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Peptidoglycan NlpC/P60 peptidases in bacterial physiology and host interactions

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SUMMARY

The bacterial cell wall is composed of a highly crosslinked matrix of glycopeptide polymers known as peptidoglycan that dictates bacterial cell morphology and protects against environmental stresses. Regulation of peptidoglycan turnover is therefore crucial for bacterial survival and growth and is mediated by key protein complexes and enzyme families. Here, we review the prevalence, structure, and activity of NlpC/P60 peptidases, a family of peptidoglycan hydrolases that are crucial for cell wall turnover and division as well as interactions with antibiotics and different hosts. Understanding the molecular functions of NlpC/P60 peptidases should provide important insight into bacterial physiology, their interactions with different kingdoms of life and the development of new therapeutic approaches.

eTOC

Enzymes that remodel the bacterial cell wall, peptidoglycan, are critical for both cell division and their interactions with host organisms, making them potentially useful targets for therapeutic

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AUTHOR CONTRIBUTIONS

M.E.G., S.K., J.E. and H.C.H. conceived and wrote the manuscript.

DECLARATION OF INTERESTS

M.E.G. and H.C.H. have filed a patent application (PCT/US2020/019038) for the commercial use of SagA-expressing bacteria to improve checkpoint blockade immunotherapy.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

applications. In this review, Griffin M et al. provide an overview of NlpC/P60 peptidoglycan hydrolases and summarize their unique functions across different microbes.

INTRODUCTION

Bacteria live in a variety of biomes from the natural environment to cohabitation with other creatures, each with their own harsh and ever-changing physiological conditions. To combat these environmental stresses, bacteria produce protective glycopeptide polymers within their cell walls known as peptidoglycan (PG).[1,2] PG consists of a repeating disaccharide unit of *N*-acetylglucosamine (NAG, GlcNAc) and *N*-acetylmuramic acid (NAM, MurNAc), with a stem peptide attached to the 3-*O*-lactoyl group of NAM (Figure 1). Stem peptide structures are highly heterogeneous within the cell wall of individual bacterial cells. In newly incorporated PG monomers, the stem peptide generally exists as a pentapeptide chain, which can be modified and crosslinked with other stem peptides during PG maturation. Across bacterial species, PG also exhibits significant structural diversity, with substitutions at multiple amino acid residues of the stem peptide and crossbridge as well as modifications to the carbohydrate backbone.

Although the rigid structure of PG can prevent cell lysis under osmotic shock, PG must be degraded and reformed to allow for cell growth and division.[3] Bacteria have developed highly regulated systems of enzymes to remodel PG.[4,5] The nomenclature of these proteins is often complicated due to historical precedence and overlapping enzyme classifications.[6] In accordance with previous literature, we will refer to remodeling enzymes as three separate groups based on their substrate specificity: glycosidases, which act on the glycosidic bonds between NAG and NAM residues; amidases, which degrade the amide bond between NAM and the stem peptide; and peptidases, which cleave amide bonds between two amino acid residues of the stem peptides or crosslinking bridges. Within the peptidase family, enzymes can be further classified by the stereochemistries of the two amino acid residues they hydrolyze (*L,D* vs. *D,L* vs. *D,D*) and their relative location within the stem peptide (endo- vs. carboxypeptidase). In addition to substrate- or function-based classifications, these proteins have also historically been categorized by structural homology of their catalytic domains. For example, PG peptidase activity is accomplished by at least 10 distinct domain types.[6] In this review, we will focus on efforts to understand the activities and biological functions of the "new lipoprotein C from *Escherchia coli* / protein of 60 kDa of *Listeria monocytogenes*" (NlpC/P60) superfamily of cysteine peptidases (Figure 1). Through the biochemical and functional characterization of these enzymes, we will highlight how this widespread family of PG hydrolases are critical mediators of both bacterial physiology and microbial-host interactions. Together, the broad roles of NlpC/P60-containing proteins underscore their untapped potential as novel targets for antibiotics and modulators of host immunity.

The NlpC/P60 family of cysteine peptidases

The NlpC/P60 domain is approximately 150 amino acid residues in length and 15 kDa in size (Figure 2A).[7] Nearly all of these domains fold akin to other papain-like proteases, with a series of three N-terminal α-helices followed by a five-stranded β-barrel. This

tertiary structural motif is similar to other cysteine peptidase families, which aided in initial predictions of the catalytic core of this domain. NlpC/P60 domains are unified by the presence of conserved cysteine and histidine residues, which are found at the N-terminus of the second, conserved α-helix and within the first two β-strands, respectively (Figure 2A). Like other cysteine proteases, the cysteine residue acts as a nucleophile to break the targeted PG amide bond, whereas the histidine residue functions as a general acid-base. In addition to the invariant catalytic diad, some NlpC/P60 domains contain a third polar amino acid residue (His, Glu, or less frequently Gln or Asn) that may act as a coordinating residue for the catalytic histidine through hydrogen bonding.

Peptidase activity of NlpC/P60 domains is most frequently specific for the amide bond between the second and third amino acid residues of the stem peptide (Figure 1).[6,7] These residues differ between bacterial species (γ-D-Glu vs. γ-D-Gln at the second position and L-Lys vs. meso-DAP vs. amidated meso-DAP at the third position), leading to peptidases with differential molecular specificities. In addition, these enzymes often have differential specificities towards substrates depending on chemical structures distal from the targeted amide bond, including the length and crosslinking of the stem peptide, the presence of the glycan residues and the extent of polysaccharide polymerization. NlpC/P60 activity has also been demonstrated on other peptide linkages including between meso-DAP and the fourth stem peptide residue, D-Ala, or between meso-DAP and a crosslinked D-Ala residue from a separate stem peptide [6,7]. Beyond strict peptidase activity, some NlpC/P60-containing proteins possess amidase activity to separate MurNAc residues from the stem peptide. Although the protein of unknown function DUF1460 family (IPR010846) is classified separately by InterPro, recent evidence has indicated that DUF1460 represents a new group of evolutionarily and structurally related NlpC/P60 amidases [8]. Together, bacteria take advantage of these diverse substrate preferences by often expressing multiple, differentially regulated, and localized NlpC/P60 proteins, allowing them to coordinate the complex process of PG remodeling.

NlpC/P60 domains have been extensively examined through structural studies, which date back to the first solution NMR structure of *E. coli* MepS (Spr) reported in 2008 (Figure 2A) [9]. To date, there are over 25 structures currently deposited in the Protein Data Bank, allowing for direct comparisons of protein architecture across the bacterial kingdom. Crucially, these studies have highlighted conserved mechanisms of substrate recognition. The NlpC/P60 substrate-binding pocket was first mapped for YkfC from *Bacillus cereus* (Figure 2B).[10] X-ray crystallography of the purified enzyme revealed unexpected electron density within the putative enzyme active site. Although no substrate was added in the purification process, this density was assigned to the L-Ala-D-Glu dipeptide that may have been derived from PG the recombinant *E. coli* host. The bound dipeptide was found to be stabilized by multiple hydrogen bonds within the substrate binding groove, including Glu83, Tyr118 and Asp256, which are highly conserved in the YkfC subfamily of NlpC/P60 enzymes. Guided by this initial structure, multiple computational docking studies of muropeptides with solved structures also aided in defining the substrate-binding cleft of NlpC/P60 proteins. Together, these data uncovered additional regions and residues that may be critical for substrate recognition. For example, docking of an mDAP-type GlcNAc-MurNAc (GM)-tetrapeptide fragment to CwlT of *Staphylococcus aureus* found

that conformational rearrangement of the flexible β4/β5 loop is required for enzymatic function and may dictate substrate specificity.[11] Docking of Lys-type GM-tetrapeptide and GM-pentapeptide to SagA from *Enterococcus faecium* revealed that two aromatic residues, Trp433 and Trp462, interact with the glycan headgroup of the muropeptide and may function as a clamp for substrate binding (Figure 2C).[12] Further in silico alanine screening and in vitro validation showed that multiple aromatic and hydrophilic residues along an extended substrate-binding groove were also crucial for enzymatic turnover, likely through stabilization of the muropeptide-bound structure.[13] Iterative superimposition and rigid-body fitting of PG fragments to Acd24020 from *Clostridium difficile* was used to generate a model of the enzyme bound to a crosslinked PG substrate.[14] This model revealed that crosslinked PG forms an H-like structure, with the stem peptide accommodated within the substrate-binding groove and the polysaccharide backbone extending perpendicular to the groove along the surface of the domain. The bound structure and subsequent in vitro characterization demonstrated that the unique hourglass shape of the Acd24020 groove enabled recognition of the direct mDAP-D-Ala crosslinks found in PG from *C. difficile*, underscoring that the composition and orientation of the substrate groove within NlpC/P60 proteins may regulate its specificity for distinct PG chemical structures.

