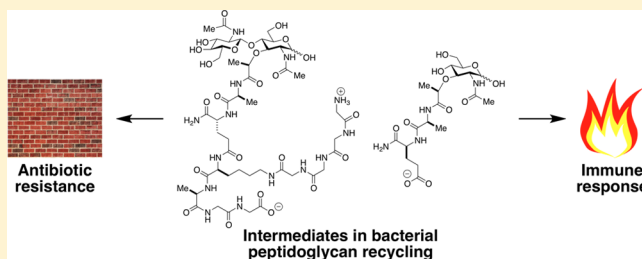


Messenger Functions of the Bacterial Cell Wall-derived Muropeptides

Marc A. Boudreau, Jed F. Fisher, and Shahriar Mobashery*

Department of Chemistry and Biochemistry, Nieuwland Science Hall, University of Notre Dame, Notre Dame, Indiana 46556, United States

ABSTRACT: Bacterial muropeptides are soluble peptidoglycan structures central to recycling of the bacterial cell wall and messengers in diverse cell signaling events. Bacteria sense muropeptides as signals that antibiotics targeting cell-wall biosynthesis are present, and eukaryotes detect muropeptides during the innate immune response to bacterial infection. This review summarizes the roles of bacterial muropeptides as messengers, with a special emphasis on bacterial muropeptide structures and the relationship of structure to the biochemical events that the muropeptides elicit. Muropeptide sensing and recycling in both Gram-positive and Gram-negative bacteria are discussed, followed by muropeptide sensing by eukaryotes as a crucial event in the innate immune response of insects (via peptidoglycan-recognition proteins) and mammals (through Nod-like receptors) to bacterial invasion.



A bacterium must engage its environment and yet preserve its structural integrity during the processes of growth and cell division. A key structural component of this integrity is the bacterial envelope. The envelope comprises the bacterial cell surface as a tightly organized series of layers, including the cell wall, membrane(s), and the intervening space(s) between these layers.^{1,2} The organization of these layers demarcates the prokaryotes. For a monoderm (single membrane) bacterium such as *Staphylococcus aureus* (a Gram-positive-staining bacterium), the outer surface of its envelope is the cell wall, and the cell wall surrounds a single membrane. For diderm bacteria such as *Mycobacterium tuberculosis* (also Gram-positive-staining), the outer surface is comprised of mycolic acid lipids attached to the cell wall, while for the Gram-negative-staining diderm *Escherichia coli*, the layer organization is an outer-membrane surface (with a lipopolysaccharide external leaflet), followed by an intervening space (the periplasm, within which is the cell wall) and then an inner membrane. Because many of the components of the bacterial envelope are structurally unique to the bacteria, the biochemical pathways relating to the bacterial envelope (and especially to the cell wall) are molecular targets of antibiotics.^{3,4} To preserve the structural integrity of this incredibly complex envelope, bacteria have developed mechanisms to detect and respond to circumstances that jeopardize its integrity. Likewise, when the food source for a bacterium is an unwilling eukaryote, the eukaryote responds and does so in part by detecting the presence of structures uniquely bacterial. One particular structural class, used in both detection events, is the bacterial cell wall-derived muropeptide.

The bacterial cell wall is a peptidoglycan polymer (a single molecule, called the sacculus) formed by the cross-linking of oligopeptide stems presented by neighboring glycan strands. A synonym for the peptidoglycan polymer is “murein”, derived

from *murus*, Latin for “wall”. The structure of the polysaccharide is a repeating β -(1 \rightarrow 4)-(*N*-acetylglucosamine- β -(1 \rightarrow 4)-*N*-acetylmuramic) disaccharide possessing interconnected oligopeptide stems attached to the lactyl group of the muramic saccharide. During cell-wall biosynthesis, the oligopeptide stem of one glycan strand is cross-linked by the transfer of an acyl group to an amine on the stem of an adjacent strand. Notwithstanding the seeming implication of a repetitive pattern for the peptidoglycan structure, the sacculus shows substantial “internal” variability in terms of glycan strand length, oligopeptide stem structure, and degree of cross-linking under different growth conditions. The term “muropeptide” refers to the ensemble of structures obtained from enzymatic digestion of the peptidoglycan polymer. This digestion uses both glycan-cleaving and stem-cleaving (amidase) enzymes, giving the representative (*N*-acetylglucosamine- β -(1 \rightarrow 4)-*N*-acetylmuramic) (GlcNAc-MurNAc) muropeptide structures of Scheme 1. The ensemble of structures that results from these enzymatic events is identified typically by mass spectrometric interpretation of the peaks obtained from high-performance liquid chromatography (HPLC) analysis.

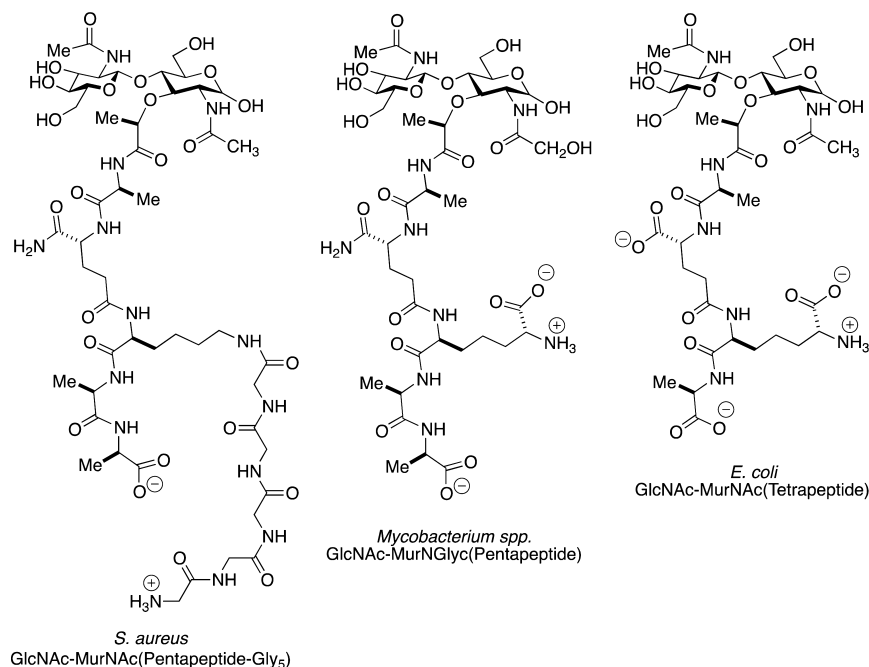
Muropeptide structures are also obligatory intermediates during cell-wall biosynthesis and remodeling by bacteria, and during infection as a result of the immune response. The biological relevance of muropeptide structures to both events has been known for some time.⁵ However, as a result of the confluence of technological advances, including an improved understanding of the molecular bases for immune recognition, the routine availability of robust methods for determining

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Scheme 1^a


^aAt the left is a representative mucopeptide of the monoderm Gram-positive bacterium *S. aureus*. The GlcNAc-MurNAc disaccharide is shown as the “free” MurNAc glycoside. This reducing saccharide is customarily transformed by chemical reduction prior to chromatographic analysis. The sequence of the peptide stem attached to the lactyl carboxylate of the MurNAc is L-Ala-D-iGln-L-Lys-D-Ala-D-Ala. A (Gly)₅ “bridge” is biosynthetically added to the ε-amine of the L-Lys. In the stem cross-linking event catalyzed by the bifunctional penicillin-binding protein enzymes, the ultimate D-Ala residue is lost as a leaving group as an acyl-enzyme is formed. This D-alanyl acyl-enzyme is transferred to the amine of the terminal glycine of the (Gly)₅ bridge of an adjacent peptidoglycan strand to complete the cross-link. In the center is a representative mucopeptide of a Gram-positive *Mycobacterium* spp., with an L-Ala-D-iGln-meso-DAP-D-Ala-D-Ala stem peptide sequence. Cross-linking occurs from the D-alanyl acyl-enzyme derived from one strand to the amine terminus of the meso-diaminopimelate of an adjacent strand. At the right is a representative mucopeptide of the diderm Gram-negative bacterium *E. coli*.

mucopeptide structure, and the development of methods for mucopeptide synthesis, the specific roles of particular mucopeptide structures as messengers are at last coming into focus. Mucopeptides serve as messenger molecules to the bacterium that cell-wall-targeting antibiotics are present, to spore formers and persists that time for outgrowth is optimal as communicated by neighboring cells, and to the eukaryote that a bacterial infection is in progress. In this work, we evaluate these roles, with particular emphasis on the relationship of mucopeptide structure to the key biochemical events within each.

