donor strand) and 3rd residue (in the acceptor strand) of two stem peptides. Penicillin and other beta-lactam antibiotics, whose beta-lactam core structurally mimics the D-$Ala_{(IV)}$-D-$Ala_{(V)}$ moiety of the acyl donor, inactivate TPs by covalently acylating the catalytic Ser residue.[71] Because of the fact that penicillin binds to these enzymes, TPs were given the name, penicillin-binding proteins (PBPs). On the other hand, LDTs are structurally distinct from TPs but catalyse a similar transpeptidation reaction with a catalytic Cys residue in place of Ser (**Figure 5, bottom**). LDTs recognize the tetrapeptide terminus of the donor strand on peptidoglycan and activate the formation of 3-3 crosslinks instead.[67] Notably, LDTs are not susceptible to beta-lactam antibiotics (except carbapenems and cephalosporins).[72,73] Correspondingly, elevated levels of 3-3 crosslinks in peptidoglycan have been found in several beta-lactam-resistant bacteria.[66–69] For more details, readers can refer to a separate review focused on LDTs by Aliashkevich and Cava.[74]

3.1. Coupling of PGT and TP activities in PBPs

In aPBPs, where the TP domain is part of a bifunctional enzyme, TP activity and PGT activity are highly coupled and coordinated. While the PGT activity is independent of TP activity and can proceed even when the TP domain is inhibited or inactivated, the TP activity of an aPBP requires ongoing glycan polymerisation by the PGT domain.[48,75–78] For instance, analogues such as Lipid I (*i.e.,* without the GlcNAc moiety) and methylene-Lipid II (*i.e.,* a methylene group replaces the oxygen atom between the muramyl C1 and phosphorus atom of the lipid tail) that are incompatible for PGT reaction, are indeed not suitable for transpeptidation by aPBPs such as *E. coli* PBP1b.[48] Consistently, the addition of either moenomycin, a potent PGT inhibitor, or lysozyme that cleaves peptidoglycan backbone can effectively inhibit TP activity as well, supporting the requirement of ongoing PGT polymerisation for TP activity in aPBPs.[48] On the other hand, bPBPs' TP activity is not dependent on their SEDS partners.[21,34] Both *E. coli* PBP2 and *Streptococcus thermophilus* PBP2x could crosslink peptidoglycan *in vitro* when incubated with non-cognate PGTs instead of their SEDS partner (*i.e.,* RodA and FtsW).[21,34] These observations imply that bPBPs could potentially perform multi-functional crosslinking in bacteria, such as during peptidoglycan repair, maintenance and synthesis of higher-order peptidoglycan multimers that are not coupled with the partner SEDS PGTs.

3.2. Substrate selectivity of TPs and LDTs

Unlike PGTs that are tolerant of stem peptide modifications on Lipid II, TPs exhibit stringent requirements for their natural acceptor substrates for crosslinking. Given the diversity of the third amino acid in peptidoglycan stem peptide across different

bacteria, the inaccessibility to native Lipid II molecules has greatly impeded biochemical reconstitutions of bacterial TPs. The commonly used synthetic $Lys_{(III)}$-Lipid II for PGT assays is an inappropriate substrate for TP crosslinking (although it is suitable for the first TP activation step[46–48]). As a result, the full reconstitution of bacterial TPs has significantly lagged the characterisation of PGTs in the past decades. In recent years, the scarce Lipid II substrates have been made available through chemical synthesis[46,79,80] or extraction methods.[81] Analysis with state-of-the-art LC-MS has replaced traditional HPLC-based assays to enable facile detection of crosslinked peptidoglycan products. Alternatively, simpler peptide substrates are also useful for the evaluation of TP activity *in vitro*.[82–84] Key insights into the substrate structural requirements of bacterial TPs and LDTs are summarised below.

3.2.1. Canonical Lipid II is required for TP crosslinking

In 2013, Lebar *et al*. reported the first chemical synthesis of $mDAP_{(III)}$-Lipid II, the canonical form (except with the shorter C35 lipid) found in Gram-negative bacteria such as *E. coli*, and developed a rapid LC-MS assay to analyse PGT and TP activities of *E. coli* aPBPs, PBP1a and PBP1b *in vitro*.[46] This work established synthetic and analytical tools for the characterisation of aPBP TP activity *in vitro*. In addition, a similar strategy with synthetic amidated-$mDAP_{(III)}$-Lipid II was applied to reconstitute the TP activity of *Bacillus subtilis* PBP1, which revealed remarkable differences between bacterial TPs towards the carboxamide acceptor substrate.[80] While such synthetic $mDAP_{(III)}$-Lipid II substrates are valuable, the diverse branch peptides in Gram-positive bacterial Lipid II, which are required for studying the activity of cognate TPs, pose a significant synthetic challenge and call for more robust preparation. In 2017, Qiao *et al.* reported a general strategy to accumulate and isolate practical quantities of native Lipid II from both Gram-positive and Gram-negative bacteria.[81] The facile access to native Lipid II has opened up opportunities for the biochemical reconstitutions of TPs from diverse bacteria.

In the biosynthesis of native Gly_5-Lipid II in *S. aureus*, the FemXAB proteins are responsible for the formation of the pentaglycine (Gly_5) branch peptide. Specifically, FemX adds the first, FemA adds the second and third, and FemB adds the last two Gly residues in Lipid II. Interestingly, deletion of *femA* and/or *femB* gene(s) was found to re-sensitise methicillin-resistant *S. aureus* (MRSA) to beta-lactam antibiotics.[85] It was proposed that the increased susceptibility in these mutants could be due to the inability of MRSA PBPs to utilize modified Lipid II with shortened branch peptides for efficient crosslinking. To explore the crosslinking preference of individual MRSA PBPs, Srisuknimit *et al.* reconstituted the TP activities of major TPs, PBP2, PBP2a and PBP4 in MRSA using Gly_1-, Gly_3-, or native Gly_5-Lipid II as substrates, which were isolated from Δ*femA*, Δ*femB, or* wildtype *S. aureus*, respectively (**Figure 6a**).[86] Interestingly, PBP2, the sole aPBP in *S. aureus*, works well with all three Lipid II variants, whereas the resistant PBP2a and PBP4 are unable to crosslink Gly_1-Lipid II, suggesting FemA might be a potential target for re-sensitisation of MRSA to beta-lactam antibiotics.[86]

Other Gram-positive bacteria such as *E. faecalis* and *E. faecium* possess L-Ala-L-Ala or D-iAsx (*i.e.,* D-Asp or D-isoAsn) branch peptide in their native Lipid II, respectively (**Figure 6b**). In searching to elucidate substrate tolerance of *Enterococcus* PBPs with respect to the branch peptide, Arbeloa *et al.* constructed *Enterococcus* mutants that display mosaic branch peptides in peptidoglycan via heterologous expression of non-native genes for branch peptide installation.[87] Analysis of the mosaic peptidoglycan composition in *E. faecalis* mutant revealed non-native branch peptides (e.g. L-Ala-Gly-Gly, Gly_5) at both donor and acceptor strands in crosslinks, implying a broad substrate tolerance of *E. faecalis* PBPs *in vivo*.[83,87,88] On the contrary, *E. faecium* PBPs appear more specific towards the native D-iAsx branch for crosslinking (**Figure 6b**).[83,87,88] However, determining the substrate specificity of individual PBPs in *Enterococcus* would require biochemical reconstitution with varying Lipid II substrates, as in the case of *S. aureus* PBPs.[86] On the other hand, the characterisation of *Enterococcus* LDTs with tripeptide substrates has established that they stringently require the canonical acceptor branch for 3-3 crosslinking.[67,84,88]

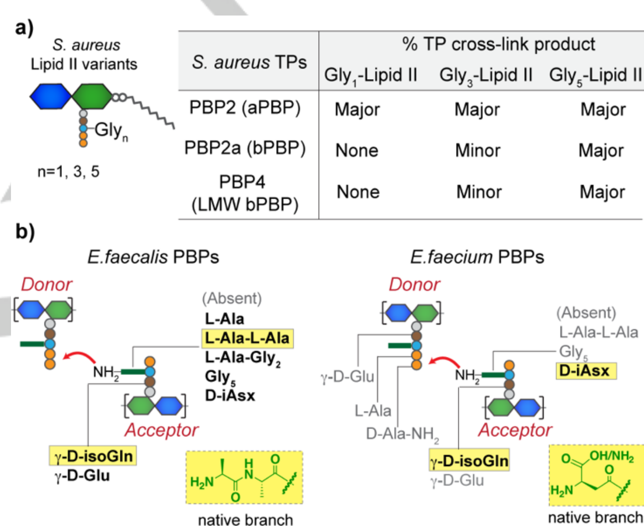


Figure 6. Bacterial TPs display substrate specificity for crosslinking. **(a)** Biochemical reconstitution illustrates that *S. aureus* TPs, PBP2, PBP2a and PBP4 show distinct crosslinking preference to the Lipid II variants bearing one, three or five glycine(s) in the branch peptide. 'Major' and 'Minor' indicates ~45-100% and ~10-20% crosslink products as observed experimentally.[86] **(b)** Analysis of mosaic peptidoglycan crosslinks in *Enterococcus* reveals different substrate specificities of *E. faecalis* PBPs and *E. faecium* PBPs.[82,83,87,88] Schematic representation of peptidoglycan substrates with identities of the coloured hexagons and circles outlined in Figure 1. Substrate specificity with regard to the branch peptide and amidation state of γ-D-isoGlx (γ-D-isoGln or γ-D-isoGlu) were investigated. The native amino acids were boxed in yellow, the bolded amino acids on donor and acceptor strands can be utilized by PBPs, and the unsuitable amino acids are shown in grey. 'Absent' indicates the absence of a branch peptide. D-iAsx means D-isoAsn or D-isoAsp. The chemical structure of the native branch peptide in each *Enterococcus* species is shown as well.

3.2.2. TPs and LDTs catalyse D-amino acid exchange

In the absence of the appropriate incoming acceptor nucleophile for TP crosslinking, both water molecules and D-amino acids can be utilized by TPs and LDTs (**Figure 5**). When a water molecule attacks the acyl-enzyme intermediate, the hydrolysis product is formed. In the event of a D-amino acid acting as the nucleophile, the reaction is essentially the reverse of the initial activation step, resulting in an exchange of D-amino acids. Using *E. coli* PBP1a as an example, Lupoli *et al.* first demonstrated TP-mediated incorporation of radiolabelled D-Ala

and other non-canonical D-amino acids (NCDAAs) into peptidoglycan during PGT polymerisation *in vitro*.[89] Around the same time, Cava *et al.* also reported that the *Vibrio cholerae* LDT, LdtA, could incorporate NCDAAs into soluble muropeptide substrates *in vitro*.[90] Concurrent with these initial biochemical studies on D-amino acid exchange by TPs and LDTs, VanNieuwenhze and co-workers demonstrated the first *in situ* application of fluorescent D-amino acid (FDAAs) for peptidoglycan labelling across different bacteria.[91] The versatility and convenience of FDAA labelling provide a facile method for bacterial imaging, whose popularity and applications have bloomed over the past decade. The development of FDAAs has been extensively reviewed.[92–94] Notably, FDAAs are incorporated into bacterial peptidoglycan *in situ* through the actions of TPs and LDTs,[95] highlighting the substrate promiscuity of these enzymes for D-amino acid exchange.

Furthermore, certain bacterial low-molecular weight (LMW) bPBPs exhibit unusual characteristics for D-amino acid exchange. For instance, *S. aureus* PBP4, an LMW PBP that has a single TP domain, could exchange NCDAA into the pentapeptide stem terminus in Lipid II, representing the first characterised PBP that utilises Lipid II monomer as a donor strand.[96] The distinct activity of *S. aureus* PBP4 is different from the previously characterised *E. coli* aPBPs, whose TP activity requires ongoing PGT activity. This has further inspired the identification of a class of functionally unique PBPs in other bacteria too, including *E. faecalis* PBPX and *Streptococcus gordonii* PBPX, which could modify Lipid II as well.[97] The unique chemo-enzymatic activities of these LMW bPBPs render them useful tools for the preparation of functional Lipid II and muropeptide probes to study different biological processes.[98–100]

3.2.4. Natural stereochemistry on substrates is required for TP and LDT activity

Both bacterial TPs and LDTs do not utilize substrates with altered stereochemistry. Pires and co-workers have developed a series of fluorescent donor and acceptor peptide substrates with unnatural stereochemistry to address their suitability for *in situ* peptidoglycan labelling, which provides a proxy readout for the substrate preferences of bacterial TPs and LDTs.[82,83] For instance, tetra- or pentapeptide donor probes that have L-Ala at either the fourth or fifth position are inappropriate for labelling *E. faecium*.[82] Similarly, the acceptor peptide probes with a D-Ala-D-Ala branch (instead of the natural L-Ala-L-Ala) could not label *E. faecalis* peptidoglycan.[83] These observations are consistent with the fact that natural stereoisomers of substrates are needed for peptidoglycan crosslinking.[48,82,83]

Apart from *in situ* labelling experiments, the substrate preferences of bacterial TPs to stereoisomers can be directly demonstrated in biochemical reconstitutions too. For instance, Catherwood *et al.* recently used the L,L-DAP$_{(III)}$ donor strand (instead of the natural L,D-DAP$_{(III)}$) to probe the activity of *E. coli* PBP1b *in vitro*.[48] Interestingly, this donor strand was activated by PBP1b to form the covalent acyl-enzyme intermediate, which was subsequently hydrolysed into a tetrapeptide, thus preventing any 4-3 crosslinking.[48] In the same vein, the TP-catalysed D-amino acid exchange reaction was stereoselective as well, since L-amino acids were unsuitable substrates.[67,88,89] Taken together, bacterial TPs and LDTs recognize natural stereochemistry in substrates for transpeptidation, which should be accounted for when designing potential inhibitors. In particular, correct stereochemistry of the residues closest to the crosslink is critical for activity (acyl donor: X$_{(III)}$-D-Ala$_{(IV)}$-D-Ala$_{(V)}$; acyl acceptor: mDAP/branch peptide).

3.2.5. Amidation of substrates affect TP and LDT activity

Although transpeptidation reaction pertains to the 4th and 5th amino acids in the donor stem peptide, bacterial TPs exhibit exquisite selectivity for the canonical amidation status in both donor and acceptor substrates. In most Gram-positive bacteria, the peptidoglycan stem peptide features γ-D-isoGln at the second position in stem peptide, which is amidated by amidotransferases MurT/GatD during Lipid II biosynthesis.[101–103] Earlier transposon studies in MRSA revealed that non-amidated stem peptides (*i.e.,* changing γ-D-isoGln$_{(II)}$ to γ-D-isoGlu$_{(II)}$) would significantly reduce the overall peptidoglycan crosslinking levels and re-sensitise MRSA to beta-lactam antibiotics, suggesting that MRSA PBPs are unable to properly crosslink non-amidated substrates.[104,105] Through biochemical reconstitution of *Streptococcus pneumoniae* PBP2a, Zapun *et al.* first demonstrated that the amidated Lipid II substrate is indeed critical for efficient TP crosslinking *in vitro*.[77] Furthermore, substrate amidation is not limited to the second residue in stem peptide; many Gram-positive bacteria showcase additional amidated moieties in peptidoglycan. For instance, *E. faecium* has an amidated D-isoAsn as the branch peptide, whereas *B. subtilis* and *Mycobacterium smegmatis* possess amidated mDAP (*i.e.*, mDAP-NH_2) at the third residue in stem peptide. Studies demonstrate that the fully amidated substrates are strongly preferred by TPs and LDTs crosslinking in these organisms.[82–84,106] Consistent with the recognition of mDAP-NH_2 acceptor for crosslinking, *B. subtilis* PBP1 efficiently catalyses D-amino carboxamide exchange reaction in addition to D-amino acid exchange *in vitro*.[80] Accordingly, fluorescent D-amino carboxamide probes appear superior to traditional FDAAs for peptidoglycan labelling in *B. subtilis*.[80] On the other hand, amidation of the donor peptide *C*-terminus, which is unnatural in bacteria, strongly disfavours *E. faecium* TP and LDT crosslinking.[82]

Overall, these studies support the notion that bacterial TPs and LDTs strongly discriminate against substrates containing non-native amidation states for transpeptidation. Recently, the crystal structure of the *S. pneumoniae* MurT/GatD complex was solved in the apo-form, which showcases several putative channels that participate in ammonia transfer.[107] While further endeavours are needed to elucidate the muropeptide substrate binding site in MurT/GatD, such structural studies offer valuable insights into rational designs of inhibitors that disrupt GatD/MurT function, which could represent a novel class of antibacterial agents.