As an additional layer of regulation, NlpC/P60 domains are frequently found in larger proteins that can contain other structural regions or defined domains (Figure 2D). For example, many NlpC/P60-containing proteins including AvPCP from *Anabaena variabilis* possess one or more bacterial Src homology 3 (SH3) domains, which have been predicted to mediate protein-protein interactions.[15] Other common coinciding features include LysM domains, which are thought to be involved in direct PG or cell wall binding,[16] as well as prodomains that may regulate catalytic activity as seen in RipA from *Mycobacterium tuberculosis*.[17] Therefore, the overall structure of NlpC/P60 domain-containing proteins may also serve as an additional determinant of biological specificity. Together with substrate preference and endogenous mechanisms of gene regulation, these differentiating factors across bacterial peptidases allow NlpC/P60 domains to exert potentially non-overlapping functions.

The NlpC/P60 domain is found broadly throughout bacteria as well as within viruses, archaea, and even eukaryotes.[7] The InterPro classification of protein families categorizes the NlpC/P60 domain (IPR000064) along with the structurally similar cysteine, histidine-dependent amidohydrolases and peptidases (CHAP, IPR007921) domain as part of the papain-like cysteine peptidase superfamily (IPR038765).[18] Via sequence analysis, NlpC/P60 domains have been separated into four major groups: (1) P60-like, (2) AcmB/LytN-like, (3) YaeF/Poxvirus G6R-like, and (4) lecithin retinol acyltransferase-like (LRAT-like). The P60-like family proper encompasses most PG-degrading D,L-endopeptidases within bacteria, whereas the AcmB/LytN-like group is a divergent and likely monophyletic clade found in a subset of Gram-positive bacteria arising from rapid sequence divergence. The YaeF/Poxvirus G6R type domain is distributed across taxonomic kingdoms in bacteria, archaea, *C. elegans*, and poxviruses, whereas the LRAT-like family is also found in viruses, eukaryotes, and rarely within bacterial species. NlpC/P60 domain organization is circularly permuted in the YaeF/Poxvirus G6R-like groups and LRAT-like groups, in which the histidine-containing

β-barrel is found prior to the cysteine-containing α-helix in the primary amino acid sequence while maintaining the overall orientation of the catalytic residues.

Functions of NlpC/P60 hydrolases in bacterial growth and division

NlpC/P60 proteins have been identified and characterized in diverse bacteria, including Gram-negative, Gram-positive, and Actinobacteria taxa. These proteins are involved in numerous cellular processes, including growth, division, competition, defense, virulence, sporulation, and biofilm formation. Their expression is controlled by regulatory elements such as two-component systems, alternative sigma factors and riboswitches, and their functionality is modulated by protein-protein interactions, post-translational modifications, and proteolysis. This information is summarized in Table 1.

A fundamental role of PG hydrolases in bacterial physiology is to aid in growth and division (Figure 3). If cells did not have the ability to degrade and reassemble their own PG, they would become trapped by their own cell walls, unable to expand, elongate, or separate during binary fission. The essential roles of PG remodeling can be seen using the antibiotics complestatin or corbomycin, which inhibit PG hydrolysis by binding to PG and occluding access by hydrolases. Treatment of *Bacillus subtilis* with either molecule results in a thickening of the cell wall, and ultimately cell death.[149] Unsurprisingly, NlpC/P60-containing enzymes have been broadly implicated in bacterial proliferation across multiple genera.

In Gram-positive bacteria, the specific roles of NlpC/P60 hydrolases in growth and division have been well studied (Figure 3A). The Gram-positive model organism *B. subtilis* contains at least four active NlpC/P60 D,L-endopeptidases: CwlO, LytF, CwlS, and LytE.[59] CwlO is localized to the cylindrical part of the rod-shaped cell and is specifically involved in elongation. LytF and CwlS are localized proximal to the septum and poles of dividing cells and are implicated in cell separation. LytE is present along the cell rod, as well as the septum and poles, and this enzyme has roles in both elongation and cell separation. Double knockout of *cwlO* and *lytE* is synthetically lethal due to the necessity for D,L-endopeptidase activity within the cell rod during growth.[59,69] This essential activity is underscored by recent results showing that all other 40 annotated PG hydrolases in *B. subtilis* can be simultaneously knocked out in combination with either *cwlO* or *lytE* without affecting survival, but loss of only these two enzymes is lethal.[61] Both CwlO and LytE are thought to be recruited by homologs of eukaryotic actin. The actin homolog Mbl directs the subcellular localization of CwlO by facilitating its interaction with FtsX in the FtsEX complex, which both activates CwlO and anchors the protein to the membrane-proximal portion of the cell wall.[65] Similarly, the actin homolog MreBH recruits LytE to the periphery of the cell, where the protein is secreted and noncovalently bound to the growing sacculus in a helical pattern.[72] As the sacculus grows, the newly incorporated LytE becomes dispersed throughout both the membrane-proximal and membrane-distal portions of the cell wall. LytF has been shown to be both necessary and sufficient for complete or near-complete cell separation, with most *lytF* deficient cells found in long septate chains.[84] However, loss of *lytE*, *lytF*, and *cwlS* completely prevents separation and causes cells to chain.[85] Interestingly, cell separation can be restored in this triple mutant strain by expression of

various PG hydrolases, including non-D,L-endopeptidases and non-NlpC/P60 hydrolases, that are targeted to the septum by fusion with the PG-binding domain of LytE.[69] This shows that unlike elongation, which specifically requires D,L-endopeptidase activity, separation can be facilitated by any type of PG hydrolysis.

Several other NlpC/P60 hydrolases from Gram-positive bacteria have also been assigned specific roles in growth and division, such as *Lactobacillus plantarum* LytA (elongation and septum positioning) and LytB (septum maturation);[101] *Lactobacillus rhamnosus* Msp1 (cell separation);[102] *Clostriodiodes difficile* CwlA (cell separation);[94] and *Enterococcus faceium* SagA (cell separation).[13] The *Streptococcus pneumoniae* CHAP protein PcsB has also been implicated in cell separation.[150,151] Notably, SagA and PcsB have the same domain architecture as *B. subtilis* CwlO, containing an N-terminal coiled-coil domain and a C-terminal NlpC/P60 or CHAP domain. The coiled-coil domain has been shown to facilitate interaction with FtsX for CwlO and PcsB.[152] Although the function of the SagA coiled-coil domain remains unclear, *E. faecium* strains encode FtsEX homologs that warrant further investigation with SagA. Interestingly, SagA, like either CwlO or LytE, has also been reported to be essential.[96]

Much less is known about the specific functions of NlpC/P60 hydrolases in Gram-negative bacteria (Figure 3B). The Gram-negative model organism *Escherichia coli* contains at least four NlpC/P60 proteins: MepS (Spr), MepH (YdhO), YafL, and NlpC.[21] Knocking out all four of these genes simultaneously inhibits growth on Nutrient Agar but not on standard Luria Broth (LB) or minimal medium. Of the four NlpC/P60 proteins in *E. coli*, only MepS has been studied thoroughly. MepS is a D,D-endopeptidase that promotes cell elongation and indirectly inhibits division.[30] Simultaneous knockout of MepS along with another non-NlpC/P60 PG hydrolase, MepM, is synthetically lethal in LB but not in minimal medium, indicating that the cell's growth program in standard conditions requires these endopeptidases in a way that growth in nutrient-poor conditions does not. Differential growth under these two conditions was found to be regulated by amino acid availability; however, the amino acids that regulate each enzyme are distinct.[24]

Five NlpC/P60 proteins have been identified in the model mycobacterium *Mycobacterium tuberculosis*: RipA, RipB, RipC, RipD, and Rv0024 (Figure 3C).[153] The first four are named "resuscitation-promoting factor (rpf)-interacting proteins" due to their complexation with proteins that were originally thought to enable latent *M. tuberculosis* infections to become virulent.[131] RipA localizes to the septum of dividing cells and is involved in cell separation. *ripA* can be deleted as long as *ripB* (which is immediately downstream of *ripA* in the same operon) is present, indicating that there is some degree of functional redundancy between the two.[130] However, the phenotypes of *ripA* and *ripB* knockout strains are different. The *ripA* knockout strain produces elongated cells that have more septa than wild-type cells, whereas the *ripB* knockout strain does not produce either of these phenotypes, indicating that RipA is more influential on overall cell morphology. RipC has been implicated in growth but not cell septation. Mutation of *ripC* results in a significant decrease in growth as measured by optical density, which is partially reversed by complementation. However, the mutant cells are still capable of forming normal septa, as observed by electron microscopy. RipA and RipC both contain an N-terminal coiled-coil domain. For RipC, this domain

allows for binding to FtsX,[145] similar to the coiled-coil domains of *B. subtilis* CwlO and *S. pneumoniae* PcsB. Conversely, this domain is cleaved off of RipA during activation, so its function in protein activity or regulation remains unknown. The importance of RipD and Rv0024 in mycobacterium biology are less well studied. The NlpC/P60 domain of RipD possesses mutations in the predicted catalytic triad and has been confirmed to be inactive against PG.[147] Rv0024 lacks a canonical signal sequence,[153] so it is unclear if and how this enzyme is directed to the extracytosolic environment. Nevertheless, ectopic expression of Rv0024 in *Mycobacterium smegmatis* results in increased biofilm formation and antibiotic tolerance due to its effects on the hydrophobicity of the cell surface,[148] suggesting that the protein is exported through an unknown mechanism.