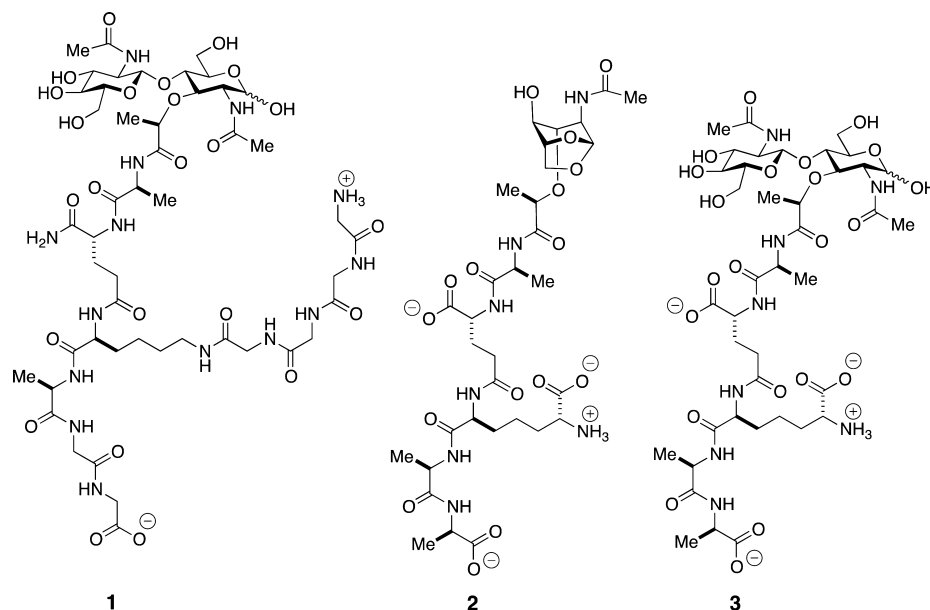
MURPEPTIDE SENSING BY GRAM-POSITIVE BACTERIA

The term antibiotic has a strong anthropocentric connotation: a molecule isolated from Nature that has the ability to engage a bacterial target so as to prevent bacterial growth, or to act as a bactericide, and thus having value for the chemotherapeutic control of infection. Efforts to understand the roles of the natural products in Nature, and of the “antibiotics” in particular, from disinterested perspectives embrace their potential additional roles in intraspecies and interspecies communication.^{6–12} Nonetheless, antibiotics are indisputably functional molecules, and a bacterium that encounters an antibiotic must respond whether for the purposes of evasion or mobilization of a defense. A consequence of the targeting by many antibiotics of the enzymes of cell-wall biosynthesis and remodeling is perturbation of the steady-state concentrations of biosynthetic intermediates, and now in two examples, the β-lactam

antibiotic-dependent induction of vancomycin resistance in the Gram-positive bacterium methicillin-resistant *S. aureus* and induction of β-lactam resistance in Gram-negative bacteria, mucopeptides act as messenger molecules.

The Mucopeptide Messenger in β-Lactam-Dependent Induction of Vancomycin Resistance in Methicillin-Resistant *S. aureus*. Vancomycin is a glycopeptide antibiotic that is most active against Gram-positive bacteria.^{13,14} Its mechanism is disruption of cell-wall biosynthesis by occlusion of the key structures in cell-wall biosynthesis, especially the biosynthetic intermediate Lipid II. This occlusion occurs by formation of a stable, noncovalent complex between vancomycin and cell-wall structures exhibiting a -D-Ala-D-Ala stem terminus (see Scheme 1) as a key recognition motif.¹⁵ Two mechanisms for resistance to vancomycin are known. In the *Enterococci*, there is an alternative biosynthetic pathway in which the -D-Ala-D-Ala stem structure is replaced with a depsipeptide -D-Ala-D-Lac structure, for which vancomycin has poor affinity.^{16,17} Vancomycin-resistant *Enterococci* result. Intermediate-level vancomycin resistance occurs in another Gram-positive bacterium, *S. aureus*, as a result of an increased level of peptidoglycan biosynthesis giving a thicker and more extensively cross-linked peptidoglycan. While vancomycin-D-Ala-D-Ala complexes are still formed, they are unproductive with respect to inhibition of cell-wall biosynthesis.¹⁸ A new variation on this second mechanism for attaining vancomycin resistance is now described for a strain of methicillin-resistant *S. aureus* (MRSA).¹⁹ MRSA has high-level resistance to the β-lactam class of antibiotics (this class includes the extensively

Scheme 2. Muropeptide Messengers Implicated in the Induction of Antibiotic Resistance^a



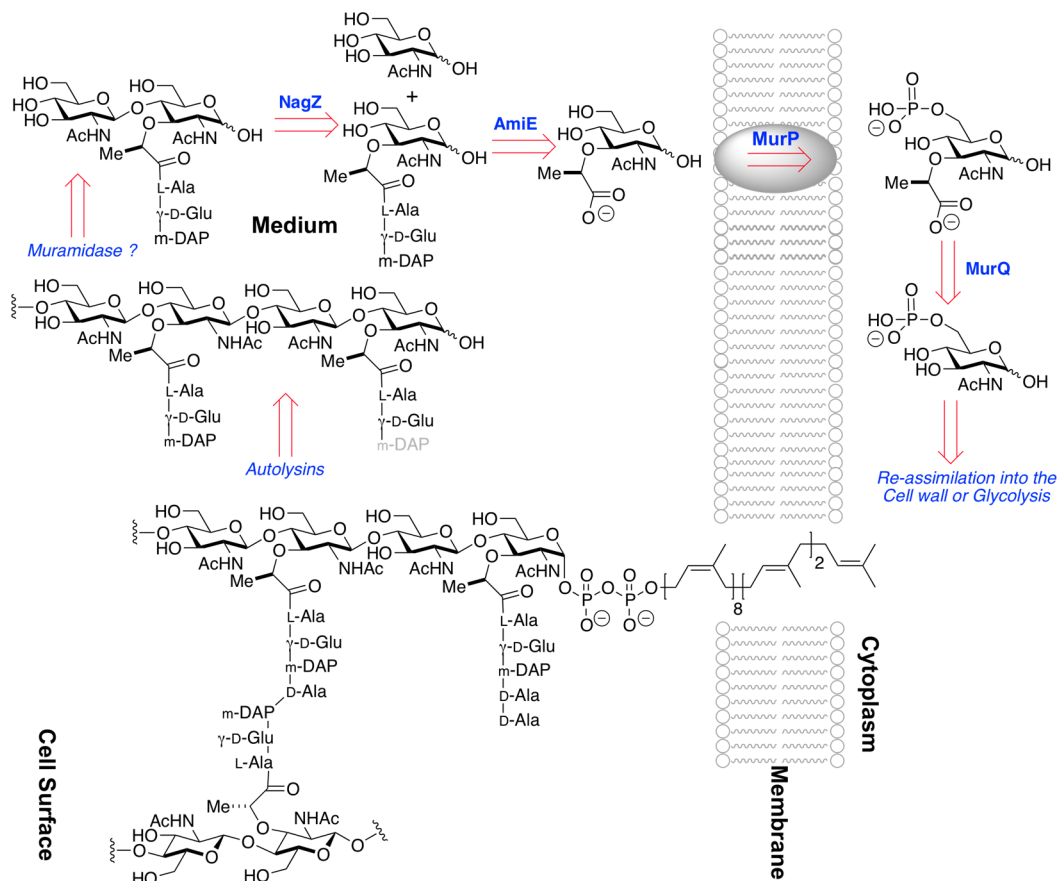
^aBIVR-MRSA *S. aureus* responds to the presence of muropeptide **1** in the medium with the creation of a cell wall that is more able to deplete vancomycin from the medium. Following β -lactam challenge of Gram-negative bacteria having a complete *ampR-ampC* system, the structural homeostasis of the free muropeptide pool entering the cytoplasm for recycling is altered. Among the enzymes controlling the structural identities of the entering muropeptides are a low-molecular mass PBP (found in the periplasm and presumed to be inhibited by the β -lactam), the NagZ β -glucosaminidase of the cytoplasm, and the AmpD amidase of the cytoplasm (see Scheme 5). Anhydromuropeptide **2** is the probable structure that engages the LysR-type transcription regulator AmpR to derepress the *ampC* gene and allow AmpC β -lactamase expression. Following β -lactam challenge of the Gram-negative bacterium *Aeromonas hydrophila*, the level of muropeptide **3** is increased (whether as part of the cell wall or as the free muropeptide). This increase is sensed by a BlrB kinase system and results in the induction of β -lactamase expression.

