Cell Wall Bioactive Molecules as Signaling and Effector Agents in Bacterial Physiology and Virulence

Fernando Baquero[1,2,*], Juan A. Ayala[3], Rafael Cantón[1,4]

1 Servicio de Microbiología. Hospital Universitario Ramón y Cajal and Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS). Madrid. Spain

2 CIBER de Epidemiología y Salud Pública (CIBERESP). Instituto de Salud Carlos III. Madrid. Spain

3 Centro de Biología Molecular Severo Ochoa, CSIC, Madrid, Spain

4 CIBER de Enfermedades Infecciosas (CIBERINFEC). Instituto de Salud Carlos III. Madrid. Spain

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Correspondence: Fernando Baquero, baquero@bitmailer.net

ABSTRACT

The molecules that make up the bacterial cell wall should be seen not only as passive structural components of the murein sacculus that protect and enclose the inner membrane containing the bacterial cytoplasm. They are also active bioactive molecules released during bacterial replication, especially after cell lysis, leading to a deconstructive process. These molecules vary in structure from simple acetylated monosaccharides or amino acids, such as D-amino acids, to more complex muropeptides and cross-linking peptides. They can be classified as Cell Wall Bioactive Molecules (CWBAMs), which have signaling and effector roles that affect bacterial physiology, including biofilm formation, sporulation, and antibiotic resistance. CWBAMs also participate in interactions with other bacteria, the microbiota, and immune cells from human and animal organs, including the central nervous system. The effects of CWBAMs released during cell wall breakdown remain largely unknown, especially since they can translocate from mucosal surfaces colonized by microbiota into the bloodstream. CWBAMs are not necessarily toxins and should be distinguished from endotoxins. Their role in bacterial-host interactions is a promising area for future research.

INTRODUCTION

The bacterial cell is an organism containing functional organ-like structures that ensure its health and resilience to changes.[1] Similar to higher forms of life, the functions provided by these structures are highly integrated to maintain homeostasis in changing environments. The organization of this integration requires signals and effectors. Signals are generated in response to detecting intracellular or extracellular changes, ultimately altering bacterial physiology and behavior. Effectors are often responsive to signals or are self-regulated, leading to adaptive responses to the detected changes.

One of the essential organs found in nearly all bacteria is the cell wall, primarily made up of a continuous and elastic layer of peptidoglycan. It is often decorated with other associated biomolecules, including various peptides, proteins, teichoic acids, and lipoproteins, and is anchored to the outer cell membrane in Gram-negative bacteria. For decades, the cell wall was regarded as merely a static sacculus that maintains the cohesion and physical interaction of all bacterial organelles within the cytoplasm. It also preserves the size, shape, osmotic protection, integrity, and individuality of the bacterial cell, as well as cellular differentiation. Although this view remains valid today, the role of the cell wall in bacterial cell biology has broadened to be understood from a more dynamic perspective.[2] This perspective highlights the constant structural changes of this macromolecular structure, which are remodeled during bacterial growth and adaptive homeostasis activities. These activities result in the continuous release of short, mostly soluble fragments, including cell wall bioactive molecules (CWBAMs), mainly, but not exclusively, dimeric or trimeric muropeptides. These fragments can act as signals and effectors that influence the bacteria's own metabolism, differentiation, and interactions with other bacteria and host organisms. They are also involved in pathogenesis in animals and plants[3]. Additionally, CWBAMs can be monomeric components of peptidoglycan, such as N-acetyl-glucosamine, the pentapeptide bridges linking peptidoglycan strands, or single amino acids, including non-canonical D-amino acids, which can be released into the extracellular environment.

At least one-third of the murein lipoprotein Lpp, Braun's lipoprotein, with a trimeric helical structure, and one of the most abundant proteins in *Escherichia coli*, with about 200,000 copies per cell, is covalently attached to murein peptide. This attachment ensures a physical-mechanical connection with the outer membrane.[4-6] LdtF, a murein endopeptidase, cleaves the linkage between peptidoglycan and the Lpp lipoprotein[7], and therefore Lpp can be released from the sacculus, allowing it to act as a potential CWBAM.[8] Finally, we cannot discard as CWBAM soluble components of other secondary cell wall polymers, covalently linked to peptidoglycan, as teichoic acids (polyol-phosphate polymers) or fragments of capsular polysaccharides.[9]

