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Reviews

The sentinel role of peptidoglycan recycling in the β-lactam resistance of the Gram-negative *Enterobacteriaceae* and *Pseudomonas aeruginosa*

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ABSTRACT

The peptidoglycan is the structural polymer of the bacterial cell envelope. In contrast to an expectation of a structural stasis for this polymer, during the growth of the Gram-negative bacterium this polymer is in a constant state of remodeling and extension. Our current understanding of this peptidoglycan "turnover" intertwines with the deeply related phenomena of the liberation of small peptidoglycan segments (muropeptides) during turnover, the presence of dedicated recycling pathways for reuse of these muropeptides, β-lactam inactivation of specific penicillin-binding proteins as a mechanism for the perturbation of the muropeptide pool, and this perturbation as a controlling mechanism for signal transduction leading to the expression of β-lactamase(s) as a key resistance mechanism against the β-lactam antibiotics. The nexus for many of these events is the control of the AmpR transcription factor by the composition of the muropeptide pool generated during peptidoglycan recycling. In this review we connect the seminal observations of the past decades to new observations that resolve some, but certainly not all, of the key structures and mechanisms that connect to AmpR.

Contents

1. Introduction

The progressive emergence of bacteria with multiple resistance determinants has compromised the ability of chemotherapy to control bacterial infection. For decades the standard antibiotic regimen for infections caused by the Gram-negative *Enterobacteriaceae* was the latest-generation cephalosporin antibiotics, important members of the β-lactam class of antibiotics. A particular resistance mechanism of concern with respect to these cephalosporins is the AmpC β-lactamase [1–3]. AmpC catalyzes the hydrolytic destruction of the β-lactam core of these antibiotics and thus prevents the β-lactam antibiotic from reaching its targets, the penicillin-binding protein (PBP) enzymes. The PBP enzymes synthesize and maintain the peptidoglycan polymer of the bacterial cell wall, and are inactivated by the β-lactam antibiotics. The

Abbreviations: HMM PBP, high molecular mass penicillin binding protein; LMM PBP, low molecular mass penicillin binding protein; LT, lytic transglycosylase; LTTR, lys-type transcription factor.

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mechanism for this inactivation is a covalent, and functionally irreversible, acylation of the same serine active-site nucleophile used for the catalytic reactions of the PBPs with the peptidoglycan. The AmpC β-lactamase is a member of the class C serine-dependent β-lactamase family. It was once a chromosomal enzyme of the *Enterobacteriaceae* but now is increasingly found as constitutively expressed from a plasmid [4,5]. The proliferation of several different β-lactamases, each with the ability to confer resistance to both the cephalosporin and carbapenem β-lactam structures [6,7], is foundational to the concern that an era of untreatable bacterial infection is nigh [8].

A curious observation was made thirty years ago, prior to the emergence of the AmpC enzymes as a clinically important resistance mechanism. The Gram-negative bacterium *Escherichia coli*, one of the *Enterobacteriaceae*, encoded a chromosomal AmpC β-lactamase that was exceedingly weakly expressed even following exposure to β-lactam antibiotics. In contrast, a dramatic increase in expression of the chromosomal AmpC β-lactamases was seen for other members of the *Enterobacteriaceae* (such as *Citrobacter freundii*, and also for *Pseudomonas aeruginosa*) following exposure of these bacteria to β-lactam antibiotics [9]. While the genomic-level basis for this contrast was quickly discerned (as discussed below), the molecular-level basis of the signal transduction has emerged much more slowly. Our current understanding intertwines the phenomena of an obligatory turnover of the bacterial peptidoglycan polymer during bacterial growth and septation, the consequent liberation of small peptidoglycan segments (muropeptides) during these processes, the existence of dedicated recycling pathways for the recovery and reuse of these muropeptides, β-lactam inactivation of specific penicillin-binding proteins as a mechanism for the perturbation of the muropeptide pool, and this perturbation as a controlling mechanism for the signal transduction leading to AmpC expression as a specific resistance mechanism. In certain bacteria (notably *P. aeruginosa*) the confluence of these phenomena—the AmpR transcription regulator—controls the expression of yet additional resistance and virulence mechanisms [10,11]. With the forceful arrival of the AmpC β-lactamase as an important resistance mechanism for problematic Gram-negative pathogens, the value of controlling the signal transduction pathway is recognized as a possible means to subvert AmpC-dependent β-lactam resistance [12–15]. In this review, we connect the seminal observations of the past decades to new observations that resolve some, but certainly not all, of the key connecting points for these phenomena.