clinically used penicillins, cephalosporins, and carbapenems). β -Lactam antibiotics act as β -D-Ala- β -D-Ala mimetics to achieve irreversible inactivation of the transpeptidase domain of the bifunctional penicillin-binding protein (PBP) enzymes of peptidoglycan biosynthesis. β -Lactam resistance by MRSA results from the replacement of a β -lactam-susceptible PBP by a PBP enzyme that is not susceptible. Because MRSA-infected patients are often also infected with Gram-negative bacteria, combined vancomycin and β -lactam therapy is common. This combination therapy has given rise to β -lactam antibiotic-induced vancomycin-resistant MRSA (BIVR-MRSA). In the presence of β -lactam antibiotics, BIVR-MRSA cells survive by a structural transformation that allows the rapid depletion of vancomycin from their environment, eventually allowing the resumption of cell growth when the vancomycin is sufficiently depleted.¹⁹ A hypothesis for explaining this phenomenon, based on the vancomycin-resistant *Enterococci*, is an increased level of Lipid II biosynthesis induced by the β -lactam antibiotics giving more abundant β -D-Ala- β -D-Ala-containing muropeptides. Ikeda et al. show that in the presence of both vancomycin and the β -lactam antibiotic ceftizoxime, BIVR cells released 10-fold more muropeptides into the medium than either MRSA or methicillin-susceptible *S. aureus*.²⁰ Induction of BIVR resistance correlated with the presence of a particular muropeptide structure, GlcNAc-MurNAc-L-Ala-D-iGln-L-Lys-(ϵ -Gly₄)- β -D-Ala-Gly₂ [**1** (Scheme 2)], identified by in vitro enzymatic degradation of the cell wall and exhaustive muropeptide purification. When this purified muropeptide is added to a vancomycin-containing culture of BIVR-MRSA in the absence of a β -lactam, the bacteria grow more rapidly than bacteria treated with vancomycin alone. In this same assay, the muropeptide derivative of **1** lacking the tetraglycine substitution

to the ϵ -amine of the lysine was equipotent compared to **1**. The ability of these muropeptides at 50–100 μ g/mL to promote growth of BIVR-MRSA cells exceeded that of 1 μ g/mL ceftizoxime.

The mechanism by which **1** induces this BIVR phenomenon is not fully understood. Ikeda et al.²⁰ exclude the simplest explanation—formation of a complex between **1** and vancomycin is not observed—and hence, **1** itself does not act as a vancomycin trap. Previous studies of the muropeptide composition of the peptidoglycan polymer of MRSA show alterations in its biosynthesis, likely as a result of the use of a different ensemble of biosynthetic muropeptides.²¹ Because the thickened cell wall observed in vancomycin-intermediate *S. aureus* correlates to a vancomycin trapping mechanism,²² a mechanism whereby released muropeptides are internalized to “activate” cell-wall biosynthesis (for example, by altering muropeptide structure through biosynthetic gene repression and expression, to favor peptidoglycans with improved vancomycin binding) is possible.²³ A muropeptide sensing mechanism is used to induce β -lactam resistance in Gram-negative bacteria (vide infra). In contrast, the signaling messenger in the VanS histidine kinase-dependent expression of the vancomycin resistance pathway of *Streptomyces coelicolor* is vancomycin itself.²⁴ Both observations attest to the ability of these Gram-positives to internalize complex structures from their environment and to act on this internalization with a resistance response. In contrast, the molecular mechanism for the expression of vancomycin resistance in enterococcal pathogens is not proven. Vancomycin resistance has a substantial fitness cost that correlates to a requirement for its inducible regulation through a vanR/vanS system.²⁵ However, there are substantial genetic variations in this operon; for

Scheme 3. Schematic Overview of MurNAc Recycling in the Gram-Positive Monoderm *B. subtilis*^a



^aThe single membrane of the bacterium is depicted as a cartoon running vertically to the right of center. The cytoplasm of the bacterium is found to the right of the membrane, while the cell surface is found to the left. In Gram-positive bacteria, the cell surface consists of a thick (relative to Gram-negative bacteria) peptidoglycan (in the example of *B. subtilis*, approximately 35 nm),³⁶ to which proteins are both covalently and noncovalently attached. The membrane-bound peptidoglycan lipid (bottom structure) represents a biosynthetic intermediate, here shown with one un-cross-linked peptide stem and with one cross-linked (to an adjacent glycan strand) peptide stem. Autolysin (including a not-yet-characterized muramidase) releases the GlcNAc-MurNAc disaccharide (top left). NagZ-dependent glycosylase activity splits the disaccharide, following which AmiE (possibly in concert with other amidases) releases the peptide. MurNAc is internalized by the MurP phosphotransferase, after which the lactyl ether is cleaved by the MurQ etherase. This schematic for *B. subtilis* should not be taken as general. Reith and Mayer have obtained strong evidence of a wholly cytoplasmic recycling pathway in the Gram-positive bacterium *Clostridium acetobutylicum*.^{37,38}

example,^{26–28} encompassing differential responses by vancomycin, televancin, and teicoplanin, leaving open the possibilities of regulation by the antibiotic, changes in the cell wall, or changes in the levels of cell-wall intermediates.²³

Muropeptide Recycling in Gram-Positive Bacteria.

The ability of Gram-negative bacteria to efficiently recycle their muropeptides is long recognized and interpreted in terms of the evolutionary advantage with respect to these nutrients, and in the minimization of the immune response of a host to bacterial infection (vide infra).^{29,30} Recognition that muropeptide recycling occurs in Gram-positive bacteria is, however, new.^{29,31} While the peptidoglycan of Gram-negative bacteria is assembled between two membranes, and thus the muropeptides they used for both cell-wall biosynthesis and cell-wall remodeling are confined to their periplasmic space, the cell wall of the monoderm Gram-positive bacterium is exposed to the medium. The apparent absence of a Gram-positive periplasmic space and the observation that as much as 50% of the cell wall is released during the growth of the model Gram-positive bacterium *Bacillus subtilis* suggested the absence of efficient muropeptide recycling.³² As a complement to a study of N-

acetylmuramic acid catabolism in *E. coli*,³³ Mayer et al. identified a cluster of six genes in *B. subtilis* encoding muropeptide-recycling enzymes. The identities of five of the gene products are known: MurQ, a MurNAc-6-phosphate etherase; MurR, the transcriptional regulator; MurP, a MurNAc-specific phosphotransferase; AmiE, an amidase hydrolytically cleaving the MurNAc-L-Ala amide bond; and NagZ, a cell-wall-associated β -N-acetylglucosaminidase.³⁴ The suggested operation of four of the enzymes in this gene cluster,³¹ in concert with other cell-wall autolysins released into the medium, is summarized in Scheme 3. The four *B. subtilis* enzymes are orthologs of enzymes found in the muropeptide recycling pathway of *E. coli*. Additional orthologs of other enzymes, and of a phosphotransferase used by *E. coli* for GlcNAc recycling, were also identified in *B. subtilis*.^{31,35} Although our present understanding suggests that nutrient recovery is a primary role, the evidence implicating a role for muropeptides in β -lactam-dependent induction of vancomycin resistance in BIVR-MRSA and Gram-positive spore resuscitation (discussed below) suggests the possibility that Gram-

positive mucopeptide recovery may be used for transcriptional regulation of resistance responses.

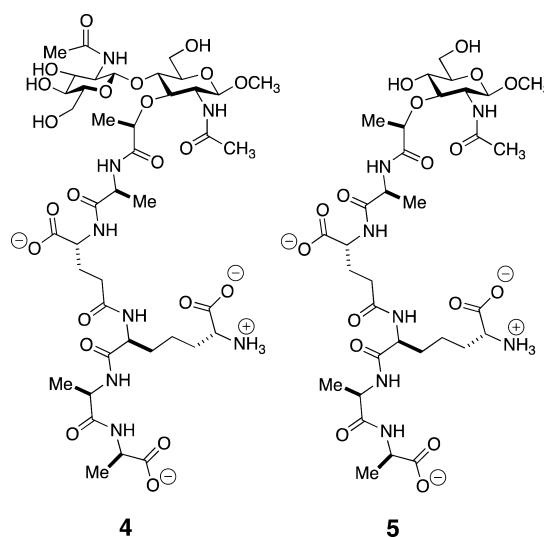
Muropeptide Sensing in Gram-Positive Resuscitation.

Bacteria have sophisticated mechanisms for coping with environmental stress, including the formation of persister³⁹ and dormant states (notably seen for mycobacteria),⁴⁰ and spore formation by other Gram-positive bacteria (notably the Gram-positive *Clostridia* and *Bacillus* sp.).^{41–43} Entry into dormant and spore states, and resuscitation from these states, is of particular interest because of the relationship of these states to human infection by (for example) *M. tuberculosis* and *Bacillus anthracis*.^{44,45} Peptidoglycan structure is a key aspect of these dormant states, and especially with respect to resuscitation. The structures of the peptidoglycans of the dormant *M. tuberculosis* and the *B. anthracis* spore are altered for the purposes of structural integrity, stability toward muramidase degradation, and possible differentiation of the structure of the dormant peptidoglycan from the peptidoglycan made during bacterial growth. A key feature of the dormant mycobacterial peptidoglycan is altered cross-linking^{46,47} of an N-glycolylmuramic acid-containing peptidoglycan,^{48,49} and for the *Bacillus* sp., the extensive appearance in the spore peptidoglycan of the so-called muramic δ -lactam.⁵⁰