THE ORIGIN AND RELEASE OF CELL WALL BIOACTIVE BIOMOLECULES

The fragmentation of the peptidoglycan into soluble fragments should be compatible with the maintenance of the cell wall macromolecule's recycling and continuity. This is ensured by the patched and often tridimensional lattice structure of the cell wall, composed of multiple cross-linked layers. The CWBAMs result from the action of amidases, which cleave the first amide bond of the stem peptide linking the N-acetylmuramic acid in the glycan strand, thereby preventing subsequent cross-linking with other glycan strands. Peptidases can attack the bonds between amino acids of these cross-linking peptides. Finally, glycan strands can also be cleaved by glycosidases (*N*-acetylmuramidases, *N*-acetylglucosaminidases).[10] The physiological reason for such local deconstruction of peptidoglycan is essentially cell wall turnover, which creates open sites where recently synthesized muropeptides can be inserted, resulting in the elongation of the cell wall required for replication. This process probably influences the shaping of the cell, thereby contributing to the production of both curvatures and filaments. It can be inferred that the newly constructed muropeptides may act as CWBAMs. The lipid-linked NAG-NAM-pentapeptide precursor (lipid II) is produced in the inner leaflet of the cytoplasmic membrane and translocated to the periplasm by flippases (as MurJ). Flippases probably works in combination with multienzyme complexes involving also polyprenyl-diphosphate phosphatase. Bacitracin growth inhibition is due to its involvement in this process. About 5,000 lipid II molecules should be flipped per second in accordance with the needs of peptidoglycan polymerization. However, it is rapidly captured by the peptidoglycan building block, polymerized by lipid II polymerases of the SEDS family, as FtsW (in the divisome) and RodA (in the elongasome), and cross-linked by complex multiprotein machines involving glycosyltransferases and transpeptidases.[11] The production, regulation, dynamics, recycling effects and cell release of a multiplicity of non-PBP enzymes associated to the cell wall is an open field of research.[12]

The release of cell wall fragments from growing cells in the environment was indicated a long time ago.[13] Therefore, the release of CWBAMs should peak during active cell growth, antibiotic exposure, and/or stressful environments. In a single cell duplication round in *E. coli*, about one-half of the peptidoglycan is excised from the cell wall as anhydromuropeptides, most of them being reused, suggesting a robust turnover of the cell wall.[14] Reaching the duplication end, most CWBAMs are captured in the cell wall mesh, become less soluble and less mobile to act as efficient signaling agents. In any case, the rate of cell wall recycling differs among bacterial species[3] and defective recycling, as occurs in pathogenic *Neisseria*, which results in a larger CWBAM release. In a significant part, peptidoglycan and other CWAS are released in microvesicles, as outer membrane vesicles in Gram negatives.

The release of CWBAMs is certainly triggered by bacterial autolytic processes. Autolysins, which provide cell wall lytic functions, include endopeptidases, amidases, carboxypeptidases, phospho-glycosidases, muramidases, or lytic glycosidases such as phospho-transglycosidases (phospho-muramidases), which may eventually act within the same protein.[15-16] Regulation of autolysin expression is a complex field, as it involves both external and internal factors, including post-translational regulatory mechanisms of these enzymes, as seen in the case of murein hydrolase.[17] CWBAMs are recycled by living bacteria to facilitate new peptidoglycan (PG) formation, through a process called PG recycling, where bacteria consume their own exoskeletons.[18] This activity varies in intensity across different growth phases among bacterial species, and these changes in pericellular CWBAM levels can influence signaling, host communication, immune stimulation, and adaptive responses.[19] *E. coli* recycling transporters include AmpG and Opp. An important ecological aspect is whether cell wall fragments from other bacteria in the nearby microbiota can contribute to peptidoglycan recycling in a specific species. Opp is an ATP-binding cassette transporter paired with a PG-specific periplasmic binding protein, Mpp, which imports cell wall fragments from other bacteria[19]. The adaptive and evolutionary implications of this type of "cell wall recombination" are certainly topics of interest. Different survival dynamics observed in experiments involving multiple punctures of the cell wall[20] may be related to this recycling process.

The physical disruption of the cell is the ultimate result of the bactericidal action of antibiotics[21]. Exposure to beta-lactam antibiotics triggers an autolytic breakdown of the cell wall, and many other antibiotics contribute to cell dis-structuration or apoptotic processes, leading to the release of CWBAMs. The same should happen with lytic phages, toxin-antitoxin systems, large bacteriocins, lantibiotics, and microcins, as well as in cases of bacterial fratricide and cannibalism or cellular penetration by predatory bacteria such as *Bdellovibrio*. Additionally, cell wall breakdown results from the innate immune response, from lysis in phagolysosomes to the action of antimicrobial peptides like host defensins. Finally, bacterial digestion by intestinal molecules capable of destroying bacteria[22] should be considered. An area that remains insufficiently explored is the dynamics of self-cell wall degradation after bacterial death, and the functioning of the enzymes involved[23]. A schema illustrating the nature and CWBAMs interactive network is presented in Figure 1.