3.3. Peptidoglycan mimetics inhibitors

Given that beta-lactams mimic the D-Ala$_{(IV)}$-D-Ala$_{(V)}$ terminus of the acyl acceptor in the transpeptidation reaction, efforts have been made to rationally modify the beta-lactam core with side chain extensions that resemble the canonical stem peptide or branch peptide. Unfortunately, the success of such 'peptidoglycan mimetics' has been mixed.[108–112] For instance,

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the introduction of two extended arms to the cephalosporin core with peptide motifs that mimic the acyl donor and the acyl acceptor, respectively, improved the compound's specificity: it inhibited the TP activity of *E. coli* PBP1b selectively *in vitro* but showed no interaction or inhibition against *E. coli* PBP5 (a carboxypeptidase, CP).[111] In another work, Josephine *et. al.* demonstrated that the addition of side chain extensions onto beta-lactams unexpectedly improved the inhibition of LMW PBPs, but not the bPBPs *in vitro*.[108] Similarly, the inclusion of stem peptide or branch peptide mimics to a carbapenem scaffold did not improve its inhibition of *E. faecium* LDT (Ldt_{FM}).[110] While these results could potentially be attributed to the different substrate recognition and specificity of different PBPs, the fact that these compounds showed limited activity against PBPs in biochemical assays dampens the excitement of evaluating their antibacterial potential against bacterial cells.

4. Peptidoglycan hydrolases

4.1. Challenges with hydrolase characterisation

Peptidoglycan hydrolases are a group of enzymes involved in cleaving various chemical bonds in peptidoglycan polymer, including glycosidases, amidases, endopeptidases (EPs), and carboxypeptidases (CPs) *etc* (**Figure 7a**). Similar to PGTs and TPs, peptidoglycan hydrolase enzymes also display functional redundancy. Deducing the biological roles of individual hydrolases is difficult, since most of them are non-essential, where single or multiple gene deletions can be compensated for by other members of the family without causing cellular defects.[10–12] For instance, Wilson and Garner demonstrated that up to 40 peptidoglycan hydrolases of *B. subtilis* could be deleted in a single Δ40 mutant, yet this mutant still displayed similar growth and morphology to the wildtype.[12] Amongst the seemingly redundant hydrolases in *E. coli* and other bacteria, Levin's group and others have identified 'pH specialist' peptidoglycan enzymes that display better activities at low pH.[113–115] Therefore, it is crucial to develop robust tools and assays for biochemical characterisations of peptidoglycan hydrolases under different conditions.

4.2. Common assays for hydrolase characterisation

Biochemical characterisation of hydrolase activity requires either complex isolated sacculi or synthetic peptidoglycan as substrates for digestion, followed by analysing the cleaved products. Common analysis assays include zymography, cleavage of fluorogenic or chromogenic probes, as well as LC-MS detection (**Figure 7b**). In zymogram methods, potential hydrolases are subjected to separation on a special SDS-PAGE gel containing sacculi. Upon renaturation, the hydrolytic activity is revealed by a zone of clearance on the gel corresponding to the size of the hydrolase (**Figure 7b, left**). However, potential limitations of zymography include the inability to detect hydrolases that do not act on intact (or modified) cell sacculi, the inability to determine the exact catalytic activity, and the detection of non-specific protein binders to peptidoglycan.[116,117] Therefore, putative hydrolases must have their activity confirmed by additional assays. Fluorogenic- or chromogenic-based probes such as *p*-nitrophenyl *β*-*N*-acetylglucosaminide (pNP-GlcNAc) are widely used to study glucosaminidases *in vitro* (**Figure 7b, right**).[118–120] Furthermore, LC-MS analysis enables structural determination of peptidoglycan cleavage products based on their *m/z* values, which provide valuable information on the activity of specific peptidoglycan hydrolase (**Figure 7b, bottom**).

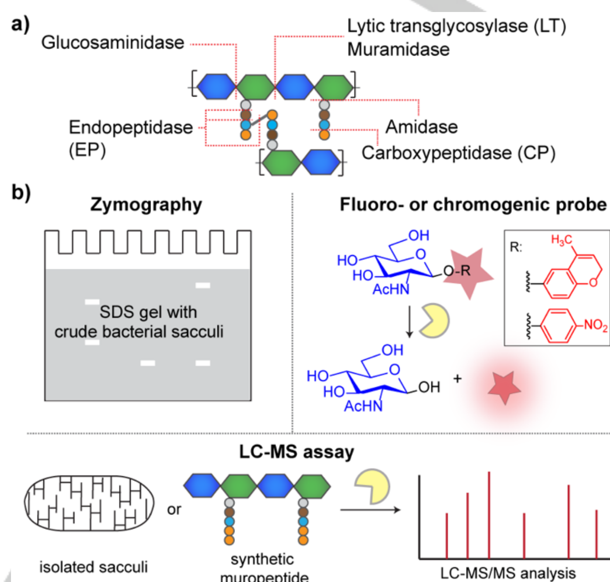


Figure 7. Major peptidoglycan hydrolase enzymes and common assays for characterisation. **(a)** Summary of the bonds cleaved by various peptidoglycan hydrolytic enzymes. Notably, lytic transglycosylase (LT) is non-hydrolytic and generates a characteristic 1,6-anhydro-MurNAc terminus in the product (*i.e.,* anhydro-muropeptide). **(b)** Common methods for characterising peptidoglycan hydrolases. In the zymogram method, the separating gel contains sacculi. Hydrolases break down the surrounding sacculi, presenting a clear band **(Left).** Cleavage of fluorogenic or chromogenic substrate analogues by hydrolases results in an increase in fluorescence or absorbance readout. *para*-Nitrophenyl (pNP) and 4-methylum-belliferyl (4-Mu) moieties are commonly used (**Right).** Hydrolytic cleavage products of sacculi or synthetic muropeptides can be determined and quantified based on their *m/z* values detected by LC-MS, providing information on peptidoglycan hydrolase activity (**Bottom**).

5. Glycosidases

The three types of peptidoglycan glycosidases include glucosaminidases, muramidases and lytic transglycosylases (LTs). Glucosaminidases hydrolyse the GlcNAc-*β*-1,4-MurNAc glycosidic bond, whereas muramidases and LTs act on the MurNAc-*β*-1,4-GlcNAc bond (**Figure 7a**). While muramidases are hydrolytic, LTs cleave non-hydrolytically to yield 1,6-anhydro-MurNAc terminating products (*i.e.,* anhydro-muropeptides) instead (**Figure 1b, right**). Peptidoglycan glycosidases are involved in a wide range of cellular processes, such as cell separation, cell motility,[121–123] toxin secretion,[124,125] and construction of secretion systems.[126–128]

Gram-positive bacteria are known to utilize glucos-aminidases and muramidases for cell separation, growth, and peptidoglycan recycling.[129–136] For instance, *E. faecalis* engages the glucosaminidase AtlA and muramidase AtlB in cell separation.[137,138] *S. aureus* requires four glucosaminidases (namely AtlA/GlcA, SagA, ScaH, SagB) to maintain normal cell morphology and growth.[139] Notably, Wheeler *et al.* showed that glucosaminidase cleavage reduces the stiffness of the cell wall.[139] In *B. subtilis*, peptidoglycan strands are broken down by

at least three enzymes, muramidase NamZ, glucosaminidase NagZ and amidase AmiE, to generate soluble saccharides and peptides (*i.e.*, GlcNAc, MurNAc and stem peptide) that are either recycled through permeases or released into the surroundings.[118,119]

On the other hand, Gram-negative bacteria encode multiple LTs: *E. coli* has at least eight LTs (*i.e.,* MltA-G, Slt70), *V. cholerae* eight, and *P. aeruginosa* eleven. LTs play important roles in cell separation, growth, and peptidoglycan recycling.[140] For instance, an *E. coli* mutant lacking six LTs displays a chaining phenotype, which is indicative of cell separation defects.[10] Similar roles of LTs in maintaining cell separation have been demonstrated in other Gram-negative bacteria including *P. aeruginosa*,[141] *Salmonella enterica* serotype Typhimurium,[142] and *V. cholerae*.[11] In addition, LTs also initiate peptidoglycan recycling in Gram-negative bacteria with the generation of periplasmic di-saccharide anhydro-muropeptides, which are subsequently transported back into the cytosol.[3]

In Gram-positive bacteria, LTs have been characterised in *Bacillus spp.,*[121,143–146] *Streptomyces spp.,*[147] and *Mycobacterium spp.*[148] Intriguingly, for each group, LTs are associated with exiting dormancy, *e.g.* resuscitation from a metabolically latent state or germination from spores.[145–148] For example, *Mycobacterium tuberculosis* LT RpfB digests intact sacculi alongside D,L-EP RipB to form anhydro-muropeptides that can resuscitate dormant *M. smegmatis* cells.[148] For other Gram-positive bacteria, identification and characterisation of putative LTs have remained elusive.[143,149,150] For example, *S. aureus* proteins IsaA and SceD, which possess putative LT domains at the *C*-terminus (PF01464 and PF06737, respectively), do not exhibit detectable cleavage activity with sacculi in zymography or HPLC assay, thus their functions are yet to be established.[150] In the sections below, we discuss efforts that elucidate substrate specificity and regulatory mechanisms of peptidoglycan glycosidases.

5.1 Substrate specificity of glycosidases

Peptidoglycan glycosidases can either be endolytic or exolytic: endo-glycosidases cleave internal glycosidic bonds to generate products of varying lengths whereas exo-glycosidases cleave glycosidic bonds at the termini to generate monomeric products (**Figure 8**). The endolytic or exolytic activity of glycosidases can be determined by LC-MS analysis (**Figure 7b, bottom**). For instance, *E. faecalis* glucosaminidase AtlA cleaves sacculi into both monomeric and multimeric products in similar ratios irrespective of enzyme concentration, a characteristic of endolytic glycosidase.[151] In contrast, *S. aureus* glucosaminidase GlcA, an exolytic glycosidase, accumulates MurNAc-GlcNAc monomers as the sole products upon digestion of sacculi.[152] Among the eight LTs in *E. coli*, MltE[153] and MltG[154] are exclusively endolytic, whereas the other LTs either showcase solely exolytic activity (*i.e.* MltA, MltF) or a combination of both (*i.e.* MltB, MltC, MltD, Slt70).[153] Structural studies are powerful in explaining the exolytic or endolytic mechanisms of individual glycosidases.[155–159] For instance, *E. coli* MltE contains a long binding groove that strongly favours the binding of MurNAc instead of anhydro-MurNAc at the +2 position in the glycan substrate, establishing the structural basis for its endolytic activity (**Figure 8**).[155,158] In a separate study, an extended substrate binding groove was identified in the crystal of structure *E. coli* MltC that allows extensive interactions with a long peptidoglycan strand, enabling subsequent exolytic cleavage.[156] On the other hand, *P. aeruginosa* Slt was shown to alter its structural conformation in order to accommodate long peptidoglycan polymers for endolytic cleavage.[159] Taken together, these examples underscore the importance of structural studies in explaining glycosidases' substrate specificities observed in biochemical assays.

Some peptidoglycan glycosidases recognize unique modifications on the glycan backbone. Denuded glycans, which are peptidoglycan strands devoid of stem peptides, are produced by cell separation amidases and are found abundantly at the cell septa in some bacteria.[141,152,160] For instance, *S. aureus* glucosaminidase GlcA acts exclusively on denuded glycans *in vitro*.[152] Similarly, *P. aeruginosa* RlpA, a cell separation LT, also shows a strong substrate preference for denuded glycan, but not intact sacculi or synthetic muropeptides.[141] Their substrate selectivity may be attributed to the peptidoglycan-binding domains, LysM and SPOR. Both domains can interact extensively with the *N*-acetyl groups on GlcNAc[161] and MurNAc[162] in the peptidoglycan backbone. While the SPOR domain was shown to exclusively recognize denuded glycans,[162] LysM domain favours binding with denuded glycans but is promiscuous to interact with typical peptidoglycan substrates too.[132,161,163]. Furthermore, *Bacillus anthracis* SleB, a germination specific LT, specifically cleaves peptidoglycan with muramic δ-lactam modifications,[145,146] which are unique glycan modifications found in spore-forming *Bacilli*.[164–166]

In addition to glycan modifications, certain glycosidases also exhibit specific requirements to peptidoglycan stem peptides. Lee *et al.* have extensively characterised the enzymatic activities of seven LTs in *E. coli,* where each recombinant LT was incubated with sacculi and crude products were analysed by LC-MS/MS.[153] Four LTs (MltD-F, Slt70) cleave only non-crosslinked peptidoglycan substrates while excluding crosslinked peptido-glycan.[153] On the other hand, Geiger *et al.* have uncovered a unique muramidase TtsA in the typhoid toxin secretion system of *S.*Typhi*,* which specifically cleaves the MurNAc-β-1,-4-GlcNAc bond in 3-3 crosslinked substrates.[125,167]

Structurally defined synthetic peptidoglycan fragments are superior for elucidations of the glycosidases' substrate specificity in greater detail. Lee *et al.* explored the substrate requirements of all eleven *P. aeruginosa* LTs using synthetic muropeptide substrates,[168] representing the most in-depth and extensive biochemical characterisation of bacterial LTs to date. Interestingly, *P. aeruginosa* MltA shows a unique substrate requirement, where its substrate needs to be an LT product itself with anhMurNAc at the +2 position (**Figure 8**).[168] This observation may suggest the intricate co-ordinations among multiple LTs in processing peptidoglycan *in vivo*. Remarkably, the LT ensemble in *P. aeruginosa* displays diverse and overlapping substrate requirements in terms of glycan length, exolytic/endolytic activity, and stem peptide, which may endow them with different biological roles under different growth conditions.[168]

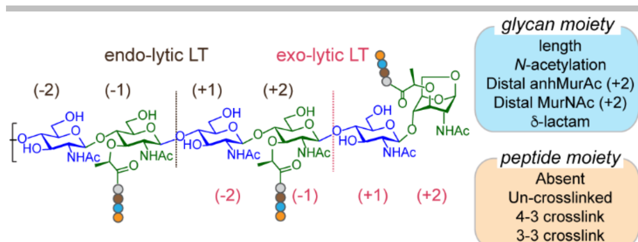


Figure 8. Tetra-saccharide peptidoglycan substrate with cleavage sites for both endolytic lytic transglycosylase (endolytic LT; brown) and exolytic lytic transglycosylase (exolytic LT; pink) shown. The saccharides on the two sides of the cleaved bond are numbered -2, -1 and +1, +2 respectively. Notably, different LTs (described in the text) exhibit a wide range of substrate preferences for glycan and peptide moieties in peptidoglycan.

