beta-lactamase induction and cell wall metabolism in Gram-negative bacteria

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INTRODUCTION

Bacteria should continuously maintain and shape their envelopes to adapt enormous stresses they encounter in different niches and to meet physiological needs, such as growth and multiplication. Bacterial envelope is highly organized as a layer structure including cell wall, membrane(s), and the possible space between them. The structure of cell envelope varies in prokaryotes. In general, Gram-positive bacteria contain a thick layer of cell wall as well as a layer of cytoplasmic membrane. However, Gram-negative bacteria (e.g., *Escherichia coli*) typically contain an outer membrane, an intervening periplasmic space where a thin layer of cell wall resides, and a layer of cytoplasmic membrane.

The bacterial cell wall is unique to bacteria and plays a critical role in maintaining cell integrity. In addition, the conserved cell wall components, such as muropeptide fragments, could serve as a signal to trigger host immunologic or pathologic responses. In Gram-positive bacteria, the expression levels of the enzyme essential for the biosynthesis of bacteria cell wall, bind and acylate active site of penicillin-binding protein (PBP), the enzyme essential for the biosynthesis of bacteria cell wall.

To counteract bactericidal effect of beta-lactams, bacteria have quickly evolved defense systems in which production of beta-lactamase is a major beta-lactam resistance mechanism. Bacterial resistance to beta-lactam antibiotics has become a worldwide health care problem, as exemplified by the recent emergence of broad-range beta-lactam resistant NDM-1 (New Delhi metallo-beta-lactamase 1) strains (*Kumarasamy et al.*, 2010). Beta-lactamase is an enzyme that could hydrolyze beta-lactam ring, consequently deactivating beta-lactam antibiotics. In Gram-negative bacteria, the beta-lactamase was usually produced at very high concentration constitutively or by induction via direct interaction of beta-lactam antibiotic with regulatory systems (e.g., MecR1/MecI in *Staphylococcus aureus*; *Kogut et al.*, 1956; *Richmond*, 1963, 1965; *Pilocic*, 1965; *Zhu et al.*, 1992; *Fuda et al.*, 2005; *Safa et al.*, 2005). In Gram-negative bacteria, the expression level of beta-lactamase is usually low, however, it has been observed that production of beta-lactamase was inducible but molecular basis for this phenomenon was not clear (*Ambler*, 1980; *Jacobs et al.*, 1997).

In the past, extensive research has focused on the structure, function, and ecology of beta-lactamases while limited efforts were placed on the regulatory mechanisms of beta-lactamases. In 1990s, the induction of beta-lactamase AmpC was observed to be correlated to the recycling process of cell wall in Gram-negative bacteria.
bacteria, which shed light on the molecular basis of beta-lactamase induction (Jacobs et al., 1994). In the past two decades, accumulating evidence has shown the relationship between muropeptide release and beta-lactamase induction in Gram-negative bacteria (Holdt et al., 1994; Jacobs et al., 1994, 1997; Koasak et al., 2005). However, in Gram-positive bacteria, there is little evidence showing the induction of beta-lactamases by liberated murein fragments. Recently, Amoroso et al. (2012) observed that a cell wall fragment could re-enter in the cytoplasm of Bacillus licheniformis and function as a signal to induce the expression of beta-lactamase. However, whether this cell wall fragment is the major signal for beta-lactamase induction in this Gram-positive bacterium still needs to be determined in the future. Given the lack of information on the relationship between beta-lactamase induction and cell wall metabolism in Gram-positive bacteria, in this review, we only summarize the relevant background information and recent research on the mechanisms of beta-lactamase induction by cell wall fragments in Gram-negative bacteria. In addition, we also discuss potential strategies to mitigate beta-lactam resistance by targeting beta-lactamase induction pathways.

PEPTIDOGLYCAN BIOSYNTHESIS AND RECYCLING

In Gram-negative bacteria, peptidoglycan (PG), also called murein, is a mesh structure with units of continuous biopolymer residing on the intervening space between the outer and inner (cytoplasmic) membrane. Specifically, PG is a polysaccharide composed of repeating β-(1→4)-GlcNAc-P-β-(1→4)-MurNAc disaccharide interconnected by oligopeptide stems via covalent bond (Glauer et al., 1988; Figure 1). The PG maintains cell integrity by sustaining internal osmotic pressure and keeps the regular bacterial shape. The glycan strand in E. coli is averagely composed of 29 disaccharide-peptide units (Glauer, 1988).