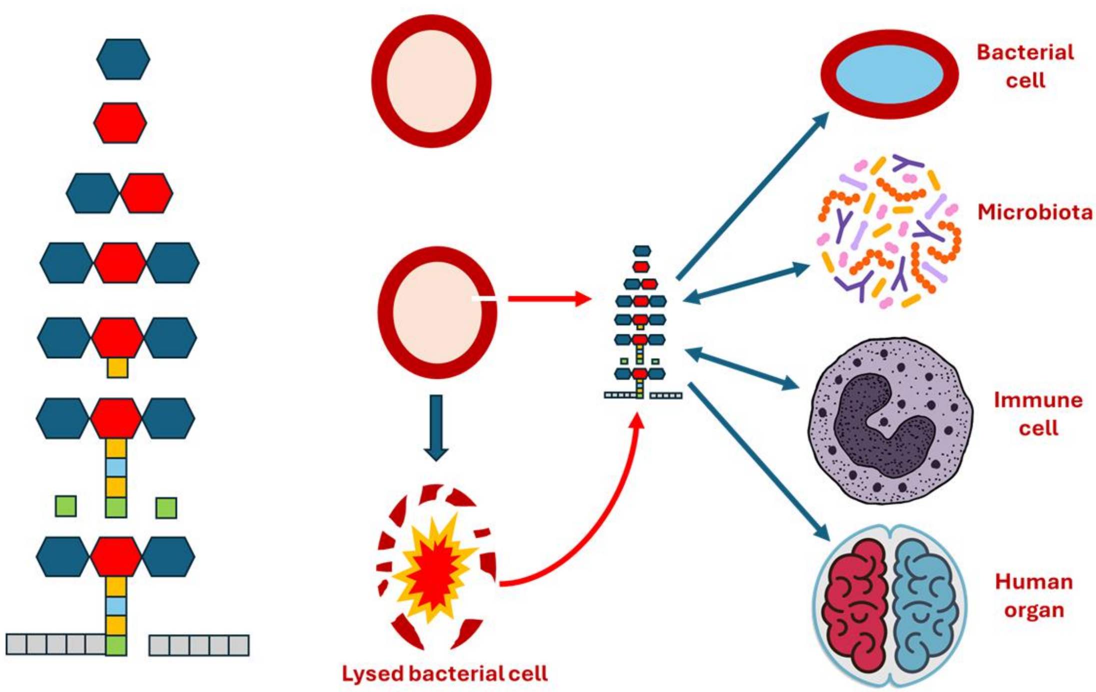

Figure 1. Cell wall bioactive molecules (CWBAMs). The left side features a schematic diagram illustrating various CWBAMs released from leaky (during replication) or dead bacterial cells. Blue and red hexagons represent N-acetyl glucosamine and N-acetyl-muramic acid in different linking conformations. Small rectangles depict amino acids and their cross-linking chains. Blue and green colors indicate D-amino

acids, while grey denotes glycine residues, which are attached or unattached to larger molecules. On the center-right, a bacterial cell with a dark red cell wall may release CWBAMs, mainly originating from lysed cells. These molecules then influence other bacteria or the microbiota, are detected and modulate immune cell activity, and also impact human organs, including the central nervous system.

EFFECTS ON BACTERIAL PHYSIOLOGY

Effects on the determination of cellular shape

Form serves as both function and sign. The shape of a bacterial cell—whether spherical, ovoid, ellipsoid, lemon-shaped, bean-shaped, cylindrical (rod, filament, bifurcated cylinder), crescent, or spire—is determined by the structure of its cell wall. These shapes can change into one another based on the microorganism's physiological or adaptive needs. Each form requires a specific three-dimensional arrangement of peptidoglycan molecules and their precursors, as well as a particular topology of CWBAMs.[24] This results from the trade-off between conflicting biosynthetic protein complexes, the elongasome and the divisome, which probably share a common evolutionary origin .[25] The conflict is evident because cell wall elongation—the extension of the lateral wall—only occurs when a division septum is not forming, and *vice versa*. Modulating this conflict depends on murein recycling genes (*mre* genes), which produce membrane-associated proteins, some resembling actin, that signal the spatial direction of peptidoglycan biosynthesis. This regulation influences the activity of transglycosidases and transpeptidases (PBP proteins). However, the precise interactions between Mre proteins and PBPs are not yet fully understood, and studying this remains challenging. These complexes can quickly associate and dissociate during the cell growth cycle, as well as in response to environmental changes, leading to the formation of specific shapes .[24,26]. An important factor in regulating rod and sphere shapes in different environmental conditions is the surface area to volume ratio.[27-28] Shape is determined by the size of cell wall building blocks and regulated by penicillin-binding proteins (PBPs), which work in coordination with SEDS (shape, elongation, division, and sporulation) transmembrane glycosyl transferases, hydrolases, and other enzymes associated with the cell envelope. There is likely a specific migration and localization of CWBAM clusters at particular cell sites. These are finally assembled by penicillin-binding proteins[16,29], which decrease their solubility, mobility, and most likely their signaling roles. Additionally, the mechanical properties of bacterial shape are modulated by the lipoprotein Lpp in Gram-negative microorganisms.[6,30] Note that cell shape and volume may have physiological consequences, as they influence the molecular and organelle intracellular density within subcellular compartments, leading to structural epistatic interactions and the emergence of new phenotypes, including antibiotic resistance.[31]

