Cell-wall recycling and synthesis in Escherichia coli and Pseudomonas aeruginosa – their role in the development of resistance

Supurna Dhar,1 Hansi Kumari,1 Deepak Balasubramanian2 and Kalai Mathee1,3,*

INTRODUCTION

The cell-wall is an essential component of bacterial architecture that confers cell shape, prevents lysis under fluctuating internal turgor pressure and protects from external assaults, thus enabling their ubiquitous existence. The cell-wall is composed of tightly cross-linked peptidoglycan (PG) which encircles the inner membrane and forms a protective layer known as the murein sacculus [1]. The murein sacculi vary in size and chemical content in different species. In Gram-negatives such as E. coli, the cell-wall is approximately 6 nm, whereas in the Gram-positives it may span up to 80 nm providing the capacity to withstand higher amounts of turgor pressure [2–4]. Extensive remodelling of the cell-wall occurs during bacterial cell growth and division. Thus, recycling of the cell-wall components is critical to conserve resources. Both Gram-positive and -negative bacteria recycle almost 40–50% of the cell-wall components [5, 6]. Cell-wall recycling and synthesis are tightly coordinated to preserve bacterial integrity. Since the survival of bacteria critically depends on their PG-based cell-walls, it is a selective target of many antibiotics. Hence, the development of antibiotic resistance in bacteria is intimately tied in with cell-wall synthesis and recycling [7]. Both these processes have been studied in Citrobacter freundii, Enterobacter cloacae and E. coli and to some extent in P. aeruginosa [7–10].

P. aeruginosa is listed as a pathogen of a serious threat (level 2) by the US Center for Disease Control and Prevention as it causes 51 000 infections per year; of which 6700 are multi-drug resistance with 440 deaths [11]. Furthermore, carbapenem-resistant P. aeruginosa is also one of the three bacterial species listed by the World Health Organization as a pathogen of critical priority [12]. This bacterium is also one of the ESKAPE (Enterococcus faecium, Staphylococcus
**CELL-WALL BIOCHEMISTRY**

The bacterial murein sacculus is composed of a heteropolysaccharide of N-acetyl muramic acid (MurNAc) and N-acetyl-glucosamine (GlcNAc) linked by β-1→4 glycosidic linkages, with a short chain peptide containing up to five amino acids, attached to the muramyl moiety [20–22]. The full-length pentapeptide side chain in Gram-negative bacteria is typically composed of L-alanine-γ-D-glutamate- meso-diaminopimelic acid-D-alanyl-D-alanine (L-Ala-γD-Glu-m-DAP-D-Ala-D-Ala) [23]. Cross-linking in Gram-negative bacteria between two adjacent peptide chains commonly occurs between the third residue of one chain (m-DAP) and the fourth residue (D-Ala) of the other [21, 24]. High-performance liquid chromatography analysis of purified murein sacculus has shown that the extent of cross-linking is comparable between *E. coli* and *P. aeruginosa* [25]. This cross-linking creates a mesh-like structure, which confers murein sacculus the strength to withstand internal pressure.

The PG in many Gram-negatives including *E. coli* and *P. aeruginosa* has linked proteins known as Braun’s lipoprotein [25–27]. The covalent linkage of this lipoprotein between the outer membrane and the murein sacculus contributes further to the stability of the cell-wall. There is a considerable variation in the thickness of the murein sacculus amongst Gram-negative bacteria. For instance, the PG thickness of *E. coli* is 6.35±0.53 nm whereas *P. aeruginosa* is at 2.41±0.54 nm [3]. Despite the variations found in the details of the structural architecture in the Gram-negatives, the cell-wall is highly conserved across bacteria promoting its use as a valuable antibiotic target.

**CELL-WALL BIOSYNTHESIS**

The biosynthesis of the cell-wall can be divided into three phases based on the localization of the processes; namely those occurring in the cytoplasm, the inner leaflet of the cytoplasmic membrane and the periplasm (Fig. 1).

**Escherichia coli**

**Cytoplasm**

Fructose-6-phosphate is converted to uridine-diphosphate (UDP)-GlcNAc through a series of steps driven by the activities of the GlmS, GlmM and GlmU [28–30]. UDP-GlcNAc is further converted to UDP-MurNAc pentapeptide by the activity of the Mur group of enzymes (MurA, MurB, MurC, MurD, MurE and MurF) [31, 32]. To this pentapeptide, a 55-carbon aliphatic chain called undecaprenyl pyrophosphate is attached by MraY [33]. This aliphatic moiety is attached to the inner face of the cytoplasmic membrane to form lipid I [34, 35].

**Inner membrane**

MurG catalyses the addition of GlcNAc to the MurNAc moiety of lipid I to yield lipid II [32, 36]. Lipid II is flipped over into the periplasmic space using a ‘flippase’ enzyme [37]. The identity of ‘flippase’ remains ambiguous and is the subject of current research [38–40]. In *E. coli*, FtsW and Mur have been independently shown to be the proteins responsible for translocation of the lipid-linked muropeptides from the cytoplasm into the periplasmic space [41–44]. Furthermore, in *E. coli* the transport and subsequent polymerization of lipid II by FtsW is regulated via PBP1B and PBP3 [44].

**Periplasm**

The final steps of the PG synthesis involve the incorporation of the GlcNAc-MurNAc pentapeptide component of lipid II into the rapidly evolving murein sacculus. High molecular mass penicillin-binding proteins (HMM PBPs) with a transglycosylase domain (PBP1A, PBP1B and PBP1C) facilitate the link between the MurNAc end of lipid II with the GlcNAc of the existing strand [45–50]. HMM PBPs with a transpeptidase domain (PBP1A, PBP1B, PBP1C, PBP2 and PBP3) catalyse the formation of cross-links between two peptide chains [47, 51–54] (Table 1). Cross-linking is composed of two steps wherein the terminal D-Ala-D-Ala of the peptide chain attached to MurNAc is cleaved with the release of one alanine [55]. The murein tetrapeptide so formed is known as the ‘donor’ peptide. Subsequently, the terminal D-alanine of the tetrapeptide attaches to the meso-diaminopimelic acid of an ‘acceptor’ peptide chain [55].