Additional roles of bacterial NlpC/P60 proteins

Competition and cellular defense ––—While PG remodeling is crucial for survival and homeostasis, the ability to cleave PG of other species presents an opportunity for niche competition. Several Gram-negative bacterial species use Type VI Secretion Systems (T6SS) to deploy NlpC/P60 hydrolases that degrade the peptidoglycan of competing bacteria (Figure 4A). For example, *Pseudomonas aeruginosa* injects the Tse1 hydrolase into its competitors' periplasm. Because this mechanism can also deliver Tse1 to other *P. aeruginosa* cells, an immunity protein called Tsi1 is employed for self-protection.[32–34] In *Salmonella enterica* subspecies Typhimurium and *Enterobacter cloacae*, the secreted hydrolase Tae4 and its immunity protein Tai4 serve a similar role, though the mechanism by which the hydrolase is inhibited by its immunity protein is distinct from that of Tse1/Tsi1.[20,42] *Vibrio cholerae* employs the secreted hydrolase TseH for competition with bacteria that occupy similar ecological niches.[47,48] The cell killing activity of TseH was inhibited by the putative *V. cholerae* immunity protein TsiH when over-expressed in *E. coli*.[47] Interestingly, *V. cholerae* with all other anti-bacterial effectors inactivated except TseH was unable to kill a Δ*tsiH V. cholerae* strain.[48] Further assays demonstrated that the WigKR envelope stress response element in *V. cholerae* can also protect against the hydrolase in the absence of TsiH, providing multiple routes to prevent T6SS-mediated cell killing. In addition to these T6SS-secreted hydrolases, the Gram-negative bacterium *Photobacterium damselae* subspecies *piscicida* has been shown to use a Type II Secretion System (T2SS) to inject the protein PnpA into the extracellular environment. PnpA does not hydrolyze *P. damselae* peptidoglycan, but it does degrade PG of two competing bacteria, *Vibrio anguillarum* and *Vibrio vulnificus*.[39] However, how PnpA accesses the cell wall of these Gram-negative organisms remains unclear and requires further investigation. These examples highlight how NlpC/P60-mediated competition can be accomplished through distinct strategies using either differential substrate specificities or effector/immunity pairs.

Virulence ––—Several NlpC/P60 hydrolases have been implicated in virulence (Figure 4B). The founding member of this protein superfamily, P60 from the Gram-positive bacterium *Listeria monocytogenes*, was originally identified as an invasion-related protein that aided in inducing phagocytosis by host cells,[109] a key step in the organism's pathogenic life cycle. This biological activity was subsequently linked to the PG hydrolase activity of P60.[111] A second protein in this organism, P45, was also shown to have a similar role and function in *L. monocytogenes* virulence.[108]

The *Bacillus cereus* and *Bacillus thurengiensis* protein CwpFM was originally identified as an enterotoxin named EntFM and was shown to be highly prevalent in outbreak-causing strains.[54,55] Subsequent analysis showed that this protein is an NlpC/P60 peptidase[52,53] that modulates cell motility, morphology, adherence to epithelial cells, biofilm formation, and macrophage vacuolization. Mortality of insect larvae after infection with *B. thurengiensis* was significantly reduced in a *cwpFM* mutant compared to wild-type.[52]

In *Corynebacterium diptheriae*, DIP1281 has been shown to be critical for adhesion and invasion into epithelial cells.[119] *dip1281* mutant cells do not separate properly and instead form chains; however, this phenotype is not related to PG hydrolysis but rather to rearrangements of the protein layer on the cell surface.[118]

The zebrafish pathogen *Mycobacterium marinum* expresses IipA and IipB, orthologs of *M. tuberculosis* RipA and RipB, respectively.[129] These proteins have been shown to be essential for bacterial invasion and intracellular persistence (*iip*) within macrophages (Figure 4C). Although zebrafish infection with wild-type *M. marinum* showed complete lethality, a mutant strain with a disrupted *iip* locus was completely tolerated. Interestingly, the authors in that study found that complementation with *M. tuberculosis* RipA (Rv1477) completely restored the lethal phenotype, while complementation with RipB (Rv1478) did not.[129] Similarly, the lethality of *M. tuberculosis* in mouse infection models was also attenuated by *ripC* mutation, and this activity was partially restored by complementation,[144] suggesting NlpC/P60 hydrolases contribute to mycobacteria virulence in different hosts.

Sporulation and germination ——Certain NlpC/P60 enzymes aid in sporulation and germination. Sporulation is a complex process in which certain bacterial species (usually Gram-positive) respond to nutrient deprivation by differentiating into a resistant endospore within a so-called mother cell. Lysis of the mother cell releases the mature spore into the environment. When nutrients improved conditions are detected, the spore undergoes germination to restore the cell to a functional state. As expected, these processes are known to require extensive PG remodeling.[154–158] For example, the *Streptomyces coelicolor* NlpC/P60 protein SwlA has been shown to be involved in both sporulation and germination. A *swlA* mutant produces defective spores that have abnormal morphologies and are hypersentisive to heat.[116] Importantly, single mutations of three other, non-NlpC/P60 hydrolase genes (*rpfA*, *swlB*, and *swlC*) also confer these phenotypes, suggesting that SwlA is just one of several enzymes responsible for proper peptidoglycan remodeling during spore formation in this organism. Spores from the *swlA* and *rpfA* mutants are also slower to germinate than wild-type spores, although they are capable of full resuscitation.[116]

The *Bacillus anthracis* protein BAS1812 was originally identified as BA1952 from a reverse-vaccinology screen of seropositive antigens[51] and was later shown to have activity in sporulation and germination. Identification and characterization of proteins related to *B. anthracis* sporulation are particularly important, as the spores of this organism are the cause of anthrax disease. Like mutation of *swlA* in *S. coelicolor*, deletion of *BAS1812* in *B. anthracis* resulted in impaired endospore formation and delayed germination.[49] Both of these phenotypes were partially reversed by complementation with a second copy of *BAS1812*. However, the *BAS1812* deletion also conferred detergent sensitivity that the *S. coelicolor*

swlA mutant did not exhibit. BAS1812 is nearly identical to the previously discussed CwpFM protein of the spore-forming organism *B. cereus*, suggesting that this protein may play a sporulation/germination role in that organism as well. In *Bacillus sphaericus*, the protein DPP VI has been identified as an NlpC/P60 hydrolase and is highly upregulated during sporulation.[57] However, the direct roles of CwpFM and DPP VI in endospore formation have not been demonstrated, indicating the need for further examination of NlpC/P60 proteins in these processes.

The mechanisms underlying the sporulation and germination-related phenotypes of the mutants listed above are not known and will require further study to elucidate. However, there is one sporulation-related NlpC/P60 enzyme in *B. subtilis,* the transglutaminase Tgl, that has been studied mechanistically. Instead of acting as a PG hydrolase, Tgl covalently crosslinks proteins that make up the spore coat.[91–93] Thus, this protein is one of several NlpC/P60 proteins that serve non-hydrolytic functions.

Other non-PG hydrolytic functions ––In some cases, NlpC/P60-containing proteins have evolved to perform additional cellular functions that are unrelated to PG hydrolysis. Some examples are the *P. aeruginosa* MagC protein, which serves to anchor the Mag complex to PG, but has not been reported to hydrolyze PG.[31] Additionally, the *Staphylococcus aureus* protein LnsA has been suggested to mediate acylation of lipoproteins[113] and the *B. subtilis* protein PgdS has been shown to degrade poly-γ-glutamic acid.[88,89]

Regulation of bacterial NlpC/P60 proteins

In general, PG hydrolysis is a highly regulated process.[4,5] Overactive PG hydrolysis can compromise the integrity of the cell wall and leave the cell vulnerable to physical stresses, whereas hypoactive PG hydrolysis can prevent cell growth and division. Moreover, the proper amount of PG hydrolysis changes over the lifetime of a cell based on factors such as nutrient availability, temperature, pH, and antibiotic stress. Cells must be able to respond to these types of environmental cues in order to ensure that the balance between peptidoglycan synthesis and hydrolysis is maintained at all times.

The mechanisms by which NlpC/P60 hydrolases are regulated are diverse (Figure 5). At the transcriptional level, two-component systems and alternative sigma factors play key roles. For example, the *B. subtilis* WalRK two-component system (previously known as YycFG) controls expression of *cwlO* and *lytE* (Figure 5A).[62] The sensor histidine kinase WalK senses the cleavage products produced by CwlO and LytE[67] and relays this information to the response regulator WalR, which in turn binds to the *cwlO* and *lytE* promoters and modulates their expression. When the cleavage products are below the proper concentration, WalRK upregulates transcription of *cwlO* and *lytE* and vice-versa. The WalRK regulon also includes *iseA*, which encodes an inhibitor of CwlO and LytE.[159] When the cleavage product concentration is too high, *iseA* transcription is upregulated and vice-versa. Another two-component system that regulates an NlpC/P60 enzyme is the PhoPQ system in *S.* Typhimurium. This system regulates many genes involved in virulence [160] including the *ecgA*, which encodes an NlpC/P60 family member.[40] Here, the sensor histidine kinase

PhoQ senses various extracellular signals, such as pH, osmolarity, and magnesium ion concentration and relays the signal to the response regulator PhoP, which then modulates expression of *ecgA*. In addition to transcription factors like WalR and PhoP, the use of alternative sigma factors allows a cell to selectively transcribe certain genes based on the cell's progression through the cell cycle. In *B. subtilis*, *lytE* is trancsribed by RNA polymerase with σI,[77] while *lytF* is transcribed by RNA polymerase with σD.[81] At least one NlpC/P60 hydrolase is regulated at the post-transcriptional level as well (Figure 5B). The RNA transcript of *cwlO* is extremely unstable,[63] and CwlO protein also has a very short half-life (~5 min),[60] which allows for tighter control of CwlO regulation through the need for constant regeneration of *cwlO* mRNA. Bioinformatic analysis has predicted that some NlpC/P60 enzymes may be regulated by riboswitches;[161] however, experiments will be needed to confirm this and to determine the mechanism of action.