Effects on central bacterial metabolism

Although the question was raised long ago, there is very little information about how cell wall biomolecules influence bacterial overall metabolism. It was reasonably assumed that cell wall biosynthesis and ongoing rearrangements during growth phases consume a significant portion of bacterial energy, which requires a regulated allocation of resources from central metabolism.[32] Peptidoglycan synthesis involves redirecting the glycolytic intermediate fructose-6-phosphate into amino sugar biosynthesis, facilitated by the branchpoint enzyme GlmS. MurA directs the downstream product, UDP-GlcNAc. Amino acids are used for structural purposes, such as forming peptidoglycan cross-bridges. Cell envelope synthesis also requires the isoprenoid carrier lipid undecaprenyl phosphate.[33] For example, the Braun Lpp lipoprotein, essential for bacterial elongation and maintaining bacterial shape, is among the most abundant bacterial proteins. This requires a high translational demand, stemming from the need for ribosomal synthesis and transfer

RNAs. Conversely, the Lpp biomolecules should be exported by dedicated export proteins such as SecY, SecD, and the specific Lol system. Generally, the conflicting trade-off between the energy needed for cell wall elongation and the expression of other vital genes to sustain bacterial fitness may be regulated by codon choice; using dissimilar codon usage to allocate transfer RNA resources can adjust the balance of expression levels, thereby preventing a catastrophic cellular burden on the host.[34-35] The effects of synthesizing non-canonical amino acids, which could harm bacterial metabolism, are mitigated through the excretion of these biomolecules. In dense bacterial populations, bacteria's need for cell wall construction may benefit from scavenging metabolites, including CWBAMs, released by neighboring cells, and eventually resources obtained from the host during symbiotic colonization or infection. Lastly, the cell wall stress response, regulated by cell wall stress stimuli[36-38], helps bacteria survive cell wall damage. In some cases, such damage, often caused by antibiotics, results in the release of CWBAMs, which may trigger adaptive mechanisms mediated by small RNAs that directly enhance sugar metabolism, leading to more efficient energy acquisition for cell wall repair.[39]

Effects on sporulation and germination

It has been proposed that the release of muropeptide fragments into the extracellular environment is a potent germinant of dormant *Bacillus subtilis* spores.[40] However, it appears that the regulation of sporulation may involve CWBAMs associated with muropeptides, such as L-alanine, which acts as a germinant, and D-alanine, the product of alanine racemase (Alr), which functions as a sporulation inhibitor. Interestingly, Alr is a key external component of the spore— or pre-spore-coat. Alanine racemase may control the unnecessary but energetically costly sporulation process. Conversely, alanine dehydrogenase (Ald), which allows growth in the presence of L-alanine, promotes both sporulation and nutrition of the developing cell. Blocking alanine dehydrogenase activity, which breaks down L-alanine, can cause endospores to undergo premature and unproductive germination.[40] Therefore, a balanced equilibrium between L-ala and D-ala is crucial for a healthy and efficient sporulation process, weighing the costs and benefits of sporulation. Additionally, *B. subtilis* produces spores *in vivo*; peptidoglycan fragments, as well as NAG (or associated CWBAMs), may stimulate eukaryotic-like kinase signals, influencing spores to exit dormancy.[41,42]

Effects on biofilm formation

N-acetylglucosamine influences biofilm formation.[43] CWBAMs, as D-amino acids, release planktonic cells from biofilms (see below). The breakdown of peptidoglycan by AmpC releases muropeptides. The *ampC* gene encodes a Class C beta-lactamase, which is related to DD-carboxypeptidases and affects the availability of pentapeptide substrates for cross-linking by DD-transpeptidases (PBPs). AmpC expression is controlled at the transcriptional level by AmpR, a LysR-type multigene regulator involved in about 500 other bacterial genes, including repression of biofilm formation. A complex interaction exists between changes in peptidoglycan composition and biofilm development.[44] It has been observed that, in the hospital environment, members of the Serratia marcescens complex, which carry the entire AmpR regulatory cluster, markedly decrease the inducible expression of AmpC. This likely results in reduced muropeptide release and may promote persistent biofilm formation in basin sinks, leading to unexpected susceptibility to beta-lactam agents.[45] In Gram-positive bacteria, abundant wall teichoic acids serve a similar role in surface attachment as lipopolysaccharides do in Gram-negative bacteria.[46]