5.2 Regulation of glycosidases

Bacteria regulate their glycosidases to prevent aberrant peptidoglycan degradation. On the one hand, many bacteria control glucosidase activity by proteolytic activation. For instance, *S. aureus* glucosaminidase GlcA is synthesised as a proprotein (AtlA), which contains both catalytic amidase and glucosaminidase domains separated by repeating glycine-tryptophan (GW) domains.[169–171] Post-translational cleavage of AltA by the secreted proteases in *S. aureus* yields mature glucosaminidase GlcA and amidase AmiA separately (**Figure 9, right**).[169,170,172] Similarly, the activity of *E. faecalis* AtlA is regulated by extracellular proteases by removal of the *N*-terminus inhibitory glycosylated domain (**Figure 9, right**).[173,174] On the other hand, some bacteria also regulate their glycosidase activities via protein degradation. The periplasmic protease Prc in *E. coli* is shown to effectively prevent MltG accumulation.[175] Furthermore, interaction with muropeptide substrates can also regulate enzymatic activity in glycosidases. For instance, Domínguez-Gil *et al.* established that *P. aeruginosa* MltF binding to a muropeptide substrate at an allosteric site triggers a conformational change to activate MltF.[176]

5.3 MltG – an LT with a novel function?

During peptidoglycan assembly, the elongating peptidoglycan polymer remains anchored onto the cell membrane with the undecaprenyl pyrophosphate lipid at the reducing end. The removal of the lipid anchor permits the termination of peptidoglycan extension, dissociation of peptidoglycan polymers from the cell membrane, and liberation of the undecaprenyl lipid, which is a scarce resource in bacterial cells. Despite the functional importance, the identities of the glycosidases (and/or other enzymes) involved in peptidoglycan termination have remained elusive. In 2016, Bernhardt and co-workers identified MltG, an inner membrane protein in *E. coli* that associates with PBP1b, which exhibits exolytic LT activity *in vitro*.[154] The observation that deletion of MltG in *E. coli* gave rise to an increase in the average length of peptidoglycan polymers led to the hypothesis that it may be the long-sought-after peptidoglycan terminase.[154,177] Notably, unlike other LTs, MltG is widely conserved among both Gram-negative and Gram-positive bacteria,[154] consistent with the proposed role of a peptidoglycan terminase. Interestingly, MltG homologues in *B. subtilis* and *S. pneumoniae* (MpgA) exhibit different activities: *B. subtilis* MltG is an endolytic LT,[144,154] whereas *S. pneumoniae* MpgA is a muramidase instead.[143,149] Moreover, a single amino acid substitution in *E. coli* MltG (D245N) would convert it from an LT into a muramidase.[143] Oddly, all three MltG homologues were shown to release nascent peptidoglycan strands about seven disaccharide units in length *in vitro*, which are seemingly too short for the native peptidoglycan strands *in vivo*, thereby casting doubts on the suggested terminase role of MltG in bacteria.[143,144] Further biochemical and genetic evidence are needed to elucidate the peptidoglycan terminase mechanisms across different bacteria, which would shed important insights into how bacteria produce/regulate peptidoglycan of different lengths in cells.

6. Amidases

Peptidoglycan amidases, also known as *N*-acetylmuramyl-L-alanine amidases, cleave the amide bond between the lactoyl moiety of MurNAc and L-Ala$_{(I)}$, separating the stem peptide from the glycan backbone (**Figure 7a**). Amidases are found in almost all bacteria. For instance, *E. coli* has three periplasmic amidases, AmiA, AmiB and AmiC, which all belong to the Amidase_3 superfamily (PF01520) and participate in cell separation by cleaving crosslinked septal peptidoglycan.[10,178,179] *E. coli* mutants lacking multiple amidases form elongated chains with thick peptidoglycan rings between adjacent cells.[10,178,179] In addition to AmiA-C, *E. coli* also contains two amidases, AmiD and AmpD that belong to the Amidase_2 superfamily (PF01510).[180,181] AmiD is a periplasmic lipoprotein, while AmpD is in the cytosol and responsible for the cleavage of anhydro-muropeptides in peptidoglycan recycling.[180,181] Mechanistically, bacterial amidases from the above two families are zinc-dependent metalloenzymes that can be inactivated by metal chelators such as EDTA. Below we highlight biochemical studies to address amidase substrate specificity.

6.1 Substrate specificity of amidases

The substrate preference of amidases can be inferred from the enzymatic degradation of crude sacculi, but conclusions are often limited due to the heterogenous and insoluble nature of sacculi that contain varying glycan and peptide lengths as well as different degrees of crosslinking. Hence, compositionally defined peptidoglycan substrates are valuable for determining amidases' substrate requirements. For instance, using synthetic peptidoglycan of different glycan lengths (*i.e.* 2, 4 and >30), Lupoli *et al.* first established that *E. coli* AmiA requires at least tetra-saccharide peptidoglycan fragments as substrates.[182] Similarly, the sole amidase in *N. gonorrhoeae*, AmiC, which is essential for proper cell separation, was shown to produce both crosslinked and non-crosslinked peptides from sacculi, indicating its active amidase activity.[183] Further characterisation using structurally defined peptidoglycan substrates revealed that it also requires at least a tetra-saccharide fragment for activity.[183] Interestingly, both *E. coli* AmiA and *N. gonorrhoeae* AmiC cleaved only one stem peptide (instead of two) per tetra-saccharide substrate.[182,183] A similar preference for tetra-saccharide substrates was found for *S. pneumoniae* LytA too, which also exhibits an extended substrate-binding interface to accommodate and interact with large peptidoglycan substrates.[184,185]

Besides insights into amidases' selectivity of glycan chain length, Do *et al.* recently revealed that *S. aureus* amidase LytH (Amidase_3 superfamily) together with its partner protein ActH specifically cleaves un-crosslinked peptidoglycan substrates, a substrate preference that is the first of its kind.[186] Notably, the access to native Lipid II enabled straightforward preparation of matched peptidoglycan substrate that is either crosslinked or non-crosslinked for detailed biochemical characterisation.[81] In addition, both *S. aureus* amidase AmiA and its homologue in *Staphylococcus epidermis* (Amidase_2 superfamily) were shown to recognize the stem peptide identity. For instance, AmiA's active site forms extensive hydrogen bonding with the α-CONH$_2$ group on γ-D-isoGln$_{(II)}$ in the stem peptide, whereas substrates containing the non-amidated γ-D-Glu$_{(II)}$ residue were unsuitable for AmiA cleavage.[187–189]

Amidases involved in peptidoglycan recycling are specific towards substrates produced by the preceding enzymes in the pathway. For instance, *B. subtilis* AmiE is specific to muramyl peptides generated from upstream glucosaminidases;[118] whereas AmpD in Gram-negative bacteria is specific to anhydro-muropeptides produced from LTs.[180,190,191] In *P. aeruginosa*, there are a total of three paralogous genes for AmpD: AmpD, AmpDh2 and AmpDh3, which all participate in the upregulation of beta-lactam resistance.[192] To decipher the substrate specificity of respective amidase, Mobashery and co-workers did a series of elegant biochemical assays and LC-MS/MS analysis using both synthetic muropeptides and crude sacculi as substrates.[193–195] *P. aeruginosa* AmpD, a cytosolic protein, recognizes only anhydro-muropeptides as substrates, which supports its role as the *bona fide* amidase in peptidoglycan recycling.[194] Its K_M with anhydro-muropeptide substrates was in the low millimolar range, which likely represents the physiological concentration of these peptidoglycan metabolites in the cytoplasm.[194] On the other hand, periplasmic AmpDh2 and AmpDh3 cleave both reducing-end muropeptides and anhydro-muropeptides *in vitro*.[193–195] Moreover, studies using bacterial sacculi (pre- or post- cleaved by LTs) as substrates revealed that AmpDh2 and AmpDh3 could act on both crosslinked and non-crosslinked substrates.[193–195] Remarkably, AmpDh3 manifests a strong preference for insoluble polymeric sacculus, whereas AmpDh2 favours soluble muropeptides.[193–195] Their distinct substrate preferences could be attributed to their different protein multimeric states and structures: AmpDh3 forms a soluble tetramer with an extensive binding surface to accommodate polymeric substrate; AmpDh2 has a membrane-bound dimer structure with a smaller binding surface suitable for soluble muropeptide substrates instead.[193,195] The complementary roles of amidases for cell wall turnover could be a common mechanism in other bacteria.

In addition, some Gram-positive amidases that contain a catalytic CHAP domain (PF05257) have been demonstrated to display both EP and amidase activity, which we discuss in more detail in section 7.2.

6.2. Regulation of amidases

The hydrolytic activity of amidases is tightly regulated in bacteria to prevent aberrant peptidoglycan cleavage that could lead to cell lysis (**Figure 9**). Interestingly, *E. coli* amidases exhibit low basal activities *in vitro*, and their peptidoglycan-splitting roles for cell separation require stimulation by LytM-domain proteins.[196,197] Bernhardt and co-workers elucidated that *E. coli* AmiA and AmiB are activated by the periplasmic protein EnvC, whereas AmiC is activated by the outer membrane lipoprotein NlpD.[196,197] In particular, activation of amidases by EnvC requires a conformational change of the cytokinesis protein FtsEX at the septal ring.[198] Structurally, amidase activation involves the displacement of a conserved inhibitory α-helix to expose the amidase active site (**Figure 9, middle**).[196,199–201] Apart from EnvC and NlpD, ActS, another outer membrane lipoprotein, was recently identified as a third amidase activator in *E. coli,* which only functions under cell wall stress and preferentially activates AmiC.[113,202] Thus, amidases have different regulatory mechanisms depending on the physiochemical environment in bacteria.

Furthermore, a common amidase activation strategy in several Gram-positive bacteria involves the allosteric regulation by a partner protein to stabilise metal chelation.[186,203] For instance, the activity of *S. aureus* LytH requires complex formation with ActH, an atypical membrane protein activator.[186] Using fluorophore-labelled peptidoglycan substrates and gel-based assays, Page *et al.* revealed that the extracellular tetra-tricopeptide (TPR) domain of ActH (ActH$_{TPR}$) is critical and sufficient for LytH activation *in vitro*.[203] Genetic studies further confirmed the activating role of ActH$_{TPR}$ *in vivo.*[203] Based on structural and biochemical investigations, the authors showed that ActH$_{TPR}$ binding with LytH stabilises Zn^{2+} or Fe^{2+} in the active site (**Figure 9, left**).[203] While the exact mechanism is unknown, it is hypothesised that ActH induces conformational changes in LytH to facilitate metal cofactor stabilisation.[203] Similarly, in *C. difficile,* amidase CwlD activity is controlled by its allosteric activator GerS, which also facilitates Zn^{2+} binding and catalysis, where the *N*-terminus of GerS was shown to directly participate in Zn^{2+} stabilisation.[204]

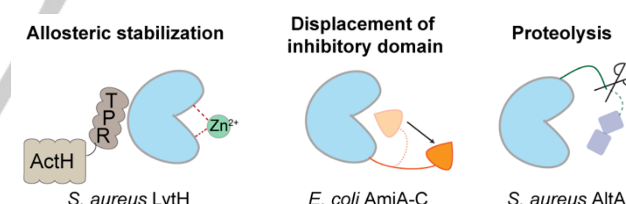


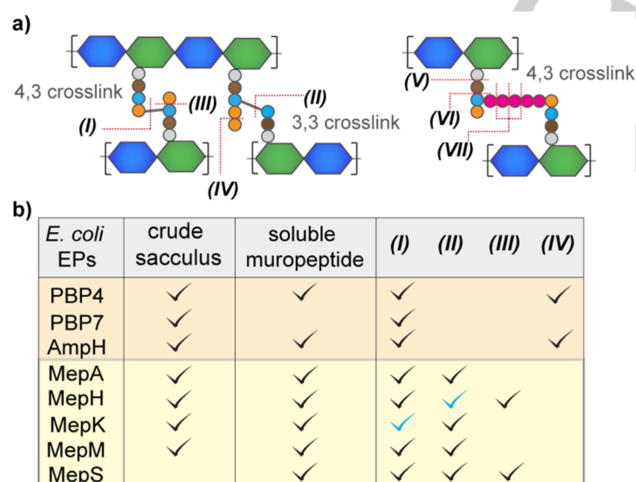
Figure 9. Peptidoglycan hydrolases (*i.e.* amidases or hydrolases, shown in blue) that degrade peptidoglycan are tightly regulated to prevent bacterial autolysis. A common strategy adopted by bacteria is to translate the hydrolase into an inactive form. Activator protein binding the allosteric site may stabilise metal co-factors chelation at the active site (**Left**); activators can displace an inhibitory domain (**Middle**); the conversion to an active form can occur via proteolytic degradation of the inhibitory domain (**Right**).

7. Endopeptidases (EPs)

Endopeptidases (EPs) are hydrolytic enzymes that specifically cleave amide bonds in peptidoglycan crosslinks or stem peptides (**Figure 7a** and **Figure 10a**). One of their primary roles is to enable insertions of new cell wall materials for cell growth, elongation and separation. In this section, we will highlight the activities and regulatory mechanisms of EPs in the model Gram-negative organism *E. coli*, contrast these with EPs of Gram-positive bacteria, as well as discuss recent findings on the significance of gut microbiota-encoded EPs in the host.

7.1 Biochemical characterisation of *E. coli* EPs

Gram-negative *E. coli* encodes eight EPs that can be broadly categorised into two groups: (1) PBP4, PBP7, and AmpH from the PBP family;[205–208] (2) metalloenzymes, comprising of MepH and MepS from the NlpC/P60 family [206,209] and MepA, MepM and MepK from the LAS, M23, M15 families, respectively.[206,209–211] Among the eight EPs, MepM appears the most important, as an *E. coli* mutant with the other seven EPs deleted was still viable.[206] Biochemical activities of *E. coli* EPs were demonstrated using either sacculi or total soluble muropeptides (*i.e.,* sacculi pre-digested with mutanolysin or lysozyme) as substrates (**Figure 10a, b**).[205–211] Notably, different EPs display distinct substrate specificity *in vitro.* While the PBP family of EPs are specific to the 4-3 crosslink (*i.e.* $Ala_{(IV)}$→$mDAP_{(III)}$ bond), the five metalloenzymes can cleave both 4-3 and 3-3 crosslinks (*i.e.,* acting as D,D-EP and L,D-EP respectively) (**Figure 10a [I, II]**).[205–211] The fact that metalloenzymes cleave both types of crosslinks suggests that they may recognize the acceptor strand, which is in common in both crosslinks.[206] Consistently, MepS only acts on soluble tetrapeptides but not tripeptide or pentapeptide substrates.[209] Moreover, four *E. coli* EPs exhibit dual activities: MepS and MepH act as L,D-CPs, converting tetrapeptides to tripeptides by cleaving the $mDAP_{(III)}$-D-$Ala_{(IV)}$ bond,[206,209] whereas PBP4 and AmpH trim pentapeptides by removing D-$Ala_{(V)}$ at the terminus (**Figure 10a [III, IV]**).[205,208] Additionally, *E. coli* also encodes a paralog of LDT, LdtF, that has two unique functions. First, LdtF acts as an L,D-EP and catalyses the detachment of Braun's lipoprotein (an outer membrane protein covalently bonded to peptidoglycan in Gram-negative bacteria).[212,213] Next, LdtF also functions as a Gly-specific CP that acts on stem tetrapeptides with terminal Gly residues.[212,213] (see section 8.2).



E. coli EPs	crude sacculus	soluble muropeptide	*(I)*	*(II)*	*(III)*	*(IV)*
PBP4	✓	✓	✓			✓
PBP7	✓		✓			
AmpH	✓	✓	✓			✓
MepA	✓	✓	✓	✓		
MepH	✓	✓	✓	✓ (blue)	✓	
MepK	✓	✓	✓ (blue)	✓		
MepM	✓	✓	✓	✓		
MepS		✓	✓	✓	✓	

Figure 10. (a) Schematic representation of specific bonds cleaved by endo-peptidases (EP) and carboxypeptidases (CP). (I) D-$Ala_{(IV)}$→$mDAP_{(III)}$ (D,D-EP); (II) $mDAP_{(III)}$→$mDAP_{(III)}$ (L,D-EP); (III) $X_{(III)}$-D-$Ala_{(IV)}$ (L,D-CP); (IV) D-$Ala_{(IV)}$-D-$Ala_{(V)}$ (D,D-CP); (V) L-$Ala_{(I)}$-γ-D-$iGlx_{(II)}$ (L,D-EP); (VI) γ-D-$iGlx_{(II)}$-$X_{(III)}$ (D,L-EP); (VII) bonds within or at the *N*-terminus of the branch peptide. Identities of the amino acids in the peptidoglycan stem peptide are outlined in Figure 1. **(b)** Selectivity of eight *E. coli* EPs involved in cell growth, cell elongation, or cell separation.[205–211] Black ticks indicate that the enzyme is capable of cleaving the specified bond whereas blue ticks indicate that cleavage of the specified bond is a minor activity of the enzyme. EPs can be broadly categorized into two groups: the PBP family (orange) and metalloenzymes (yellow). Most EPs can act on both intact sacculus and soluble muropeptides, and a few EPs also exhibit CP activity.