**Pseudomonas aeruginosa**

Bioinformatics analyses suggest the presence of *E. coli* homologues in *P. aeruginosa*; however, the enzymatic characterization for many of these proteins is yet to be performed (Table 1).
Cytoplasm

Sequence comparison reveals homologues of GlmM (PA4749), GlmS (PA5549) and GlmU (PA5552) that aids in the synthesis of UDP-GlcNAc from fructose-6-phosphate (Table 1). In *P. aeruginosa* glucosamine-6-phosphate synthetase GlmS is under the regulation of a repressor GlmR (PA5550) [56]. A transcriptional factor NagR regulates both GlmS and GlmU in *E. coli*, highlighting the difference in...
regulation of PG saccharide GlcNAc between the two organisms [57]. In addition to protein homology, the functional activity of GlmM as a phosphoglucosamine mutase in *P. aeruginosa* has also been established [58]. Homologues of the Mur enzymes that lead to the formation of UDP-MurNAc pentapeptide from UDP-GlcNAc namely an enolpyruvyl transferase MurA (PA4450), a reductase MurB (PA2977), as well as the Mur ligases, MurC (PA4411), MurD (PA4414), MurE (PA4417) and MurF (PA4416) are also encoded in the *P. aeruginosa* genome (Table 1). The activity of the *P. aeruginosa* Mur enzymes were analysed by providing the initial substrate for MurA, UDP-GlcNAc and detecting the formation of the final product UDP-MurNAc-pentapeptide [59]. The enzymatic properties of all the Mur ligases of *P. aeruginosa* were also heterologously characterized in *E. coli* [60, 61]. *P. aeruginosa* also encodes a homologue of Mpl ligase (PA4020) which facilitates ligation of tri-, tetra- and pentapeptides to UDP-MurNAc (Table 1). The cytosolic translocase MraY (PA4415) then transfers the UDP-MurNAc pentapeptide to an undecaprenol carrier group that anchors the muropeptide to the inner membrane (Table 1) [62].

### Inner membrane

*P. aeruginosa* harbours inner membrane glycosyltransferase MurG (PA4412) and flipases FtsW (PA4413) and MviN (PA4562) (Table 1). A crystal structure of *P. aeruginosa* MurG has been solved in complex with its substrate, UDP-GlcNAc [63].

### Periplasm

The periplasmic cell-wall synthesis enzymes for polymerization and cross-linking of the muropeptides include the HMM PBPs, namely PBP1A (PA5045), PBP1B (PA4700), PBP2 (PA4003) and PBP3 (PA4418) (Table 1) [64–67].

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**Table 1. List of genes involved in cell-wall synthesis**

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<th>Proteins</th>
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<th>Protein homology (%)</th>
<th>Functional annotation</th>
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The data is compiled from the EcoCyc and Pseudomonas Genome database website [260, 261].

*The value for protein sequence identity and similarity of *E. coli* and *P. aeruginosa* was obtained using CLUSTALW2 ‘pairwise sequence alignments’.

NF, not found.
sequence homology to *E. coli*, PBP1A (PA5045) and 1B (PA4700) contain both transglycosylase and transpeptidase domains whereas PBP2 (PA4003) and PBP3 (PA4418) have the domain for transpeptidase activity [68, 69]. In addition, *P. aeruginosa* also contains an additional transpeptidase, PBP3B/3X (PA2272), which is not found in *E. coli* [70]. *P. aeruginosa* PBP3 and 3B expression differs temporally, the former being upregulated during the exponential and the latter during stationary growth-phase [70].

**PEPTIDOGLYCAN GROWTH**

To ensure bacterial growth (elongation and division) while maintaining cellular integrity, both PG degradative enzymes such as low molecular mass penicillin-binding proteins (LMM PBP), lytic transglycosylases (LT), and amidases and PG synthesize enzymes such as HMM PBPs must perform in a coordinated fashion along with the various bacterial cytoskeletal elements to avoid cell lysis [71]. The growth of the murein sacculus has been studied extensively in *E. coli* by Park *et al*. [72, 73]. Using radioactively labelled PG they demonstrated that newly formed PG strands are cross-linked with the existing strand at multiple sites. At the same time, the cross-links in the existing murein strand are degraded by endopeptidases [73]. A three-for-one growth model suggests that the existing strand acts as a dock that incorporates three newly synthesized murein chains [74]. The soluble PG fragments that are generated during this progressive replacement of the existing murein with the new strand are recycled [75].

**CELL-WALL RECYCLING**

As the cells divide, the walls break and rejoin at every cycle, thus generating muropeptides of varying lengths. Gram-positives such as *Bacillus* sp., *Lactobacillus acidophilus* release close to 25–30% of their muropeptides into the culture media [6, 76, 77]. Only 6–8% extracellular muropeptides were detected for *E. coli* [75]. The latter finding gave rise to the hypothesis that the Gram-negatives recycle their muropeptides. The hypothesis was confirmed by the detection of tritiated-DAP reuse by the bacteria during cell growth [5]. Extensive studies in *E. coli* have provided the knowledge on cell-wall recycling in Gram-negatives [78] (Fig. 1). Although *P. aeruginosa* harbours the orthologous genes, their functional roles remain to be elucidated [79] (Table 2). The following section compares cell-wall recycling in Gram-negative *E. coli* and *P. aeruginosa*. The pathway is broken down into processes occurring in the subcellular compartments; namely the periplasm, inner membrane and cytoplasm (Figs 1 and 2).

**Periplasm**

Mainly, three types of lytic enzymes are found in the periplasmic space that creates the muropeptide intermediates (Figs 1 and 2). These include the LTs, the LMM PBPs and the amidases [78, 80].

**Lytic transglycosylases (LTs)**

The LTs act on the murein sacculus to release their signature product GlcNAc 1,6-anhydro-MurNAc [81] (Fig. 1). The LTs facilitate PG remodelling, insertion of membrane-associated structures such as flagella and the secretion systems, and help in cell separation during division [82–84].