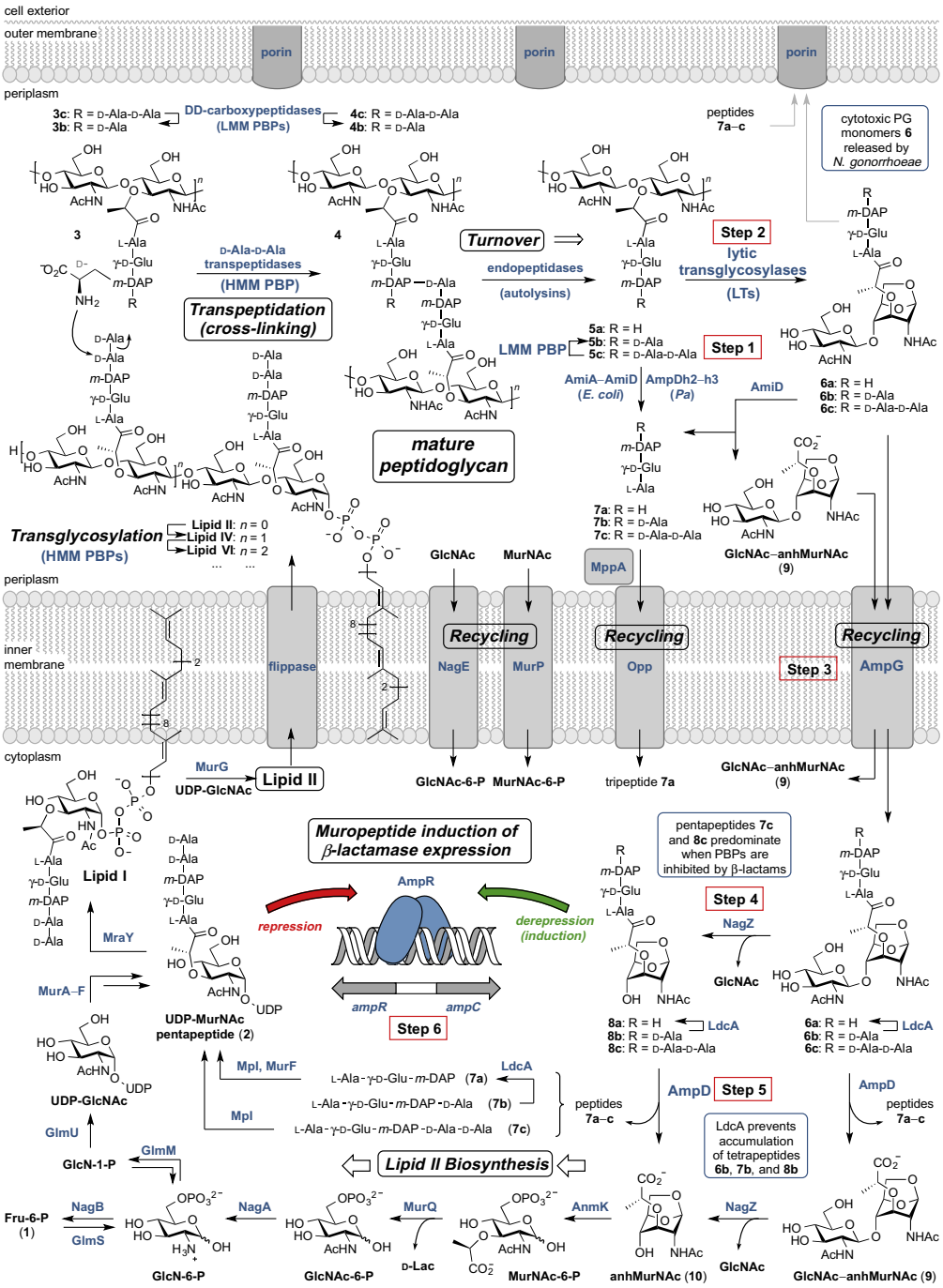
2. The central role of the *ampR* gene in AmpC β-lactamase expression