7.2. Diverse substrate specificity of EPs in Gram-positive bacteria

Given the diversity of branch peptides in Gram-positive bacterial peptidoglycan, these organisms encode EPs that exhibit distinct substrate specificity. In particular, spore-forming *Bacilli* possess a vast number of EPs for peptidoglycan remodelling, which has been extensively characterised by Sekiguchi's group[214–221] and others. For instance, *B. subtilis* CwlK and LytH are L,D-EPs that specifically cleave the L-$Ala_{(I)}$-γ-D-$Glu_{(II)}$ bond (**Figure 10a [V]**),[164,216] and generate products with only L-$Ala_{(I)}$ in the stem peptide, a unique peptidoglycan feature in *Bacillus* spores.[164–166] In addition, *B. subtilis* also encodes seven D,L-EPs (including a bifunctional D,L-EP/muramidase CwlT), which specifically cleave the bond between γ-D-$Glu_{(II)}$-$mDAP_{(III)}$ (**Figure 10a [VI]**). [184–190] Amongst these, CwlO and LytE localize at the lateral cell wall and are associated with cell elongation, whereas CwlS, LytF and LytE localize at cell septa and poles to participate in cell separation.[215,219] D,L-EPs are also widespread in Gram-positive bacteria including *Lactobacillus,*[222–226] *Mycobacterium*,[227–229] *Enterococcus*,[230–232].

In *S. aureus*, several EPs are known to cleave the pentaglycine crosslinks. For instance, *S. aureus* LytM and the recently discovered LytU belong to the lysostaphin family of EPs that cleave between the second and third glycine in the pentaglycine branch peptide (**Figure 10a [VII]**).[233,234] *S. aureus* amidase EssH, which contains a CHAP domain, also acts as an EP that cleaves the pentaglycine peptide at these two positions.[235] Similarly, LytN is another CHAP-containing hydrolase that exhibits both amidase and EP activity, cleaving peptide bonds at the end of the pentaglycine peptide to break crosslinks and release cell wall proteins.[236] Lastly, *S. thermophilus* Cse is a CHAP domain L,D-EP that specifically breaks crosslinks, cleaving the bond between D-$Ala_{(IV)}$ on the donor peptide and L-Ala in the acceptor branch peptide.[237]

7.3. Cellular regulatory mechanism of EPs

In searching to understand the cellular regulatory mechanisms of *E. coli* EPs, Reddy and co-workers first reported that the protein level of MepS alters significantly with the growth phase in *E. coli*, where it is abundantly expressed during the exponential phase but rapidly diminishes at the onset of stationary phase.[238] This intriguing observation led to the discovery of a proteolytic system that involves a TPR-containing outer membrane lipoprotein, NlpI and a periplasmic protease, Prc that modulates the cellular level of MepS in *E. coli*.[238] Biochemical pulldown study suggested that NlpI likely acts as an adaptor protein that facilitates degradation of MepS by Prc.[238] In separate studies, it was discovered that both MepH and MepM are also Prc substrates.[239,240] Building upon earlier studies, Banzhaf *et al.* performed global proteomics-based screening to establish that *E. coli* NlpI is implicated in the abundance and thermostability of many cell envelope proteins including LTs and amidases, indicating a broader regulatory role of NlpI in *E. coli* peptidoglycan biogenesis.[241] Moving forward, identifying the upstream signals that NlpI senses may open up new avenues to intervene in bacterial peptidoglycan homeostasis. Intriguingly, Darwin and co-workers have identified a proteolytic complex in *P. aeruginosa*, consisting of lipoprotein LbcA and protease CtpA that perform similar regulatory functions.[242,243] The fact that

P. aeruginosa LbcA-CtpA is non-homologous to *E. coli* NlpI-Prc suggests such regulatory proteins in different bacteria may arise by convergent evolution.[242,243]

Besides post-translational degradation, conformational rearrangement represents another regulatory mechanism for EPs in Gram-negative bacteria (**Figure 9, middle**). For instance, the primary EP in *V. cholerae*, ShyA was found to exist in two drastically different conformations: a closed form with an inhibitory domain blocking the active site, and an open form that permits substrate binding. [244] Correspondingly, mutant variants of ShyA that favour the open conformation leads to increased EP activity both *in vitro* and *in vivo*.[244] This conformational switch appears a common regulatory mechanism for EPs of similar architecture in other bacteria, including *E. coli* MepM.[244] Nevertheless, the molecular signals that trigger the conformational activation of EPs are yet to be elucidated.[244]

In most Gram-positive bacteria, the widely conserved WalK/WalR two-component system controls peptidoglycan homeostasis (reviewed here[245]). For instance, the expression levels of *B. subtilis* elongation D,L-EPs, CwlO and LytE, are regulated by WalK/WalR system, which senses peptidoglycan EP products.[246–248] Similarly, *Mycobacterium* and *Streptomyces* also possess the MtrA/MtrB two-component system that performs similar roles to WalK/WalR.[245] For example, MtrA/MtrB controls the expression of the mycobacterial cell-separating D,L-EP, RipA.[249] Apart from two-component systems, bacterial EPs are also under other forms of regulation. For instance, *Mycobacterium* RipA features an auto-inhibitory domain and requires either a partner protein or proteolysis for activation (**Figure 9, middle and right**).[229] Moreover, RipA also interacts with and is regulated by the peptidoglycan synthase, PBP1.[250] Taken together, the expression and activity of bacterial EPs are extensively controlled by different mechanisms.

Hijacking the cellular regulatory mechanisms of bacterial EPs or constructing recombinant EPs containing peptidoglycan binding domains might result in uncontrolled bacterial autolysis.[251–253] For instance, *S. aureus* peptidoglycan hydrolase LytM was fused with the peptidoglycan binding domain of lysostaphin, affording an effective anti-staphylococcal peptide *in vitro*.[251,252] Such designs are attractive for the development of novel antibacterial agents.

7.4. EPs in the gut microbiota

Recent studies have shed insights into the roles of EPs produced by host gut microbiota.[7] Commensal bacteria *Lactobacillus* and *Enterococcus* express EPs such as SagA,[223–226,230–232] that generate soluble muropeptides, which are agonistic to the host NOD2 innate immune sensor (**Figure 11**).[254,255] These NOD2-active muropeptides are shown to act as adjuvants to potentiate cancer immunotherapy,[256] alleviate Crohn's disease (CD) progression in mouse models,[224,225] and protect mice against pathogenic species.[230,257] Remarkably, a recent metagenomic study by Gao *et. al* has revealed that CD patients manifest a significantly lower abundance of gut microbial D,L-EP genes in their stool,[224] highlighting the potential implications of the gut microbiota EPs and their muropeptide products in human disease.

E. faecium SagA and its homologues in *Enterococcus,* which were discovered and have been extensively characterised by Hang and co-workers,[230–232,256,257] exhibit interesting substrate specificity *in vitro*. The NlpC/p60 domain of SagA cleaves the γ-D-isoGln(II)-L-Lys(III) bond in the stem peptide,[231] a conserved moiety in peptidoglycan of most Gram-positive bacteria (**Figure 11**). SagA strictly discriminates against muropeptides from other bacteria, suggesting the particular branch peptide in *Enterococcus* (*i.e.* D-iAsx) may impart substrate specificity.[230] Moreover, SagA preferentially acts on soluble, crosslinked muropeptides isolated from *E. faecium*, but is unable to cleave crude sacculi.[230] This observation may imply that the activity of SagA *in vivo* requires close coordination with muramidases to generate bioactive soluble muropeptides in the host gut niche (yet the exact order of the SagA and muramidase cleavage remains unclear). However, this contrasts with a recent finding that showed that both SagA and a homologue found in *Lactobacillus salivarius*, could generate NOD2-active muropeptides *in vitro* when their recombinant forms were incubated with *E. coli* or *S. aureus*.[224] Lastly, while SagA has been found to localize at the septa in *E. faecium*, its exact biological function in *E. faecium* is still unclear.[231] The exciting yet intriguing roles of gut microbiota-encoded EPs in host warrant further study in order to harness their therapeutic potential.

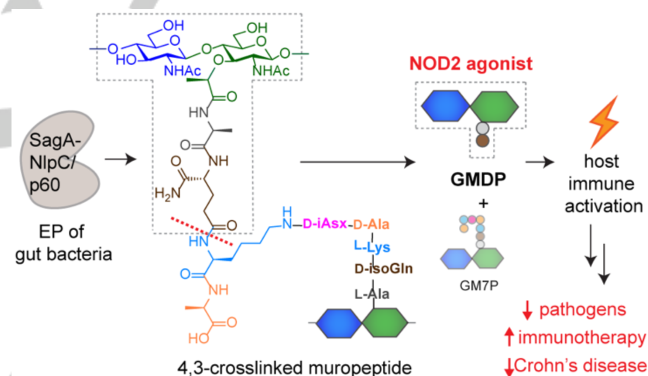


Figure 11. L,D-endopeptidase SagA in gut microbiota digest crosslinked peptidoglycan substrates and release NOD2-activating muropeptides into the host gastrointestinal tract. NOD2 recognizes the minimal MurNAc-L-Ala-γ-D-isoGln motif and triggers downstream immune activity. The presence of these muropeptides can alleviate Crohn's disease,[224,225] act as an immunotherapy adjuvant,[256] and protect the host against pathogenic infections.[232,257]

8. Carboxypeptidases (CP)

Bacteria possess many carboxypeptidases (CPs) such as D,D-CPs, D,L-CPs and L,D-CPs that remove the terminal amino acid from penta-, tetra- or tripeptide stems (**Figure 10a [III, IV]** and **Figure 12**). CPs are crucial for the maintenance of bacterial shape and cell integrity,[115,258–261] since the cleavage activity of CPs controls the amount of available donor substrate for crosslinking. For example, a trio of orthologous CPs, Pgp1-3 in *Campylobacter jejuni* and Csd 3-4,6 in *Helicobacter pylori*, are critical for maintaining bacterial helical cell shape. In each trio, *C. jejuni* Pgp1 and *H. pylori* Csd4 are D,L-CPs,[262,263] Pgp2 and Csd6 are L,D-CPs,[264,265] Pgp3 and Csd3 are bifunctional enzymes with D,D-EP and D,D-CP activities,[266,267] which act sequentially in trimming the stem peptides in these helical

bacteria. In *E. coli*, among the seven CPs, both PBP5 and PBP6a are D,D-CPs that generate tetrapeptide donor precursors for LdtD-mediated 3-3 crosslinking during outer membrane stress.[69,70] Similarly, *E. faecium* DdcY[268] (a D,D-CP) and *M. smegmatis* DacB2[64] (a dual functional D,D-EP/D,D-CP) cleave pentapeptide stems to form tetrapeptide precursors for 3-3 crosslinking as well.

CPs are also implicated in other cellular processes such as virulence,[269,270] recycling,[269,271–273] and metabolism of NCDAA-containing peptidoglycan in bacteria.[212,274] For instance, *E. coli* MpaA is a cytosolic D,L-CP that specifically degrades tripeptide stems (generated by LdcA) during peptidoglycan recycling.[272,273] Moreover, *N. gonorrhoeae* L,D-CP LdcA participates in peptidoglycan recycling and contributes to the release of bioactive tripeptide anhydro-muropeptides into the niche.[269] Similarly, the periplasmic D,L-CP EcgA in *S.* Typhimurium acts on tripeptide substrates, thus altering the pools of muropeptide agonists for host immune activation and contributing to bacterial virulence.[270]

8.1. Functional non-redundancy of CPs under different conditions

The model organism *E. coli* encodes at least seven D,D-CPs, including PBP4 (DacB), PBP4b, PBP5 (DacA), PBP6a (DacC), PBP6b (DacD), PBP7 (PbpG), and AmpH. Among them, PBP4, PBP7 and AmpH also have EP functions (see section 7.1). Deletions of multiple CPs in *E. coli* yield no morphological defects (except for PBP5).[259,261] To establish that PBP5 is the main CP in *E. coli* under typical laboratory conditions, Nelson and Young thoroughly examined the morphology of >20 PBP single and multiple deletion mutants of *E. coli*.[259,261] Using a similar approach, Peters *et. al* compared the peptidoglycan composition of mutants grown in normal or acidic conditions.[115] Intriguingly, under acidic conditions, mutants lacking PBP6b contained a larger proportion of pentapeptides compared to other mutants.[115] Further *in vitro* characterisation of *E. coli* PBP5 and PBP6b with intact sacculi revealed that PBP6b unexpectedly displays higher catalytic activity under acidic conditions. Thus, PBP6b could act as a 'pH specialist' that ensures normal rod morphology of *E. coli* at low pH.[115] Therefore, the redundant set of *E. coli* CPs may allow bacterial survival under different growth conditions.

8.2. Distinct substrate specificity of bacterial CPs.

CPs do not require complex peptidoglycan substrates for activity, since simple peptides, such as diacetyl-L-Lys-D-Ala-D-Ala, have been commonly used as model substrates for the characterisation of D,D-CPs.[274–278] Addition of a chromogenic moiety (*e.g.,* acetyl-D-amino acid-*p*-nitroaniline) onto the model peptide enables a convenient absorbance assay for measuring CP activity.[274] Using these substrates, Miyamoto *et al.* established that *E. coli* PBP4a and *B. subtilis* PBP5 can remove NCDAAs (e.g. D-Cys, D-Arg, D-Leu) from the stem peptide terminus, suggesting their potential biological roles in regulating NCDAA levels in peptidoglycan in cells.[274] Additionally, the LDT responsible for detachment of the Braun's lipoproteins in *E. coli*, LdtF, also functions as an L,D-CP specific to the tetrapeptide substrate with a terminal Gly residue.[212]

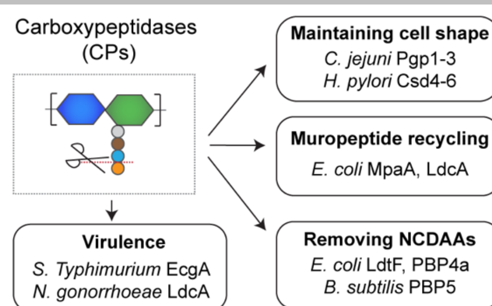


Figure 12. Carboxypeptidases (CPs) play important biological roles in bacteria. CPs cleave the terminal amino acid in tetra-, penta- or tripeptide stems in peptidoglycan. The above figure shows examples of specific bacterial CPs that are involved in different aspects of their physiology.