Investigation of the *E. coli* open reading frames led to the identification of eight genes encoding LTs; *mltA*, *mltB*, *mltC*, *mltD*, *mltE* (*emtA*), *mltF*, *mltG* and *slt70* [81, 85–90]. The enzymatic activities of all the *E. coli* LTs were demonstrated using the murein sacculus isolated from the log and stationary phase as a substrate [85]. All the LTs are exolytic and cleave at a terminus of the PG strand. However, MltE, MltD, MltB, Slt70 (SltY) and MltC are endolytic and cleave in the middle of a PG strand [85]. Of these, MltA was reported to have the highest activity on purified murein sacculus. All the enzymes show a higher activity on the murein sacculus isolated during the log phase than the stationary phase. The higher activity is in accordance with the extensive remodelling that occurs at the log phase [85]. It may also be due to the structural differences in the sacculus during bacterial growth, and as well as the lack of cell division at stationary phase [23]. Deletion analyses demonstrate the presence of extensive functional redundancy within this group of eight genes encoding LTs [83]. *E. coli* tolerates the loss of up to three LTs, but deletion of all eight leads to cell death [83, 91, 92].

The LTs in *P. aeruginosa* were first identified using renaturing PAGE and zymograms [93, 94]. A total of 11 LTs, MltA (PA1222), MltB (PA4444), MltD (PA1812), MltF (PA3764), MltF2 (PA2865), MltG (PA2963), Slt (PA3020), SltB2 (PA1171), SltH/Slt3 (PA3992) and RlpA (PA4000) were reported in *P. aeruginosa* [95–99]. A few studies separately enumerate the enzymatic activities of all the LTs [97, 99, 100]. *In vitro* analyses with purified enzymes demonstrated that all the *P. aeruginosa* LTs have both endolytic and exolytic activity. MltD, MltF2, RlpA and Slt exhibited higher endolytic activity whereas MltA, SltB1, SltB2 and SltB3 had higher exolytic activity [99]. The most and least active enzymes are SltB1 and MltF2, respectively [99]. Some of these enzymes exhibit substrate preference – MltB specifically requires a peptide chain on the muropeptide to demonstrate its lytic activity whereas RlpA acts on the degradation products of amidases, namely GlcNAc-anh-MurNAc without the attached peptide chains [97, 99] (Fig. 2).

**Low molecular mass penicillin-binding proteins (LMM PBPs)**

The LMM PBPs act mostly as endopeptidases and or carboxypeptidases [24, 51, 101]. The endopeptidase activity of PBPs leads to hydrolysis of the cross-bridge between m-DAP and d-Ala, whereas the carboxypeptidases remove the terminal amino acid from a pentapeptide during cross-linking [24, 101, 102].
E. coli has five LMM PBPs, namely PBP4/DacB, PBP5/DacA, PBP6/DacC, PBP6b/DacD and PBP7/8/PbpG [74, 79, 103, 104] (Table 2). The PBP4/DacB is a bifunctional peptidase with both carboxy and endopeptidase activities [105]. Together with PBP5/DacA, PBP4/DacB is involved in maintaining normal cell morphology [105–107]. PBP5/DacA is found attached to the inner membrane [108]. It is the major carboxypeptidase in E. coli; deletion of dacA is associated with cell-shape defects and a striking increase in free pentapeptides [109–112]. PBP6/DacC and PBP6b/DacD exhibit carboxypeptidase activities [113–115]. An increase in expression levels of dacC is noted during the stationary phase [116].

Another endopeptidase PBP7/PbpG and its proteolytic product PBP8 have been identified in E. coli [117]. Loss of pbpG did not result in any morphological aberrations, however, the absence of both dacA and pbpG encoding PBP5 and PBP7,
### Table 2. List of genes involved in cell-wall recycling

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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MupP</td>
<td>NF PA3172</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MurU</td>
<td>NF PA0597</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NagA</td>
<td>nagA PA3758</td>
<td>30</td>
<td>47</td>
</tr>
<tr>
<td>Mpl</td>
<td>mpl PA4020</td>
<td>59</td>
<td>72</td>
</tr>
<tr>
<td>NagB</td>
<td>nagB NF</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
respectively resulted in increased cell-shape defects as compared to the loss of dacA alone [106, 118].

Expression of dacA and dacC is regulated by the BolA protein, which is highly conserved in eukaryotes and prokaryotes [119, 120]. BolA maintains cell morphology in both a PBP5/PBP6-dependent and -independent manner [121]. BolA regulates the carboxypeptidase activities of DacA/PBP5 and DacC/PBP6, and overexpression of bolA showed a decreased rate of growth which was lost upon deletion of both dacA and dacC [119, 120]. Overexpression of bolA also impaired PBP2-mediated cell elongation and formation of cytoskeleton due to spatial disorganization of MreB [121].

LMM PBPs in *P. aeruginosa* include PBP4/DacB (PA3047), PBP5/DacC (PA3999) and PBP7/PbpG (PA0869) [66, 122–125]. Loss of dacC results in increased pentapeptides establishing its role as the primary carboxypeptidase in *P. aeruginosa* [123, 125, 126]. *P. aeruginosa* encodes a homologue of BolA (PA0857), which shows a 46% identity to their *E. coli* counterpart. However, its role as a regulator of carboxypeptidases in *P. aeruginosa* is yet to be investigated. An additional increase of pentapeptides is also seen upon deletion of dacB and pbpG, confirming the presence of multiple carboxypeptidases [125]. PBP4/DacB and PBP7/PbpG also act as endopeptidases [122, 124, 125]. *P. aeruginosa* appears to not harbour PBP6. However, *P. aeruginosa* PBP5 shares 48% homology with *E. coli* PBP6.

**Amidases**

The periplasmic amidases remove the amino acid chain from the muramyl moieties of the sacculus as well as from the recycling by-products such as GlcNAc-ahNurNAc peptides [127–131]. *E. coli* expresses four periplasmic enzymes AmiA, AmiB, AmiC and AmiD that have N-acetylmuramyl-L-alanine amidase activity [129, 132, 133]. AmiA, AmiB and AmiC have a high sequence homology and assist in cell separation during division [133]. Loss of these three amidases affects septal formation resulting in a chaining phenotype with aggregates of three to six cells [83, 130]. The activity of these three enzymes is regulated spatio-temporally by proteins with LytM domains, which have conserved sequences and are collectively known as dLytM factors [134]. EnvC and NlpD are dLytM proteins [134]. EnvC belongs to the divisome group of proteins that participate in cytokinesis and activates AmiA and AmiB, whereas NlpD regulates AmiC activity [135]. AmiD is tethered to the outer membrane and does not contribute to cell separation, and its loss has no effect on the cell-wall morphology [131, 136]. AmiD cleaves at both N-acetylmuramyl and 1,6-anhydro-N-acetylmuramyl peptides and plays a critical role in generating fragments for recycling [80, 130, 131]. The loss of amiD resulted in an accumulation of GlcNAc-ahNurNAc tri- and tetrapeptides [131].