Effects of cross-linking peptides and non-canonical D-amino acids on bacterial interactions

The production of non-canonical D-amino acids (NCDAA), such as D-Alanine, by epimerases and racemases to form peptidic bridges in peptidoglycan, can negatively impact bacterial cell metabolism. As a result, the excess D-Alanine is expelled outside the cell via a secretion system. [47] The released D-Alanine may have both toxic and potentially beneficial regulatory effects on the cell wall synthesis of neighboring bacteria within microbial communities. Generally, D-amino acids influence microbial growth[48] and have been considered among non-peptidic microcins.[49] D-Amino acids also aid in biofilm disassembly, supporting the hypothesis that reduced muropeptide release promotes biofilm formation.[50-52]. However, the activity of racemases can be inhibited by peptidoglycan peptides, indicating a negative regulatory mechanism to prevent excessive NCDAA production.[53] The reason for their presence in the cell wall stem peptides may be that D-Amino acids help protect bacteria from extracellular proteases, which typically cleave between two L-isomers[54], or they may contribute to resistance against certain antimicrobial agents targeting the stem peptide (see below). Additionally, D-Amino acids might serve regulatory roles among members of the intestinal and respiratory microbiota.[55-56] Finally, D-homoserine-lactones could be involved in interbacterial quorum sensing.[57]

Antimicrobial resistance, effects on bacterial fitness, and antibiosis

Antibiotics contribute, either directly or indirectly, to the destruction of the cell wall. For example, beta-lactams bind to PBPs (glycosyltransferases, transpeptidases, and DD-carboxypeptidases), blocking these enzymes involved in the polymerization of glycan strands and the cross-linking of peptide stems. This results in an accumulation of muropeptides and causes changes in bacterial shape, ultimately leading to partial or complete destructuration of the cell wall and cell lysis.

The cell wall stress stimulon (CWSS) is a multi-gene inducible response to the inhibition of cell wall synthesis. CWSS induction is regulated by the VraSR two-component system, which detects an unknown signal, most likely CWBAMs, since the CWSS response is not specific to different cell wall-altering antibiotics.[58-59] VraS histidine kinase, part of the VraSR two-component system in *S. aureus*, detects signals that upregulate gene expression for cell wall synthesis. Mutations may develop that increase the efficiency of VraS kinase activity, leading to changes that favor bacterial survival.[60]

Another survival mechanism, separate from CWSS but also driven by CWBAMs, addresses the need for peptidoglycan recycling processes. These processes influence the induction of endopeptidase enzymes, such as the protein AmpC or AmpH[60-62], which help maintain bacterial shape. It has been stated that the regulation of AmpC is finely tuned to detect defects in cell wall synthesis caused by beta-lactam drugs, likely by creating space in the wall matrix for the Insertion of new material during cell growth.[63-64] However, the physiological role of AmpC is a critical area that remains scarcely explored. AmpC is more commonly known as a serine beta-lactamase, which detoxifies beta-lactam agents by acting as a beta-lactam ring peptidase. AmpC was, in fact, the first enzyme reported to have a beta-lactamase function in *Escherichia coli*, as early as 1940.[65] In a group of Gamma-Proteobacteria, including pathogens such as *Enterobacter*, *Serratia*, *Citrobacter,* and *Pseudomonas*, AmpR activates beta-lactamase production by sensing high levels of intracellular muropeptides in the presence of a broad range of beta-lactam agents, including penicillins, oxyiminocephalosporins, monobactams, and, to a lesser extent, carbapenems.[66] The rate of induction and beta-lactamase production varies among different bacterial species and antibiotics. Some genera, such as *Salmonella* or *Proteus*, lack AmpC, or AmpC is not induced, as with *E. coli*. Mutations leading to constitutive AmpC hyperproduction frequently occur in the *ampD* genes, which encode an N-acetyl-anhydromuramyl-L-alanine amidase, influencing the levels of *ampC*-activating muropeptides. However, inactivating mutations in *ampD* amidases—and consequently AmpC derepression—might reduce fitness, negatively affecting growth, motility, and cytotoxicity.[67] In *Pseudomonas*, signals derived from peptidoglycan, such as CWBAM, resulting from cefoxitin exposure, are elusive, probably because, despite being a good inducer, cefoxitin exhibits poor activity on *Pseudomonas aeruginosa* AmpC-activating potency for CWBAM 1,6-anhydro-N-acetylmuramyl-pentapeptide. This is likely influenced by various pathways resulting from signaling trade-offs between AmpC inducers and repressors, such as UDP-N-acetylmuramyl-pentapeptide.[68] In *Salmonella*, experimental hyperproduction of AmpC (where the *ampC* gene was introduced along with *ampR* via transformation) results in reduced growth rates, changes in cellular and colony morphology, and a decreased ability to invade eukaryotic cells. In this case, AmpC may reduce levels of L-D dimers, lipoprotein-bound muropeptides, and anhydrous muropeptides.[69] Therefore, antibiotic resistance may decrease the release of CWBAMs. There is a possible antagonistic relationship between antibiotic resistance and virulence mediated by CWBAMs[70], but we cannot dismiss the evolution of a dangerous balance between these traits in a highly antibiotic-polluted world.[71-72] Fosfomycin blocks *de novo* UDP-MurNAc biosynthesis by inhibiting UDP-N-acetylglucosamine enol pyruvyl transferase (MurA). In several bacterial organisms, NAM exposure, which increases the cellular pool of UDP-NAM, triggers a salvage pathway, conferring resistance to fosfomycin.[73-74]