The timing and amount of NlpC/P60 hydrolase production are key to their ability to perform their functions properly. This complex form of transcriptional and translational regulation is highlighted by work showing that the synthetic lethality of *cwlO* and *lytE* in *B. subtilis*[64] can be overcome by *lytF* expressed from the *cwlO* promoter (P*cwlO-lytF*).[68] However, the P*cwlO-cwlS* construct is unable to rescue the *cwlO* and *lytE* double knockout strain, despite CwlS being very similar to LytF.[68] Although the exact molecular mechanism underlying this partial redundancy between NlpC/P60 proteins is unknown, this example underscores the importance of examining both protein activity and regulation to determine their roles in bacterial physiology.

NlpC/P60 enzymes are also regulated at the post-translational level (Figure 5C). Protein-protein interactions such as those described above for hydrolases and actin homologs, FtsEX, inhibitors like the immunity proteins for T6SS-secreted hydrolases or IseA, and putative tuberculoidal resuscitation factors, constitute one mechanism of post-translational regulation. A second mechanism is post-translational modification. For example, *C. difficile* CwlA is differentially localized based on whether it is phosphorylated. Unphosphorylated CwlA is exported to the extracytosolic environment where it can hydrolyze PG, whereas phosphorylated CwlA is retained in the cytoplasm and is thus unable to hydrolyze PG.[94] The *L. rhamnosus GG* protein Msp1 undergoes O-glycosylation at two serine residues. This modification allows for proper localization of the protein and confers protection against proteases, though it does not directly affect hydrolase activity.[103] Two hydrolases in *M. tuberculosis*, RipA and RipB, are post-translationally modified by cleavage of a pro-domain. Only after this cleavage do the NlpC/P60 domains of these enzymes become active. Finally, some NlpC/P60 hydrolases are degraded through regulated proteolysis. *E. coli* MepS, for example, is degraded by the NlpI/Prc system,[22] while *P. aeruginosa* PA1198 is degraded by the LbcA/CtpA system.[35–38]

Functions of bacterial NlpC/P60 domains in eukaryotic biology

NlpC/P60 enzymes can also play a more complex role in communication across kingdoms through the generation of immune active molecules (Figure 6). The hydrolysis of PG via NlpC/P60 domains yields small muramyl peptides.[162] These fragments can in turn be sensed by eukaryotic pattern recognition receptors to elicit nonspecific immune responses.[163]

This central pillar of innate immunity allows animals to rapidly respond to a variety of microbial threats. PG fragments are detected by the nucleotide-binding domain, leucine-rich containing (NLR) proteins, NOD1 and NOD2.[164–166] NOD1 recognizes fragments containing the dipeptide isoglutamate-meso-DAP (iE-DAP), which is present primarily in Gram-negative bacteria. Conversely, NOD2 binds to fragments containing the glycopeptide MurNAc-alanine-isoglutamate/isoglutamine or muramyl dipeptide (MDP). This structure is present in both Gram-negative and Gram-positive bacteria and can be selectively generated from an intact stem peptide through the D,L-endopeptidase activity of NlpC/P60 enzymes. Therefore, NlpC/P60 proteins may uniquely be able to activate host immunity via their PG remodeling capacity.

The activity of probiotic bacteria may be mediated by NlpC/P60 catalytic activity. Species of *Lactobacillus* including *L. rhamnosus* and *L. casei* express and secrete two proteins (p40 and p75) that contain CHAP and NlpC/P60 domains, respectively (Figure 6A).[99,167] Both of these proteins possess hydrolytic activity against PG, and the p75 protein is specific for the amide bond between isoglutamine and lysine as expected for other NlpC/P60 D,L-endopeptidases.[102] Interestingly, the presence of these two hydrolytic enzymes also had significant effects on intestinal epithelial cell (IEC) biology. Treatment of IECs with conditioned medium or purified proteins activated survival signaling via Akt, inhibited cytokine-induced apoptosis, and improved cell proliferation. Nevertheless, it remains unknown whether the probiotic effects of these enzymes were due to direct generation of immune active muramyl peptides or other mechanisms, such as their ability to bind to mammalian extracellular matrix proteins.

PG remodeling by NlpC/P60s may be an adaptive advantage used by infectious bacteria. For example, *Salmonella enterica* serovar Typhimurium selectively expresses the NlpC/P60 protein EcgA in nutrient-limiting conditions such as intracellular vesicles during infection of mammalian cells (Figure 6B).[40,41] In biochemical assays, EcgA was shown to have specialized D,L-endopeptidase activity only on uncrosslinked PG fragments. The exact function of the specific modification remains unknown; however, in vivo experiments revealed that a Δ*ecgA* mutant strain showed a significant competitive disadvantage using an infection model with intraperitoneal injection of *Salmonella* but not with oral infection. Together, these data suggest that NlpC/P60 enzymes may play a role in pathogen colonization of and dissemination within the host.

Beyond its bacteria-intrinsic activities, the *E. faecium* enzyme SagA has profound effects on host physiology (Figure 6C).[97,168] Colonization of both *C. elegans* and mice with SagA-expressing *E. faecium* led to protection against infection by *S.* Typhimurium. The pro-survival activity of this NlpC/P60 enzyme was confirmed by direct administration of the enzyme itself, as well as colonization with other, nonprotective species, *E. faecalis* and *L. plantarum*, that had been engineered to stably express SagA. Using a mutant variant of SagA where its putative catalytic residues were converted to alanine, catalytically dead SagA was no longer able to rescue worms from *Salmonella* pathogenesis. In mice, colonization with SagA-expressing bacteria showed an increase in the expression of the barrier protein mucin 2 as well as antimicrobial peptides cryptdin 2 and REGIIIγ. Survival upon *Salmonella* challenge required the host PG receptor NOD2 as well as epithelial cell-specific MYD88, a

signaling adaptor for Toll-like receptors. Finally, the pro-survival activity of SagA was found to protect against *Clostridium difficile* pathogenesis, highlighting the broad host effects of SagA against enteric infections.

The genus *Enterococcus* has also been linked to a variety of systemic immune disease phenotypes, including increased susceptibility to graft-versus-host disease[169] and increased efficacy of cancer chemo- and immunotherapies.[170,171] In addition to *E. faecium*, other species such as *E. durans*, *E. hirae*, and *E. mundtii* were recovered from cancer patients who responded to anti-PD-1 immune checkpoint inhibitor therapy.[172,173] Monocolonization with these bacteria using a murine model of melanoma confirmed the causal link between *Enterococcus* and immunotherapy response.[98] Bioinformatic and biochemical analyses revealed that the active *Enterococcus* species expressed and secreted orthologs of *E. faecium* SagA. In vitro enzyme assays showed that the SagA orthologs all possessed D,L-endopeptidase activity with no discernable differences in substrate specificity. Importantly, colonization with *L. lactis* strains engineered to express wild-type or catalytically dead SagA demonstrated that the PG remodeling activity of SagA was required for anti-tumor activity. Responsiveness to immunotherapy was lost in animals lacking the NOD2 receptor, indicating that PG sensing was necessary for improved drug efficacy. Finally, colonization with SagA-expressing bacteria led to increased immune cell infiltration into tumors, with higher levels of effector and tumor antigen-specific CD8 T cells, providing a cellular basis for the observed anti-tumor activity of SagA.

More broadly, PG remodeling by other NlpC/p60 enzymes from gut microbiota with D,L-endopeptidase activity may function as a general mechanism to modulate host immunity. Recent evidence has found that overall D,L-endopeptidase activity in fecal samples was anti-correlated with Crohn's disease in humans and led to predisposition to colitis in mice upon fecal microbiota transplantation.[105] Oral teatment of animals with either the purified NlpC/p60 protein UC118 or the UC118-expressing bacterium *Lactobacillus salivarius* ameliorated DSS-induced colitis. However, these beneficial effects were not restored in mice treated with a catalytically dead mutant of UC118, suggesting that the phenotype was driven by the catalytic activity of UC118. Screening of almost 50 commercially available bacterial species found that only a subset were able to strongly activate NOD2, and importantly, all NOD2-activating isolates expressed D,L-endopeptidases. Thus, NlpC/p60 enzymes with this specific activity may serve as both a prognostic biomarker and therapeutic treatment for colitis in NOD2-sufficient patients.