The PG biosynthesis involves multi-stage enzymatic activities. First, the PG monomer unit (disaccharide with oligopeptide stem) is attached to a lipid in the cytoplasmic leaf of inner membrane (van Heijenoort, 2001b; Barreteau et al., 2008; Bouhss et al., 2008). Second, the PG monomer-lipid intermediate is flipped into periplasm and catalyzed into the end of extending glycan chain by glycosyltransferases (Goffin and Ghysen, 1998; van Heijenoort, 2001a; Sauvage et al., 2008). Finally, the stem oligopeptides (L-Ala-γ-D-Glu-meso-A2pm-(L)-D-Ala-D-Ala pentapeptide) are liberated from PG. The main muropeptides are GlcNAc-anhMurNAc-L-Ala-γ-D-Glu-meso-A2pm-d-Ala (GlcNAc-anhydroMurNAc-tetrapeptide), with small amount of tri-, pentapeptides (Glauer, 1988). Second, these muropeptides are transported into cytoplasm through the inner membrane transporter AmpG (Park and Uehara, 2008). Subsequently, in cytoplasm, the GlcNAc sugar residue is removed by the glycoside hydrolase NacZ (Cheng et al., 2000; Votsch and Templin, 2000). The resulting population of L-1-anthroMurNAc-oligopeptides are further transformed to UDP-MurNAc-pentapeptide (Park and Uehara, 2008), a PG precursor that can be reincorporated into the PG biosynthesis pathway (Park and Uehara, 2008). The muropeptides could also serve as a signal to induce the production of beta-lactamase, which will be discussed below in Section “Mechanisms of Beta-lactamase Induction.”

BETA-LACTAM ANTIBIOTICS AND BETA-LACTAMASE

In 1928, Alexander Fleming observed the bactericidal effect of Penicillium notatum, leading to the identification of the first beta-lactam antibiotic, penicillin (Fleming, 1929). Since then, a variety of beta-lactam antibiotics with different antimicrobial profiles have been discovered or synthesized, such as penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems. All beta-lactam antibiotics share a common core containing a four-member beta-lactam ring (Figure 2). This beta-lactam ring displays phenomenal structural mimicry with the backbone of the D-alanyl-D-alanine, the substrate of PBPs (including PBP1a, PBP1b, PBP1c, PBP2, and PBP3; Goffin and Ghysen, 1998; Sauvage et al., 2008). Thus, PBPs are involved in the final stage of PG synthesis. Each bacterial cell may produce different PBPs, leading to various types of cross-linkage, such as β-Ala → (D)-meso-A2pm, (L)-meso-A2pm → (D)-meso-A2pm, and so on (van Heijenoort, 2011), for making a rigid mesh structure of PG.

Notably, PG is not a static biological structure. The structural units of PG changes dynamically during bacterial growth and doubling, with old units degraded and new materials added. Instead of starting over the complete de novo synthesis as described above, large quantities of the new materials added are recycled from the degraded PG units. It’s estimated that up to 60% of the parental cell wall is made of the recycled PG units during active bacterial growth (de Pedro et al., 2001; Park and Uehara, 2008).