D-amino acids in the stem peptides linking peptidoglycan chains may help bacteria resist antibiotics, as seen with the dipeptide D-alanyl-D-serine or D-alanyl-D-lactate, which blocks the activity of glycopeptide antibiotics like vancomycin. On the other hand, some D-amino acids make avian *E. coli* more vulnerable to tetracycline and aminoglycosides, likely due to increased expression of outer membrane proteins[75]. DD-carboxypeptidases such as PBP6b from *E. coli*[76] are targets for certain antibiotics.[77] However, little is known about how beta-lactams interact with modified stem peptides, though a synergistic effect of D-amino acids and glycine with β-lactams has been suggested, based on inhibition of carboxypeptidase.[78-79] In *Staphylococcus*, D-amino acids contribute to resistance against daptomycin, a lipopeptide antibiotic that forms a tripartite complex with lipid II and phosphatidylglycerol, especially when combined with teichoic acid overproduction.[80]

Finally, the presence of D-amino acids is generally a hallmark of peptides biosynthesized via non-ribosomal peptide synthetases (NRPSs), and D-amino acids are incorporated into novel antimicrobial peptide structures with enhanced activity.[81] The occurrence of D-amino acids is rare in microcins and, in general, in ribosomally synthesized post-translationally modified peptides (RiPPs).[82] However, some lassopeptides contain a D-amino acid at the C-terminus[83] . Other RiPPs, which are common in Gram-positive bacteria, such as lanthionine-containing antimicrobial peptides (lanthipeptides), may also include D-amino acids.[84]

The development of lipoprotein biosynthesis inhibitors, such as LpsA signal peptidase, suggests that increased lipoprotein levels may confer a heteroresistance phenotype affecting antibiotic action.[85-86]

Revisiting the effect of antibiotics as signaling agents

Two decades ago, we suggested that at low concentrations of antibiotics in the environment, which result from local antibiotic producers, these substances should not be viewed solely as bacterial weapons for competing. Instead, they might serve as signaling molecules that help regulate the homeostasis of microbial communities, affecting traits such as biofilm formation, motility, and even eukaryotic cytotoxicity.[87] However, we can now reinterpret these signaling effects by attributing them to CWBAMs released by the action of antibiotics on bacterial cells, rather than to antibiotics themselves. Very low concentrations of beta-lactam antibiotics can influence cell morphology and biofilm formation, eventually leading to bacterial lysis with DNA release.[88]

EFFECTS ON HOST INNATE BACTERIAL IMMUNITY AND DISEASE PATHOGENESIS

The host recognition of cell wall active biomolecules

More than 30 years ago, Alexander Tomasz contributed to the discovery that the membrane glycoprotein CD14 serves as a receptor used by mammalian cells to recognize and signal responses to a wide range of bacterial components. This was a key finding in developing the concept of innate immune response, ultimately leading to serious outcomes such as septic shock.[89] Hosts have evolved mechanisms to recognize alien signals released by bacteria, generally called "microbial-associated molecular patterns" (MAPS or Pathogen-AMPS). Typically, CWBAMs are unique structures targeted by the host pattern recognition receptors (PRRs).