The key difference between *E. coli* compared to other *Enterobacteriacae* (such as *C. freundii* and *Enterobacter cloacae*) is found in the respective genomes. *C. freundii* and *E. cloacae* (and the other *Enterobacteriaceae*, with the exception of *E. coli* and *Shigella* species) have a linked genetic locus (*ampR*) that encodes the AmpR transcription regulator. The AmpR protein is a member of the large LysR-type transcription regulator (LTTR) family [16] and has the characteristic structure—an N-terminal helix-turn-helix DNA-binding domain and C-terminal co-inducer (effector) binding domain—of this family [17]. The *ampR* gene is found between the divergently expressed *ampC* gene and the *frd* operon. The *ampR* gene is separated by a bifunctional transcription terminator. Control of AmpC expression follows the customary mechanism for LTTR regulation of gene expression. Binding of an effector molecule to the effector-binding domain of the LTTR results in dissociation of the effector-LTTR complex from the promoter, allowing gene transcription (here, expression of AmpR). The promoters for *ampR* and *ampC* have substantial overlap [18]. The expressed AmpR may (or may not) then bind a co-inducer structure as it relocates to a binding site upstream of the promoter for the divergently-transcribed target gene, to activate its transcription [16]. AmpR therefore regulates expression of both genes. In the absence of a β-lactam, AmpC expression is repressed (in *C. freundii*, by 2.5-fold), while in the presence of a β-lactam, AmpC expression is induced (in *C. freundii*, by 10- to 200-fold) [9,18]. The inability of *E. coli* to induce expression of its AmpC β-lactamase is the consequence of the loss from the *E. coli* genome of its *ampR* gene [19]. Notwithstanding the subtlety (and complexity) of LTTR regulation of AmpC expression, the relationship between β-lactam exposure as the initiating event and AmpC expression as the culminating event is sensible. This understanding gives two outstanding questions: the events interconnecting β-lactam exposure to AmpC expression, and the structures of the co-effector molecule (that depresses AmpR expression) and the co-inducer molecule (that induces AmpC expression).

3. Peptidoglycan recycling

For a bacterium the preservation of the structural integrity of its peptidoglycan during its growth and eventual division is paramount. An early assumption was that preservation of this integrity during peptidoglycan growth corresponded to a growth mechanism of accretion (progressive elongation). It was therefore a surprise to discover that growth (both of the sidewalls and the new septum) of the Gram-negative bacterium *E. coli* coincided with a continuous release of the existing peptidoglycan [20] as the new peptidoglycan was incorporated [21], and the dynamic recycling of the released peptidoglycan. Peptidoglycan turnover and recycling also occurs in Gram-positive bacteria, but is less well characterized [22]. While the phenomenon of extensive release of existing peptidoglycan as new peptidoglycan is added has stimulated several creative proposals for the biosynthesis of the peptidoglycan polymer, none of these proposals is proven. Given the extensive peptidoglycan release during growth, the recovery and reuse of the released peptidoglycan has a probable fitness advantage. The mechanistic complexity of the events that occurring in the cytosol, the inner membrane, the periplasm, and the outer membrane of the Gram-negative bacterium relating to peptidoglycan synthesis (approximated as an accretion event), turnover, and recycling is illustrated in Scheme 1 [23]. As the mechanism for peptidoglycan release during its biosynthesis remains unknown, this aspect is not presented in this Scheme.

Peptidoglycan turnover (Scheme 1, top center) occurs through the simultaneous action of endopeptidases (autolysins) and the lytic transglycosylases (Scheme 1, top right). Within both enzyme families—Gram-negative bacteria contain multiple enzymes of each—their respective activities are spatially controlled (such as through lipobox sequence delivery of many of these enzymes to the inner leaflet of the outer membrane, and the PBP activities directed by signal peptides to the outer leaflet of the inner membrane) as well as controlled through incorporation into multi-enzyme assemblies. The breadth of the enzyme components within these assemblies, especially as they relate to growth (elongosome) and septation (divisome) has been suggested [24–26]. The consequence of the enzyme assembly with respect to defining the substrate and product of the individual enzymic reactions is unknown. The low-molecular-mass (LMM) PBPs exert both endopeptidase activity with respect to the crosslinked stems of the peptidoglycan, and exolytic D,D-carboxypeptidase activity (removal of the terminal D-Ala residue of the stem) with respect to the uncrosslinked peptidoglycan as a substrate. This activity controls the extent of D,D-crosslinking of the peptidoglycan, and one of these



Scheme 1. Principal events in the synthesis, turnover, and recycling of the peptidoglycan of the Gram-negative bacterium, segregated with respect to the events of the cytoplasm (lower), inner membrane (center), and the periplasmic space between the inner membrane and outer membrane (upper). Peptidoglycan turnover and recycling comprises events that proceed clockwise from the periplasm (upper center), through the inner membrane (center right) and into the cytoplasm (lower right). The key steps (labeled as Step 1 through Step 6) are further illustrated in Scheme 2. This figure is adapted from Johnson et al. [23] and is used with permission.