The PG recycling also involves multi-stage enzymatic activities. First, the lytic transglycosylase (LT) cleaves the glycan strand between the MurNAc and GlcNAc, and forms the 1,6-anhydro bond at the newly exposed MurNAc end in the mean time. With the aid of the endopeptidases (e.g., PBP2) that could break the cross-linkage between stem oligopeptides, anhydro muropeptide monomers (GlcNAc-anhydroMurNAc-peptides) are liberated from PG. The main muropeptides are GlcNAc-anhMurNAc-L-Ala-γ-D-Glu-meso-A2pm-d-Ala (GlcNAc-anhydroMurNAc-tetrapeptide), with small amount of tri-, pentapeptides (Glauer, 1988). Second, these muropeptides are transported into cytoplasm through the inner membrane transporter AmpG (Park and Uehara, 2008). Subsequently, in cytoplasm, the GlcNAc sugar residue is removed by the glycoside hydrolase NacZ (Cheng et al., 2000; Votsch and Templin, 2000). The resulting population of L-1-anthroMurNAc-oligopeptides are further transformed to UDP-MurNAc-pentapeptide (Park and Uehara, 2008), a PG precursor that can be reincorporated into the PG biosynthesis pathway (Park and Uehara, 2008). The muropeptides also could serve as a signal to induce the production of beta-lactamase, which will be discussed below in Section “Mechanisms of Beta-lactamase Induction.”
Figure 1 | Schematic structure of PG and target sites of different enzymes (pointed by color arrows). The synthetic enzyme (PBP) is highlighted in red while the lytic enzymes (NagZ, AmpD, and LT) are highlighted in blue. Notably, NagZ and AmpD catalyze the liberated muropeptides instead of intact PG. Hexagons denote sugars while rectangles denote stem amino acids. The cross-linkage between the top and bottom glycan strands is D-Ala → meso-A2pm. LT, lytic transglycosylase; PBP, penicillin-binding protein; m-A2pm, meso-diaminopimelic acid; AnhMurNAc, 1,6-anhydro-MurNAc; β1→4, β-(1,4)-glycosidic bond.

Figure 2 | The mimicry of beta-lactam antibiotics to D-alanyl-D-alanine (D-Ala-D-Ala). The four-member lactam ring in penicillin was highlighted in red.

Although numerous efforts have been placed on the discovery of new generation of beta-lactam antibiotics to further improve their clinical efficacy, bacteria have been evolving with an unbeatable pace to fail those new beta-lactams (Culotta, 1994). To address this serious public health issue, it is imperative to study the molecular basis of beta-lactam resistance so that we can overcome beta-lactam resistance by targeting resistance mechanisms.

The molecular mechanisms of beta-lactam resistance have been widely studied (Ogawara, 1981; Fuda et al., 2004; Jovetic et al., 2010; Harris and Ferguson, 2012). To evade the bactericidal effects of beta-lactam antibiotics, Gram-negative bacteria have evolved multiple strategies, such as production of beta-lactamases (Korfmann and Wiedemann, 1988; Jacoby, 2009), production of novel PBPs with reduced affinity to beta-lactam antibiotics (Fuda et al., 2004), reducing beta-lactam antibiotics entry through mutations in porins, and expelling beta-lactam antibiotics out of cells using multi-drug efflux pumps (Kohler et al., 1999). Of these mechanisms, producing beta-lactamases, the enzymes that could hydrolyze beta-lactam ring, is still the most efficient strategy (Abraham and Chain, 1940; Jacoby and Munoz-Price, 2005). It has been proposed that beta-lactamases and the PBPs may share a common ancestor due to the presence of certain sequence homology (Massova and Mobashery, 1998). Recently, Fernandez et al. (2012) observed that overexpression beta-lactamases changed the
As mentioned above, in many bacteria belonging to Enterobacteriaceae family, AmpC expression is induced by beta-lactam antibiotics. Since beta-lactam antibiotics treatment can trigger the release of large amount of muropeptides in periplasm, which could be subjected to cell wall recycling process, the relationship between cell wall recycling and beta-lactamase induction has been examined and confirmed in recent studies. Briefly, in the AmpG–AmpR–AmpC pathway, beta-lactam antibiotics treatment breaks the balance of PG biosynthesis (e.g., due to the inhibited PRP and the functional LT), consequently liberating GlcNAc-anhydro-MurNAc-oligopeptides in periplasm (Templin et al., 1992). The GlcNAc-anhydro-MurNAc-oligopeptides are further transported into cytoplasm through AmpG transporter (Park and Uchiara, 2008). The GlcNAc moiety is removed by enzyme NagZ, leading the accumulated PG products (mainly anhydro-MurNAc-tetrapeptides). In cytoplasm, anhydro-MurNAc-oligopeptide are the inducer of beta-lactamase expression through the interaction with AmpR (Lindquist et al., 1989; Jacobs et al., 1997).