Functions of eukaryotic NlpC/P60 domains

Although NlpC/P60 domains are most frequently found in prokaryotes, rare instances of NlpC/P60 proteins are found within Eukarya.[7] Their evolutionary origin(s) remains unclear; however, it has been suggested that some of these enzymes were acquired by horizontal gene transfer from bacteria. For example, the human parasite responsible for trichomoniasis, *Trichomonas vaginalis*, possesses at least two NlpC/P60 domain-containing proteins (TVAG_457240 and TVAG_209010) with hydrolytic activity against bacterial PG (Figure 7A).[174] Like bacterial enzymes, the proteins are exported to the cell surface of the pathogen, and their catalytic activity required the active site cysteine residue.

Interestingly, the NlpC/P60 enzymes were active against intact *E. coli* PG, and co-incubation of the pathogen over-expressing one NlpC/P60 enzyme with *E. coli* led to bacterial lysis. Since NlpC/P60 enzymes do not typically penetrate the outer membrane of Gram-negative bacteria, further investigation of how these *Trichomonas vaginalis* NlpC/P60 domain-containing proteins gain access to the PG layer will be important. Expression levels of the two enzymes was also responsive to environmental conditions. If these genes were acquired by horizontal gene transfer, then their genomic incorporation was followed by the evolution of functional secretion signals and regulated expression to best utilize the adaptive advantage provided by NlpC/p60 activity during mucosal colonization.

Another potential instance of horizontal gene transfer occurred in the evolutionary ancestors of the house dust mite, genus *Dermatophagoides*. First discovered in the clinically relevant species *D. pteronyssinus* and *D. farinae*, multiple species of dust mite express and secrete a 14 kDa protein that shows homology to bacterial NlpC/P60 enzymes (Figure 7B).[175] Termed *lytFM*, this protein is capable of lysing Gram-positive bacteria, and further biochemical characterization by zymography indicated that the protein is capable of hydrolyzing PG. The enzyme is expressed under the control of a bacterial promoter, suggesting that *lytFM* may derive from a prokaryotic endosymbiont. The presence of highly similar NlpC/P60-containing proteins in dust mite-associated *Bacillus* and *Staphylococcus* species further support the endosymbiont gene transfer hypothesis.[176] Although the bacteriocidal activity of *lytFM* may help the dust mite to maintain its associated microbiota, the biological importance of this eukaryotic NlpC/P60 protein has not been fully elucidated.

Other eukaryotic NlpC/P60 proteins are often members of the LRAT-like group (Figure 7C). LRAT-like NlpC/P60 domains do not hydrolyze PG; instead, they often act as phospholipases/acyltransferases (PLA/ATs).[177] For example, the mammalian protein and founding member of this group, lecithin retinol acyltransferase (LRAT), catalyzes the esterification of Vitamin A (all-*trans*-retinol) via acyl transfer from phosphatidylcholine, which enables retention of Vitamin A in peripheral tissues.[178] Mammals also express H-REV107, which functions as a tumor suppressor through negative regulation of the proto-oncogene H-RAS.[179] Similar to LRAT, this protein acts as a PLA/AT using phopsholipid substrates. H-REV107 inhibits H-RAS palmitoylation and its downstream signaling, which can be reversed via chemical inhibition of H-REV107 acyltransferase activity. However, whether H-REV107 directly modifies H-RAS or an associated palmitoyl transferase to mediate this activity remains unclear. LRAT-like enzymes are also found in lower organisms, including the protein EGL-26 from the nematode *Caenorhabditis elegans*.[180] Although the molecular function of EGL-26 catalysis is unknown, loss of this protein or mutagenesis of its putative active site residues led to atypical vulval cell morphogensis and decreased egg laying, highlighting the broad biological functions of eukaryotic LRAT-like NlpC/p60 proteins.

Outlook and future directions

The effects of microbial biology on their resident hosts will remain a key concern for human health in the foreseeable future. The battlefronts of host-microbial interactions are quite diverse, from the ongoing emergence of antibiotic-resistant microbial strains[181] to the

intimate roles of microbes on immune development and homeostasis.[182] Nevertheless, these myriad effects are often difficult to target due to a lack of information regarding exact molecular connections between microbial activity and host outcomes. The biosynthesis, remodeling, and degradation of PG is an essential function of virtually all bacteria, and these crucial and bacterial-specific processes have been targeted throughout the history of antibiotic development from its birth with penicillin-based beta lactams to today's glycopeptide derivatives such as vancomycin and corbomycin.[183] Nevertheless, much remains unknown about the molecular intricacies of PG turnover, including the roles of many seemingly redundant remodeling proteins.

As summarized in this review, NlpC/P60 enzymes are widespread components of the PG remodeling machinery that are often essential for the survival and growth of many bacterial species. Thus, we propose that these near ubiquitous cysteine proteases may be a functional target for next-generation antibiotics. Importantly, the development of enzyme-specific inhibitors may allow for precise control of individual species and strains within complex communities, avoiding broad changes to microbiota communities often associated with wide-spectrum antibiotics. Moreover, the presence of catalytic cysteine residues within the active sites of these enzymes opens the door for the development of covalent-based inhibitors, which have recently seen widespread success in the targeting of mammalian proteins.[184] Alternatively, compounds that activate endogenous NlpC/P60 activity may serve as bacterial lysis agents.

Finally, the intrinsic endopeptidase activity and substrate specificity of NlpC/P60 enzymes may also offer avenues to direct host immune modulation. The ability of NlpC/P60 proteins like SagA to generate muropeptides may be exploited to activate innate immune pathways using engineered probiotics.[98] Rather than relying on the black box of existing, poorly defined strains, the development of live biotherapeutic products with known enzymatic activity and small molecule output may be a feasible strategy for next-generation anti-infectives and immunotherapies. Together, these studies and potential applications highlight the necessity for a better molecular-level understanding of host-microbial interactions through the further study of PG remodeling NlpC/P60 enzymes.

SIGNIFICANCE

Microbes residing upon or within larger organisms can be beneficial or antagonistic for host biology. Understanding the molecular determinants of these interactions may provide new targets to control microbial activity and their effects on the host. This review summarizes how the broad family of NlpC/P60 enzymes play critical roles in bacterial cell-intrinsic and -extrinsic phenomena. Multiple studies have found that the ability to degrade peptidoglycan by NlpC/P60 proteins is essential for bacterial survival, growth, and division. The generation of fragmented peptidoglycan metabolites by these enzymes can also initiate host immune signaling to improve intestinal barrier function and elicit adaptive immune responses. Therefore, a better fundamental understanding of NlpC/P60 structure, activity, and function may provide targets to control prokaryotic growth via next-generation anti-infectives and new routes to improve host immunity through defined, biotherapeutic adjuvants.

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Highlights

NlpC/P60 hydrolases degrade bacterial peptidoglycan and generate muropeptides.

These enzymes are important for proper bacterial growth and cell division.

NlpC/P60 proteins influence bacterial-host interactions including immune stimulation.

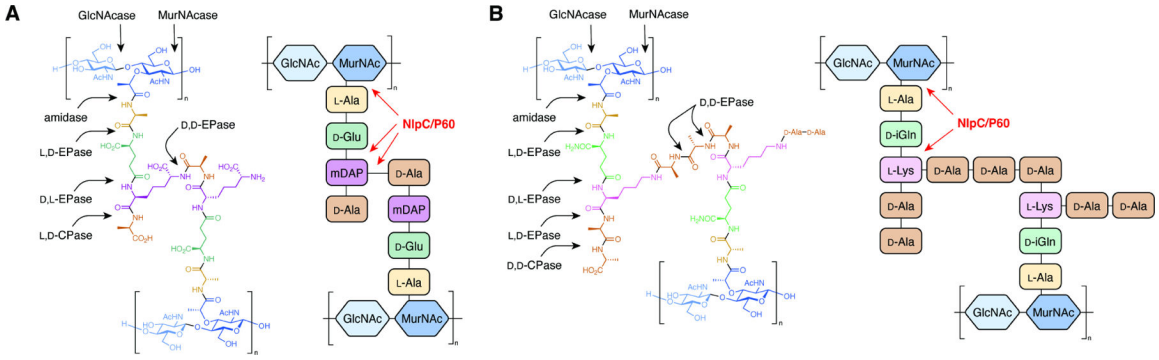

Figure 1. Peptidoglycan structure and remodeling.

Generalized chemical structure of a crosslinked peptidoglycan monomer with the potential bonds cleaved by different classes of peptidoglycan remodeling enzymes (arrows) for (A) Gram-negative *E. coli* and (B) Gram-positive *E. faecalis*.