On the other hand, the cytosolic L,D-CP LdcA in *E. coli* that is involved in peptidoglycan recycling strongly prefers canonical tetrapeptides compared to NCDAA-containing tetrapeptides.[271] Cytosolic accumulation of NCDAA tetrapeptides in bacteria during the stationary phase in turn impacts peptidoglycan crosslinking levels and precursor concentrations.[271] Thus, the respective substrate specificity of PBP4, PBP5 and LdcA ensure their key roles in regulating peptidoglycan homeostasis in *E. coli*. In addition, recent studies have characterised the L,D-CP LdcB in several Gram-positive bacteria as well. Both *S. pneumoniae* LdcB and *B. subtilis* LdcB actively cleave the tetrapeptide end in both sacculi and synthetic tetrapeptide substrates.[260,279] Expectedly, they exhibit stereospecificity toward the D-Ala at the tetrapeptide end and do not cleave the L-Ala terminus.[279]

9. Summary and outlook

Bacterial peptidoglycan is a conserved and essential structure across all bacteria. Enzymes in the peptidoglycan biosynthesis pathway (*i.e.,* PGTs and TPs) are well-recognized excellent targets of antibiotics, where characterisation of their substrate specificity and development of *in vitro* assays has greatly facilitated new antibiotics discovery. On the other hand, inhibition of enzymes involved in peptidoglycan remodelling and breakdown (*i.e.,* glycosidases, amidases, endopeptidases, and carboxypeptidases etc.) to perturb the well-regulated balance of peptidoglycan homeostasis is potentially lethal to bacteria.[280] Indeed, corbomycin and complestatin, which are inhibitors of most, if not all, peptidoglycan hydrolases, are effective against MRSA.[280] In another example, bulgecin, a natural product inhibitor of several LTs, shows no antibacterial activity alone but is capable of potentiating beta-lactams in *P. aeruginosa*.[281] Studies in the past two decades have offered key insights into the apparent functional redundancy of these major families of enzymes in peptidoglycan metabolic pathways, especially in model bacterial organisms such as *E. coli*, *S. aureus*, *B. subtilis* and *E. faecalis* etc. The seemingly redundant peptidoglycan enzymes do exhibit differences in their substrate preferences and regulation mechanisms, which may be exploited for the potential development of narrow-spectrum antibiotics against specific bacteria. Moreover, the tools and reagents developed for biochemical reconstitutions are highly valuable for screening potential inhibitors targeting the peptidoglycan pathway.

In recent years, the trillions of bacteria in the host gut niche are demonstrated to shed a large abundance of soluble peptidoglycan fragments, which are ubiquitously present in the host gut and systemic circulation.[282,283] These peptidoglycan fragments are increasingly recognized for their importance as key effector molecules in mediating host health and physiology, including controlling host appetite and body temperature, promoting cancer immunotherapy and impacting fungal pathogenesis *etc*.[7,98,256,257,282,284–286] Thus, understanding the peptidoglycan pathways in gut bacteria is necessary to elucidate the underlying mechanisms. Aptly, the multitude of studies on peptidoglycan enzymes in the model bacteria has paved the way for in-depth mechanistic investigations of peptidoglycan assembly and release processes in the gut microbiota. Altogether with advances in molecular genetics and improved culturing conditions, we anticipate a wealth of knowledge on the enzymatic activities of gut microbiota peptidoglycan enzymes in the years to come.

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[1] W. Vollmer, *FEMS Microbiol. Rev.* **2008**, *32*, 287–306.
[2] W. Vollmer, D. Blanot, M. A. De Pedro, *FEMS Microbiol. Rev.* **2008**, *32*, 149–167.
[3] C. Mayer, R. M. Kluj, M. Mühleck, A. Walter, S. Unsleber, I. Hottmann, M. Borisova, *Int. J. Med. Microbiol.* **2019**, *309*, 151326.
[4] J. M. Chan, J. P. Dillard, *J. Bacteriol.* **2017**, *199*, e00354-17.
[5] R. S. Rosenthal, W. Nogami, B. T. Cookson, W. E. Goldman, W. J. Folkening, *Infect. Immun.* **1987**, *55*, 2117–2120.
[6] O. Irazoki, S. B. Hernandez, F. Cava, *Front. Microbiol.* **2019**, *10*, 500.
[7] C. Li, Y. Liang, Y. Qiao, *Front. Microbiol.* **2022**, *13*, 863407.
[8] K. Emami, A. Guyet, Y. Kawai, J. Devi, L. J. Wu, N. Allenby, R. A. Daniel, J. Errington, *Nat. Microbiol. 2017 23* **2017**, *2*, 1–9.
[9] D. C. McPherson, D. L. Popham, *J. Bacteriol.* **2003**, *185*, 1423–1431.
[10] C. Heidrich, A. Ursinus, J. Berger, H. Schwarz, J.-V. Höltje, *J. Bacteriol.* **2002**, *184*, 6093–6099.
[11] A. I. Weaver, V. Jiménez-Ruiz, S. R. Tallavajhala, B. P. Ransegnola, K. Q. Wong, T. Dörr, *Mol. Microbiol.* **2019**, *112*, 1100–1115.
[12] S. Wilson, E. Garner, 2021, bioRxiv preprint DOI: 10.1101/2021.02.18.431929.
[13] W. Vollmer, U. Bertsche, *Biochim. Biophys. Acta - Biomembr.* **2008**, *1778*, 1714–1734.
[14] P. Reed, H. Veiga, A. M. Jorge, M. Terrak, M. G. Pinho, *J. Bacteriol.* **2011**, *193*, 2549–2556.
[15] A. J. Meeske, E. P. Riley, W. P. Robins, T. Uehara, J. J. Mekalanos, D. Kahne, S. Walker, A. C. Kruse, T. G. Bernhardt, D. Z. Rudner, *Nature* **2016**, *537*, 634–638.
[16] S. Atwal, S. Chuenklin, E. M. Bonder, J. Flores, J. J. Gillespie, T. P. Driscoll, J. Salje, *mBio* **2021**, *12*, e01342-21.
[17] C. Fraipont, S. Alexeeva, B. Wolf, R. van der Ploeg, M. Schloesser, T. den Blaauwen, M. Nguyen-Distèche, *Microbiology* **2011**, *157*, 251–259.
[18] S. Leclercq, A. Derouaux, S. Olatunji, C. Fraipont, A. J. F. Egan, W. Vollmer, E. Breukink, M. Terrak, *Sci. Rep.* **2017**, *7*, 43306.
[19] X. Liu, J. Biboy, E. Consoli, W. Vollmer, T. den Blaauwen, *PLOS Genet.* **2020**, *16*, e1009276.
[20] M. Sjodt, K. Brock, G. Dobihal, P. D. A. Rohs, A. G. Green, T. A. Hopf, A. J. Meeske, V. Srisuknimit, D. Kahne, S. Walker, D. S. Marks, T. G. Bernhardt, D. Z. Rudner, A. C. Kruse, *Nature* **2018**, *556*, 118–121.
[21] A. Taguchi, M. A. Welsh, L. S. Marmont, W. Lee, M. Sjodt, A. C. Kruse, D. Kahne, T. G. Bernhardt, S. Walker, *Nat. Microbiol. 2019 44* **2019**, *4*, 587–594.
[22] M. Sjodt, P. D. A. Rohs, M. S. A. Gilman, S. C. Erlandson, S. Zheng, A. G. Green, K. P. Brock, A. Taguchi, D. Kahne, S. Walker, D. S. Marks, D. Z. Rudner, T. G. Bernhardt, A. C. Kruse, *Nat. Microbiol.* **2020**, *5*, 813–820.
[23] P. D. A. Rohs, J. Buss, S. I. Sim, G. R. Squyres, V. Srisuknimit, M. Smith, H. Cho, M. Sjodt, A. C. Kruse, E. C. Garner, S. Walker, D. E. Kahne, T. G. Bernhardt, *PLOS Genet.* **2018**, *14*, e1007726.
[24] Y. Li, H. Gong, R. Zhan, S. Ouyang, K.-T. Park, J. Lutkenhaus, S. Du, *PLOS Genet.* **2021**, *17*, e1009366.
[25] D. L. Perlstein, Y. Zhang, T.-S. Wang, D. E. Kahne, S. Walker, *J. Am. Chem. Soc.* **2007**, *129*, 12674–12675.
[26] M. A. Welsh, K. Schaefer, A. Taguchi, D. Kahne, S. Walker, *J. Am. Chem. Soc.* **2019**, *141*, 12994–12997.
[27] T.-S. A. Wang, T. J. Lupoli, Y. Sumida, H. Tsukamoto, Y. Wu, Y. Rebets, D. E. Kahne, S. Walker, *J. Am. Chem. Soc.* **2011**, *133*, 8528–8530.
[28] D. Barrett, T.-S. A. Wang, Y. Yuan, Y. Zhang, D. Kahne, S. Walker, *J. Biol. Chem.* **2007**, *282*, 31964–31971.
[29] Y. Zhang, E. J. Fechter, T.-S. A. Wang, D. Barrett, S. Walker, D. E. Kahne, *J. Am. Chem. Soc.* **2007**, *129*, 3080–3081.
[30] Y. Yuan, D. Barrett, Y. Zhang, D. Kahne, P. Sliz, S. Walker, *Proc. Natl. Acad. Sci.* **2007**, *104*, 5348–5353.
[31] T.-S. A. Wang, S. A. Manning, S. Walker, D. Kahne, *J. Am. Chem. Soc.* **2008**, *130*, 14068–14069.
[32] B. Schwartz, J. A. Markwalder, S. P. Seitz, Y. Wang, R. L. Stein, *Biochemistry* **2002**, *41*, 12552–12561.
[33] Y. Rebets, T. Lupoli, Y. Qiao, K. Schirner, R. Villet, D. Hooper, D. Kahne, S. Walker, *ACS Chem. Biol.* **2014**, *9*, 459–467.
[34] M. Banzhaf, B. van den Berg van Saparoea, M. Terrak, C. Fraipont, A. Egan, J. Philippe, A. Zapun, E. Breukink, M. Nguyen-Distèche, T. den Blaauwen, W. Vollmer, *Mol. Microbiol.* **2012**, *85*, 179–194.
[35] M. Lee, D. Hesek, J. Zajíček, J. F. Fisher, S. Mobashery, *Chem. Commun.* **2017**, *53*, 12774–12777.
[36] X.-Y. Ye, M.-C. Lo, L. Brunner, D. Walker, D. Kahne, S. Walker, *J. Am. Chem. Soc.* **2001**, *123*, 3155–3156.
[37] D. L. Perlstein, T.-S. Andrew Wang, E. H. Doud, D. Kahne, S. Walker, *J. Am. Chem. Soc.* **2010**, *132*, 48–49.
[38] C.-Y. Liu, C.-W. Guo, Y.-F. Chang, J.-T. Wang, H.-W. Shih, Y.-F. Hsu, C.-W. Chen, S.-K. Chen, Y.-C. Wang, T.-J. R. Cheng, C. Ma, C.-H. Wong, J.-M. Fang, W.-C. Cheng, *Org. Lett.* **2010**, *12*, 1608–1611.
[39] S.-H. Huang, W.-S. Wu, L.-Y. Huang, W.-F. Huang, W.-C. Fu, P.-T. Chen, J.-M. Fang, W.-C. Cheng, T.-J. R. Cheng, C.-H. Wong, *J. Am. Chem. Soc.* **2013**, *135*, 17078–17089.
[40] K.-T. Chen, C.-K. Lin, C.-W. Guo, Y.-F. Chang, C.-M. Hu, H.-H. Lin, Y. Lai, T.-J. R. Cheng, W.-C. Cheng, *Chem. Commun.* **2017**, *53*, 771–774.
[41] C. Fraipont, F. Sapunaric, A. Zervosen, G. Auger, B. Devreese, T. Lioux, D. Blanot, D. Mengin-Lecreulx, P. Herdewijn, J. Van Beeumen, J.-M. Frère, M. Nguyen-Distèche, *Biochemistry* **2006**, *45*, 4007–4013.
[42] K. E. DeMeester, H. Liang, M. R. Jensen, Z. S. Jones, E. A. D'Ambrosio, S. L. Scinto, J. Zhou, C. L. Grimes, *J. Am. Chem. Soc.* **2018**, *140*, 9458–9465.
[43] Y. Xu, V. M. Hernández-Rocamora, J. H. Lorent, R. Cox, X. Wang, X. Bao, M. Stel, G. Vos, R. M. van den Bos, R. J. Pieters, J. Gray, W. Vollmer, E. Breukink, *iScience* **2022**, *25*, 104753.
[44] H.-W. Shih, Y.-F. Chang, W.-J. Li, F.-C. Meng, C.-Y. Huang, C. Ma, T.-J. R. Cheng, C.-H. Wong, W.-C. Cheng, *Angew. Chem. Int. Ed.* **2012**, *51*, 10123–10126.
[45] V. M. Hernández-Rocamora, N. Baranova, K. Peters, E. Breukink, M. Loose, W. Vollmer, *eLife* **2021**, *10*, e61525.
[46] M. D. Lebar, T. J. Lupoli, H. Tsukamoto, J. M. May, S. Walker, D. Kahne, *J. Am. Chem. Soc.* **2013**, *135*, 4632–4635.