activities also contributes to AmpR regulation, as discussed below. The lytic transglycosylases (LTs) are present in both the elongosome and divisome assemblies, and with respect to the former have been thought to be the catalytic activity that terminate the elongation of the nascent glycan strands assembled by the transglycosylase activity of the PBPs (Scheme 1, center left). Their catalytic activity, the non-hydrolytic cleavage of the *N*-acetylmuramic–*N*-acetylglucosamine glycosidic bond of the peptidoglycan, is also irrefutably implicated in the AmpR regulation of resistance, as also discussed below. The cleavage reaction of the lytic transglycosylases may be either endolytic or exolytic [27]. The muropeptides released by the LTs (exemplified by the disaccharide structures **6a–c** of Scheme 1) are translocated from the periplasm to the cytoplasm by passage through the AmpG permease protein (Scheme 1, center right) in a proton-motive-force-dependent event [28,29]. In the cytoplasm the glycosidic bond of the disaccharide is hydrolytically cleaved by the NagZ enzyme [30,31] and the peptide stem hydrolytically removed by the AmpD enzyme [32–35]

(Scheme 1, lower right). These structures are reused as they enter the biosynthetic pathway to Lipid II (Scheme 1, bottom to center right).

4. Undecaprenol recycling

A no less important aspect of peptidoglycan recycling is the preservation by the bacterium of its small (in *E. coli* approximately 1.5×10^5 molecules per cell [36]) steady-state pool of the polyprenol phosphate and diphosphate lipids [37,38]. The polyprenol lipids are the universal carriers, in all life forms, for the saccharides required for glycopeptide biosynthesis [39]. The undecaprenol pyrophosphate-linked NAG-NAM disaccharide intermediate of peptidoglycan biosynthesis, Lipid II, is assembled in the cytoplasm [40,41] and is translocated from the cytoplasm to the periplasmic space to function as a substrate for the periplasmic PBP enzymes. The concurrent (with the peptidoglycan) assembly of the lipopolysaccharides of the outer leaflet of the Gram-negative outer membrane also uses polyprenol-linked polysaccharide biosynthetic intermediates [42]. Both glycosyl transfer events release undecaprenol diphosphate as a product. To sustain the steady-state pool of undecaprenol lipids required for continued biosynthesis, the head group of the undecaprenol must translocate back to the cytoplasm. The chemistry of this final translocation is not well understood (and hence is not depicted in Scheme 1). Circumstantial evidence implicates hydrolytic cleavage of the released undecaprenyl pyrophosphate to the undecaprenyl phosphate (the substrate used by the cytoplasmic enzyme MraY for the synthesis of Lipid I) as the event preceding translocation [43,44]. Whether translocation occurs at this stage (reverse transport by the Lipid II flippase?) [38], or whether further hydrolysis occurs to give the free alcohol to enable a spontaneous translocation of the head group, is unproven. The former process is the more probable as it would be both catalytic and would deliver undecaprenyl phosphate to the cytoplasm to directly act as substrate for MraY. Moreover, the velocity required for undecaprenyl phosphate translocation during growth of the cell envelope well exceeds estimates for spontaneous head group movement [38]. The difficulty in assigning the breadth of functional identities to the flippase(s) (MurJ, FtsW, RodA...) involved in the translocation of the "head" group of Lipid II [24] may rest in part for these proteins to accommodate the necessarily concurrent event of reverse translocation of undecaprenyl phosphate. Not surprisingly, the greater mass of peptidoglycan that is found in the Gram-positive bacteria corresponds to a larger undecaprenol (in *Mycobacteria*, a decaprenol) pool [36]. The importance of polyprenol recycling to the bacterium, and the greater accessibility of the undecaprenol intermediates in Gram-positive bacteria, is underscored by an astonishing breadth of antibiotics that exert their mechanism by interfering with peptidoglycan biosynthesis and/or undecaprenol recycling through complexation of the glycosylated undecaprenyl pyrophosphate moiety (as exemplified by Lipid II itself [45,46]). The list of antibiotics recognizing this structural motif includes many antibiotics with a preferential Gram-positive spectrum such as vancomycin [47], bacitracin [48], the bacteriocins (colicins) [49], the lantibiotics [50–53] and the defensins [54].