*P. aeruginosa* harbours periplasmic AmiA (PA5538) and AmiB (PA4947). Both these proteins share homology with their *E. coli* counterparts and have N-acetylmuramyl-L-alanine amidase activity [137]. Deletion of amiA exhibited no significant effect on *P. aeruginosa* cell viability or morphology [138]. AmiB (PA4947) in contrast to *E. coli* AmiB was found to be essential for *P. aeruginosa* survival [138]. *P. aeruginosa* AmiB is required for cell separation during division, as deletion resulted in filamentous growth with a marked deficiency in the invagination of the inner membrane. Additionally, AmiB depletion decreased outer membrane impermeability for which the mechanism is unclear [138]. *P. aeruginosa* AmiB requires the presence of additional activator dLytM proteins such as NlpD, NlpS and EnvC [138].

*P. aeruginosa* also has two periplasmic amidases, AmpDh2 (PA5485) and AmpDh3 (PA0807), that play a critical role in cell-wall recycling [139]. Both enzymes have significant homology with *E. coli* AmiD (Table 2). AmpDh2, akin to AmiD is anchored to the outer membrane [131, 140]. Both AmpDh2 and AmpDh3 process the peptide chain on the polymeric murein sacculus as well as 1,6-anhydromuramyl peptides generated through the activities of LTs [141]. The preferred substrate, however, for both these amidases is the murein sacculus [141]. Comparing the activity between these two amidases, AmpDh3 has a higher specific activity on the murein sacculus [140–142]. Crystal structures and catalytic activities of all the *P. aeruginosa* cell-wall recycling amidases have been reviewed elsewhere [143].

**Inner membrane**

The muropeptides that are generated by the periplasmic enzymes are transported into the cytoplasm via the inner membrane proteins which play an indispensable role in the recycling process. The inner membrane AmpG permease facilitates the diffusion of muropeptides from the periplasm into the cytoplasm [7, 144] (Figs 1 and 2). The *ampG* gene was first identified in *Enterobacter cloacae* [145]. Since then, it has been found in other members of Enterobacteriaceae including *C. freundii*, *Serratia marcescens* and *Salmonella* sp. The earliest indications that AmpG may be the primary permease in cell-wall recycling came from Normark’s lab during the investigation of its role in β-lactamase expression [146]. Its role as a permease was confirmed when an *ampG* deletion resulted in the increased release of muropeptides in the media which were rescued upon complementation in *trans* [7]. *E. coli* AmpG transports muropeptides that contain the disaccharide units GlcNAc-ahNurNAc across the inner membrane into the cytoplasm [147]. AmpG has 10 transmembrane helices, and *ampG* is the second open
reading frame in a two-gene operon which may be post-
transcriptionally regulated [146, 148]. Besides AmpG, E. coli
also has another unique permease system, OppBCDF, which
complexes with the muropeptide-binding protein MppA and
plays a minor role in PG recycling [144, 149] (Fig. 1).

P. aeruginosa harbours two AmpG homologues, PA4393
(AmpG) and PA4218 (AmpP) (Table 2) [150, 151]. Both
AmpG and AmpP are inner membrane permeases with 14
and 10 transmembrane helices, respectively [151]. The
expression of ampG and ampP is regulated by the transcrip-
tional regulator AmpR (PA4109) in an inducer (sub-MIC of
β-lactam)-dependent and -independent manner, respec-
tively [18]. Both ampG and ampP are the second genes in
independent two-gene operons [151]. An uptake assay using
fluorophore-conjugated muropeptides demonstrated that
P. aeruginosa AmpG transports any muropeptides contain-
ing GlcNAc-1,6-anhydro-MurNAc [152]. However, the
in vivo role of AmpG and AmpP in muropeptide transport
remains to be elucidated. AmpG activity can be inhibited by
the proton gradient uncoupler, carbonyl cyanide m-chloro-
phenyl hydrazine, suggesting that its activity is dependent
on single proton motive force [153].

An alternative route of transfer of GlcNAc into the cyto-
plasm using NagE exists for both E. coli and P. aeruginosa
[154]. The NagE phosphotransferase phosphorylates and
imports GlcNAc into the cytoplasm, adding to the cyto-
plasmic pool of GlcNAc-6-P [154]. In E. coli, the absence of
nagE resulted in a decrease of 50% of GlcNAc-6-P in the
cytoplasm [154]. Whether this phosphotransferase plays a
prominent role in cell-wall recycling in P. aeruginosa is yet
to be explored. However, P. aeruginosa nagE (PA3761)
mutants cannot grow using GlcNAc as the sole carbon
source, suggesting a significant role for NagE in GlcNAc
uptake [155]. In E. coli, the uptake and metabolism of Mur-
NAc are mediated by a specific phosphotransferase MurP
[156]. This transporter is required for the growth of this
bacterium in the presence of MurNAc as the sole carbon
source [156]. MurP has not been identified in P. aeruginosa.

Cytoplasm

Once in the cytoplasm, the muropeptides are processed by
diverse enzymes to form lipid II composed of UDP-
GlcNAcMurNac pentapeptide attached to a hydrophobic
undecaprenol-pyrophosphate group [33, 36]. Lipid II is
flipped across the cytoplasmic membrane into the periplas-
mic space, where GlcNAcMurNAc pentapeptide is reincor-
porated into the growing cell-wall (Figs 1 and 2). There is
an overlap between the enzymes mediating these reactions in
E. coli and P. aeruginosa; however, certain enzymes remain
unique to each bacterial system. These enzymes are
detailed below.

NagZ

In the cytoplasm, the β-N-acetylglucosaminidase NagZ pro-
cesses the muropeptides [157] (Fig. 1). The enzymatic ac-
cticity of NagZ was first identified in E. coli K-12 mutant strains
which showed a deficiency in β-N-acetylglucosaminidase
[157]. The mutation was mapped to ycfO that was renamed
as nagZ following functional characterization of the protein
[157–159]. NagZ cleaves the bond between GlcNAc and
1,6-anhydro-MurNAc, leading to the formation of inde-
pendent units of GlcNAc and 1,6-anhMurNAc peptides
[157]. Deletion of the nagZ gene results in the accumulation
of GlcNAc-anhMurNAc peptides [157, 160]. Bioinformatics
analyses of nagZ sequences led to its identification in
numerous Gram-negative pathogens such as Klebsiella
pneumonia, Vibrio cholera, P. aeruginosa, Haemophilus
influenza and Bordetella pertussis among others [150].