[47] D. Mengin-Lecreulx, T. Falla, D. Blanot, J. Van Heijenoort, D. J. Adams, I. Chopra, *J. Bacteriol.* **1999**, *181*, 5909–5914.
[48] A. C. Catherwood, A. J. Lloyd, J. A. Tod, S. Chauhan, S. E. Slade, G. P. Walkowiak, N. F. Galley, A. S. Punekar, K. Smart, D. Rea, N. D. Evans, M. J. Chappell, D. I. Roper, C. G. Dowson, *J. Am. Chem. Soc.* **2020**, *142*, 5034–5048.
[49] C.-Y. Huang, H.-W. Shih, L.-Y. Lin, Y.-W. Tien, T.-J. R. Cheng, W.-C. Cheng, C.-H. Wong, C. Ma, *Proc. Natl. Acad. Sci.* **2012**, *109*, 6496–6501.
[50] A. L. Lovering, L. De Castro, N. C. J. Strynadka, *J. Mol. Biol.* **2008**, *383*, 167–177.
[51] J. Nakamura, H. Yamashiro, H. Miya, K. Nishiguchi, H. Maki, H. Arimoto, *Chem. Weinh. Bergstr. Ger.* **2013**, *19*, 12104–12112.
[52] A. Severin, S. W. Wu, K. Tabei, A. Tomasz, *Antimicrob. Agents Chemother.* **2004**, *48*, 4566–4573.
[53] J. D. Chang, E. E. Foster, H. Yang, S. J. Kim, *Biochemistry* **2017**, *56*, 612–622.
[54] A. Bouhss, N. Josseaume, A. Severin, K. Tabei, J.-E. Hugonnet, D. Shlaes, D. Mengin-Lecreulx, J. Van Heijenoort, M. Arthur, *J. Biol. Chem.* **2002**, *277*, 45935–45941.
[55] B. Ostash, R. Makitrynskyy, O. Yushchuk, V. Fedorenko, *BBA Adv.* **2022**, *2*, 100065.
[56] N. A. Caveney, S. D. Workman, R. Yan, C. E. Atkinson, Z. Yu, N. C. J. Strynadka, *Nat. Commun.* **2021**, *12*, 2775.
[57] D. T. King, G. A. Wasney, M. Nosella, A. Fong, N. C. J. Strynadka, *J. Biol. Chem.* **2017**, *292*, 979–993.
[58] A. L. Lovering, L. H. de Castro, D. Lim, N. C. J. Strynadka, *Science* **2007**, *315*, 1402–1405.
[59] M.-T. Sung, Y.-T. Lai, C.-Y. Huang, L.-Y. Chou, H.-W. Shih, W.-C. Cheng, C.-H. Wong, C. Ma, *Proc. Natl. Acad. Sci.* **2009**, *106*, 8824–8829.
[60] Y. Yuan, S. Fuse, B. Ostash, P. Sliz, D. Kahne, S. Walker, *ACS Chem. Biol.* **2008**, *3*, 429–436.
[61] C. M. Gampe, H. Tsukamoto, E. H. Doud, S. Walker, D. Kahne, *J. Am. Chem. Soc.* **2013**, *135*, 3776–3779.
[62] J. Zuegg, C. Muldoon, G. Adamson, D. McKeveney, G. Le Thanh, R. Premraj, B. Becker, M. Cheng, A. G. Elliott, J. X. Huang, M. S. Butler, M. Bajaj, J. Seifert, L. Singh, N. F. Galley, D. I. Roper, A. J. Lloyd, C. G. Dowson, T.-J. Cheng, W.-C. Cheng, D. Demon, E. Meyer, W. Meutermans, M. A. Cooper, *Nat. Commun.* **2015**, *6*, 7719.
[63] M. Lavollay, M. Arthur, M. Fourgeaud, L. Dubost, A. Marie, N. Veziris, D. Blanot, L. Gutmann, J. L. Mainardi, *J. Bacteriol.* **2008**, *190*, 4360.
[64] C. Baranowski, M. A. Welsh, L. T. Sham, H. A. Eskandarian, H. C. Lim, K. J. Kieser, J. C. Wagner, J. D. McKinney, G. E. Fantner, T. R. Ioerger, S. Walker, T. G. Bernhardt, E. J. Rubin, E. H. Rego, *eLife* **2018**, *7*, e37516.
[65] J. Peltier, P. Courtin, I. El Meouche, L. Lemée, M.-P. Chapot-Chartier, J.-L. Pons, *J. Biol. Chem.* **2011**, *286*, 29053–29062.
[66] J. L. Mainardi, V. Morel, M. Fourgeaud, J. Cremniter, D. Blanot, R. Legrand, C. Fréhel, M. Arthur, J. Van Heijenoort, L. Gutmann, *J. Biol. Chem.* **2002**, *277*, 35801–35807.
[67] J. L. Mainardi, M. Fourgeaud, J. E. Hugonnet, L. Dubost, J. P. Brouard, J. Ouazzani, L. B. Rice, L. Gutmann, M. Arthur, *J. Biol. Chem.* **2005**, *280*, 38146–38152.
[68] J.-L. Mainardi, R. Legrand, M. Arthur, B. Schoot, J. van Heijenoort, L. Gutmann, *J. Biol. Chem.* **2000**, *275*, 16490–16496.
[69] J.-E. Hugonnet, D. Mengin-Lecreulx, A. Monton, T. den Blaauwen, E. Carbonnelle, C. Veckerlé, Y. Brun V., M. van Nieuwenhze, C. Bouchier, K. Tu, L. B. Rice, M. Arthur, *eLife* **2016**, *5*, e19469.
[70] N. Morè, A. M. Martorana, J. Biboy, C. Otten, M. Winkle, C. K. G. Serrano, A. Montón Silva, L. Atkinson, H. Yau, E. Breukink, T. den Blaauwen, W. Vollmer, A. Polissi, *mBio* **2019**, *10*, e02729-18.
[71] D. J. Tipper, J. L. Strominger, *Proc. Natl. Acad. Sci.* **1965**, *54*, 1133–1141.
[72] S. Triboulet, V. Dubée, L. Lecoq, C. Bougault, J.-L. Mainardi, L. B. Rice, M. Ethève-Quelquejeu, L. Gutmann, A. Marie, L. Dubost, J.-E. Hugonnet, J.-P. Simorre, M. Arthur, *PLOS ONE* **2013**, *8*, e67831.
[73] J.-L. Mainardi, J.-E. Hugonnet, F. Rusconi, M. Fourgeaud, L. Dubost, A. N. Moumi, V. Delfosse, C. Mayer, L. Gutmann, L. B. Rice, M. Arthur, *J. Biol. Chem.* **2007**, *282*, 30414–30422.
[74] A. Aliashkevich, F. Cava, *FEBS J.* **2022**, *289*, 4718–4730.
[75] U. Bertsche, E. Breukink, T. Kast, W. Vollmer, *J. Biol. Chem.* **2005**, *280*, 38096–38101.
[76] B. Schwartz, J. A. Markwalder, Y. Wang, *J. Am. Chem. Soc.* **2001**, *123*, 11638–11643.
[77] A. Zapun, J. Philippe, K. A. Abrahams, L. Signor, D. I. Roper, E. Breukink, T. Vernet, *ACS Chem. Biol.* **2013**, *8*, 2688–2696.
[78] P. Born, E. Breukink, W. Vollmer, *J. Biol. Chem.* **2006**, *281*, 26985–26993.
[79] C. M. Vacariu, M. E. Tanner, *Chem. – Eur. J.* **2022**, *28*, e202200788.
[80] M. D. Lebar, J. M. May, A. J. Meeske, S. A. Leiman, T. J. Lupoli, H. Tsukamoto, R. Losick, D. Z. Rudner, S. Walker, D. Kahne, *J. Am. Chem. Soc.* **2014**, *136*, 10874–10877.
[81] Y. Qiao, V. Srisuknimit, F. Rubino, K. Schaefer, N. Ruiz, S. Walker, D. Kahne, *Nat. Chem. Biol.* **2017**, *13*, 793–798.
[82] S. E. Pidgeon, A. J. Apostolos, J. M. Nelson, M. Shaku, B. Rimal, M. N. Islam, D. C. Crick, S. J. Kim, M. S. Pavelka, B. D. Kana, M. M. Pires, *ACS Chem. Biol.* **2019**, *14*, 2185–2196.
[83] A. J. Apostolos, S. E. Pidgeon, M. M. Pires, *ACS Chem. Biol.* **2020**, *15*, 1261–1267.
[84] F. Ngadjeua, E. Braud, S. Saidjalolov, L. Iannazzo, D. Schnappinger, S. Ehrt, J. E. Hugonnet, D. Mengin-Lecreulx, D. Patin, M. Ethève-Quelquejeu, M. Fonvielle, M. Arthur, *Chem. - Eur. J.* **2018**, *24*, 5743–5747.
[85] B. Ling, B. Berger-Bächi, *Antimicrob. Agents Chemother.* **1998**, *42*, 936–938.
[86] V. Srisuknimit, Y. Qiao, K. Schaefer, D. Kahne, S. Walker, *J. Am. Chem. Soc.* **2017**, *139*, 9791–9794.
[87] A. Arbeloa, J.-E. Hugonnet, A.-C. Sentilhes, N. Josseaume, L. Dubost, C. Monsempes, D. Blanot, J.-P. Brouard, M. Arthur, *J. Biol. Chem.* **2004**, *279*, 41546–41556.
[88] S. Magnet, A. Arbeloa, J. L. Mainardi, J. E. Hugonnet, M. Fourgeaud, L. Dubost, A. Marie, V. Delfosse, C. Mayer, L. B. Rice, M. Arthur, *J. Biol. Chem.* **2007**, *282*, 13151–13159.
[89] T. J. Lupoli, H. Tsukamoto, E. H. Doud, T. A. Wang, S. Walker, D. Kahne, *J. Am. Chem. Soc.* **2011**, *133*, 10748–10751.
[90] F. Cava, M. A. De Pedro, H. Lam, B. M. Davis, M. K. Waldor, *EMBO J.* **2011**, *30*, 3442–3453.
[91] E. Kuru, H. V. Hughes, P. J. Brown, E. Hall, S. Tekkam, F. Cava, M. A. de Pedro, Y. V. Brun, M. S. VanNieuwenhze, *Angew. Chem. Int. Ed.* **2012**, *51*, 12519–12523.
[92] A. D. Radkov, Y.-P. Hsu, G. Booher, M. S. VanNieuwenhze, *Annu. Rev. Biochem.* **2018**, *87*, 991–1014.
[93] H. Lin, C. Yang, W. Wang, *RSC Chem. Biol.* **2022**, *3*, 1198–1208.
[94] Y. P. Hsu, G. Booher, A. Egan, W. Vollmer, M. S. Vannieuwenhze, *Acc. Chem. Res.* **2019**, *52*, 2713–2722.
[95] E. Kuru, A. Radkov, X. Meng, A. Egan, L. Alvarez, A. Dowson, G. Booher, E. Breukink, D. I. Roper, F. Cava, W. Vollmer, Y. Brun, M. S. VanNieuwenhze, *ACS Chem. Biol.* **2019**, *14*, 2745–2756.
[96] Y. Qiao, M. D. Lebar, K. Schirner, K. Schaefer, H. Tsukamoto, D. Kahne, S. Walker, *J. Am. Chem. Soc.* **2014**, *136*, 14678–14681.
[97] M. A. Welsh, A. Taguchi, K. Schaefer, D. Van Tyne, F. Lebreton, M. S. Gilmore, D. Kahne, S. Walker, *J. Am. Chem. Soc.* **2017**, *139*, 17727.
[98] L. Li, A. W. R. Ng, C. Adamson, H. Hayashi, C. Li, H. Lim, Y. Qiao, *ACS Chem. Biol.* **2022**, *17*, 2538–2550.
[99] A. García-Heredia, A. A. Pohane, E. S. Melzer, C. R. Carr, T. J. Fiolek, S. R. Rundell, H. C. Lim, J. C. Wagner, Y. S. Morita, B. M. Swarts, M. S. Siegrist, *eLife* **2018**, *7*, e37243.
[100] D. M. Missiakas, O. Schneewind, *Curr. Protoc. Microbiol.* **2013**, *28*, 9C.1.1-9C.1.9.
[101] D. Münch, T. Roemer, S. H. Lee, M. Engeser, H. G. Sahl, T. Schneider, *PLOS Pathog.* **2012**, *8*, e1002509.
[102] E. R. Nöldeke, T. Stehle, *Int. J. Med. Microbiol.* **2019**, *309*, 151334.
[103] T. A. Figueiredo, R. G. Sobral, A. M. Ludovice, J. M. F. de Almeida, N. K. Bui, W. Vollmer, H. de Lencastre, A. Tomasz, *PLoS Pathog.* **2012**, *8*, e1002508.

[104] J. Gustafson, A. Strässle, H. Hächler, F. H. Kayser, B. Berger-Bächi, *J. Bacteriol.* **1994**, *176*, 1460–1467.
[105] A. M. Strandén, M. Roos, B. Berger-Bächi, *Microb. Drug Resist.* **1996**, *2*, 201–207.
[106] A. J. Apostolos, J. M. Nelson, J. R. A. Silva, J. Lameira, A. M. Achimovich, A. Gahlmann, C. N. Alves, M. M. Pires, *ACS Chem. Biol.* **2020**, *15*, 2966–2975.
[107] C. Morlot, D. Straume, K. Peters, O. A. Hegnar, N. Simon, A.-M. Villard, C. Contreras-Martel, F. Leisico, E. Breukink, C. Gravier-Pelletier, L. Le Corre, W. Vollmer, N. Pietrancosta, L. S. Håvarstein, A. Zapun, *Nat. Commun.* **2018**, *9*, 3180.
[108] H. R. Josephine, P. Charlier, C. Davies, R. A. Nicholas, R. F. Pratt, *Biochemistry* **2006**, *45*, 15873–15883.
[109] H. R. Josephine, I. Kumar, R. F. Pratt, *J. Am. Chem. Soc.* **2004**, *126*, 8122–8123.
[110] S. Saidjalolov, Z. Edoo, M. Fonvielle, L. Mayer, L. Iannazzo, M. Arthur, M. Etheve-Quelquejeu, E. Braud, *Chem. - Eur. J.* **2021**, *27*, 3542–3551.
[111] M. Lee, D. Hesek, M. Suvorov, W. Lee, S. Vakulenko, S. Mobashery, *J. Am. Chem. Soc.* **2003**, *125*, 16322–16326.
[112] I. Kumar, H. R. Josephine, R. F. Pratt, *ACS Chem. Biol.* **2007**, *2*, 620–624.
[113] E. A. Mueller, A. G. Iken, M. Ali Öztürk, M. Winkle, M. Schmitz, W. Vollmer, B. Di Ventura, P. A. Levin, *Mol. Microbiol.* **2021**, *116*, 311–328.
[114] E. A. Mueller, P. A. Levin, *mBio* **2020**, *11*, e02456-20.
[115] K. Peters, S. Kannan, V. A. Rao, J. Biboy, D. Vollmer, S. W. Erickson, R. J. Lewis, K. D. Young, W. Vollmer, *mBio* **2016**, *7*, e00819-16.
[116] J. E. M. M. Wong, M. Blaise, *Biochimie* **2020**, *177*, 25–29.
[117] C. A. Escobar, T. A. Cross, *Anal. Biochem.* **2018**, *543*, 162–166.
[118] S. Litzinger, A. Duckworth, K. Nitzsche, C. Risinger, V. Wittmann, C. Mayer, *J. Bacteriol.* **2010**, *192*, 3132–3143.
[119] M. Müller, M. Calvert, I. Hottmann, R. M. Kluj, T. Teufel, K. Balbuchta, A. Engelbrecht, K. A. Selim, Q. Xu, M. Borisova, A. Titz, C. Mayer, *J. Biol. Chem.* **2021**, *296*, 100519.
[120] C. E. Serrano-Maldonado, I. García-Cano, A. González-Canto, E. Ruiz-May, J. M. Elizalde-Contreras, M. Quirasco, *J. Mol. Microbiol. Biotechnol.* **2018**, *28*, 14–27.
[121] S. Sanchez, C. M. Dunn, D. B. Kearns, *J. Bacteriol.* **2021**, *203*, e00029-21.
[122] S. Roure, M. Bonis, C. Chaput, C. Ecobichon, A. Mattox, C. Barrière, N. Geldmacher, S. Guadagnini, C. Schmitt, M.-C. Prévost, A. Labigne, S. Backert, R. L. Ferrero, I. G. Boneca, *Mol. Microbiol.* **2012**, *86*, 845–856.
[123] F. A. Herlihey, P. J. Moynihan, A. J. Clarke, *J. Biol. Chem.* **2014**, *289*, 31029–31042.
[124] K. A. Cloud-Hansen, K. T. Hackett, D. L. Garcia, J. P. Dillard, *J. Bacteriol.* **2008**, *190*, 5989–5994.
[125] T. Geiger, M. Pazos, M. Lara-Tejero, W. Vollmer, J. E. Galán, *Nat. Microbiol.* **2018**, *3*, 1243–1254.
[126] Y. G. Y. Chan, M. B. Frankel, D. Missiakas, O. Schneewind, *J. Bacteriol.* **2016**, *198*, 1123–1136.
[127] D. Zahrl, M. Wagner, K. Bischof, M. Bayer, B. Zavecz, A. Beranek, C. Ruckenstuhl, G. E. Zarfel, G. 2005 Koraimann, *Microbiology* **2005**, *151*, 3455–3467.
[128] B. J. Burkinshaw, W. Deng, E. Lameignère, G. A. Wasney, H. Zhu, L. J. Worrall, B. B. Finlay, N. C. J. Strynadka, *J. Biol. Chem.* **2015**, *290*, 10406–10417.
[129] N. Inagaki, A. Iguchi, T. Yokoyama, K. Yokoi, Y. Ono, A. Yamakawa, A. Taketo, K.-I. Kodaira, *Gene* **2009**, *447*, 61–71.
[130] C. Eckert, S. Magnet, S. Mesnage, *FEBS Lett.* **2007**, *581*, 693–696.
[131] C. Huard, G. Miranda, Y. Redko, F. Wessner, S. J. Foster, M.-P. Chapot-Chartier, *Appl. Environ. Microbiol.* **2004**, *70*, 3493–3499.
[132] A. Steen, G. Buist, G. J. Horsburgh, G. Venema, O. P. Kuipers, S. J. Foster, J. Kok, *FEBS J.* **2005**, *272*, 2854–2868.
[133] A. Dhalluin, I. Bourgeois, M. Pestel-Caron, E. Camiade, G. Raux, P. Courtin, M.-P. Chapot-Chartier, J.-L. 2005 Pons, *Microbiology* **2005**, *151*, 2343–2351.
[134] E. Camiade, J. Peltier, I. Bourgeois, E. Couture-Tosi, P. Courtin, A. Antunes, M.-P. Chapot-Chartier, B. Dupuy, J.-L. Pons, *J. Bacteriol.* **2010**, *192*, 2373–2384.
[135] C. Huard, G. Miranda, F. Wessner, A. Bolotin, J. Hansen, S. J. Foster, M.-P. 2003 Chapot-Chartier, *Microbiology* **2003**, *149*, 695–705.
[136] B. R. Johnson, T. R. Klaenhammer, *Appl. Environ. Microbiol.* **2016**, *82*, 5687–5697.
[137] S. Mesnage, F. Chau, L. Dubost, M. Arthur, *J. Biol. Chem.* **2008**, *283*, 19845–19853.
[138] C. Eckert, M. Lecerf, L. Dubost, M. Arthur, S. Mesnage, *J. Bacteriol.* **2006**, *188*, 8513–8519.
[139] R. Wheeler, R. D. Turner, R. G. Bailey, B. Salamaga, S. Mesnage, S. A. S. Mohamad, E. J. Hayhurst, M. Horsburgh, J. K. Hobbs, S. J. Foster, *mBio* **2015**, *6*, e00660.
[140] D. A. Dik, D. R. Marous, J. F. Fisher, S. Mobashery, *Crit. Rev. Biochem. Mol. Biol.* **2017**, *52*, 503–542.
[141] M. A. Jorgenson, Y. Chen, A. Yahashiri, D. L. Popham, D. S. Weiss, *Mol. Microbiol.* **2014**, *93*, 113–128.
[142] C. Monteiro, X. Fang, I. Ahmad, M. Gomelsky, U. Römling, *J. Bacteriol.* **2011**, *193*, 6443–6451.
[143] A. Taguchi, J. E. Page, H.-C. T. Tsui, M. E. Winkler, S. Walker, *Proc. Natl. Acad. Sci.* **2021**, *118*, e2103740118.
[144] J. Sassine, M. Pazos, E. Breukink, W. Vollmer, *Cell Surf.* **2021**, *7*, 100053.
[145] J. D. Heffron, B. Orsburn, D. L. Popham, *J. Bacteriol.* **2009**, *191*, 2237–2247.
[146] J. D. Heffron, N. Sherry, D. L. Popham, *J. Bacteriol.* **2011**, *193*, 125–131.
[147] D. L. Sexton, F. A. Herlihey, A. S. Brott, D. A. Crisante, E. Shepherdson, A. J. Clarke, M. A. Elliot, *J. Biol. Chem.* **2020**, *295*, 9171–9182.
[148] V. D. Nikitushkin, G. R. Demina, M. O. Shleeva, S. V. Guryanova, A. Ruggiero, R. Berisio, A. S. Kaprelyants, *FEBS J.* **2015**, *282*, 2500–2511.
[149] H. C. T. Tsui, J. J. Zheng, A. N. Magallon, J. D. Ryan, R. Yunck, B. E. Rued, T. G. Bernhardt, M. E. Winkler, *Mol. Microbiol.* **2016**, *100*, 1039–1065.
[150] M. R. Stapleton, M. J. Horsburgh, E. J. Hayhurst, L. Wright, I. M. Jonsson, A. Tarkowski, J. F. Kokai-Kun, J. J. Mond, S. J. Foster, *J. Bacteriol.* **2007**, *189*, 7316–7325.
[151] V. Roig-Zamboni, S. Barelier, R. Dixon, N. F. Galley, A. Ghanem, Q. P. Nguyen, H. Cahuzac, B. Salamaga, P. J. Davis, Y. Bourne, S. Mesnage, F. Vincent, *J. Biol. Chem.* **2022**, *298*, 101915.
[152] M. Nega, P. M. Tribelli, K. Hipp, M. Stahl, F. Götz, *Commun. Biol.* **2020**, *3*, 1–10.
[153] M. Lee, D. Hesek, L. I. Llarrull, E. Lastochkin, H. Pi, B. Boggess, S. Mobashery, *J. Am. Chem. Soc.* **2013**, *135*, 3311–3314.
[154] R. Yunck, H. Cho, T. G. Bernhardt, *Mol. Microbiol.* **2016**, *99*, 700–718.
[155] C. Artola-Recolons, C. Carrasco-López, L. I. Llarrull, M. Kumarasiri, E. Lastochkin, I. Martínez de Ilarduya, K. Meindl, I. Usón, S. Mobashery, J. A. Hermoso, *Biochemistry* **2011**, *50*, 2384–2386.
[156] C. Artola-Recolons, M. Lee, N. Bernardo-García, B. Blázquez, D. Hesek, S. G. Bartual, K. V. Mahasenan, E. Lastochkin, H. Pi, B. Boggess, K. Meindl, I. Usón, J. F. Fisher, S. Mobashery, J. A. Hermoso, *ACS Chem. Biol.* **2014**, *9*, 2058–2066.
[157] J. Vijayaraghavan, V. Kumar, N. P. Krishnan, R. T. Kaufhold, X. Zeng, J. Lin, F. van den Akker, *PLOS ONE* **2018**, *13*, e0197136.
[158] G. Fibriansah, F. I. Gliubich, A.-M. W. H. Thunnissen, *Biochemistry* **2012**, *51*, 9164–9177.
[159] M. Lee, M. T. Batuecas, S. Tomoshige, T. Domínguez-Gil, K. V. Mahasenan, D. A. Dik, D. Hesek, C. Millán, I. Usón, E. Lastochkin, J. A. Hermoso, S. Mobashery, *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 4393–4398.
[160] C. Morlot, T. Uehara, K. A. Marquis, T. G. Bernhardt, D. Z. Rudner, *Genes Dev.* **2010**, *24*, 411–422.
[161] S. Mesnage, M. Dellarole, N. J. Baxter, J.-B. Rouget, J. D. Dimitrov, N. Wang, Y. Fujimoto, A. M. Hounslow, S. Lacroix-Desmazes, K. Fukase, S. J. Foster, M. P. Williamson, *Nat. Commun.* **2014**, *5*, 4269.
[162] M. Alcorlo, D. A. Dik, S. De Benedetti, K. V. Mahasenan, M. Lee, T. Domínguez-Gil, D. Hesek, E. Lastochkin, D. López, B. Boggess, S. Mobashery, J. A. Hermoso, *Nat. Commun.* **2019**, *10*, 5567.
[163] M. B. Frankel, O. Schneewind, *J. Biol. Chem.* **2012**, *287*, 10460–10471.
[164] G. J. Horsburgh, A. Atrih, S. J. Foster, *J. Bacteriol.* **2003**, *185*, 3813–3820.