5. Peptidoglycan recycling as a sentinel event leading to resistance enzyme expression

Recent studies on the relationship between peptidoglycan recycling and AmpC induction have focused on *P. aeruginosa*. Although *P. aeruginosa* is not a member of the *Enterobacteriaceae*, *P. aeruginosa* is an aggressive and opportunistic Gram-negative pathogen that also has an intact *ampR–ampC* system [10]. Its AmpC β-lactamase is a primary mechanism for the clinical resistance of *P. aeruginosa* to the β-lactam antibiotics, and indeed the optimization of β-lactam structures that are efficacious against *P. aeruginosa* coincides with those structures that poorly induce AmpC expression. Moreover, the *ampR* gene of *P. aeruginosa* regulates additionally a host of virulence mechanisms [55,56]. These mechanisms include expression of a second (PoxB) β-lactamase and the MexEF efflux pump that confers cross-resistance to both the quinolones and the aminoglycosides [57]. "Pan-β-lactam" resistance in *P. aeruginosa* combines efflux, OprD porin deletion, and PBP profile alteration with AmpC expression [58]. A direct connection between peptidoglycan recycling by *P. aeruginosa* and AmpR regulation was inferred from the strong similarity for the structural and regulatory gene sequence of *ampR–ampC* [59] and subsequently by extensive experiments including the observation of *ampD* inactivation resulting in constitutive AmpC expression [60], *ampG* inactivation restoring β-lactam susceptibility [61], and NagZ inhibition likewise restoring β-lactam susceptibility [62]. Incapacitation of peptidoglycan recycling in *P. aeruginosa* imposes a fitness cost [63]. Two notable differences are found between the *P. aeruginosa* and the *Enterobacteriaceae* peptidoglycan-recycling systems. One difference is the presence in *P. aeruginosa* of two AmpG permeases, the *ampR*-independent AmpG and the *ampR*-dependent AmpP. The use of both permeases gives greater AmpC induction [64,65]. A second difference is the presence of three AmpD amidases [66]. While all three of these amidases contribute to the synthesis of muropeptide structures having the ability to ultimately repress AmpC expression [67], one (AmpD) has the primary role [68]. High-level β-lactam resistance in *P. aeruginosa* coincides [69] to concurrent mutations (some with fitness cost) in AmpC (to optimize activity against a particular β-lactam structure) and in AmpR (to increase AmpC expresion). These observations suggest strong similarities between the peptidoglycan recycling and AmpC expression systems in *P. aeruginosa* and the *Enterobacteriaceae*.

Several recent studies in *P. aeruginosa* have clarified the molecular mechanisms in this relationship to an extent not yet established for the *Enterobacteriaceae*. The first of these studies identified the single step deletion of a particular (and by *in vitro* assessment, non-essential) low-molecular-mass (LMM) PBP (PBP4, encoded by *dacB*) as an activity intimately involved in both AmpC overexpression and activation of the BlrAB (formerly CreBC) two-component regulator of other resistance pathways [70]. The mechanistic basis for the BlrAB-dependent component to β-lactam resistance is not known, but does not involve β-lactamase expression [71]. Nonetheless, the induction of multiple β-lactamases in the *Aeromonas* spp. occurs exclusively in response to BlrAB two-component signaling as a result of perturbation of peptidoglycan due to PBP4 inactivation [72]. This PBP, as well as the PBP4 of *P. aeruginosa*, otherwise were presumed to possess mere "housekeeping" DD-carboxypeptidase and DD-endopeptidase activities—the routine assignment made to all LMM PBPs—with respect to peptidoglycan biosynthesis and maintenance. This observation implicates PBP4 as having at least one specific function beyond peptidoglycan housekeeping function: creation of a muropeptide that controls AmpR resulting in repression of AmpC β-lactamase expression. A β-lactam that results in the strong expression of AmpC in *P. aeruginosa* is one with a high affinity for PBP4 inactivation. Loss of PBP4 activity results in the disappearance of specific muropeptide(s) from the recycling pool, resulting in a transition in the AmpR regulation so as to induce AmpC expression. An intimate role for NagZ in the biosynthesis of this same muropeptide—whose structure is discussed below—is confirmed (again) by the ability of a NagZ inhibitor to restore β-lactam susceptibility [73].