PRRs include oligomerization domain proteins like NOD-1, the primary peptidoglycan receptor, and NOD-2, both containing a C-terminal leucine-rich repeat, a central nucleotide-binding site (NBD/NOD), and an N-terminal caspase activation and recruitment domain. Among PRRs, there are bactericidal agents targeting the bacterial cell wall, such as peptidoglycan recognition proteins (PGRP1, PGRP2, PGRP3, PGRP4), which can kill invading microbes in human tissues and cellular phagosomes. All PGRPs have a carboxy-terminal type 2 amidase domain used for recognizing peptidoglycan. Typically, PGRP-2 is an N-acetylmuramyl-L-alanine amidase that cleaves the lactyl bond between NAM and the stem amino acid peptide. Another group of PRRs is the C-type lectin receptors (CLRs), which recognize bacterial glycan backbones. CLRs capable of recognizing bacterial cell wall components include dectins, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), and the Gram-positive bactericidal Regenerating gene family protein 3A (REGIII3A). Mannose-binding lectin (MBL) binds to peptidoglycan and inhibits the formation of proinflammatory cytokines. Toll-like receptors (TLRs) detect bacterial peptidoglycan, lipoteichoic acid (LTA), and lipoproteins (LPP). Additionally, lysozymes are small proteins that recognize and cleave the glycosidic bond between NAG and NAM, resulting in bacterial lysis. Overall, the role of PRRs detecting CWBAMs is to activate the innate immune response, which serves as the first line of defense against invading bacteria. For comprehensive reviews, see Sukhithasri et al[90], Irazoki et al[3], and Juan et al.[12] . Wall teichoic acids exposed or released by Gram-positive bacteria are recognized by various human immune receptors, including surface-expressed receptors on immune cells such as langerin and macrophage galactose-type lectin, scavenger receptors (SREC-1), and soluble serum receptors like specific antibodies and mannose-binding lectin.[91] Bacterial resistance to the host defense mechanisms sensing or acting on CWBAMs has evolved along with historical bacterial-host interactions. In essence, the mechanisms of resistance include modifications of peptidoglycan glycan chain, as acetylation of the C-6 position of NAM, for instance, involving peptidoglycan O-acetyltransferase A (PatA) and peptidoglycan O-acetyltransferase B (PatB), in Gram-negatives. The N-deacetylation of peptidoglycan (PgdA), as well as the O-acetyl transferase-A (OatA), or the N-glycosylated modification of C-2 position of NAM (NamH) results in resistance to lysozyme. Additionally, bacteria can modify the stem pentapeptide by amidating amino acid residues, contributing to resistance to PRR recognition.[89] Apparently, the undecaprenyl-phosphate involved in the translocation of cell wall components has poor or no biological effects in the host.[92]

Effects of the peptidoglycan fragments and their basic bricks N-acetyl-glucosamine (NAG) and N-acetyl-muramic acid (NAM)

In Gram-positive bacteria, fragments of thick peptidoglycan trigger cytokine release via CD14. However, the Gram-positive peptidoglycan is about 1000 times less active than LPS in promoting inflammation on a weight-for-weight basis, suggesting that only part of it may be proinflammatory. Long, soluble peptidoglycan chains (around 125 kDa) are poorly active. Hydrolyzing these chains to their minimal units (two sugars and a stem peptide) completely eliminates inflammation[93]. In fact, NAM exhibits anti-inflammatory properties.[94] Apparently, the optimal constraint for activation might involve three cross-linked stem peptides. The composition of these peptides appears important: replacing the first L-alanine in the stem peptide with D-alanine completely abolished inflammation in experimental meningitis.[93] NAG may play a significant biological role in mammals, including humans. For example, it has been linked to neurodegenerative diseases such as multiple sclerosis, acting as a modulator of inflammation, myelination, and neurodegeneration, thereby improving patient health.[95] Among bioactive muropeptides resulting from cell wall degradation, the NAG-NAM-tetrapeptide fragment of peptidoglycan functions as a toxin (tracheal toxin), causing death of tracheal (*Bordetella*) or vaginal (*Neisseria*) ciliated epithelial cells, and seemingly inducing slow-wave sleep; muramyl-dipeptides influence host immune response activation.[96] In *Staphylococcus aureus*, a specific endopeptidase degrades the pentaglycine bridge linking peptides.[97] However, the non-degraded portion remains antigenic[98], but its effect on cell viability is poorly understood.