[165] D. L. Popham, J. Helin, C. E. Costello, P. Setlow, *J. Bacteriol.* **1996**, *178*, 6451–6458.
[166] A. Atrih, P. Zöllner, G. Allmaier, S. J. Foster, *J. Bacteriol.* **1996**, *178*, 6173–6183.
[167] T. Geiger, M. Lara-Tejero, Y. Xiong, J. E. Galán, *eLife* **2020**, *9*, e53473.
[168] M. Lee, D. Hesek, D. A. Dik, J. Fishovitz, E. Lastochkin, B. Boggess, J. F. Fisher, S. Mobashery, *Angew. Chem. Int. Ed.* **2017**, *56*, 2735–2739.
[169] T. Oshida, M. Sugai, H. Komatsuzawa, Y. M. Hong, H. Suginaka, A. Tomasz, *Proc. Natl. Acad. Sci.* **1995**, *92*, 285–289.
[170] M. Sugai, H. Komatsuzawa, T. Akiyama, Y. M. Hong, T. Oshida, Y. Miyake, T. Yamaguchi, H. Suginaka, *J. Bacteriol.* **1995**, *177*, 1491–1496.
[171] R. Biswas, L. Voggu, U. K. Simon, P. Hentschel, G. Thumm, F. Götz, *FEMS Microbiol. Lett.* **2006**, *259*, 260–268.
[172] C. Chen, V. Krishnan, K. Macon, K. Manne, S. V. L. Narayana, O. Schneewind, *J. Biol. Chem.* **2013**, *288*, 29440–29452.
[173] B. Salamaga, T. K. Prajsnar, A. Jareño-Martinez, J. Willemse, M. A. Bewley, F. Chau, T. B. Belkacem, A. H. Meijer, D. H. Dockrell, S. A. Renshaw, S. Mesnage, *PLOS Pathog.* **2017**, *13*, e1006526.
[174] E. K. Stinemetz, P. Gao, K. L. Pinkston, M. C. Montealegre, B. E. Murray, B. R. Harvey, *PloS One* **2017**, *12*, e0186706.
[175] P.-C. Hsu, C.-S. Chen, S. Wang, M. Hashimoto, W.-C. Huang, C.-H. Teng, *Front. Microbiol.* **2020**, *11*, 2000.
[176] T. Domínguez-Gil, M. Lee, I. Acebrón-Avalos, K. V. Mahasenan, D. Hesek, D. A. Dik, B. Byun, E. Lastochkin, J. F. Fisher, S. Mobashery, J. A. Hermoso, *Structure* **2016**, *24*, 1729–1741.
[177] J. L. Bohrhunter, P. D. A. Rohs, G. Torres, R. Yunck, T. G. Bernhardt, *Mol. Microbiol.* **2021**, *115*, 1170–1180.
[178] C. Heidrich, M. F. Templin, A. Ursinus, M. Merdanovic, J. Berger, H. Schwarz, M. A. De Pedro, J.-V. Höltje, *Mol. Microbiol.* **2001**, *41*, 167–178.
[179] R. Priyadarshini, M. A. de Pedro, K. D. Young, *J. Bacteriol.* **2007**, *189*, 5334–5347.
[180] C. Jacobs, B. Joris, M. Jamin, K. Klarsov, J. van Beeumen, D. Mengin-Lecreulx, J. van Heijenoort, J. T. Park, S. Normark, J.-M. Frère, *Mol. Microbiol.* **1995**, *15*, 553–559.
[181] T. Uehara, J. T. Park, *J. Bacteriol.* **2007**, *189*, 5634–5641.
[182] T. J. Lupoli, T. Taniguchi, T.-S. Wang, D. L. Perlstein, S. Walker, D. E. Kahne, *J. Am. Chem. Soc.* **2009**, *131*, 18230–18231.
[183] J. D. Lenz, E. A. Stohl, R. M. Robertson, K. T. Hackett, K. Fisher, K. Xiong, M. Lee, D. Hesek, S. Mobashery, H. S. Seifert, C. Davies, J. P. Dillard, *J. Biol. Chem.* **2016**, *291*, 10916–10933.
[184] P. Mellroth, T. Sandalova, A. Kikhney, F. Vilaplana, D. Hesek, M. Lee, S. Mobashery, S. Normark, D. Svergun, B. Henriques-Normark, A. Achour, *mBio* **2014**, *5*, e01120-13.
[185] T. Sandalova, M. Lee, B. Henriques-Normark, D. Hesek, S. Mobashery, P. Mellroth, A. Achour, *Mol. Microbiol.* **2016**, *101*, 954–967.
[186] T. Do, K. Schaefer, A. G. Santiago, K. A. Coe, P. B. Fernandes, D. Kahne, M. G. Pinho, S. Walker, *Nat. Microbiol.* **2020**, *5*, 291–303.
[187] N. Lützner, B. Pätzold, S. Zoll, T. Stehle, H. Kalbacher, *Biochem. Biophys. Res. Commun.* **2009**, *380*, 554–558.
[188] F. M. Büttner, S. Zoll, M. Nega, F. Götz, T. Stehle, *J. Biol. Chem.* **2014**, *289*, 11083–11094.
[189] S. Zoll, B. Pätzold, M. Schlag, F. Götz, H. Kalbacher, T. Stehle, *PLOS Pathog.* **2010**, *6*, e1000807.
[190] M. Lee, W. Zhang, D. Hesek, B. C. Noll, B. Boggess, S. Mobashery, *J. Am. Chem. Soc.* **2009**, *131*, 8742–8743.
[191] C. Carrasco-López, A. Rojas-Altuve, W. Zhang, D. Hesek, M. Lee, S. Barbe, I. André, P. Ferrer, N. Silva-Martin, G. R. Castro, M. Martínez-Ripoll, S. Mobashery, J. A. Hermoso, *J. Biol. Chem.* **2011**, *286*, 31714–31722.
[192] I. Rivera, R. Molina, M. Lee, S. Mobashery, J. A. Hermoso, *Microb. Drug Resist.* **2016**, *22*, 470–476.
[193] S. Martínez-Caballero, M. Lee, C. Artola-Recolons, C. Carrasco-López, D. Hesek, E. Spink, E. Lastochkin, W. Zhang, L. M. Hellman, B. Boggess, S. Mobashery, J. A. Hermoso, *J. Am. Chem. Soc.* **2013**, *135*, 10318–10321.
[194] W. Zhang, M. Lee, D. Hesek, E. Lastochkin, B. Boggess, S. Mobashery, *J. Am. Chem. Soc.* **2013**, *135*, 4950–4953.
[195] M. Lee, C. Artola-Recolons, C. Carrasco-López, S. Martínez-Caballero, D. Hesek, E. Spink, E. Lastochkin, W. Zhang, L. M. Hellman, B. Boggess, J. A. Hermoso, S. Mobashery, *J. Am. Chem. Soc.* **2013**, *135*, 12604–12607.
[196] T. Uehara, K. R. Parzych, T. Dinh, T. G. Bernhardt, *EMBO J.* **2010**, *29*, 1412–1422.
[197] T. Uehara, T. Dinh, T. G. Bernhardt, *J. Bacteriol.* **2009**, *191*, 5094–5107.
[198] D. C. Yang, N. T. Peters, K. R. Parzych, T. Uehara, M. Markovski, T. G. Bernhardt, *Proc. Natl. Acad. Sci.* **2011**, *108*, E1052–E1060.
[199] D. C. Yang, K. Tan, A. Joachimiak, T. G. Bernhardt, *Mol. Microbiol.* **2012**, *85*, 768–781.
[200] M. Rocaboy, R. Herman, E. Sauvage, H. Remaut, K. Moonens, M. Terrak, P. Charlier, F. Kerff, *Mol. Microbiol.* **2013**, *90*, 267–277.
[201] N. T. Peters, C. Morlot, D. C. Yang, T. Uehara, T. Vernet, T. G. Bernhardt, *Mol. Microbiol.* **2013**, *89*, 690–701.
[202] C. K. Gurnani Serrano, M. Winkle, A. M. Martorana, J. Biboy, N. Morè, P. Moynihan, M. Banzhaf, W. Vollmer, A. Polissi, *Mol. Microbiol.* **2021**, *116*, 329–342.
[203] J. E. Page, M. A. Skiba, T. Do, A. C. Kruse, S. Walker, *Proc. Natl. Acad. Sci.* **2022**, *119*, e2201141119.
[204] C. A. Feliciano, B. E. Eckenroth, O. R. Diaz, S. Doublié, A. Shen, *PLOS Genet.* **2021**, *17*, e1009791.
[205] S. M. González-Leiza, M. A. de Pedro, J. A. Ayala, *J. Bacteriol.* **2011**, *193*, 6887–6894.
[206] H. Voedts, D. Dorchêne, A. Lodge, W. Vollmer, M. Arthur, J. Hugonnet, *EMBO J.* **2021**, *40*, 1–19.
[207] T. Romeis, J.-V. Höltje, *Eur. J. Biochem.* **1994**, *224*, 597–604.
[208] B. Korat, H. Mottl, W. Keck, *Mol. Microbiol.* **1991**, *5*, 675–684.
[209] S. K. Singh, L. SaiSree, R. N. Amrutha, M. Reddy, *Mol. Microbiol.* **2012**, *86*, 1036–1051.
[210] P. K. Chodisetti, M. Reddy, *Proc. Natl. Acad. Sci.* **2019**, *116*, 7825–7830.
[211] H. Engel, A. van Leeuwen, A. Dijkstra, W. Keck, *Appl. Microbiol. Biotechnol.* **1992**, *37*, 772–783.
[212] R. Bahadur, P. K. Chodisetti, M. Reddy, *Proc. Natl. Acad. Sci.* **2021**, *118*, e2101989118.
[213] M. Winkle, V. M. Hernández-Rocamora, K. Pullela, E. C. A. Goodall, A. M. Martorana, J. Gray, I. R. Henderson, A. Polissi, W. Vollmer, *mBio* **2021**, *12*, e00836-21.
[214] I. P. Sudiarta, T. Fukushima, J. Sekiguchi, *Biochem. Biophys. Res. Commun.* **2010**, *398*, 606–612.
[215] H. Yamamoto, S. Kurosawa, J. Sekiguchi, *J. Bacteriol.* **2003**, *185*, 6666–6677.
[216] T. Fukushima, Y. Yao, T. Kitajima, H. Yamamoto, J. Sekiguchi, *Mol. Genet. Genomics* **2007**, *278*, 371–383.
[217] H. Yamaguchi, K. Furuhata, T. Fukushima, H. Yamamoto, J. Sekiguchi, *J. Biosci. Bioeng.* **2004**, *98*, 174–181.
[218] R. Ohnishi, S. Ishikawa, J. Sekiguchi, *J. Bacteriol.* **1999**, *181*, 3178–3184.
[219] M. Hashimoto, S. Ooiwa, J. Sekiguchi, *J. Bacteriol.* **2012**, *194*, 796–803.
[220] T. Fukushima, A. Afkham, S.-I. Kurosawa, T. Tanabe, H. Yamamoto, J. Sekiguchi, *J. Bacteriol.* **2006**, *188*, 5541–5550.
[221] S. Ishikawa, Y. Hara, R. Ohnishi, J. Sekiguchi, *J. Bacteriol.* **1998**, *180*, 2549–2555.
[222] M.-C. Duchêne, T. Rolain, A. Knoops, P. Courtin, M.-P. Chapot-Chartier, Y. F. Dufrêne, B. F. Hallet, P. Hols, *Front. Microbiol.* **2019**, *10*, 713.
[223] I. J. J. Claes, G. Schoofs, K. Regulski, P. Courtin, M.-P. Chapot-Chartier, T. Rolain, P. Hols, I. von Ossowski, J. Reunanen, W. M. de Vos, A. Palva, J. Vanderleyden, S. C. J. D. Keersmaecker, S. Lebeer, *PLOS ONE* **2012**, *7*, e31588.
[224] J. Gao, X. Zhao, S. Hu, Z. Huang, M. Hu, S. Jin, B. Lu, K. Sun, Z. Wang, J. Fu, R. K. Weersma, X. He, H. Zhou, *Cell Host Microbe* **2022**, *30*, 1435-1449.e9.
[225] E. M. Fernandez, V. Valenti, C. Rockel, C. Hermann, B. Pot, I. G. Boneca, C. Grangette, *Gut* **2011**, *60*, 1050–1059.
[226] K. Regulski, P. Courtin, M. Meyrand, I. J. J. Claes, S. Lebeer, J. Vanderleyden, P. Hols, A. Guillot, M.-P. Chapot-Chartier, *PLOS ONE* **2012**, *7*, e32301.
[227] D. Böth, G. Schneider, R. Schnell, *J. Mol. Biol.* **2011**, *413*, 247–260.
[228] D. J. Martinelli, M. S. Pavelka, *J. Bacteriol.* **2016**, *198*, 1464–1475.