A central issue to the understanding of both dormant bacteria and bacterial spores is the mechanism(s) by which they sense the presence of an advantageous environment for the resumption of growth. While there is no doubt that the sensing process is multistep and involves concurrence of multiple signaling pathways, substantial evidence now implicates muropeptides as key signaling molecules during the resuscitation pathways of both dormant mycobacteria and *Bacilli* spores.^{51,52} The targets of these muropeptides are specific bacterial membrane-bound eukaryotic-like serine/threonine protein kinases (STKs).^{53–55} Dworkin et al. have correlated environmental muropeptides, released as a result of peptidoglycan digestion by a secreted peptidoglycan hydrolase,⁵⁶ acting as germinants toward *B. subtilis* spores through activation of the PrkC STK.^{57,58} Evaluation of a set of synthetic muropeptides⁵⁹ as stimulants of the germinant activity of *B. subtilis* spores identified GlcNAc-MurNAc muropeptide **4** (Scheme 4) as being substantially more effective ($EC_{50} = 10$ nM) than either MurNAc monosaccharide **5** or GlcNAc-anhMurNAc disaccharide **2** (Scheme 2). A tetrasaccharide (GlcNAc-MurNAc)₂ muropeptide was equipotent to **4**. The molecular target of muropeptide **4** as the PrkC STK was proven elegantly by substitution of the *B. subtilis* PrkC STK gene with that of the homologous gene for *S. aureus* PrkC. *B. subtilis* synthesizes a meso-diaminopimelate-containing peptidoglycan, and its PrkC STK does not recognize lysine-containing muropeptides. In contrast, *S. aureus* synthesizes a lysine-containing peptidoglycan, and its PrkC recognizes both meso-diaminopimelate- and lysine-containing muropeptides. Thus, spores obtained from *B. subtilis* with the *S. aureus* STK would be expected (as long as the *S. aureus* STK remains competent for signaling in *B. subtilis*) to respond to a lysine-containing muropeptide. Indeed, the *S. aureus* STK is competent in *B. subtilis*, and the spores with the *S. aureus* STK resuscitate in response to the *S. aureus* muropeptide.⁵⁹ Among the pathways presumed to be activated in spore germination are four (in *B. anthracis*) lytic enzymes required for the remodeling of the cortex peptidoglycan of the spore.^{60–62}

A complete understanding of the relationship between binding of the muropeptide to the STK and activation of the

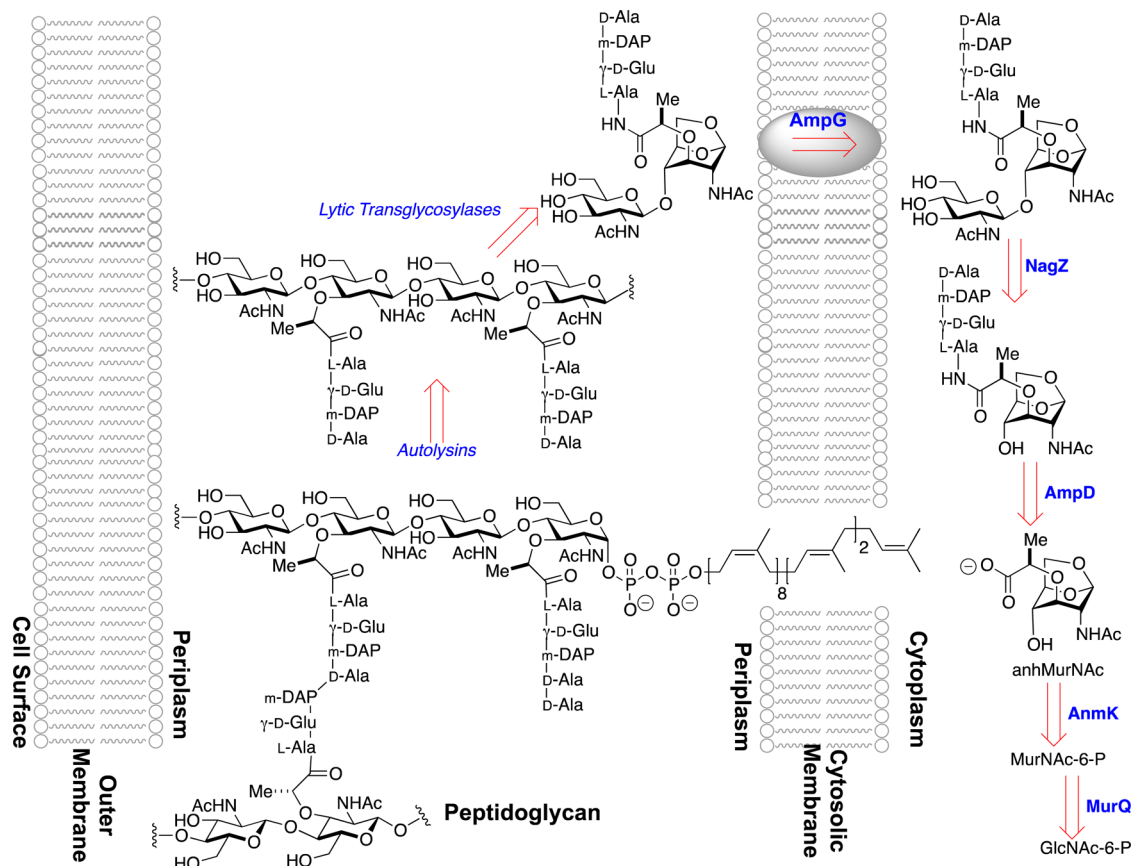
Scheme 4. Structure of Active GlcNAc-MurNAc Muropeptide **4 for Muropeptide-Dependent Resuscitation of *B. subtilis* Spores, via Activation of the *B. subtilis* STK, and Compared to the Much Less Active MurNAc Muropeptide **5**^a**



^aThe two synthetic muropeptides^{59,63} show β -methyl glycosidic termini at their MurNAc saccharides.

STK is not available. The structural organization of these two PrkC proteins is identical: an intracellular serine/threonine kinase domain, a transmembrane domain, and an extracellular domain consisting of a threefold repeat of a penicillin binding-associated and serine/threonine kinase-associated (PASTA) domain.^{53,64} PASTA domains were first encountered in the structure of the highly β -lactam antibiotic-resistant penicillin-binding protein 2x,^{65,66} an enzyme used by methicillin-resistant *Streptococcus pneumoniae* to complete the transpeptidation step of cell-wall biosynthesis in the presence of otherwise inhibitory concentrations of β -lactam antibiotics. The presumption that the muropeptide (and polymeric peptidoglycan)⁶⁷ binds to the PASTA domain(s) is supported by numerous studies.^{58,59} In contrast to the compacted PASTA domains seen in the penicillin-binding protein 2x crystal structures, recent STK structural studies of the *M. tuberculosis* PknB kinase,⁶⁸ the *S. aureus* Stk1 kinase,⁶⁹ and the *S. aureus* PrkC kinase⁷⁰ implicate an extended orientation of the PASTA domains. Notwithstanding the submicromolar muropeptide activity of synthetic muropeptides in *B. subtilis* spore resuscitation, the in vitro affinity of these STKs for muropeptide structure is not high. A GlcNAc-MurNAc-pentapeptide muropeptide showed millimolar affinity for an engineered tryptophan reporter group-containing *S. pneumoniae* StkP kinase,⁶⁷ millimolar affinity for the *B. subtilis* PrkC,⁵⁸ and millimolar binding of more than one MurNAc tripeptide to the three PASTA domains of the *S. aureus* PrkC kinase.⁶⁹ Given the limited diversity of available muropeptide structures, and our ignorance about the exact structures their respective kinases have evolved to respond, these initial determinations of relatively weak in vitro affinity cannot be interpreted. This challenge is exemplified by the observation that peptidoglycan from growing bacteria, as opposed to the peptidoglycan from stationary phase bacteria or the peptidoglycan from the spore cortex, is optimal as a germinant.⁵² The expected mechanism for STK activation is muropeptide-induced dimerization,^{71–73} and proposals about

Scheme 5. Schematic Overview of Peptidoglycan Recycling in Gram-Negative Bacteria^a



^aThe cytoplasmic membrane of the bacterium is depicted as a cartoon at the right, and the outer membrane at the left. The periplasmic space between these two membranes contains the peptidoglycan. After initial processing by autolysins, lytic transglycosylases cleave the glycosidic bond in a nonhydrolytic fashion, giving a GlcNAc-anhydromuramic acid disaccharide. Transport into the cytoplasm by the AmpG permease is followed by NagZ-dependent glycosylase activity, which splits the disaccharide. AmpD then cleaves the peptide stem as shown, AnmK hydrolyzes the acetal and phosphorylates position 6 of MurNAc, and MurQ removes the lactyl moiety.

the role of the PASTA domains in this dimerization have been made.^{68–70} Muropeptide binding is suggested to occur at the hinge region between individual PASTA domains.⁷⁰