Cavallari et al. [74] confirmed PBP4 as the sentinel enzyme used to detect the presence of β-lactam antibiotics, and verified that its activity with respect to this function was not shared by any other

PBP. Additional experiments clarified the participation in peptidoglycan recycling by the lytic transglycosylase enzymes of *P. aeruginosa*. Five of the nine lytic transglycosylases (MltA, MltD, MltF2, SltG, SltH) have no role in peptidoglycan recycling (AmpC expression). Inactivation of the genes for two (Slt and MltF) of the remaining four lytic transglycosylases of *P. aeruginosa* increased the β-lactam susceptibility of the bacterium, by an unknown mechanism (as "wall-impaired" phenotypes). The mechanism here is unrelated to AmpC (whose expression level was unchanged) [74]. In contrast, genetic deletion of the remaining two lytic transglycosylases (MltB and SltB1) gave high-level β-lactam resistance (similar to *dacB* inactivation) as a result of ampC expression [74]. These latter two enzymes are family 3 LTs, and have a calcium-binding EF hand domain that is used in LT complex formation with the high-molecular-mass PBPs [75]. Moreover, while *dacB* gene inactivation gave constitutive AmpC expression, gene inactivation of MltB or SltB1 gave AmpC expression only upon β-lactam challenge. Cavallari et al. conclude that an alternate pathway is present in *P. aeruginosa* to control AmpC expression, and emphasize that the presumption that lytic transglycosylases function only in peptidoglycan housekeeping is simplistic [74].

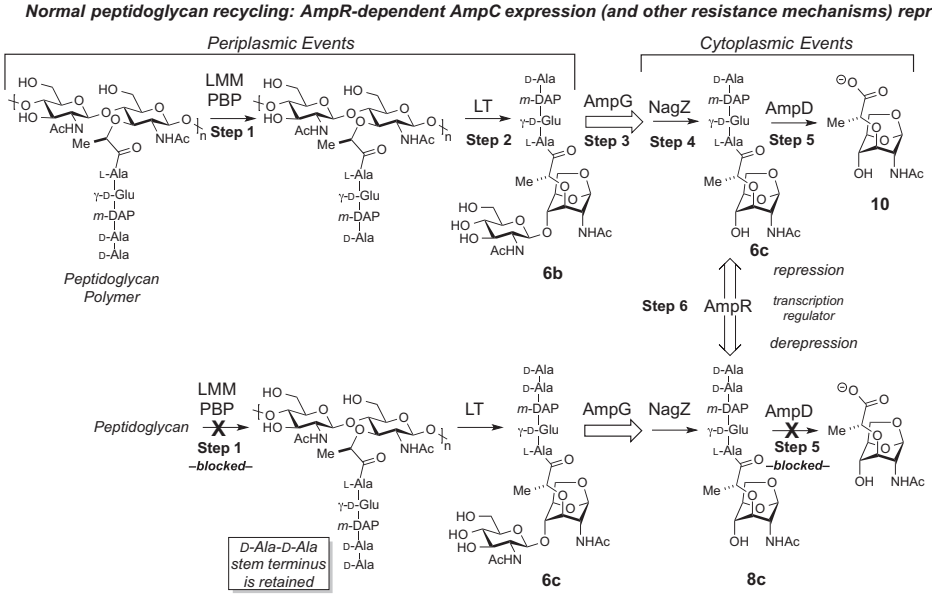
An identical conclusion with respect to LT function—that notwithstanding extensive study establishing functional redundancy among the LT enzymes of *E. coli* [76], specific LTs must have specific functions—was arrived at independently by the studies of Dillard et al. on LT function within the Gram-negative pathogen *Neisseria gonorrhoeae* [77]. While the *ampR* and *ampC* genes are absent in the *N. gonorrhoeae* genome, genes for the AmpD amidase and AmpG permease are present. *N. gonorrhoeae* shares with a second Gram-negative bacterium, *Bordetella pertussis*, the characteristic of secreting muropeptides during infection so as to result in a cytotoxic (to the host) inflammatory response [78]. These muropeptides [79,80] derive from lytic transglycosylase catalysis in the course of the recycling by *N. gonorrhoeae* of its peptidoglycan [81]. The *N. gonorrhoeae* genome encodes five LT enzymes (two additional LT enzymes often are found on genetic islands). Chan et al. [77] evaluated by gene deletion the contributions of six of the seven LTs to the synthesis of cytotoxic muropeptide(s). One of the five genomic LT enzymes (LtgC, homologous to *E. coli* MltA) functions in cell separation following division and does not contribute significantly to muropeptide release. Two of the remaining four genomic LTs—LtgB (homologous to *E. coli* MltC) and LtgE (homologous to *E. coli* MltD)—have an unknown function and also do not contribute. One of the LTs on the genomic island functions in Type IV secretion system attachment to the peptidoglycan, and also does not contribute. The remaining two genomic LTs, LtgA (homologous to *E. coli* Slt70) and LtgD (homologous to *E. coli* MltB/Slt35), each contribute to the synthesis of the cytotoxic muropeptide [82]. Lastly, Garcia and Dillard [81] provide strong circumstantial evidence for an ability of *N. gonorrhoeae* to regulate, by an unknown mechanism, the efficacy of peptidoglycan recycling. Diminished recycling efficacy would correlate to greater muropeptide release to the medium. Companion studies evaluating the role of AmpG in peptidoglycan recycling by *Neisseria meningitidis* compared to *N. gonorrhoeae* [83] are consistent with the possibility of regulatory control of the AmpG permeases.