[229] A. Ruggiero, D. Marasco, F. Squeglia, S. Soldini, E. Pedone, C. Pedone, R. Berisio, *Structure* **2010**, *18*, 1184–1190.
[230] B. Kim, Y.-C. Wang, C. W. Hespen, J. Espinosa, J. Salje, K. J. Rangan, D. A. Oren, J. Y. Kang, V. A. Pedicord, H. C. Hang, *eLife* **2019**, *8*, e45343.
[231] J. Espinosa, T.-Y. Lin, Y. Estrella, B. Kim, H. Molina, H. C. Hang, *Biochemistry* **2020**, *59*, 4470–4480.
[232] K. J. Rangan, V. A. Pedicord, Y.-C. Wang, B. Kim, Y. Lu, S. Shaham, D. Mucida, H. C. Hang, *Science* **2016**, *353*, 1434–1437.
[233] L. Ramadurai, K. J. Lockwood, J. Lockwood, M. J. Nadakavukaren, R. K. Y. 1999 Jayaswal, *Microbiology* **1999**, *145*, 801–808.
[234] V. Raulinaitis, H. Tossavainen, O. Aitio, J. T. Juuti, K. Hiramatsu, V. Kontinen, P. Permi, *Sci. Rep.* **2017**, *7*, 6020.
[235] M. Bobrovskyy, S. E. Willing, O. Schneewind, D. Missiakas, *J. Bacteriol.* **2018**, *200*, e00268-18.
[236] M. B. Frankel, A. P. A. Hendrickx, D. M. Missiakas, O. Schneewind, *J. Biol. Chem.* **2011**, *286*, 32593–32605.
[237] S. Layec, J. Gérard, V. Legué, M.-P. Chapot-Chartier, P. Courtin, F. Borges, B. Decaris, N. Leblond-Bourget, *Mol. Microbiol.* **2009**, *71*, 1205–1217.
[238] S. K. Singh, S. Parveen, L. SaiSree, M. Reddy, *Proc. Natl. Acad. Sci.* **2015**, *112*, 10956–10961.
[239] W.-J. Jeon, H. Cho, *Front. Microbiol.* **2022**, *13*, 878049.
[240] Y. J. Kim, B. J. Choi, S. H. Park, H. B. Lee, J. E. Son, U. Choi, W.-J. Chi, C.-R. Lee, *Front. Microbiol.* **2021**, *12*, 677739.
[241] M. Banzhaf, H. C. Yau, J. Verheul, A. Lodge, G. Kritikos, A. Mateus, B. Cordier, A. K. Hov, F. Stein, M. Wartel, M. Pazos, A. S. Solovyova, E. Breukink, S. van Teeffelen, M. M. Savitski, T. den Blaauwen, A. Typas, W. Vollmer, *EMBO J.* **2020**, *39*, e102246.
[242] D. Chakraborty, A. J. Darwin, *J. Bacteriol.* **2021**, *203*, e00393-21.
[243] D. Srivastava, J. Seo, B. Rimal, S. J. Kim, S. Zhen, A. J. Darwin, *mBio* **2018**, *9*, e00972-18.
[244] J. H. Shin, A. G. Sulpizio, A. Kelley, L. Alvarez, S. G. Murphy, L. Fan, F. Cava, Y. Mao, M. A. Saper, T. Dörr, *Proc. Natl. Acad. Sci.* **2020**, *117*, 11692–11702.
[245] H. Takada, H. Yoshikawa, *Biosci. Biotechnol. Biochem.* **2018**, *82*, 741–751.
[246] G. S. Dobihal, Y. R. Brunet, J. Flores-Kim, D. Z. Rudner, *eLife* **2019**, *8*, e52088.
[247] P. Bisicchia, D. Noone, E. Lioliou, A. Howell, S. Quigley, T. Jensen, H. Jarmer, K. M. Devine, *Mol. Microbiol.* **2007**, *65*, 180–200.
[248] L. I. Salzberg, L. Powell, K. Hokamp, E. Botella, D. Noone, K. M. Devine, *Mol. Microbiol.* **2013**, *87*, 180–195.
[249] R. Plocinska, G. Purushotham, K. Sarva, I. S. Vadrevu, E. V. P. Pandeeti, N. Arora, P. Plocinski, M. V. Madiraju, M. Rajagopalan, *J. Biol. Chem.* **2012**, *287*, 23887–23899.
[250] E. C. Hett, M. C. Chao, E. J. Rubin, *PLOS Pathog.* **2010**, *6*, e1001020.
[251] D. C. Osipovitch, K. E. Griswold, *FEMS Microbiol. Lett.* **2015**, *362*, 1–7.
[252] D. C. Osipovitch, S. Therrien, K. E. Griswold, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 6315–6326.
[253] A. Vermassen, R. Talon, C. Andant, C. Provot, M. Desvaux, S. Leroy, *Microorganisms* **2019**, *7*, 559.
[254] J. Mo, J. P. Boyle, C. B. Howard, T. P. Monie, B. K. Davis, J. A. Duncan, *J. Biol. Chem.* **2012**, *287*, 23057–23067.
[255] C. L. Grimes, L. D. Z. Ariyananda, J. E. Melnyk, E. K. O'Shea, *J. Am. Chem. Soc.* **2012**, *134*, 13535–13537.
[256] M. E. Griffin, J. Espinosa, J. L. Becker, J. D. Luo, T. S. Carroll, J. K. Jha, G. R. Fanger, H. C. Hang, *Science* **2021**, *373*, 1040–1046.
[257] V. A. Pedicord, A. A. K. Lockhart, K. J. Rangan, J. W. Craig, J. Loschko, A. Rogoz, H. C. Hang, D. Mucida, *Sci. Immunol.* **2016**, *1*, aai7732.
[258] P. Courtin, G. Miranda, A. Guillot, F. Wessner, C. Mézange, E. Domakova, S. Kulakauskas, M. P. Chapot-Chartier, *J. Bacteriol.* **2006**, *188*, 5293–5298.
[259] D. E. Nelson, K. D. Young, *J. Bacteriol.* **2000**, *182*, 1714–1721.
[260] S. M. Barendt, L.-T. Sham, M. E. Winkler, *J. Bacteriol.* **2011**, *193*, 2290–2300.
[261] D. E. Nelson, K. D. Young, *J. Bacteriol.* **2001**, *183*, 3055–3064.
[262] D. Esson, S. Gupta, D. Bailey, P. Wigley, A. Wedley, A. E. Mather, G. Méric, P. Mastroeni, S. K. Sheppard, N. R. Thomson, J. Parkhill, D. J. Maskell, G. Christie, A. J. Grant, *Microb. Pathog.* **2017**, *104*, 202–211.
[263] L. K. Sycuro, T. J. Wyckoff, J. Biboy, P. Born, Z. Pincus, W. Vollmer, N. R. Salama, *PLOS Pathog.* **2012**, *8*, e1002603.
[264] H. S. Kim, H. N. Im, D. R. An, J. Y. Yoon, J. Y. Jang, S. Mobashery, D. Hesek, M. Lee, J. Yoo, M. Cui, S. Choi, C. Kim, N. K. Lee, S.-J. Kim, J. Y. Kim, G. Bang, B. W. Han, B. I. Lee, H. J. Yoon, S. W. Suh, *J. Biol. Chem.* **2015**, *290*, 25103–25117.
[265] E. Frirdich, J. Vermeulen, J. Biboy, F. Soares, M. E. Taveirne, J. G. Johnson, V. J. DiRita, S. E. Girardin, W. Vollmer, E. C. Gaynor, *J. Biol. Chem.* **2014**, *289*, 8007–8018.
[266] K. Min, D. R. An, H.-J. Yoon, N. Rana, J. S. Park, J. Kim, M. Lee, D. Hesek, S. Ryu, B. M. Kim, S. Mobashery, S. W. Suh, H. H. Lee, *Nat. Commun.* **2020**, *11*, 458.
[267] M. Bonis, C. Ecobichon, S. Guadagnini, M.-C. Prévost, I. G. Boneca, *Mol. Microbiol.* **2010**, *78*, 809–819.
[268] E. Sacco, J.-E. Hugonnet, N. Josseaume, J. Cremniter, L. Dubost, A. Marie, D. Patin, D. Blanot, L. B. Rice, J.-L. Mainardi, M. Arthur, *Mol. Microbiol.* **2010**, *75*, 874–885.
[269] J. D. Lenz, K. T. Hackett, J. P. Dillard, *mBio* **2017**, *8*, e01464-17.
[270] G. Rico-Pérez, A. Pezza, M. G. Pucciarelli, M. A. de Pedro, F. C. Soncini, F. García-del Portillo, *Mol. Microbiol.* **2016**, *99*, 546–556.
[271] S. B. Hernández, T. Dörr, M. K. Waldor, F. Cava, *Cell Rep.* **2020**, *31*, 107578.
[272] A. Maqbool, M. Hervé, D. Mengin-Lecreulx, A. J. Wilkinson, G. H. Thomas, *Biochem. J.* **2012**, *448*, 329–341.
[273] T. Uehara, J. T. Park, *J. Bacteriol.* **2003**, *185*, 679–682.
[274] T. Miyamoto, M. Katane, Y. Saitoh, M. Sekine, H. Homma, *Amino Acids* **2020**, *52*, 487–497.
[275] V. V. Nemmara, S. A. Adediran, K. Dave, C. Duez, R. F. Pratt, *Biochemistry* **2013**, *52*, 2627–2637.
[276] D. Vega, J. A. Ayala, *Arch. Microbiol.* **2006**, *185*, 23–27.
[277] C. Duez, M. Vanhove, X. Gallet, F. Bouillenne, J. Docquier, A. Brans, J. Frère, *J. Bacteriol.* **2001**, *183*, 1595–1599.
[278] V. V. Nemmara, L. Dzhekieva, K. Subarno Sarkar, S. A. Adediran, C. Duez, R. A. Nicholas, R. F. Pratt, *Biochemistry* **2011**, *50*, 10091–10101.
[279] C. N. Hoyland, C. Aldridge, R. M. Cleverley, M.-C. Duchêne, G. Minasov, O. Onopriyenko, K. Sidiq, P. J. Stogios, W. F. Anderson, R. A. Daniel, A. Savchenko, W. Vollmer, R. J. Lewis, *Structure* **2014**, *22*, 949–960.
[280] E. J. Culp, N. Waglechner, W. Wang, A. A. Fiebig-Comyn, Y.-P. Hsu, K. Koteva, D. Sychantha, B. K. Coombes, M. S. Van Nieuwenhze, Y. V. Brun, G. D. Wright, *Nature* **2020**, *578*, 582–587.
[281] S. Tomoshige, D. A. Dik, M. Akabane-Nakata, C. S. Madukoma, J. F. Fisher, J. D. Shrout, S. Mobashery, *ACS Infect. Dis.* **2018**, *4*, 860–867.
[282] C. T. Tan, X. Xu, Y. Qiao, Y. Wang, *Nat. Commun.* **2021**, *12*, 2560.
[283] Z. Huang, J. Wang, X. Xu, H. Wang, Y. Qiao, W. C. Chu, S. Xu, L. Chai, F. Cottier, N. Pavelka, M. Oosting, L. A. B. Joosten, M. Netea, C. Y. L. Ng, K. P. Leong, P. Kundu, K. P. Lam, S. Pettersson, Y. Wang, *Nat. Microbiol.* **2019**, *4*, 766–773.
[284] A. Gonzalez-Santana, R. Diaz Heijtz, *Trends Mol. Med.* **2020**, *26*, 729–743.
[285] P. A. D. Bastos, R. Wheeler, I. G. Boneca, *FEMS Microbiol. Rev.* **2021**, *45*, 1–25.
[286] A. J. Wolf, D. M. Underhill, *Nat. Rev. Immunol. 2017 184* **2018**, *18*, 243–254.

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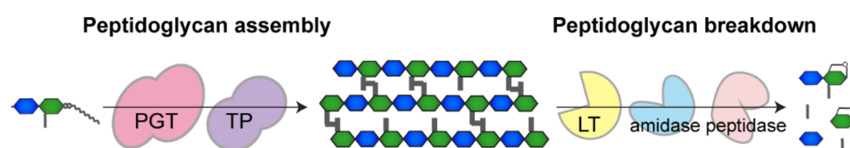

Bacteria encode multiple enzymes to build and break down the essential peptidoglycan polymer. We discuss recent biochemical characterizations of major peptidoglycan enzymes, which elucidate their activities, substrate preferences, regulation and potential inhibition strategies. These insights are valuable for the development of peptidoglycan-targeting antibiotic drugs, as well as for harnessing the immune-stimulatory effects of bacterial peptidoglycan fragments.

Institute and/or researcher Twitter usernames: @yuanqiao1002

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