A conceptually similar pathway may operate in the transition from dormancy to active growth in the nonsporulating actinobacteria, exemplified by *Micrococcus luteus* and *M. tuberculosis*. As noted above, *M. tuberculosis* has a PASTA domain-containing STK, PknD. A possible relationship of muropeptide signaling to its activation is the resuscitation-promoting (Rpf) class of enzymes, of which *M. luteus* has one and *M. tuberculosis* has five. In contrast to spore resuscitation, however, both the Rpf and STK enzymes function within the broad scope of dormancy rescue, growth, cell division, and pathogenicity.^{45,53} A molecular understanding of the presumed involvement of muropeptide-dependent signaling is less developed. The seminal observation was the identification of the *M. luteus* Rpf as a secreted protein⁷⁴ with muralytic activity.^{51,75,76} Neither its requirements with respect to its peptidoglycan as a substrate nor the products of its catalytic activity (whether lysozyme-like or lytic transglycosylase-like) has been established. A similar uncertainty about molecular mechanisms carries forward to five Rpf proteins of *M. tuberculosis*, notwithstanding structural studies of the one of the five (RpfB) that is absolutely essential to resuscitation.^{77,78} Adding to the challenge of understanding the roles for RpfB is the discovery of its complexation with RipA, an enzyme

essential for cell division and believed to have amidase activity toward the D-Gln–m-DAP amide bond of the peptidoglycan stem,⁷⁹ which also interacts with the high-molecular mass biosynthetic penicillin-binding protein of this bacterium to antagonize the muralytic activity of RpfB.⁸⁰ Given the importance of the *M. tuberculosis* Rpf proteins to both the resuscitation and virulence (but not growth) of this bacterium,⁸¹ there is a compelling opportunity to clarify the molecular basis of their peptidoglycan-dependent signaling. This effort might coincide either with inhibiting their activity⁸² or alternatively with stimulating the Rpf-dependent pathways, following the observation that nutrient resuscitation of persister bacteria can restore antibiotic susceptibility.⁸³

MUROPEPTIDE SENSING BY GRAM-NEGATIVE BACTERIA

A characteristic of the growth of many Gram-negative bacteria is robust turnover of their cell wall via muropeptide formation.³⁰ A single generation of growth liberates approximately 40–45% of the *E. coli* peptidoglycan as anhydromuropeptides, which subsequently are efficiently recovered and reused. The dynamics of cell-wall growth and turnover are relevant to the understanding of cell-wall biosynthesis during growth and division, and to the mechanism used by many Gram-negative bacteria to mount a resistance response to β -

lactam antibiotics (antibiotics that interfere with peptidoglycan synthesis by inactivation of the transpeptidase catalytic domain of the penicillin-binding proteins). Many Gram-negative bacteria sense a disruption in the recycling pathway as a consequence of the β -lactams as antibiotics and, as a result of this sensing, initiate expression of resistance enzyme(s). In particular, the high-level β -lactamase expression that results from this disruption achieves clinical levels of β -lactam resistance.⁸⁴ The seemingly paradoxical observation that the integrity of the peptidoglycan recycling pathway is not essential to cell growth under laboratory conditions and does not correlate to an observable in vitro phenotype suggests that a preeminent purpose for muropeptide recycling is the regulation of resistance and virulence responses.⁸⁵

The high-level expression of these inducible AmpC β -lactamases confers high-level β -lactam resistance to many Gram-negative pathogens. AmpC expression is induced by the presence of β -lactam antibiotics, regulated by the *ampR-ampC* system. AmpR is the 32 kDa LysR-type transcriptional regulator of this system. The *ampR* gene is absent from the chromosome of some Gram-negative bacteria, notably *E. coli*. As a result, expression of the *E. coli* AmpC β -lactamase (the gene for this enzyme is present) is constitutive but produces only very low levels of AmpC. The complete *ampR* system is present in *Citrobacter freundii*, where it has been best studied. The structure of the effector binding domain (lacking the N-terminal DNA binding domain) of *C. freundii* AmpR has been determined in a conformational state that, although it lacks a bound effector, is suggested by circumstantial evidence to correspond to the state in which AmpC expression is repressed.⁸⁶ This same study confirmed the sensitivity of *ampR* to point mutation, which can result either in the loss of AmpC expression or in its constitutive expression.

The central question is the regulatory relationship between the *ampR-ampC* system and peptidoglycan biosynthesis and recycling. Peptidoglycan biosynthesis uses a biosynthetic precursor (Lipid II) that is fully assembled in the cytoplasm and exported to the periplasm to serve as the substrate of the PBP enzymes for the sequential glycan strand elongation and peptide stem cross-linking events of cell-wall biosynthesis. New peptidoglycan is incorporated uniformly into existing peptidoglycan during sidewall elongation of the rod-shaped *E. coli* bacterium,^{87,88} and this incorporation coordinates with substantial liberation of portions of the existing peptidoglycan as soluble anhydromuropeptides.^{30,89} While the identity of the full ensemble of enzyme catalysts used for cell-wall biosynthesis is uncertain, anhydromuropeptide release involves both non-hydrolytic glycan cleavage catalyzed by the lytic transglycosylase enzymes^{90,91} and hydrolytic amidase-catalyzed cleavage of the peptide stem cross-links.^{92,93} The resulting anhydromuropeptides (Scheme 5) (the anhydro prefix refers to the bridging ether ring that results from the action of the lytic transglycosylases toward the MurNAc–GlcNAc glycosidic bond) and oligopeptides are released into the periplasm. A portion of the amidase-released oligopeptides is released from the periplasm to the medium.⁸⁹ These oligopeptides are recovered for reuse by complexation to the MppA binding protein for subsequent import through the Opp oligopeptide permease system.^{94–96} The anhydromuropeptides are themselves recovered for reuse by entry into the cytoplasm through the AmpG permease protein. Indeed, the central roles for both the lytic transglycosylases^{97,98} and the AmpG permease^{99–101} in

muropeptide recycling were first identified through correlation to β -lactam-dependent β -lactamase expression.^{102,103}

As it is now understood, the muropeptide recycling pathway coordinates to de novo peptidoglycan biosynthesis by a concise series of events. Muropeptides liberated during peptidoglycan biosynthesis are “sized” by the lytic transglycosylases localized to the inner leaflet of the outer membrane to give the GlcNAc-anhydroMurNAc structures shown in Scheme 5.¹⁰⁴ Translocation of this muropeptide to the cytoplasm occurs through the AmpG permease. Within the cytoplasm, the glycosidic bond of this muropeptide is hydrolytically cleaved by the NagZ β -N-acetylglucosaminidase.^{105,106} The action of NagZ to provide anhydroMurNAc products—in all of their tri-, tetra-, and pentapeptide forms—constitutes the entry point to the pool of effector structures believed to govern the structure and/or affinity of the DNA–*ampR* complex. The precise composition of this effector pool is further controlled by at least three other enzymes.¹⁰⁷ NagZ is a cytosolic β -glucosaminidase that acts on the anhydromuropeptide as it enters the cytoplasm (Scheme 5). AmpD is a cytosolic zinc-dependent amidohydrolase that cleaves the lactyl amide of the muropeptides, releasing as products the peptide stem and an anhydroMurNAc muropeptide. Both products enter the de novo Lipid II biosynthesis pathway. The third enzyme is a low-molecular mass PBP, which in vitro (and possibly in vivo) acts as a D,D-carboxypeptidase with respect to the D-Ala-D-Ala residues of the peptide stems found on some of the muropeptides of the peptidoglycan polymer, and on some of the anhydromuropeptides released by the lytic transglycosylases. As this PBP activity is not central to the peptidoglycan recycling pathway, its role with respect to the composition of the effector pool is considered following a closer consideration of NagZ and AmpD.¹⁰⁷

The hypothesis that the relative levels of the catabolic and anabolic muropeptides, as directly influenced by AmpG and these enzymes, govern AmpC transcription is supported by complementary experiments from several laboratories. Muropeptides originate through sequential lytic transglycosylase and amidase excision in the periplasm and pass into the cytoplasm through the AmpG permease. Genetic deletion of the lytic transglycosylases of *E. coli*,⁹⁸ or of the permease in β -lactam-resistant *Pseudomonas aeruginosa*,^{108,109} restores significant β -lactam susceptibility as a result of the inability of these mutants to induce AmpC expression through loss of muropeptides in the cytoplasm. The structural basis of AmpC expression is suggested, but not proven, by additional observations. NagZ and AmpD enzymes act upon muropeptides that enter the cytoplasm through the AmpG permease. Mutational loss of function of AmpD is a long-known clinical event that results in high-level constitutive AmpC expression.^{110,111} Our current mechanistic understanding assigns AmpD the role of dissipating structures within the anhydromuropeptide pool that would otherwise bind to AmpR and repress AmpC expression. A detailed examination of the substrate specificity of the *C. freundii* AmpD enzyme shows a strong preference for an anhydromuramic acid (monosaccharide) substrate, and insensitivity (comparing the tripeptide to the pentapeptide) with respect to peptide stem length.¹¹² A crystallographic study of this same AmpD shows an open conformation capable of substrate accommodation and verifies its close structural similarity to the peptidoglycan-recognition proteins (PGRPs) of eukaryotic innate immunity.¹¹³ A corollary to the observation that AmpD null mutations

confer a resistance phenotype is that inhibition of NagZ should potentiate β -lactam efficacy. This expectation is confirmed.^{114–118} The optimization of NagZ inhibitors with respect to potency, selectivity (relative to human enzymes), and accessibility (with respect to access to the cytoplasmic NagZ enzyme) to attain clinical relevancy is a promising strategy against high-level Gram-negative β -lactam resistance.¹⁰⁷ Moreover, these same inhibitors will allow clarification of the possible relationship between muropeptide recycling and resistance in Gram-positive bacteria.