AmpR is a LysR type transcriptional regulator and is encoded immediately upstream of ampC with opposite direction (Lindquist et al., 1989; Jacobs et al., 1997). AmpR was demonstrated as an activator for ampC using in vitro transcription assay (Jacobs et al., 1997). However, production of ampC was still repressed even if bacterial host contains functional AmpR, unless exogenous beta-lactam antibiotic was added (Honore et al., 1989; Lindquist et al., 1989, Lodge et al., 1999; Jacobs et al., 1999). Therefore, it has been hypothesized that the activator function of AmpR was inhibited by certain cellular metabolite, which was demonstrated as the cell wall synthesis precursor, Udp-MurNAc-pentapeptide (Jacobs et al., 1997). This inhibition was abolished in the mutant with point mutation in AmpR (G102E; Bartowsky and Normark, 1991), indicating the role of the residue G for the association of Udp-MurNAc-pentapeptide. Upon the treatment of beta-lactam antibiotics, the accumulated intracellular anhydro-MurNAc-oligopeptides could displace the AmpR-associated Udp-MurNAc-pentapeptide, triggering conformational change of AmpR, and subsequently activating the transcription of ampC (Jacobs et al., 1997). The DNase-I-protection assay showed the binding site of AmpR was in a 39-bp region upstream of the ampC transcription start site (−40 to −88; Jacobs et al., 1997). Interestingly, AmpR in P. aeruginosa is a global transcriptional factor whose regulon includes beta-lactamasases, proteases, quorum sensing, and other virulence factors (Kong et al., 2005; Balasubramanian et al., 2012).

Among the PG cycling process, there is a negative effector to fine-tune the expression of AmpC. A cytoplasmic N-acetylmuramoyl-l-alanine amidase, named AmpD (Holite et al., 1994), could dissociate stem peptides from the anhydro-MurNAc or GlcNAc-anhydro-MurNAc, therefore, reducing concentrations of the inducing muropeptides and mitigating the overexpression of AmpC (Jacobs et al., 1994).

Consistent with these observations on the relationship between PG recycling and beta-lactamase induction, perturbation of PG recycling also affected AmpC induction, suggesting potential pharmaceutical targets. For example, overproduction of the LT MRB stimulated beta-lactamase induction whereas specific inhibition of LT Slt70 by bulgecin repressed AmpC expression (Kraft et al., 1999). In addition, mutation of all six LT enzymes (Slt70, MltA, MltB, MltC, MltD, and EmtA) in E. coli decreased the beta-lactamase activities (Korsak et al., 2005).
Different versions of AmpG–AmpR–AmpC regulatory pathways exist in bacteria. For example, E. coli and Shigella spp. lacks an ampR gene (Bergstrom et al., 1982; Honore et al., 1986), leading to the low level, non-inducible expression of AmpC. The AmpC gene in E. coli was primarily regulated by an attenuator sequence in promoter region (Jaurin et al., 1981). The overexpression of AmpC can be achieved either by mutating attenuator (Jaurin et al., 1981) or by introducing an AmpR regulator (Kraft et al., 1999); the similar pathway was also observed in Acinetobacter baumannii (Boy and Martinez-Beltran, 2000). In Salmonella, the chromosomal AmpC–AmpR is usually absent, which may be due to unbearable production cost of AmpC (Morosini et al., 2000). However, clinical Salmonella strains can acquire AmpC–AmpR through horizontally transferred mobile elements (Barraud et al., 1998). In Serratia marcescens, besides AmpR regulation, the post-transcriptional regulation also influences the expression of AmpC. Specifically, the half-life of ampC transcript could be affected by a 126-bp, non-encoding region that forms a stem-loop structure (Mahlen et al., 2003). In P. aeruginosa PAO1, interestingly, there are three copies of ampD genes, which contributed to the stepwise up-regulation of AmpC with the discrete mutation of each copy of ampD (Juan et al., 2006).