P. aeruginosa nagZ (PA3005) is predicted as the first gene in
a two-gene operon along with PA3004, which encodes a
phosphorylase. Similar to E. coli, NagZ (PA3005) was dem-
onstrated to act as a β-N-acetylglucosaminidase [161].
Crystal structures of NagZ were solved along with its ligand.
Using X-ray structures and molecular dynamics simulation,
the enzymatic details of NagZ activity was elucidated [162].
Most of the studies with NagZ (PA3005) have been carried
out on its role in regulating antibiotic resistance, which will
be discussed in the following sections.

AmpD and LdcA

E. coli AmpD cleaves the peptide chain exclusively attached
to the 1,6-anhydromuramoyl moieties [163, 164]. The first
indication that this gene may play a critical role in cell-wall
recycling stemmed from observations of its influence on β-
lactamase induction [165]. Its specific role as an amidase in
recycling was confirmed when it was noticed that the loss of
ampD resulted in the accumulation of GlcNAc-anhMurNAc
with peptide chains [163, 164]. Subsequently, a major increase of anhydromuramyl tripeptides in ampD-deficient
E. coli cells led to the identification of an LD-carboxypepti-
dase LdcA [166]. This enzyme removes the terminal D-alan-
ine from the muramyl-tetrapeptides, thereby creating the
tripeptides [166]. To this tripeptide, MurF attaches a dipep-
tide D-alanyl-D-alanine (D-Ala-D-Ala) forming a pentapep-
tide that participates in cross-linking [131]. The loss of ldcA
results in the formation of GlcNAc-MurNAc-tetrapeptides
as the donor muropeptide, which is incapable of cross-link-
ing as it does not contain the terminal D-Ala-D-Ala moiety.
This deficient PG backbone eventually leads to cell lysis dur-
ing the stationary phase [166].

P. aeruginosa also harbours an AmpD homologue (PA4522)
(Table 2). The enzymatic activity of purified P. aeruginosa
AmpD was demonstrated using synthetic muramyl and 1,6-
anhydromuramyl derivatives [141]. AmpD activity was higher
with the 1,6-anhydromuramopentapeptides as substrates as opposed to the muramyl derivatives [141, 143]. The cytoplasmic LD-car-
boxypeptidase in P. aeruginosa PA5198 was also identified as
a functional equivalent of E. coli LdcA [167].

In the final steps of recycling processes, the PG sugars,
GlcNAc, MurNAc, anhMurNAc and the PG peptide L-Ala-
γ-D-Glu-meso-DAP-D-Ala-D-Ala rejoin the PG biosynthesis
pathway in the cytoplasm.
CONVERGENCE OF RECYCLING AND BIOSYNTHESIS

The recovery of PG intermediates and its convergence into the biosynthesis pathway is a widely conserved process in bacteria and demonstrates their efficiency in preserving critical energy resources. The muropeptides generated in the cytoplasm through the activities of NagZ, AmpD and LdcA rejoin the biosynthesis pathway through the sequential activity of AnmK, MurQ, NagA and NagB in E. coli [168–171] (Figs 1 and 2).

In E. coli, AnmK kinase phosphorylates anhMurNAc to MurNAc-6-P which is processed by MurQ eetherase to form GlcNAc-6-P [168]. GlcNAc generated in the cytoplasm after the NagZ activity is phosphorylated to GlcNAc-6-P by NagK [172]. NagA deacetylates GlcNAc-6-P to GlcN-6-P [169, 173]. GlcN-6-P undergoes deamination by NagB to form fructose-6-phosphate, the initial substrate for glycolysis, PG and lipopolysaccharide synthesis [171]. GlcN-6-P can also be converted to GlcN α–1P by GlnM which is an intermediate in the PG synthesis pathway [29] (Fig. 2).

The set of enzymes for the reutilization of the PG sugars GlcNAc and MurNAc differ among P. aeruginosa and E. coli (Figs 1 and 2). In P. aeruginosa, an AnmK homologue PA0666 phosphorylates anhMurNAc to form MurNAc-6-P; subsequently, the phosphate is removed by MupP (PA3172) resulting in MurNAc [174–177]. MurNAc is further processed by two enzymes AmgK (PA0596) and MurU (PA0597) unique to pseudomonads encoded in the same operon [178]. AmgK (PA0596) converts MurNAc to MurNAc α–1P [174]. A uridylyltransferase MurU (PA0597) acts on MurNAc α–1P to form UDP-MurNAc [178]. The presence of these two proteins bypasses the need for MurQ, NagA, NagB, NagK, GlnM and GlnS that are otherwise required for the synthesis of UDP-MurNAc in E. coli (Fig. 2). Homologues of NagA (PA3758), GlmM (PA4749) and GlnS (PA5549) are found in P. aeruginosa. This novel alternative pathway was identified in P. aeruginosa and P. putida [174, 178]. Homologues of AmgK and MurU have been identified in Proteobacteria but not in Enterobacteria [174].

In the case of the peptide components of PG, AmpD amidas e cleaves the peptide side chain from UDP-MurNAc resulting in the formation of free tri-, tetra- and pentapeptides (L-Ala-γ-D-Glu-meso-DAP-D-Ala-D-Ala) [164]. LdcA carboxypeptidase cleaves the terminal d-alanine from a tetrapeptide resulting in tripeptides [166]. In E. coli, murein peptide ligase (Mpl) recycles the tri-, tetra- and pentapeptides by ligating them to UDP-MurNAc [179, 180]. Another enzyme, MurF can also ligate dipeptide D-Ala-D-Ala (a product of ATP-dependent ligase Ddl), to a UDP-MurNAc tripeptide to form a UDP-MurNAc pentapeptide [181–183]. Homologues of Mpl (PA4020) and MurF (PA4416) and Ddl (PA4201/PA4410) are found in P. aeruginosa; however, their role in cell-wall recycling has not been elucidated.