The simplest explanation for control of AmpR regulation of AmpC expression through the ebb and flow of the metabolic–catabolic muropeptide pool in the cytoplasm cannot be, however, fully correct. The direct participation of a low-molecular mass PBP suggests the likelihood of specific, regulatory muropeptide structures. It has been long known to medicinal chemists that optimization of β -lactam antibiotic structure required awareness of their spectrum of PBP inhibition, to secure preferential inactivation of high-molecular mass PBPs (antibiotic activity) while avoiding inactivation of low-molecular mass PBPs (induction of β -lactamase expression).¹¹⁹ Oliver et al. have directly connected PBP4 of *P. aeruginosa* to the muropeptide recycling pathway.^{109,120,121} The presumption is that the enzymatic activity of this PBP alters the -D-Ala-D-Ala pentapeptide stem structures to give an anhydromuropeptide–AmpR complex that represses AmpC expression. The precise anhydromuropeptide structure used in AmpR complexation is uncertain.^{107,122} When a β -lactam inactivates PBP4, the enzymatic activity is abolished, and the composition of the entering muropeptides is altered, resulting in an AmpR complex that derepresses the *ampC* gene. PBP4 (and presumably a low-molecular mass PBP of other Gram-negative bacteria with the *ampR-ampC* system) thus signals the presence of β -lactam antibiotics through control of the anhydromuropeptide structures entering the cytoplasm. A candidate for the effector structure that binds to AmpR, to derepress the *ampC* gene encoding the AmpC β -lactamase, is shown as structure 2 in Scheme 2.

Although the assignment of a role as a sentinel enzyme to this PBP is attractive (an assignment that harmonizes fully with the consequences of AmpG deletion, AmpD mutational inactivation, and NagZ inhibition), other observations suggest a more complicated β -lactamase induction pathway in these Gram-negatives. As one unexplained observation, most Gram-negatives have several low-molecular mass PBPs (*E. coli* has seven and *P. aeruginosa* at least three), all of which are ascribed D,D-carboxypeptidase and/or -endopeptidase activities. The *P. aeruginosa* PBP4 is not the most abundant. While there are temporal patterns with respect to the appearance of these enigmatic low-molecular mass PBPs during culture, it seems unlikely that the abrupt loss of the activity of one minor PBP would substantively alter the composition of the robust stream of muropeptides entering the cytoplasm for recycling. Uncertainty about the true enzymatic activity (and the preferred substrates) of these enigmatic low-molecular mass PBPs¹²³ complicates efforts to assign to one of these PBPs full responsibility for a specific structural transformation in the muropeptide recycling pathway. One emerging possibility is clearer recognition that bacterial peptidoglycans may incorporate D-amino acids other than D-alanine, and that the -D-Ala-D-Ala terminus of the peptidoglycan stem may not be as unique as was once thought.^{124–126} Modulation of the *ampR* gene may

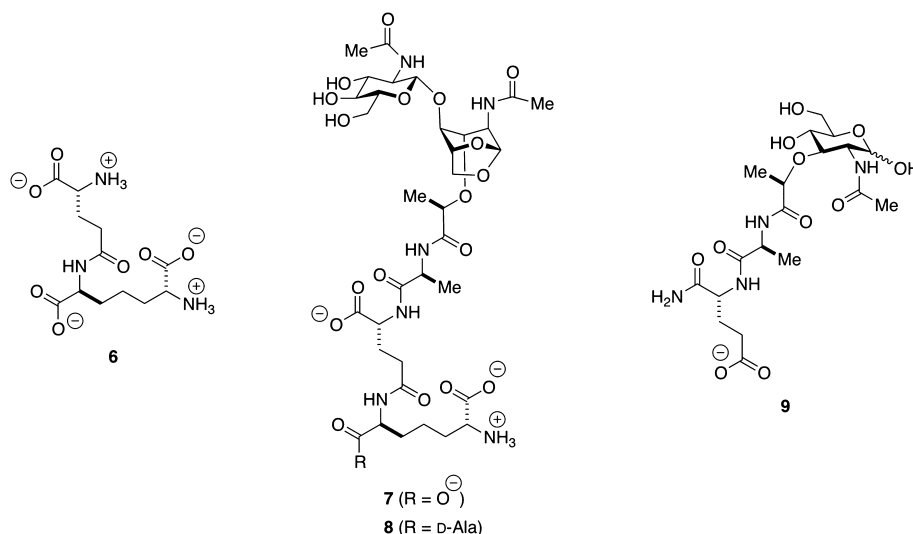
occur through interaction of AmpR with structurally unprecedented anhydromuropeptides of the recycling pool.

Additional observations attest to the complexity of Gram-negative muropeptide recycling. *P. aeruginosa* has muropeptide permeases apart from AmpG.^{108,127} Although *Neisseria gonorrhoeae* has a fully functional AmpG (but not apparently *ampR*), it has an unknown pathway that it uses to divert its muropeptides into the medium as an apparent virulence mechanism.¹¹⁰ As discussed below, muropeptide-induced toxicity is characteristic of yet other Gram-negative pathogens such as *Shigella* and *Bordetella*.¹²⁸ An interrelationship, by an as-yet-unproven mechanism, between the *ampR-ampC* and BlrAB (CreBC) resistance systems also occurs in *P. aeruginosa*¹²⁰ and *Aeromonas hydrophila*.¹²⁹ In *P. aeruginosa*, the BlrAB two-component regulatory system and the AmpR system are simultaneously activated as a result of PBP4 inactivation.¹²⁰ *A. hydrophila* PBP inactivation by β -lactam antibiotics alters the homeostasis of the muropeptide pool. This alteration is sensed by a periplasmic BlrB two-component kinase and is expressed through autophosphorylation of the BlrB cytoplasmic domain. The transfer of phosphate to the cytoplasmic BlrA protein allows phospho-BlrA binding upstream from the *blr* regulon gene promoters so as to recruit an RNA polymerase to initiate β -lactamase expression.¹²⁹ Analysis of the concurrent change in the muropeptide composition of the *A. hydrophila* peptidoglycan polymer showed a 4-fold increased abundance of a single muropeptide substructure 3 (Scheme 2). Verification of a direct interaction between this muropeptide (whether as the free muropeptide or as a substructure within the murein polymer) and BlrB is awaited. Last, *Stenotrophomonas maltophilia* has two chromosomal β -lactamases whose expression is controlled by an *ampR-ampC*-type system. While the expression of these two β -lactamases is muropeptide-dependent (expression requires the AmpG permease, and with high-level expression upon AmpD deletion), evaluation of different β -lactam structures and deletion of a high-molecular mass PBP implicate a NagZ-independent pathway for β -lactamase induction in addition to induction through its intact *ampR-ampC* system.¹³⁰

■ MUROPEPTIDES IN EUKARYOTIC IMMUNE RECOGNITION OF BACTERIA

The germline-encoded innate immune system detects and defends against microbial pathogens and as the first line of defense in vertebrates precedes activation of the acquired immune system. The innate immune system uses immune sensors, known as pattern-recognition receptors (PRRs),¹³¹ that are activated by microbially derived structures known as pathogen-associated molecular patterns (PAMPs).¹³² Mammalian PRRs include the extracellular Toll-like receptors,^{133–137} the intracellular nucleotide-binding domain/leucine-rich repeat receptors (NLRs),^{138–140} and the retinoic acid-inducible gene-I-like receptors (RLRs).^{141,142} The immune responses that follow activation of the PRRs reflect extensive receptor cross-talk, presumably to validate the focus and extent of an immune response.^{143,144} Because of the relevance of PRR recognition with respect to (for example) infection, autoimmune disease, and microbiome equilibrium with differential commensal organism versus pathogen recognition,¹⁴⁵ an understanding of the PRRs is an emerging area of immunology. These aspects are beyond the scope of this review. Our focus is the structural aspects of PRR recognition of muropeptides, and how this recognition is relevant to the ensuing (or absence of an) immune response.¹⁴⁶

Scheme 6. Structures Recognized by Intracellular Receptors NOD1 (6–8) and NOD2 (9)