THE BlrAB-LIKE TWO-COMPONENT REGULATORY SYSTEM

The TCRS, which involves sensing specific environmental stimuli (Capra and Laub, 2012), was also observed to be involved in the induction of beta-lactamase. In Aeromonas spp., the AmpC and two other chromosomally encoded beta-lactamases were regulated by the response regulator BlrA of a TCRS instead of an AmpR-type regulator (Alksne and Rasmussen, 1997). Complementation study demonstrated that overexpression of BlrA in E. coli enhanced the expression of the Aeromonas-derived beta-lactamase in E. coli MC1061 while the beta-lactamase was expressed at low level in the absence of BlrA (Alkone and Rasmussen, 1997).

The closest TCRS homolog of BlrAB in E. coli is CreBC (Anemura et al., 1996; Wanner and Wilmes-Riesenbeck, 1992). Interestingly, the beta-lactamases from Aeromonas hydrophila could be regulated by the CreBC TCRS system in the Cre+ E. coli strain such as DH5α (Avison et al., 2000, 2001). The “cre/blr-tag” signature, which is the “TTCACnnnnnnTTCAC” motif located in the promoter of Cre-regulon, was identified in E. coli (Avison et al., 2001). These “cre/blr-tag” also reside in promoters of Aeromonas-derivative beta-lactamases (Niumsup et al., 2003), and the induction of those beta-lactamases by overexpressed BlrA was dependent on the presence of “cre/blr-tag” (Avison et al., 2004).
In *P. aeruginosa*, inactivation of a non-essential PBP was shown to trigger overproduction of a chromosomally AmpC gene and this overproduction is dependent on CreBC/TGRS (Moya et al., 2009).

Interestingly, among the 52 tested *E. coli* TGRS response regulators, overexpression of *FinZ* conferred increased level of beta-lactam resistance through the action of AmpC in *E. coli* (Hirakawa et al., 2003).

Despite above evidence showing that TGRS is also involved in the induction of beta-lactamase, the identity of the corresponding cues to which the TGRS respond for beta-lactamase induction is still unknown. We speculate that specific degraded PG components may serve as a signal for the response regulator to induce the production of beta-lactamase. This hypothesis needs to be examined in the future.

**OTHER MECHANISMS**

Another novel beta-lactamase induction pathway was discovered in *Ralstonia pickettii* (Girlich et al., 2006). The chromosomally encoded beta-lactamases (OXA-22 and OXA-60) were regulated by ORF-RP3 (short for RP3), a gene located at 192-bp upstream of the AGG codon of oxa-61. Inactivation of RP3 resulted in the abolishment of induction of both beta-lactamases; complementation of the RP3 restored the inducible expression of OXA-22 and OXA-60 (Girlich et al., 2006). DNase I footprinting showed that RP3 specifically bound to tandem repeats upstream at the transcriptional start sites of OXA-22 and OXA-60 genes, suggesting RP3 is a novel positive-regulator for beta-lactamase induction (Girlich et al., 2009).

**PHARMACEUTICAL IMPLICATIONS OF BETA-LACTAMASE INDUCTION MECHANISM**

Discovery of beta-lactamase inhibitors is a promising strategy to combat the prevalent beta-lactam resistance (Bush and Macielag, 2010; Harris and Ferguson, 2012). However, this approach is challenged by the variable affinity of the inhibitors to different beta-lactamases and by the overwhelming quantity of the beta-lactamases produced in resistant cells. Based on the information reviewed here, we propose that the signaling pathways of beta-lactamase induction offer a broad array of promising targets for the discovery of new antibacterial drugs used for combination therapies. The inhibitors targeting beta-lactamase induction pathway may prevent the emergence of beta-lactam resistance and enhance the efficacy of clinical beta-lactam antibiotics, as what we have observed for the efflux pump inhibitors (Lomovskaya and Bostian, 2006). In supporting this hypothesis, the frequency of emergence of ceftazidime resistance in *P. aeruginosa* was below the detection limit ($<10^{-11}$), which is far below that for the wild-type parent strain ($3 \times 10^{-5}$; Moya et al., 2009).