The tripeptide, L-Ala-γ-D-Glu-m-DAP, can be broken down into individual amino acids. The E. coli MpaA amidas e removes the meso-DAP from L-Ala-d-Glu [184]. E. coli YcG epimerizes d-Glu to L-Glu forming L-Ala-L-Glu, which is hydrolysed to individual amino acids by PepD [185, 186]. This pathway of amino acid degradation has not been studied in P. aeruginosa and bioinformatics analyses of the protein sequences of MpaA, YcG and PepD shows the absence of these in the P. aeruginosa PA01 strain.

CELL-WALL RECYCLING AND ANTIBIOTIC RESISTANCE

Resistance is a distinctive quality of pathogens that has ensured the long tug of war between the discovery of novel antibiotics and bacterial survival. Antibiotics act at several checkpoints in the bacterial life cycle such as DNA replication, RNA synthesis, protein synthesis and their activity as well as cell-wall biogenesis and recycling [187]. Being unique to bacteria, cell-wall synthesis and recycling are often the target for many widely used antibiotics such as β-lactams, fosfomycin and glycopeptides [188]. Consequently, the components of the cell-wall recycling process play a key role in the development of resistance to the targeting antibiotics. Through a complex network of regulators, some of the cell-wall recycling components are also involved in the elaboration of cross-resistance to other classes of antibiotics such as fluoroquinolones, aminoglycosides and macrolides [19]. Here, we describe the role of cell-wall components in promoting antibiotic resistance in Gram-negative bacteria, focusing on P. aeruginosa.

β-lactam resistance involving the Amp pathway

β-lactams are one of the earliest discovered and most commonly administered antibiotics worldwide. β-lactams act by disrupting the cell-wall synthesis in bacteria [189]. Specifically, β-lactam antibiotics mimic the terminal D-Ala-D-Ala residues of the amino acid side chains that serve as the substrates for the transpeptidase domains of HMM PBPs such as PBP1A, PBP1B, PBP2 and PBP3, locking them into an inactive state, thereby preventing PG cross-linking leading to cell lysis and death [189, 190]. Thus, it is not surprising that with the intracellular activation of ampC, encoding a β-lactamase, the expression is tightly coordinated with the cell-wall recycling process in Gram-negative bacteria [191, 192]. The AmpR-AmpC system has been investigated thoroughly in Enterobacteriaceae. In many members of the Enterobacteriaceae, the induction of ampC expression in response to β-lactam antibiotics is triggered through the activation of a transcriptional regulator AmpR by the cell-wall degradation products [191, 193–196]. The induction of ampC by AmpR also requires the products of ampG, ampD, ampDh2, ampDh3 and nagZ, all of which are involved in PG recycling, as described in previous sections. Studies from the Mathee lab suggest that the AmpR-AmpC (PA4109-PA4110) system might operate differently in P. aeruginosa [197]. Expression of the genes ampG (PA4393), ampP (PA4218), ampD (PA4522), ampDh2 (PA5485), ampDh3 (PA8087) and nagZ (PA3005) in P. aeruginosa are
differentially regulated by AmpR in the presence and absence of β-lactam [18]. AmpG is the permease that transports PG degradation products from the periplasm to the cytoplasm. In Enterobacteriaceae members, an ampG mutant is unable to recycle PG and loses the ability to induce ampC expression [7, 198] (Table 3). Inactivation of ampG can restore susceptibility to β-lactams even in pan-resistant P. aeruginosa clinical isolates [199]. P. aeruginosa also has an additional AmpG homologue known as AmpP (PA4218), both of which are presumed to be involved in ampC induction [151, 153].

The AmpD amidase was identified as being important for β-lactamase induction in C. freundii and Enterobacter cloacae [194]. The absence of ampD leads to the accumulation of anhMurNAc-tripeptide in the cytosol and constitutive overproduction of β-lactamase even in the absence of induction, resulting in a high resistance to β-lactams [163]. The most common mechanism for constitutive ampC overexpression in clinical strains leading to β-lactam resistance in Enterobacteriaceae as well as P. aeruginosa is to mutate ampD [200–203] (Table 3). P. aeruginosa has three different AmpD homologues, AmpD (PA4522), AmpDh2 (PA5485) and AmpDh3 (PA8078) that are responsible for de-repression of ampC expression in a step-wise manner [139, 204]. Deletion of all three ampD genes results in increased resistance due to complete de-repression of ampC expression resulting in very high MIC for the β-lactams drugs including cephalosporins and monobactams [139, 205].

NagZ (PA3005) is a β-N-acetylglucosaminidase that removes GlcNAc to generate the 1,6-anhydromuramopeptides, the putative activators of AmpR that are required for the induction of ampC [206]. Mutations in nagZ result in low levels of ampC expression that enhances the susceptibility to β-lactam antibiotics [206, 207]. Deletion of nagZ also reversed the resistance due to ampC hyper-expression in ampD and dacB mutants [207]. Based upon these findings, to increase the bacterial susceptibility to β-lactams, small molecule inhibitors gluco-nagstatin, LOGNAC and PUGNAc which are analogous to the NagZ substrates that have oxocarbenium-like transition states have been synthesized (Fig. 3) [208, 209]. Among the three, PUGNAc was the most potent inhibitor of β-N-acetylglucosaminidase [210]. However, all three were found to inhibit human glucosaminidases rendering them less useful [208].

Details of PG recycling and their ties with β-lactam resistance have been studied in Enterobacteriaceae members. The role of many orthologous proteins including AmpR, AmpC, AmpD and AmpG have been demonstrated in P. aeruginosa and appear to have similar roles to their Enterobacteriaceae counterparts [151, 200, 211]. In addition, new players such as ampP, ampDh2 and ampDh3 have been identified in P. aeruginosa [139, 151]. Moreover, the localization of P. aeruginosa AmpR as an inner membrane protein with the helix-turn-helix domain in the cytoplasm and effector-binding domain in the periplasm leads to unique questions as to how the effector molecules are transported to their binding sites on AmpR [212].