Bacterial PAMPs include peptidoglycan-derived muropeptides from both Gram-positive and Gram-negative bacteria,¹⁴⁷ lipoprotein-derived lipopeptides,¹⁴⁸ lipopolysaccharide segments derived from the outer membrane of Gram-negative bacteria (LPS),¹⁴⁹ and lipoteichoic acid segments derived from the peptidoglycan of Gram-positive bacteria.¹⁵⁰ While the immunostimulatory activities of muropeptides have been known for decades,⁵ it has only been recently that the molecular basis for muropeptide immune recognition has emerged, in parallel with methodologies allowing the synthesis of muropeptides (as well as the other PAMPs) having defined structure. A critical limitation of prior efforts using materials from biological sources was insufficient reagent purity. The extraordinary progress with respect to advances in muropeptide synthesis and improved methods for the isolation and purification of endogenous muropeptides is exemplified by the correlation of specific chemical structure to the activation of the NOD1 and NOD2 NLRs.^{151–154} NOD1 and NOD2 recognize the muropeptides resulting from degradation of bacterial peptidoglycan. These receptors have three domains: a C-terminal leucine-rich repeat (LRR) responsible for ligand recognition, a central NOD domain that facilitates oligomerization and exhibits ATPase activity, and an N-terminal caspase-recruitment domain (CARD). Interest in the NOD receptors is driven by numerous studies correlating their expression with human diseases, such as intestinal homeostasis and other allergic diseases,¹⁵⁵ and more recently to their critical role in the autophagic response to bacterial infection.^{156,157}

The minimal structure recognized by NOD1 is γ -D-glutamyl-*meso*-diaminopimelic acid [6 (Scheme 6)].^{158,159} This structure is characteristic of the peptidoglycan of Gram-negative bacteria, as further exemplified by muropeptides 7 and 8. NOD2 recognizes muramyl dipeptide (MDP, 9).^{160,161} 1,6-Anhydromuramyl moieties such as 7 and 8 are generated by the action of lytic transglycosylases, discussed previously with respect to their central role in peptidoglycan recycling. Fukase and co-workers showed that a synthetic sample of 7 exhibits human NOD1 stimulatory activity that is higher than that of synthetic 6.^{132,162} They also showed that the compound obtained from the most active fraction from RP-HPLC purification of the supernatant of *E. coli* K-12 cells is identical to 7.¹⁶³ Muropeptide 8 was originally isolated from the Gram-negative

organism *Bordetella pertussis*,¹⁶⁴ the causative agent of pertussis or whooping cough, and is known as tracheal cytotoxin (TCT) for its causative role in the destruction of the cilia of tracheal cells during *Bo. pertussis* infection. Synthetic 8 displayed very weak human NOD1 stimulatory activity,^{132,162} which was consistent with results using a sample of 8 from natural sources.¹⁶⁵ Interestingly, 8 is able to stimulate the innate immune systems of other species. It activates murine NOD1¹⁶⁵ and peptidoglycan-recognition protein LC (PGRP-LC) from *Drosophila*.^{166,167} Muramyl dipeptide (9) is a product of enzymatic degradation of the peptidoglycan and represents a minimal structural motif for both Gram-positive and Gram-negative bacteria. It has been used as an adjuvant since the 1970s when it was shown to be the minimal active structure of peptidoglycan enhancing the immune response to Freund's incomplete adjuvant. Stimulation of NOD2 by MDP activates the proinflammatory NF- κ B signaling pathway.

Although the molecular nature of the interaction between MDP and NOD2 is not known, several conclusions can be drawn in terms of the structural requirements for stimulation of the receptor. NOD2 can detect an MDP variant in which D-*i*Gln has been substituted with D-Glu. However, substitution of D-*i*Gln for L-*i*Gln or L-Ala for D-Ala abrogates activity.^{158,160,161} This result strongly suggests that recognition of MDP by NOD2 is stereoselective. The dipeptide chain is also crucial for recognition, as synthetic GlcNAc-MurNAc dimers or tetramers missing the peptide portion do not stimulate NOD2-dependent NF- κ B activation.¹⁶⁰ Interestingly, NOD2 is also able to detect muramyl tripeptides containing either lysine or ornithine at the third position but is unable to detect a muramyl tripeptide or tetrapeptide in which the third amino acid is *meso*-diaminopimelic acid (*meso*-DAP).^{158,161} Finally, NOD2 sensing requires an intact MurNAc ring, as reduction of this moiety by NaBH₄ abolishes NOD2 recognition of the peptidoglycan fragment. In contrast, NOD1 can detect both native and reduced peptidoglycan fragments.¹⁶¹

Recent results suggest that NOD2 may detect other peptidoglycan fragments. It was shown by Volz et al. that a "peptidoglycan monomer" from *S. aureus* may be a ligand for NOD2, as NOD2-deficient dendritic cells fail to exhibit an increase in interleukins-12p70 and -23 upon activation with this monomer in concert with lipoteichoic acid or lipopolysacchar-

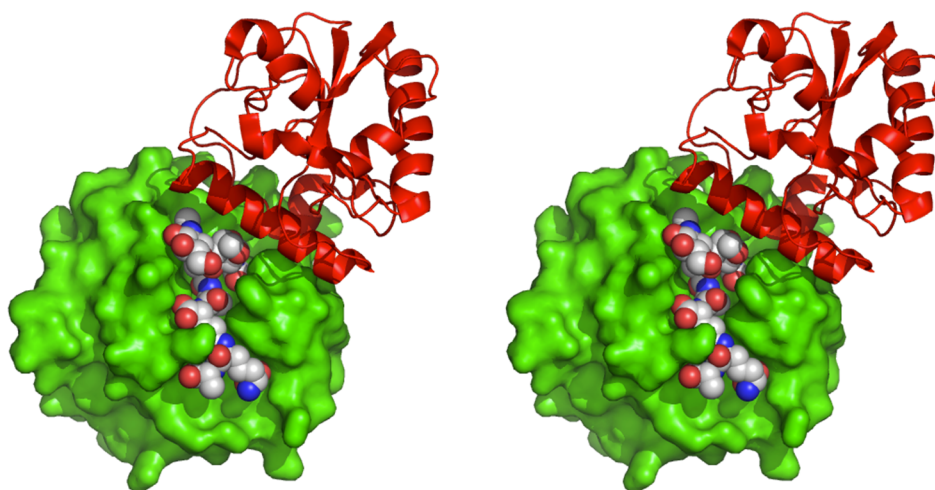


Figure 1. Stereoview of the complex of TCT with the extracellular domains of PGRP-LCx (green, space-filling) and PGRP-LCa (red, ribbon). Adapted from Protein Data Bank entry 2F2L.¹⁸⁸

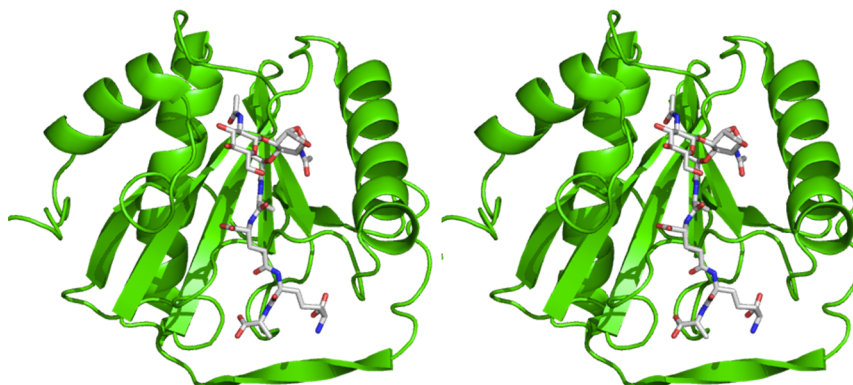


Figure 2. Stereoview of the complex of TCT with PGRP-LE. Adapted from Protein Data Bank entry 2CB3.¹⁹⁰

ide.¹⁶⁸ Mass spectral analysis suggests the structure of this monomer to be that of mucopeptide 1 (and not the structure as suggested), thus identical to the active component in the BIVR phenomenon in MRSA. The *N*-glycolyl MDP derivative (as is generated by mycobacteria), is more potent than the *N*-acetyl parent in activation of NOD2-mediated signaling *in vitro*.¹⁶⁹