The potential targets in the beta-lactamase induction pathway as well as the known inhibitors are summarized in Table 1. Several inhibitors have been identified for LTs that play a critical role in the initializing the PG cycling. The LT inhibitor bulgecin could induce cell lysis and morphology changes in the presence of beta-lactam antibiotics although bulgecin alone did not show any antibacterial activity against *E. coli* (Imada et al., 1982; Nakao et al., 1986; Bonis et al., 2012). The major molecular target of bulgecin was the soluble LT SI70 (Templin et al., 1992). In a 2.8-Å resolution crystallographic structure of SI70-bulgecin complex, one single bulgecin molecule was found to be located in the active site of SI70, indicating that bulgecin may act as an analog of an oscurcinon ion intermediate in the reaction catalyzed by SI70 (Thunnissen et al., 1993). The beta-hexasaminidase inhibitor $N$-acetylglucosamine thiazoline (NAG-thiazoline) was also found to inhibit the LT sLTB from *P. aeruginosa* (Reid et al., 2004a,b). Another inhibitor, hexa-$N$-acetylichitohexaose, can inhibit the LT from bacteriophage lambda (Leung et al., 2001). Interestingly, a proteinaceous inhibitor of vertebrate lysozymes (Ivy), which has conserved CXPHDC motif, was also found to control the autolytic activity of bacterial Lts (Clarke et al., 2010).

Regarding other targets in beta-lactamase induction pathway, PUGNac and modified EtBuPUG can inhibit the function of NagZ by the mimicry of the oxocarbenium ion-like transition state (Stubbs et al., 2007). Unlike PUGNac that is also a potent inhibitor against human O-GlcNAcase and beta-hexasaminidase, EtBuPUG displayed 100-fold selectivity toward to NagZ. The function of inner membrane permease AmpG in laboratory strains of *P. aeruginosa* can be inhibited by carbonyl cyanide m-chlorophenylhydrazone (CCCP), a general inhibitor of proton motive force, consequently leading to an increased susceptibility to beta-lactam antibiotics (Cheng and Park, 2002; Zhang et al., 2010). However, it is important to mention that CCCP also targets other energy-dependent systems, such as drug efflux pump; thus, the linkage between reduced beta-lactam resistance and AmpG inhibition was not clearly demonstrated in these studies.

Although a panel of inhibitors that target the PG recycling pathway have been identified (Table 1), it is still largely unknown if these inhibitors repress the inducible beta-lactam resistance effectively in Gram-negative bacteria, consequently enhancing the

| Target | Function | Inhibitor |
|--------|----------|-----------|
| LT     | Non-hydrolytic cleave PG with the concomitant formation of 1,6-anhydro-MurNAc | Bulgecin A (Templin et al., 1992); NAG-thiazoline (Reid et al., 2004a,b); hexa-$N$-acetylichitohexaose (Leung et al., 2003); Ivy (Clarke et al., 2010)* |
| NagZ   | Cleave disaccharide oligopeptides to release 1,6-anhydro-MurNAc-peptide | PUGNac; EtBuPUG (Stubbs et al., 2007) |
| AmpG   | Inner membrane permease of the 1,6-GlcNAc-anhydro-MurNAc-peptides | CCCP (Cheng and Park, 2002) |
| AmpR   | Binary regulator of AmpG | UDP-$N$-acetylmuramic acid peptides (Jacobs et al., 1997) |

*Proteinaceous inhibitor, also the inhibitor of vertebrate lysozymes.
efficacy of clinical beta-lactam antibiotics. This knowledge gap needs to be filled in the future. In addition, similar to all infections, disease drug developments, discovery of A. prophage protein, inhibitor targeting the beta-lactamase induction pathway and conversion such inhibitor into a clinically useful therapeutic agent are likely a lengthy and challenging process. Some key issues, such as toxicity, stability, adhesion, and production cost, must be addressed. Despite these challenges, it is imperative to develop clinically useful inhibitors to suppress beta-lactamase induction and enhance "shield-life" of a broad spectrum of beta-lactam antibiotics against bacterial pathogens. To achieve this goal, in-depth structural and functional studies are needed for the potential targets (Table 1), which is critical for identifying corresponding inhibitor(s) without increasing the antibiotic resistance issue modern approaches, such as high throughput screening of chemical compound library, homology modeling and molecular docking.

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