A further variation on the relationship between peptidoglycan recycling and the *amp* system is observed in recent studies with *Stenotrophomonas maltophilia*, an emerging multidrug-resistant Gram-negative pathogen [84]. High-level β-lactam resistance in *S. maltophilia* is associated with acquisition of additional β-lactamases, such as the NDM-1 metallo-β-lactamase, to complement its two chromosomal β-lactamases, termed the L1 metallo-β-lactamase and the L2 serine-dependent class A β-lactamase. In most strains L1 and L2 are expressed, albeit independently, under AmpR control in response to β-lactam exposure [85,86] and the ensuing perturbation of peptidoglycan recycling [87]. In the absence of an inducer, AmpR activates L1 and represses L2 expression. AmpR activates both in the presence of an inducer [88]. Proteomic analysis of such a clinical *S. maltophilia* strain using imipenem as the β-lactam inducer indicated that L1 expression no longer was under AmpR control; L2 expression was fully under AmpR control, and the imipenem-induction gave higher expression levels of NDM-1 [89]. The basis for imipenem regulation of NDM-1 is uncertain, given substantial genetic differences in the *amp* system of *S. maltophilia* and explicit evidence for at least one additional β-lactam-resistance regulatory network operating through two-component signaling and/or PBP inhibition (here involving, either separately or together, inactivation of two PBPs: the HMM PBP1A encoded by *mcrA* and the LMM PBP3) [71,90–92]. As is seen with other Gram-negative bacteria, inactivation of the (here, a constitutively-expressed) $ampD_1$ amidase gene resulted in L1 and L2 hyperexpression [93], while inactivation of the NagZ gene prevented L1 and L2 hyperexpression through AmpR (perturbation of peptidoglycan recycling) regulatory pathway but not through the PBP1A (*mrcA*) pathway [92]. Avison et al. [71] conclude that *S. maltophilia* may be representative of pathogenic bacteria where strategies to suppress peptidoglycan-recycling-dependent induction of β-lactamase expression, such as by small-molecule NagZ inhibition, may be ineffective.

6. The identity of the muropeptide messenger controlling AmpR

Six events are embedded in Scheme 1 (as Steps 1–6) that are believed to directly interconnect peptidoglycan recycling to the regulation of AmpR, as a consequence of the appearance in the cytoplasm of muropeptide structure(s) directly interacting with AmpR as either a co-effector or co-inducer [94–98]. These events are LMM PBP action on the peptide stem (Step 1); lytic transglycosylase catalysis (Step 2); entry of the muropeptide into the cytoplasm (Step 3); glycosidic cleavage by NagZ (Step 4), hydrolytic truncation of the peptide stem by AmpD to give the anhydro-MurNAc structure **10** (Step 5), and as a result of the flux dynamics between Steps 4 and 5 the generation of muropeptide structure(s) that regulate AmpR (as Step 6). Our understanding of the possible identity of these structure(s) is defined by four pivotal observations pertaining to these six steps (Scheme 2).

The first observation is that the structure-determining event with respect to ultimate AmpR regulation arises from inactivation of the sentinel LMM PBP (in *P. aeruginosa*, PBP4) by a β-lactam antibiotic. This LMM PBP may be presumed to have both endopeptidase and exopeptidase (D,D-carboxypeptidase) activities, by analogy with the PBP4 of *E. coli* [99]. By application of Occam's razor the D,D-carboxypeptidase activity has been presumed to be the structure-determining event. The danger of such a presumption requires emphasis: in biology it is not the simple event that is selected, but the event that works. Nonetheless, the structures shown in Scheme 2 derive from the presumption of D,D-carboxypeptidation by the sentinel LMM PBP as the defining structural event. Whether this reaction occurs during peptidoglycan synthesis, or occurs to the mature peptidoglycan, or occurs to the released muropeptide, is unknown. The second observation is the absolute requirement for lytic transglycosylase activity [100,101]. Hence, the structure that engages AmpR is an anhydroMurNAc structure. The third observation is that the common genetic event leading to high-level AmpC-dependent β-lactam resistance, acting through AmpR, is inactivation of the *ampD* gene [32,102,103]. Hence, the anhydroMurNAc structure engaging AmpR has an intact peptide stem. The fourth observation is that small molecule iminosaccharide inhibition of NagZ reduces AmpC-dependent resistance in many (but not all: in *S. maltophilia* for example, there are two