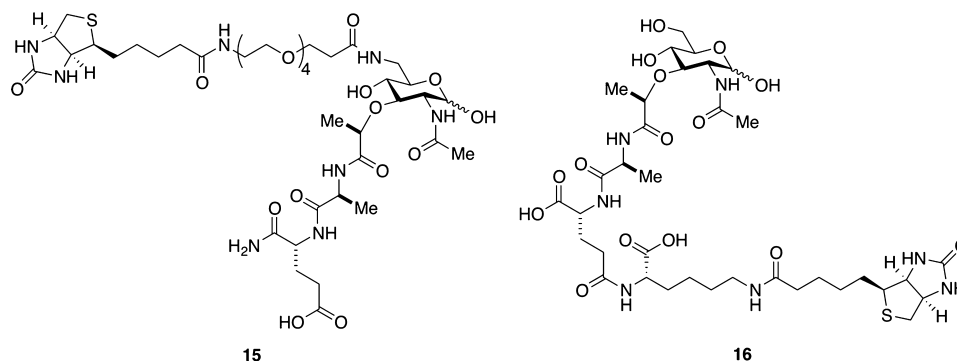
Because MDP corresponds to the minimal structural motif found in peptidoglycan, NOD2 can be regarded as a general sensor of peptidoglycan from Gram-positive and Gram-negative bacteria. In contrast, NOD1 detects γ -D-Glu-*meso*-DAP (6), a structure mostly found in peptidoglycan from Gram-negative bacteria and suggesting a specific role for NOD1 in Gram-negative innate immune response. Two hypotheses are emerging regarding the mechanisms leading to PRR activation: one proposing a direct interaction between the PAMP and its associated PRR, leading to NF- κ B activation, and the second arguing that the innate immune response to PAMPs casts a wider shadow, requiring perturbations of host metabolic and homeostatic functions and converging on the mitochondria as a signaling hub.¹⁷⁰ In support of the former proposal, surface plasmon resonance and atomic force microscopy show a direct interaction between L-Ala-D-iGln-*meso*-DAP and NOD1, with a K_d value of 35 μ M.¹⁷¹ This interaction is abolished when a truncated NOD1 lacking its LRR is used. Furthermore, binding between NOD1 and receptor-interacting serine/threonine protein kinase 2 (RICK) was strengthened when the tripeptide was preincubated with NOD1, as well as RICK phosphor-

ylation activity. RICK is thought to be recruited by NOD1 upon binding by the tripeptide and to mediate NF- κ B activation.¹⁷²

The emergence of mitochondria as a checkpoint for innate immune signaling is in part due to the identification of several innate immune receptors associated with the mitochondria. In particular, NLRX is a member of the NLR family that localizes to the mitochondrial matrix,¹⁷³ as the first and only PRR in this environment. The function of NLRX is not yet understood, but it has been argued that because of its confined localization it may not be involved in the direct detection of PAMPs.¹⁷⁰ Another class of PRRs, known as the peptidoglycan-recognition proteins (PGRPs), also plays a role in the innate immune response to invading bacteria.^{174,175} PGRPs are conserved from invertebrates to vertebrates. Unlike NOD receptors, which bind to peptidoglycan monomers, mammalian PGRPs bind to polymeric peptidoglycan.^{176–180} Several studies elucidate specific interactions between human PGRPs and peptidoglycan using synthetic peptidoglycan monomers.^{178,181–183}

In insects, PGRPs act through signaling pathways mediated by Toll receptors (in response to Gram-positive bacteria) and the immune-deficiency pathway (as a result of Gram-negative bacterial infection) to induce expression of antimicrobial peptides.^{174,175,184} A thorough discussion of insect PGRPs is beyond the scope of this review. However, there are certain instances in which mucopeptides are involved in this signaling, namely, the extracellular binding of the Gram-negative cell-wall

Scheme 7. Biotinylated Muramyl Dipeptides for Studies of NOD2 Binding



recycling product TCT (8) to a heterodimer formed by the *Drosophila melanogaster* PGRPs PGRP-LCx and PGRP-LCa^{166,185–188} and its intracellular binding to full-length PGRP-LE (PGRP-LE β).^{189–191} Both these binding events lead to activation of the immune deficiency pathway.

TCT induces the heterodimerization of PGRP-LCx and PGRP-LCa upon binding to their extracellular domains,^{186,192} and the structure of the TCT–heterodimer complex has been reported.¹⁸⁸ The structure reveals that TCT binds in the LCx docking groove. Its exposed GlcNAc-1,6-anhydroMurNAc moiety and several residues of LCx engage the surface of LCa (Figure 1). The crystal structure reveals why this signaling pathway is specific for DAP-type peptidoglycan: the *meso*-DAP of TCT forms an electrostatic interaction with the side chain of Arg413 of LCx and a hydrogen bond to the main chain amide of its Asp395.¹⁸⁸ Along with Trp394, the residues form a binding pocket to accommodate the *meso*-DAP moiety.

The crystal structure of TCT with PGRP-LE (Figure 2) reveals interactions similar to those in its complex with the PGRP-LCx and PGRP-LCa heterodimer.¹⁹⁰ TCT induces oligomerization of PGRP-LE, and this is achieved through the interactions of the GlcNAc-1,6-anhydroMurNAc group of bound TCT with a second molecule of PGRP-LE. Furthermore, the *meso*-DAP moiety of TCT forms a salt bridge with Arg254 of PGRP-LE, underscoring its preference for DAP-type peptidoglycan. The results of these structural studies are consistent with previous reports that both the GlcNAc-1,6-anhydroMurNAc and DAP moieties are required for activation of the immune deficiency pathway.^{166,167} As with other areas covered in this review, studies of the immune recognition of bacteria have been hampered by the lack of well-defined, homogeneous muropeptide samples, as isolates from natural sources may contain traces of other immunostimulatory compounds (e.g., LPS). With the advent of new synthetic methodologies, access to these ligands in pure form is now possible.^{149,193} Although a clearer understanding of how muropeptide structure affects its interactions with PGRPs is slowly emerging, less is known in the case of NOD receptors. What is the nature of the interactions of these ligands with their putative receptors? How do the subtle changes in ligand structure modulate their activity and specificity? The answers to these questions will be obtained in part with the synthesis of immunostimulatory peptidoglycan-based structures. For example, a recent report detailed the structure–activity relationship of γ -D-Glu-*meso*-DAP (6) through the synthesis of several analogues and evaluation of their activity as NOD1 agonists.¹⁹⁴ With the goal of determining whether MDP interacts directly with NOD2, syntheses of several active biotinylated versions of

this muropeptide, such as 15¹⁹⁵ and 16¹⁹⁶ (Scheme 7), have been reported. Similar MDP derivatives have already demonstrated value to the study of the cell biology of NOD signaling.¹⁹⁷

CONCLUSIONS

The relevance of cell wall-derived muropeptides as messengers in processes such as bacterial detection of the presence of antibiotics and eukaryotic detection of bacterial infections is now well established. Although the structures of many of the muropeptides responsible for these phenomena have been elucidated, much remains to be discovered in terms of the precise mechanisms of action of these molecules, exemplified by their interactions with putative receptors. For example, understanding the binding of muropeptides to the PASTA domain of STKs in Gram-positive sporulation resuscitation requires additional structural information. A similar scenario exists in the case of eukaryotic immune recognition of bacterial muropeptides, where the putative muropeptide receptors, NOD1 and NOD2, have been identified, but a clear picture of the specific interactions of the cell-wall fragments with these proteins is not yet in focus. Finally, the mechanism of the muropeptide-induced BIVR phenomenon in MRSA may not be a direct result of muropeptides themselves but may occur because of sequestration of vancomycin, for example, by the extra Lipid II that is biosynthesized in the presence of these cell-wall fragments. As a first step, the syntheses of muropeptides and their analogues have allowed correlations between structure and activity to be made. Further syntheses of muropeptides containing affinity tags or fluorescent reporters will provide new tools with which to study the interactions of these small molecules with their putative receptors, providing a more complete picture of their roles as second messengers.

AUTHOR INFORMATION

Corresponding Author

*E-mail: mobashery@nd.edu. Phone: (574) 631-2933.

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ABBREVIATIONS

BIVR-MRSA, β -lactam antibiotic-induced vancomycin-resistant MRSA; *meso*-DAP, *meso*-diaminopimelic acid; LPS, lipopolysaccharide; MDP, muramyl dipeptide; MRSA, methicillin-

resistant *S. aureus*; NLR, intracellular nucleotide-binding domain/leucine-rich repeat receptor; NLRX, mitochondrial NLR; NOD, nucleotide oligomerization domain; PAMP, pathogen-associated molecular pattern; PASTA, penicillin binding-associated and serine/threonine kinase-associated; PBP, penicillin-binding protein; PGRP, peptidoglycan-recognition protein; PRR, pattern-recognition receptor; RICK, receptor-interacting serine/threonine protein kinase 2, also known as RIP-2; STK, serine-threonine kinase; TCT, tracheal cytotoxin.

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NOTE ADDED IN PROOF

Since editorial review, three discoveries pertinent to the theme of this review have been published. In many Gram-positive bacteria, expression of β -lactamase occurs following β -lactam

antibiotic acylation of the sensor domain of the transmembrane BlaR1 protein.¹⁹⁸ Amoroso et al.¹⁹⁹ now provide evidence that a consequence of β -lactam-dependent acylation of both BlaR1 and PBPs is the formation in the cytoplasm of a mucopeptide-derived γ -D-Glu-m-A₂pm dipeptide. This dipeptide binds to the BlaI repressor so as to allow BlaZ expression. As a similar regulatory mechanism controls expression of a PBP resistant to β -lactam acylation in methicillin-resistant *S. aureus*,²⁰⁰ these data implicate an importance to Gram-positive mucopeptide recycling with respect to resistance that is no less than its proven importance in Gram-negatives. Second, a recent publication by Gee et al. describes the pseudokinase MviN as a key player in peptidoglycan-dependent STK regulation of *M. tuberculosis* cell-wall biosynthesis.^{201,202} Last, Fujimoto et al. give a comprehensive review of the structure–activity relationship for mucopeptide binding to Nod1.²⁰³