β-lactam resistance through other cell-wall-related genes

Other members involved in cell-wall recycling, namely the LTs and the LMM PBPs, also modulate β-lactam resistance. The periplasmic LTs are the major PG degradative enzymes that generate 1,6-anhydro-MurNAc muropeptides [85]. In E. coli, a double deletion of LT-encoding genes mltA-mltB, slt70-mltA or slt70-mltB and a triple deletion mltA-mltB-slt70 exhibit a higher loss in β-lactamase activities than any one single deletion that correlates with the increasing decline in murein turnover [91]. In P. aeruginosa, the loss of Slt (PA3020) and MltF (PA3764) is associated with a decrease in β-lactam resistance [98]. However, the loss of SltB1 (PA4001) and MltB1 (PA4444) led to an AmpC-independent increase in resistance to β-lactams, pipercillin and cefotaxime [98, 213]. The loss of the other P. aeruginosa LTs’ MltA (PA1222), MltD (PA1812), MltF2 (PA2865), SltG (PA1171) and SltH (PA3992) did not change the resistance profile [98] (Table 3).

The LMM PBPs comprise a group of enzymes that have endopeptidase and carboxypeptidase activity [24]. Three of the five LMM PBPs (PBP4, PBP5, PBP6, PBP6b, PBP7/8) were investigated for their role in β-lactam resistance in E. coli [214, 215]. Loss of PBP5 reduces the MIC for penicillins and cephalosporins whereas PBP6 and PBP6b removal have no resistance phenotype [215]. However, the lower MIC in PBP5 mutants can be partially restored by PBP6b. This suggests that PBP5 is important for β-lactam resistance followed by PBP6b [214]. However, the role of PBP4, PBP6 and PBP7/8 in antibiotic resistance is not well understood.

P. aeruginosa has three LMM PBPs (PBP4, PBP5 and PBP7) whose role in β-lactam resistance has been investigated [125, 216]. Clinical mutants of PBP4 (PA3047) are associated with increased β-lactam resistance [216]. In fact, a mutation in PBP4 leads to a one-step upregulation of ampC resulting in clinical β-lactam resistance [216]. Similar to PBP6b in E. coli, the role of PBP5 (PA3999) is not evident, unless in the PBP4 background where the double mutant further increases β-lactam resistance (Table 3).

Interestingly, the PBPs in E. coli and P. aeruginosa play contradicting roles in β-lactam resistance. This may be reflective of the different mechanism of resistance, as E. coli has a non-inducible ampC [217]. One speculation is that E. coli PBP5 could act as a ‘trap’ for β-lactams thereby preventing access to their targets, the HMM PBPs whereas in P. aeruginosa loss of PBP4 results in increased production of the AmpR effector which escalates ampC β-lactamase expression and thereby the MIC [124, 215, 216, 218]

Indirect regulation of β-lactam resistance through AmpR

Although P. aeruginosa AmpR acts as the nexus for AmpC β-lactamase induction and cell-wall recycling processes, it also modulates the activity of other proteins that regulate β-lactam resistance. One of these is the second chromosomally
encoded β-lactamase, PoxB (PA5514) in *P. aeruginosa* [219]. PoxB is a carbapenemase, which affects β-lactam resistance, in the absence of the major β-lactamase AmpC and outer membrane porin OprD (PA0958) [220]. Although no direct relation is seen between cell-wall recycling and *poxB*, AmpR is found to be a negative regulator of *poxB* through a yet unknown mechanism [220, 221].

Another mechanism of β-lactam resistance is through expulsion via efflux pumps namely MexAB-OprM (PA0425–PA0427) [222]. Expression of *mexAB* is repressed by a regulator MexR (PA0424) [223]. AmpR is required for *mexR* expression, suggesting an opposite impact on β-lactam resistance [18]. Moreover, deleting *ampC* and/or *ampR* in PAO1 abolishes β-lactam resistance despite having a functional MexAB

### Table 3. MIC of mutant strains of *P. aeruginosa*

MIC levels of PAO1 and the corresponding deletion strains are indicated on the left. The MIC values of PAO1, shown in yellow, is an average of the values found in the cited references. Any values greater than PAO1 are shown in gradation of orange-red. Values lower than PAO1 are shown in gradations of yellow-green. AMP, ampicillin; PIP, piperacillin; CTX, cefotaxime; CAZ, ceftazidime; CEF, cefepime; FOX, cefoxitin; ATM, aztreonam; IMI, imipenem; MER, meropenem; MIC: Minimum Inhibitory Concentration.

<table>
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<tr>
<th>Strains</th>
<th>AMP (μg ml⁻¹)</th>
<th>PIP</th>
<th>CTX</th>
<th>CAZ</th>
<th>CEF</th>
<th>FOX</th>
<th>ATM</th>
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<td>7</td>
<td>1.4</td>
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<td>4</td>
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<tr>
<td><em>dacC</em></td>
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<td>8</td>
<td>1</td>
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<td>1024</td>
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<tr>
<td><em>pbbG</em></td>
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<td>16</td>
<td>1</td>
<td>1</td>
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<td>4</td>
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<tr>
<td><em>ampC</em></td>
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<tr>
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<td>8</td>
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*MIC determined by broth microdilution.
†MIC determined by E-test.
system or PoxB, suggesting that the AmpC is the major determinant of β-lactam resistance [18, 19, 220].

Another mechanism of β-lactamase induction is mediated through the two-component system CreBC (PA0463–PA0464) and its effector, an inner membrane protein CreD (PA0465) [216]. In E. coli, CreB has been identified as a transcriptional factor that differentially regulates up to eight genes under diverse nutritional conditions [224]. E. coli CreB was also found to regulate the expression of Aeromonas hydrophila β-lactamase [225]. This two-component system in P. aeruginosa plays a pivotal role in β-lactam resistance and bacterial fitness [226, 227]. The inactivation of dacB (PBP4) leads to an increased expression of creBC, creD and ampC. The increased expression of ampC is accompanied by elevated MIC of β-lactams, which reverts to wild-type levels upon deletion of creBC [226]. The association of creBC and β-lactam resistance was seen only in dacB mutants and not with other mutations that confer high resistance, namely ampD, ampDh2 and ampDh3 [216]. Moreover, these resistance phenotypes regulated through creBC were lost in the absence of NagZ and AmpG [207, 216, 226] (Table 3). Although the interplay of the cell-wall recycling components, ampC expression and the creBC-creD system is found to regulate β-lactam resistance, the underlying details remain to be elucidated.

**Cell-wall components contributing to non-β-lactam resistance**

In addition to the various mechanisms by which the different components involved in PG biosynthesis and recycling confer β-lactam resistance, some cell-wall components acting through AmpR also play a role in resistance to non-β-lactam antibiotics [19]. Some specific examples are discussed below.