Effects of cross-linking peptides and non-canonical D-amino acids

In principle, D-amino acids, mostly CWBAMs, originated in bacteria and are toxic to life on Earth; however, bacteria themselves counteract this toxicity by converting D-amino acids into their normal enantiomers, the L-amino acids, using racemases. In a previous section, we examined the effects of non-canonical D-amino acids (NCDAA) produced by racemases on bacterial cell metabolism and biofilm structure, as well as the regulation of their production and excretion. Because of this, the cumulative excess of D-alanine is expelled outside the cell by a secretion system and may influence bacterial-host interactions. D-alanine acts as an inhibitor of proinflammatory processes, suppressing interleukin production by macrophages.[100] Altering the D-alanine decorations of lipoteichoic acids with L-alanine or removing them from their diacylglycerol lipid anchor also reduces inflammatory responses.[93] However, several D-amino acid-containing peptides (DAACPs) have been isolated from patients with cataracts, Alzheimer's, and other diseases, mainly in elderly individuals.[53,101] D-amino acids, often acquired with bacterial-contaminated foods or absorbed from the microbiota but not exclusively produced by microorganisms, are recognized as toxins by most humans and other mammals. Detoxification is carried out by transport and degradation systems, often involving flavoproteins such as D-amino acid oxidases and D-amino acid dehydrogenases, which are responsible for oxidizing neutral and acidic D-amino acids, respectively.[82] However, it cannot be rejected the hypothesis that these CWBAMs, interacting with glutamate-gated Ca^{2+} channels, might have signaling functions in most organs, especially the kidney, brain, and the intestine.[102] As mentioned before, they seem to have a signaling role influencing the gut microbiota and its relationship with intestinal mucosa defence.[54] An extensive account of the possible signaling effects of D-aminoacids in biological systems can be found in the review from Aliashkevich et al.[103] In the next section, the role of D-aminoacids in the pathogenicity of wall teichoic acids is briefly mentioned.

Effects of teichoic acids

Wall teichoic acids are polyribitol- or polyglycerol phosphate anionic polymers cross-linked to NAM residues of the peptidoglycan, eventually modified with D-alanine and NAG residues Typically present in Gram positive bacteria, they can represent up to 50% of the dry weight of staphylococcal walls.[104] Teichoic acids also facilitate the adhesion of Gram-positive bacteria to surfaces and the formation of biofilms, which also applies to colonization of mucosal surfaces of the respiratory tract.[91] In addition, cell wall teichoic acids create in the bacterial envelope a gradient of electrostatic charge, allowing the extracellular release of several staphylococci cytolytic toxins for eukaryotic cells, including hemolysins and leukocidins.[105] The D-Ala decoration of wall teichoic acids and lipoteichoic acids (associated with the cell membrane) is primarily thought to be a virulence factor, as it facilitates colonisation, invasion, immune response activation, inflammation, and abscess.[106]

Effects of Lpp, the Braun's lipoprotein

Lpp, probably the most abundant protein in *E. coli*, appears to be a crucial factor in pathogenicity. Deletion mutants producing less Lpp tend to show decreased pathogenicity.[107] The reason might be that Lpp inhibits ROS production in neutrophils, thereby preventing bacterial killing.[108] Purified Lpp synergizes with lipopolysaccharide (LPS), eventually leading to septic shock, by increasing the production of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6).[109] In addition, Lpp and L-D-transpeptidases regulate the master virulence regulator AggR in enteroaggregative *E. coli.*[110] However, Lpp is a main target of antimicrobial peptides, which partially offsets its pathogenic role.[111]

The antibiotic release of endotoxins: a reappraisal from an old concept

The term "endotoxin" was introduced by German scientist Richard Pfeiffer (1858-1945) in 1892. [112] His work was built upon the scientific foundation laid by Robert Koch (1843-1910), who provided the key definition of the term, describing endotoxins as "the toxins linked to the bacterial cell substance," not the classic excreted toxins known as exotoxins. Since Pfeiffer was studying the pathogenesis of *Vibrio cholerae*, he used the term to distinguish a heat-stable, toxic substance derived from the cell walls of gram-negative bacteria from actively secreted, traditional exotoxins. [113] As a result, during the following century, the term "cell wall endotoxin" was mainly associated with the lipopolysaccharide (LPS) found in the outer membrane of Gram-negative bacteria, which is typically released after bacterial death. Between the 1980s and 2000s, there was a surge in research highlighting the potential dangers of antibiotics, which could induce endotoxin release. [114] In some cases, the "endotoxic effect" was linked to molecules other than LPS, with Alexander Tomasz noting the pro-inflammatory role of peptidoglycan fragments.[115-116] In recent years, this perspective has largely faded. However, it may now be time to reevaluate the role of the array of biomolecules covalently attached to the bacterial cell wall (peptidoglycan) as signals and effectors involved in bacterial physiology, ecology, infectious diseases, and even in human and animal health.[22].Particularly, peptidoglycans translocated into the bloodstream from the gut microbiota may serve as signaling molecules. molecules influencing general immunological responses and even brain functions.[117-119] In summary, we are exposed not only to bacterial organisms but also to their individual molecular constituents, which are not necessarily toxins but just biological signals, active microbiomolecules. They can be named bacterial endopraxins (from the Greek πράξη, práxi, to act), as those reviewed in this work in relation to cell wall bioactive molecules. Endotoxins are only a part of endopraxins, those that produce harmful effects.

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This work honors the seminal work and vast legacy of Prof. Alexander Tomasz in the field of bacterial cell wall envelope.

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