Perturbed peptidoglycan recycling: AmpR-dependent AmpC expression (and other resistance mechanisms) derepressed

Scheme 2. The key events known to occur in peptidoglycan recycling that directly affect control of the expression of resistance pathways through the AmpR transcription factor network, especially expression of the AmpC β-lactamase. The steps (also shown in Scheme 1) illustrate the probable events leading to the muropeptide structure **6c** as a repressor of AmpC expression acting through AmpR (normal peptidoglycan recycling), and muropeptide **8c** as a depressor of AmpC expression acting through AmpR (perturbed peptidoglycan recycling leading to induction of resistance pathways).

ligands only one of which is NagZ-dependent [92]) of the Gram-negative bacteria with *amp* systems [12,104–106]. As summarized in Scheme 2, these observations are interpreted in terms of structure **8c** (anhydroMurNAc-pentapeptide) as the signal structure for β-lactamase induction (the co-inducer that derepresses *ampC*). In keeping with LMM PBP4 inactivation as the signal molecule-defining structural event, by inference structure **8b** (as a muropeptide product of unaltered peptidoglycan recycling) as the co-effector structure (that gives *ampC* repression). While this conclusion remains fully consistent with experimental observation, and with continuing muropeptide analyses with respect to AmpR regulation [86], there is as yet no direct experimental evidence in support of these assignments. Chromatographic analysis of the muropeptides released from the cell wall has proven value to the understanding of peptidoglycan structure and assembly [107] and to the mechanisms of the enzymes acting on the peptidoglycan (exemplified [27,108,109]). The application of such studies to define the detailed character of the enzymes newly identified as pivotal in Gram-negative peptidoglycan recycling, such as the PBP4 of *P. aeruginosa* [74], will surely follow.

7. Challenge and opportunity

Future chemotherapy of Gram-negative infections will require antibiotics that combines efficacy with a focused spectrum of activity, and in all likelihood by the use of multi-agent regimens. Current single agent β-lactam therapy (whether cephalosporin or carbapenem) is threatened. Strategies that interfere with critical bacterial pathways so as to restore β-lactam safety and efficacy are attractive [12–15]. Both historical data (where structure–activity development was empirically optimized to avoid induction of resistance mechanisms) and current data (NagZ inhibition to suppress induction of AmpR-dependent resistance mechanisms) support the assertion that interfering with peptidoglycan turnover and recycling involves critical bacterial pathways. Fundamental aspects of the enzymology within these pathways are still being discovered, with each step offering potential opportunity [110]. Nor are these peptidoglycan pathways the only pathways that synergize with the β-lactam antibiotics [111]. Exploratory validation of β-lactam synergy has been achieved in Gram-positive bacteria by the pairing of β-lactams with an inhibitor of peptidoglycan biosynthesis [112], an inhibitor of cytoskeleton assembly [113], and inhibitors of wall teichoic acid assembly [114–118]. Similar points of intervention certainly will be found with respect to the envelope of the Gram-negative bacterium. Within the peptidoglycan recycling pathway there is clearly opportunity for further optimization of NagZ inhibitor design. Inhibitor design against the specific lytic transglycosylase that functions on the sentinel pathway of peptidoglycan recycling (while there is every reason to believe that this will be difficult, there is no reason to believe that it is not possible), and the discovery of inhibitors that disrupt either the function or the regulation of other key proteins (such as AmpR and AmpG) in peptidoglycan recycling. Exploitation of these opportunities, however, suffers from our woeful ignorance of the full breadth of structure and function of both the proteins and the pathways. For example, while exceptional progress has been made toward understanding lytic transglycosylase structure [75,119], the experimental analysis of their catalytic activity remains laborious [27]. While the integration of the lytic transglycosylases into the pathways of peptidoglycan synthesis and recycling is complex, probing with inhibitors of these enzymes may reveal the alternate resistance pathways [74]. We have one structure for AmpR, and this structure is only that of its effector domain in the absence of a ligand [17]. The structure and regulation of the transmembrane AmpG permease is unknown. The identity of the protein responsible for a key event in undecaprenol recycling eludes us. Each of these answers is a possible point of intervention. The challenges are difficult, but the opportunity and need are immense.

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