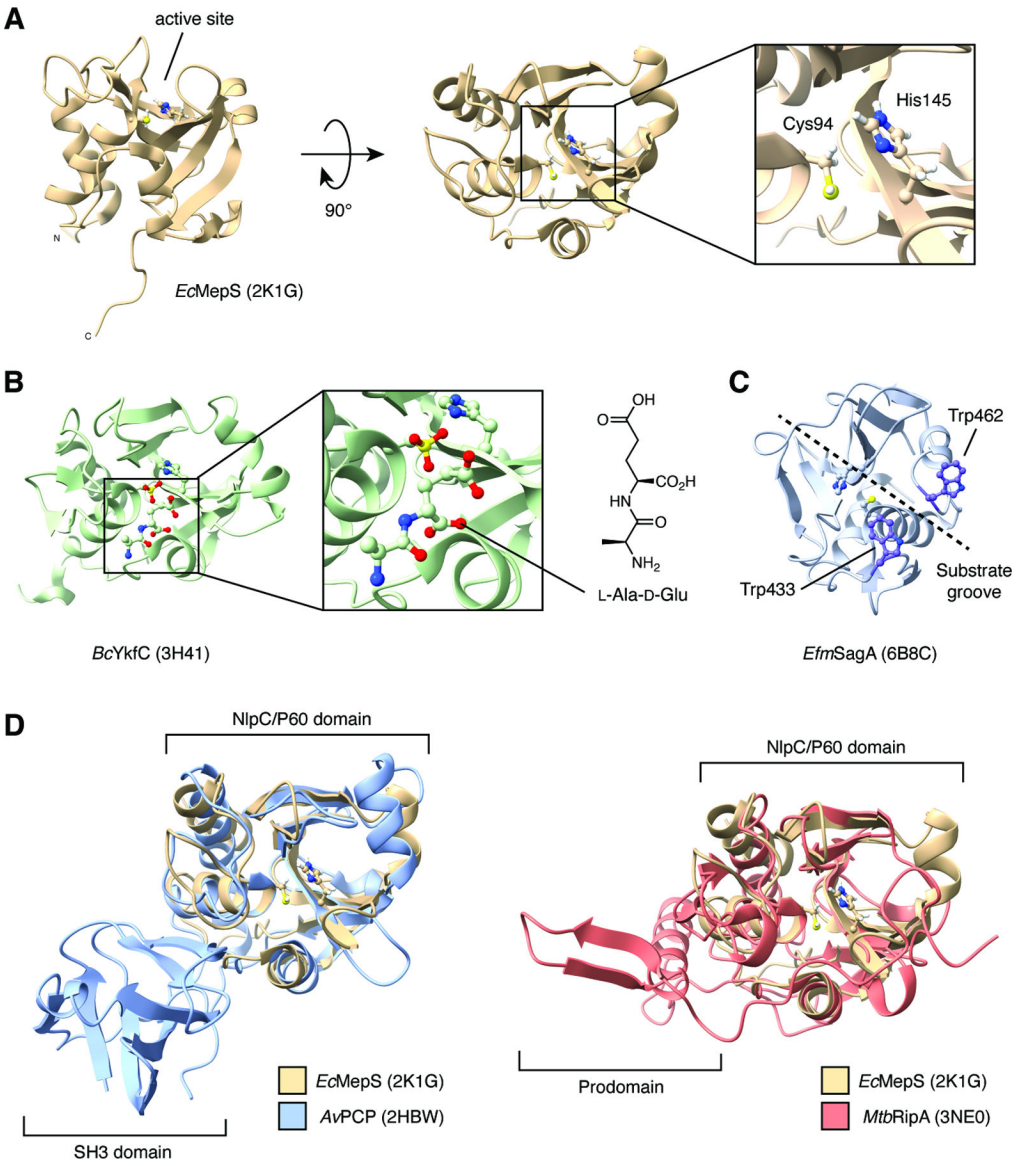

Figure 2. Biochemical structures of NlpC/P60 proteins.

(A) NMR structure and catalytic dyad of *E. coli* MepS (Spr).

(B) Crystal structure of *B. cereus* YkfC with dipeptide product of peptidoglycan hydrolysis bound to the active site.

(C) Crystal structure of *E. faecium* SagA with catalytic dyad and putative substrate-binding Trp residues highlighted.

(D) Overlay of *E. coli* MepS and *A. variabilis* AvPCP and *M. tuberculosis* RipA showing additional regulatory domains.

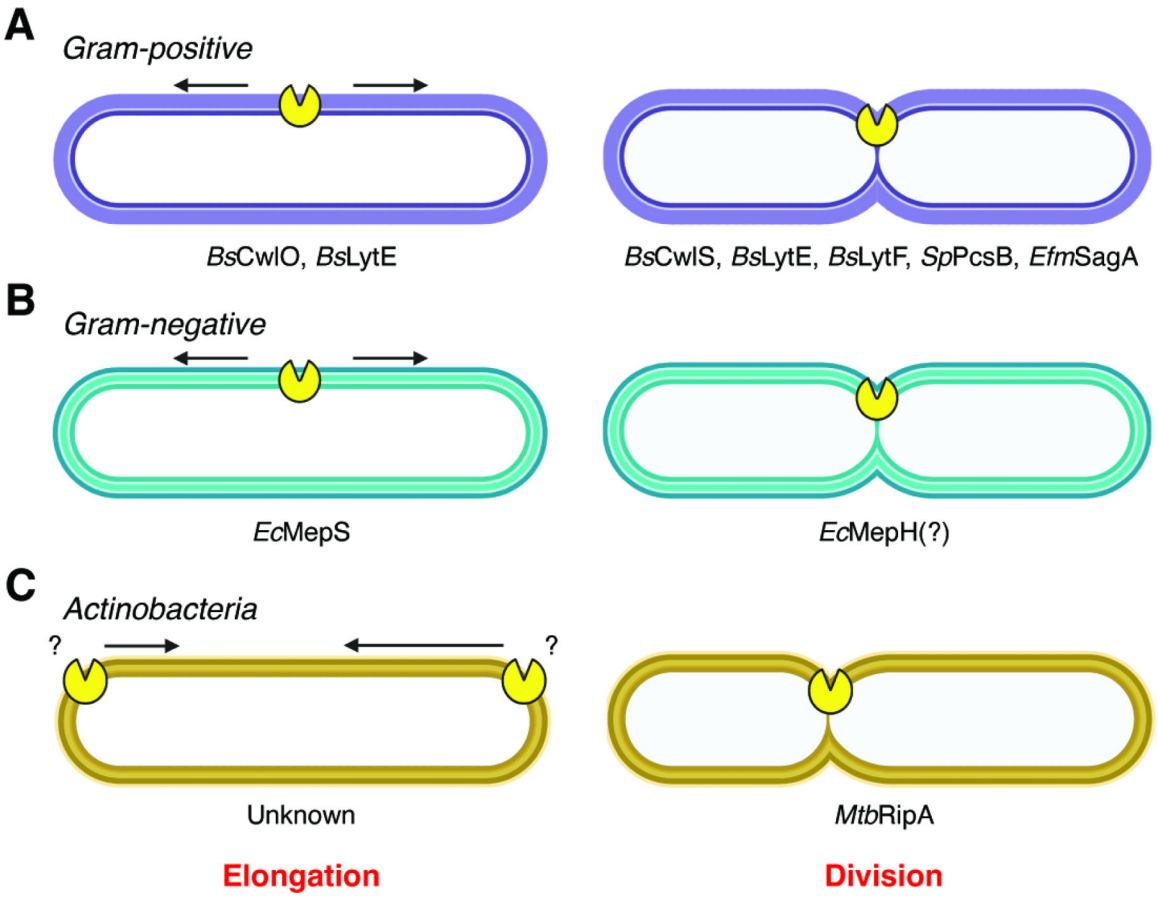

Figure 3. Roles of NlpC/P60 proteins in growth and division.

Characterized NlpC/P60 proteins involved in the elongation and division of (A) Gram-positive bacteria, (B) Gram-negative bacteria, and (C) Actinobacteria.

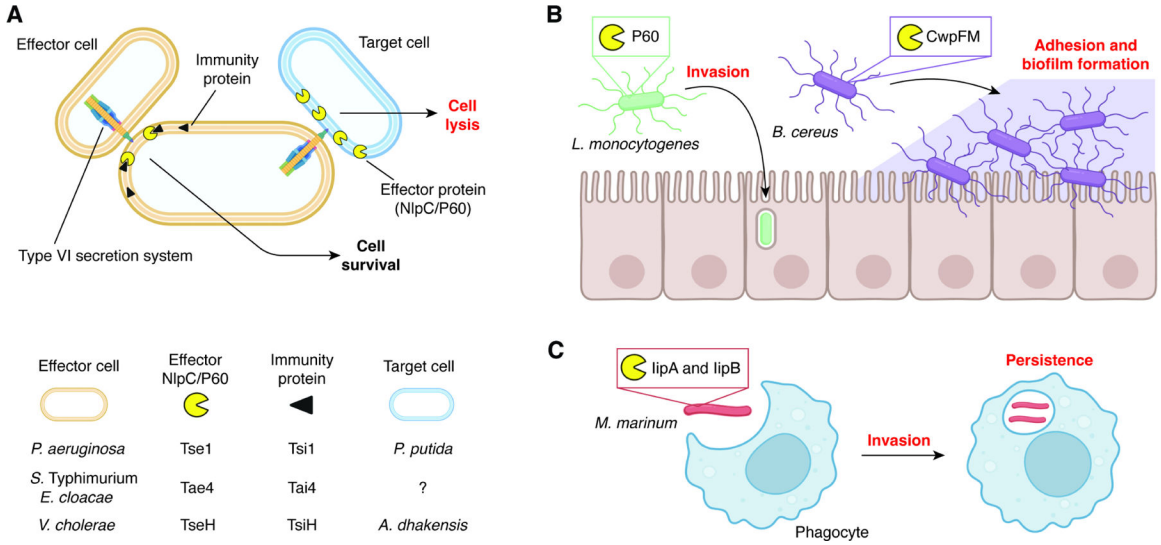

Figure 4. Functions of prokaryotic NlpC/P60 proteins in survival and infection.