**Fluoroquinolone resistance**

Quinolones, specifically fluoroquinolones, are potent, broad-spectrum synthetic antibiotics that inhibit DNA replication by targeting bacterial DNA gyrase (Gram-negatives) or topoisomerase II (Gram-positives) [228]. Due to their effectiveness against both Gram-positive and Gram-negative bacteria, extensive use of fluoroquinolones has led to a rapid rise in resistance [229–231]. Development of resistance to quinolones entails multiple mechanisms [232, 233]. The primary mechanism of quinolone resistance in Gram-negative bacteria is through mutations in target genes gyrA and gyrB [234–237]. Overexpression of MDR efflux pumps can also add significantly to the quinolone resistance especially in the presence of target mutations [238]. Unexpectedly for synthetic antibiotics, horizontal gene transfer also plays a role in quinolone resistance. A plasmid-mediated resistance gene qnr encoding a pentapeptide repeat protein protects the bacterial topoisomerases (gyrase and topoisomerase IV) from quinolone activity [239, 240].

In P. aeruginosa, the MexEF-OprN (PA2493–PA2495) system is involved in efflux of fluoroquinolones, chloramphenicol and trimethoprim [241]. This efflux system is under the control of three different regulators; the positive regulator MexT (PA2492), and the negative

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**Fig. 3.** Small molecule inhibitors of N-acetyl-β-glucosaminidase NagZ. (a) O-(2-acetamido-2-deoxy-D-glycopyranosylidene) amino N-phenylcarbamate (PUGNAc), (b) 1, N-acetylglucosaminono-1,5-lactone oxime (LOGNAc), (c) gluco analogue of 8-acetamido-5,6,7,8-tetrahydro-6,7-dihydroxy-5-(hydroxymethyl)imidazo[1,2-a] pyridine-2-acetic acid nagstatin (gluco-Nagstatin), (d) transition state of NagZ substrate analogous to the small molecule inhibitors [208, 210].
regulators MexS (PA2491) and MvaT (PA4315) [242–244]. *P. aeruginosa* AmpR negatively regulates the expression of the mexEF-oprN operon and MexT [18]. Consequently, PAOΔampR mutants are more resistant to fluoroquinolones compared to the wild-type [18]. Repression of the mexEF operon by AmpR is β-lactam-independent [18], suggesting that AmpR functions diversified from the cell-wall synthesis pathway. It is important to note that AmpR positively and negatively regulates resistance to β-lactams and quinolones, respectively, highlighting the intricate and complex balance of regulation of antibiotic resistance [18, 197].

**Aminoglycosides resistance**

Traditionally, the aminoglycoside class of antibiotics is used to treat Gram-negative infections. They bind to the 16S ribosomal subunit causing it to mistranslate leading to the synthesis of aberrant proteins [245, 246]. Aminoglycosides, such as tobramycin, along with β-lactams form the first line of *P. aeruginosa* treatment for cystic fibrosis patients [247]. One of the primary determinants of aminoglycoside resistance in *P. aeruginosa* and other Gram-negative pathogens are the presence of active efflux pumps [248, 249]. The upregulation of the MexXY efflux pump in *P. aeruginosa* is considered the most common mechanism of aminoglycoside resistance [250]. Aminoglycoside resistance can also be conferred by drug-modifying enzymes such as aminoglycoside acetyl-, nucleotidyl- and phospho-transferases [251–255]. Mutations in *aac*, *aphA1* and *aadB* that encode the aminoglycoside modifying genes have been identified in clinical *P. aeruginosa* isolates [256–258].

In the presence of β-lactams, *P. aeruginosa* AmpR negatively regulates the expression of mexX, mexY and *aph;* the latter encodes the aminoglycoside phosphotransferase [259]. However, this negative regulation is not translated into an observable phenotype. In contrast, loss of *ampR* increases aminoglycoside (amikacin and tobramycin) susceptibility in *P. aeruginosa* suggesting a positive regulation [19]. Thus, even with increased expression of efflux and drug-modifying proteins, the *ampR* mutants are more susceptible to aminoglycosides, suggesting the existence of post-translational modifications in this complex regulatory network.

**CONCLUDING REMARKS**

The growing antibiotic resistance among pathogenic bacteria is quickly escalating into a global health crisis. This is further aggravated by the hiatus in the discovery of new classes of antibiotics. Multi-drug and extreme drug resistances in clinical isolates of *P. aeruginosa* are challenging the existing treatment options. It is imperative to exploit possible new strategies to augment the effectiveness of currently used antibiotics. Every so often taking a step back to understand the problem at hand can position you to spring forward. In this review, we focus on the *E. coli* and *P. aeruginosa* bacterial cell-wall, target for many antibiotics. Deciphering the cell-wall recycling pathway in *E. coli* has been a long and arduous process due to the many experimental limitations. The genes and enzymes along with their functional role in cell-wall recycling have been well-described in *E. coli*. However, the evidence confirming the functional roles of the cell-wall recycling genes in *P. aeruginosa* is largely incomplete. Although the pathways are similar in *E. coli* and *P. aeruginosa*, there are significant differences such as the existence of multiple amidases AmpDh2 and AmpDh3, multiple permeases AmpG and AmpP and an alternative pathway for UDP-MurNac formation. Despite these differences, some of the key players in both the organisms remain the same. Both bacteria possess multiple, redundant LTs as well as LMM and HMM PBPs. NagZ and AmpD along with AmpG are also recognized as some of the significant players that regulate β-lactam resistance. The complete characterization of the recycling pathway would help us to better understand the mechanisms of antibiotic resistance.

The interplay between the cell-wall recycling pathway and the induction of β-lactamase expression has been recognized in many Gram-negative as well as in the Gram-positive bacteria. AmpR is among the very few transcriptional regulators in *P. aeruginosa* that modulates resistance to different classes of antibiotics in addition to regulating critical virulence factors. Targeting AmpR will thus render the strain less virulent and enhance sensitivity to β-lactams. Absence of human homologs for AmpR and other cell-wall related related enzymes make them attractive novel antibiotic targets. At the heart of this induction process are the muramyl peptides that bind to AmpR and alter the AmpC β-lactamase expression. A collaborative approach involving muropeptide analogues in combination with current antibiotics may be an encouraging alternative to counter antibiotic resistance.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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