(A) Multiple bacterial species use NlpC/P60 proteins as secretion system effectors to induce cell lysis of other competing microbes.

(B) Expression of bacterial NlpC/P60s enable host colonization at and within cells at the epithelial interface in the gut.

(C) NlpC/P60s produced by mycobacteria improve both invasion and persistence during infection in phagocytes.

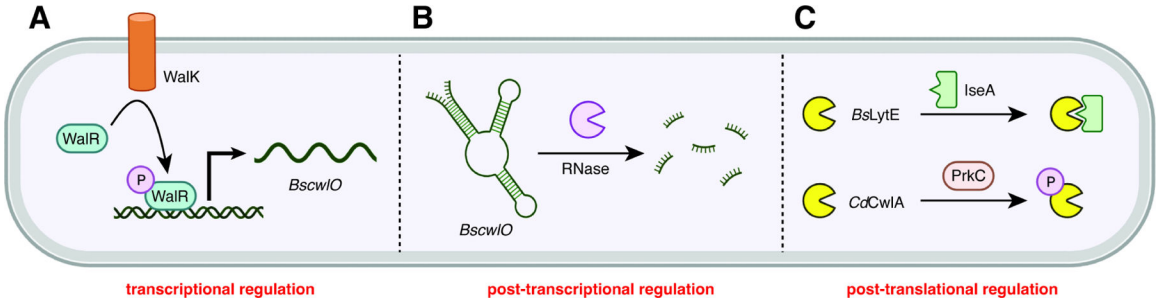

Figure 5. Diverse mechanisms of NlpC/P60 regulation in prokaryotes.

(A) The two-component WalRK signal transduction system in *B. subtilis* controls expression of the NlpC/P60 *cwlO* gene.

(B) The *cwlO* leader sequence forms a secondary structure that is cleaved by RNase Y, leading to degradation of transcript.

(C) Post-translational regulation can occur through protein-protein interactions including immunity proteins and posttranslational modifications like phosphorylation.

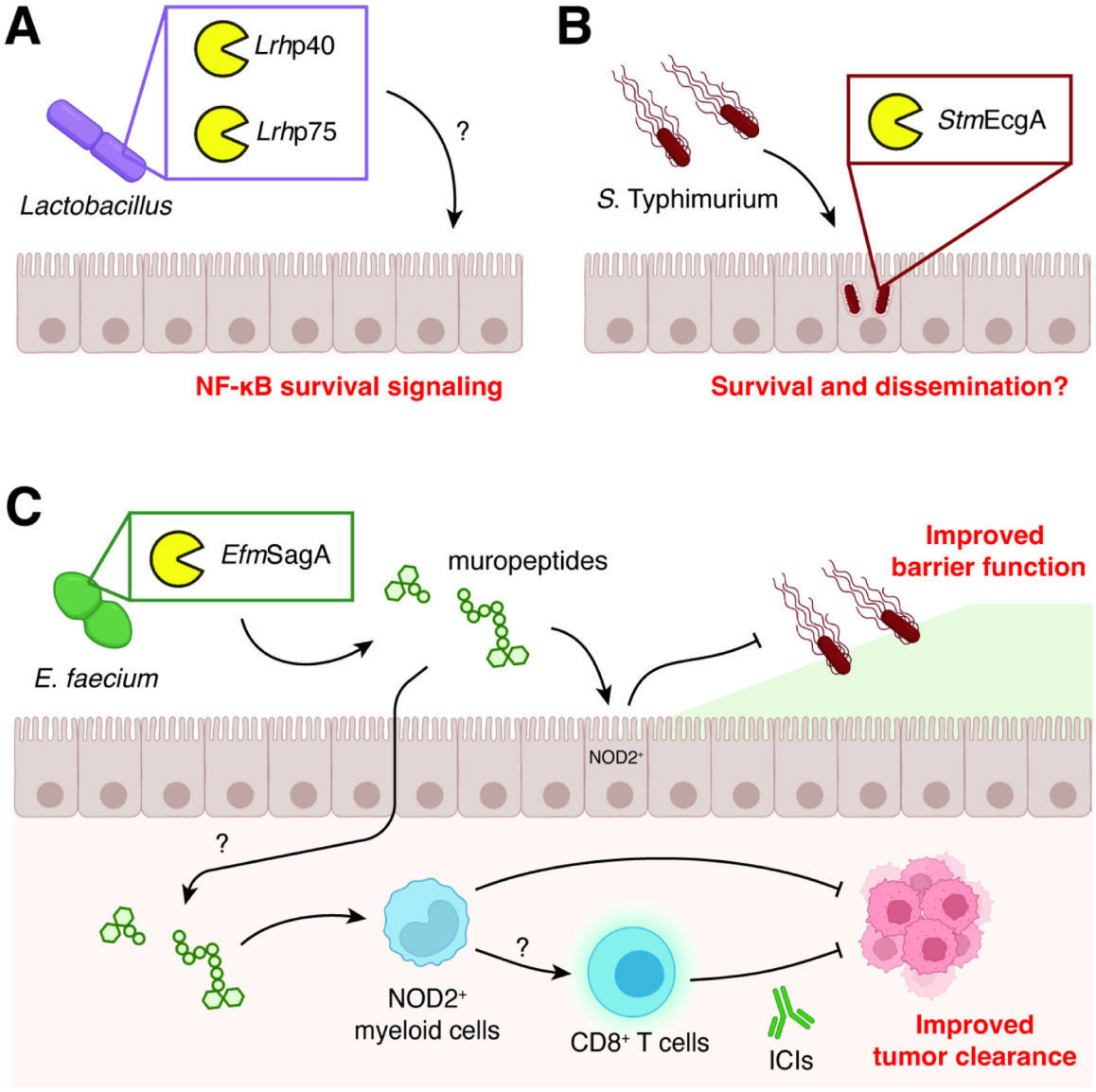

Figure 6. Functions of prokaryotic NlpC/P60 proteins in host-microbial interactions.

(A) *Lactobacillus* species produce secreted PG degrading enzymes that induce host immune signaling through NF-κB.

(B) *S.* Typhimurium selectively expresses EcgA while residing in intracellular vesicles, which increases overall survival and dissemination.

(C) *Enterococcus* species like *E. faecium* secrete SagA, which can stimulate NOD2-dependent responses in the host during infection and cancer immunotherapy through the production of muropeptides.

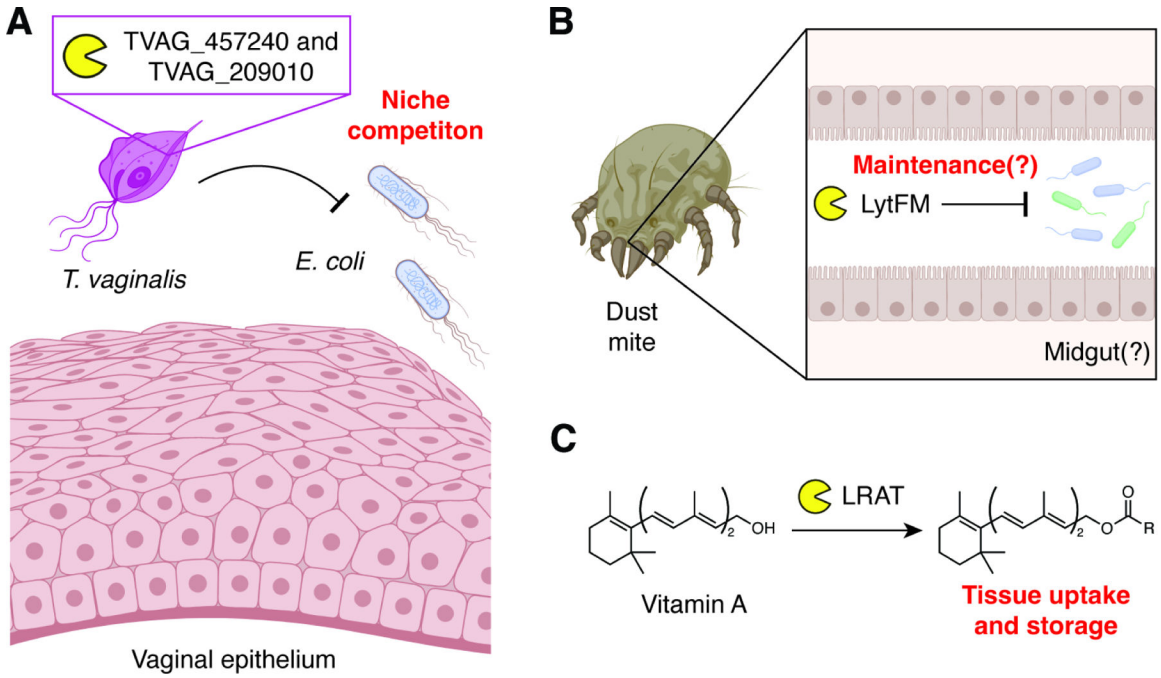

Figure 7. Functions of eukaryotic NlpC/P60s

(A) The protozoan *T. vaginalis* expresses two NlpC/P60s that may improve niche competition at the vaginal epithelium.

(B) Dust mites express LytFM, which may be involved in maintenance of its midgut microbiota.

(C) Mammalian LRAT-like NlpC/P60s like LRAT itself exhibit acyltransferase activities on non-peptidoglycan substrates.

Table 1:

Summary of bacterial NlpC/P60 proteins

Summary of bacterial NlpC/P60 proteins that have been biochemically or biologically characterized beyond sequence homology.

Organism	NlpC/P60 Protein	Specific Function(s)*	Notes	Reference(s)
Gram-Negative Bacteria				
Anabaena variabilis	AvPCP		Structure determined (PDB 2HBW)	15
Bacteriodes ovatus	YkfC		Structure determined (PDB 3NPF)	19
Bacteriodes thetaiotaomicron	YkfC		Structure determined (PDB 3PVQ & 4R0K)	19
Bacteriodes uniformis	AmiA		Structure determined (PDB 4H4J [AmiA alone], 4Q68 [AmiA/GlcNAc], and 4Q5K [AmiA/GlcNAc-AnhMurNAc])	8
Desulfovibrio vulgaris	DvLysin		Structure determined (PDB 3M1U)	19
Enterobacter cloacae	Tae4	Cellular defense	Secreted by a T6SS; self-protection is accomplished by the immunity protein Tai4	20
Escherechia coli	NlpC			21
	MepS (Spr)	Virulence	DD-endopeptidase; regulated by proteolysis (degraded by NlpI/Prc system); synthetic lethality with MepM (a non-NlpC/P60 hydrolase) in rich media but not minimal; contributes to kidney infections; deletion confers sensitivity to EDTA; structure determined (PDB 2K1G)	22 21 9 23 24 25 26 27 28 29 30
	MepH (YdhO)		Potentially regulated by nitric oxide	21 24 26 28
	YafL			21
Nostoc punctiforme	NpPCP		Structure determined (PDB 2EVR & 2FG0)	15
Pseudomonas aeruginosa	MagC	PG binding	Anchors Mag complex to PG; NlpC/P60 domain is inactive	31
	Tse1	Cellular defense	Secreted by a T6SS; self-protection is accomplished by the immunity protein Tsi1; structures determined for Tse1 (PDB 3VPI) and Tse1/Tsi1 complex (PDB 3VPJ)	32 33 34
	PA1198		Homologous to *E. coli* MepS (a DD-endopeptidase); regulated by proteolysis (degraded by LbcA/CtpA system)	35 36 37 38
	PA1199		Also homologous to E. coli MepS; C-terminal end plays a role in degradation	
Photobacterium damselae subspecies *piscicida*	PnpA	Cellular defense	Secreted by a T2SS; Is specific for peptidoglycan of competing bacteria, does not degrade *P. damselae* peptidoglycan	39
Salmonella enterica serovar Typhimurium	EcgA	Virulence	Regulated by PhoP; induced in non-growing cells	40 41

Organism	NlpC/P60 Protein	Specific Function(s)*	Notes	Reference(s)
	Tae4	Cellular defense	Secreted by a T6SS; self-protection is accomplished by the immunity protein Tai4	42 20
	MepS		Regulated by ScwA	43 44
	MepH		Regulated by ScwA	43 44
	NlpC		Regulated by ScwA	43 44
Thermus thermophilus	P60_tth		Structure determined (PDB 4XCM, 4UZ2, and 4UZ3)	45
Vibrio cholerae	NlpC			46
	TseH	Cellular defense	Structure determined (PDB 6V98)	47 48
Gram-Positive Bacteria				
Bacillus anthracis	BAS1812 (BA1952)	Sporulation	Seropositive/immunogenic; contains three SH3b domains; mutant has impared endospore formation and germination, and increased susceptibility to detergent; nearly identical to *B. cereus* CwpFM	49 50 51
Bacillus cereus	CwpFM (EntFM)	Adhesion, biofilm formation, and virulence		52 53 54 55
	YkfC		Structure determined (PDB 3H41)	10
	BcPPNE			56
Bacillus sphaericus	DPP VI	Sporulation	Upregulated during sporulation, but no direct evidence of participation	57 15
Bacillus subtilis	CwlO (YvcE)	Cell elongation	Synthetic lethality with LytE; regulated by WalRK; localizes to lateral cell wall; highly unstable transcript; binds to and depends on FtsEX	58 59 60 61 62 63 64 65 66 67 62 68 69 70 71
	LytE (CwlF)	Cell elongation and cell separation	Synthetic lethality with CwlO; regulated by WalRK; localizes to poles, septa, and lateral cell wall; upregulated when *cwlO* is deleted	59 60 61 72 73 74 75 76 62 64 67 62 77 78

Organism	NlpC/P60 Protein	Specific Function(s)*	Notes	Reference(s)
				68 69 65 71
	LytF (CwlE)	Cell separation	Non-essential; localizes to septa and poles; can be used to overcome the synthetic lethality of LytE/CwlO if expressed from the *cwlO* promoter; regulated by sigma factor D.	59 61 75 79 80 81 82 83 76 68 69 84
	CwlS (YojL)	Cell separaton	Non-essential; localizes to septa and poles; cannot be used to overcome the synthetic lethality of LytE/CwlO even if expressed from the *cwlO* promoter	59 61 85 68 16 69
	YkfC		Non-essential	61
	CwlT (YddH)		Bifunctional (has an NlpC/P60 domain as well as a lysozyme domain); part of an integrative and conjugative element (ICE)	86 87
	PgdS	Poly-γ-glutamic acid degradation	Does not degrade peptidoglycan	88 89 61 90
	Tgl	Sporulation	Acts by crosslinking proteins to the spore coat	91 92 93
Clostridiodes difficile	CwlA	Cell separation	Phosphorylation dependent	94
	CwlT			11
	Cwl0971	Virulence, sporulation		95
	Acd24020		Structure determined (PDB 7CFL)	14
Enterococcus faecium	SagA	Septum formation and host immune modulation	Essential; localizes to septum; generates muropeptides that enhance host immunity to other bacteria (PDB 6B8C)	96 13 12 97 98
Lactobacillus casei	P40			99 100
	P75			99 100
Lactobacillus paracasei	P40			100
	P75			100
Lactobacillus plantarum	LytA	Cell elongation		101
	LytB	Septum maturation		101

Organism	NlpC/P60 Protein	Specific Function(s)*	Notes	Reference(s)
	LytC			101
	LytD			101
Lactobacillus rhamnosus GG	Msp1 (P75, LGG_00324)	Cell separation	Accumulates at septum; is O-glycosylated	102 103
	LGG_02016		Strongly upregulated in acidic conditions	104
Lactobacillus salivarius	UC118	Host immune modulation		105
Lactococcus lactis	PgpA (YjgB)		Non-essential, mutants have no phenotype	106
Listeria monocytogenes	P45 (Spl)			107 108
	P60	Motility and virulence		107 109 110 111
Nocardia seriolae	NlpC/P60	Virulence	Induces apoptosis	112
Staphylococcus aureus	CwlT		Structure determined (PDB 4FDY)	11
	LnsA	Lipoprotein acylation		113
Streptococcus pneumoniae phage Dp-1	Pal	Phage virulence		114 115
Streptomyces coelicolor	SwlA	Sporulation		116 117
Actinobacteria				
Corynebacterium diptheriae	DIP1281	Virulence		118 119
	DIP1621			120 119
Corynebacterium glutamicum	RipC (CgR_1596, CgP_1735)	Cell separation	Nomenclature note: *In C. glutamicum* R, this protein is referred to as CgR_1596. In *C. glutamicum* MB001, it is CgP_1735, which was named RipC by Bernhardt *et al.*	121 122 123 124
	CgR_2070	Cell separation		121
Mycobacterium avium subspecies *paratuberculosis*	MAP_1272c		NlpC/P60 domain is inactive	125 126 127 128
	MAP_1203			125
	MAP_1204			125
	MAP_1928c			125
	MAP_0036			125
Mycobacterium marinum	IipA	Virulence	Ortholog of *M. tuberculosis* RipA	129
	IipB	Virulence	Ortholog of *M. tuberculosis* RipB	129
Mycobacterium smegmatis	RipA		Synthetic lethality with RipB	130

Organism	NlpC/P60 Protein	Specific Function(s)*	Notes	Reference(s)
	RipB		Synthetic lethality with RipA	130
Mycobacterium tuberculosis	RipA (Rv1477)	Septum formation	Synthetic lethality with RipB; localizes to septum and occasionally poles; interacts with RpfB and PBP1 (via PonA1); processed by MarP; structure determined (PDB 6EWY & 3NE0)	131 132 133 134 135 136 137 17 138 139 140 141 142 143
	RipB (Rv1478)		Synthetic lethality with RipA; structure determined (PDB 3PBI)	139 138
	RipC (Rv2190c)	Growth and virulence	Activated by FtsX	144 145 146
	RipD (1566c)		NlpC/P60 domain is inactive	147
	Rv0024	Biofilm formation and drug resistance	Expression in Mycobacterium smegmatis causes increased biofilm formation and antibiotic resistance	148

*Specific functions beyond growth and division are noted in the table.