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Regulation of peptidoglycan hydrolases: localization, abundance, and activity

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Most bacteria are surrounded by a cell wall composed of peptidoglycan (PG) that specifies shape and protects the cell from osmotic rupture. Growth, division, and morphogenesis are intimately linked to the synthesis of this exoskeleton but also its hydrolysis. The enzymes that cleave the PG meshwork require careful control to prevent aberrant hydrolysis and loss of envelope integrity. Bacteria employ diverse mechanisms to control the activity, localization, and abundance of these potentially autolytic enzymes. Here, we discuss four examples of how cells integrate these control mechanisms to finely tune cell wall hydrolysis. We highlight recent advances and exciting avenues for future investigation.

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Introduction

Most bacteria are encased within a peptidoglycan (PG) exoskeleton composed of glycan strands with the repeating disaccharide units *N*-acetylglucosamine and *N*-acetylmuramic acid that are cross-linked together by attached peptides. This three-dimensional meshwork encapsulates the cytoplasmic membrane, specifies cell shape, and protects the cell from osmotic lysis [1]. Bacterial growth, division, and morphogenesis are intimately linked to the synthesis *and* hydrolysis of this covalently closed macromolecule. To enlarge the PG meshwork during growth, bonds connecting the glycan strands must be broken to allow its expansion and/or to incorporate new strands between the existing ones. Similarly, during cell division, bonds must be cleaved in the nascent septal PG to allow outer membrane constriction in Gram-negative bacteria and to promote cell separation in Gram-positives. Finally, during bacterial differentiation, PG synthesis and hydrolysis remodel the meshwork to generate new morphologies or to shed existing protective layers. Cell wall hydrolases are central to these processes, but their activities must be carefully regulated to prevent excessive or inappropriate cleavage and the generation of lethal breaches in this protective layer. The mechanisms by which these autolytic enzymes are regulated are only beginning to be elucidated and are the subject of this minireview.

Most bacteria contain dozens of distinct cell wall hydrolases that are capable of cleaving virtually all the bonds that hold the PG meshwork together [2]. Several target the glycosidic linkages in the polymeric glycan strands, while others cleave distinct bonds in the peptide cross-links. Owing to a high degree of functional redundancy, in many cases, PG hydrolase mutants have modest or lack discernible phenotypes. Further complicating their characterization, specific hydrolases and their biological roles are often not conserved among bacteria. Despite these challenges, in a growing number of bacteria, sets of autolytic enzymes that participate in growth, division, or morphogenesis have been identified. Understanding the mechanisms by which these hydrolases are regulated and coordinated with PG synthesis is a major focus in the field.

The control of PG hydrolases can be subdivided into three broad categories: (1) direct activation or inhibition by regulatory proteins or small molecules, (2) spatial regulation by proteins or surface polymers or through modification of the PG substrate, and (3) control of PG hydrolase abundance at the transcriptional or post-transcriptional level. In virtually all cases, the regulation of these autolytic enzymes incorporates more than one of these control mechanisms to finely tune activity and to provide spatial and temporal control. Here, we devote our attention to four well-characterized examples that feature distinct combinations of control. We suggest that each provides principles that will inform how virtually all PG hydrolases are regulated. We point the reader to several excellent reviews for a more general discussion of cell wall biogenesis [1,3], the different types and activities of PG hydrolases [2,4], and the many cellular processes in which PG hydrolases have been implicated [5].

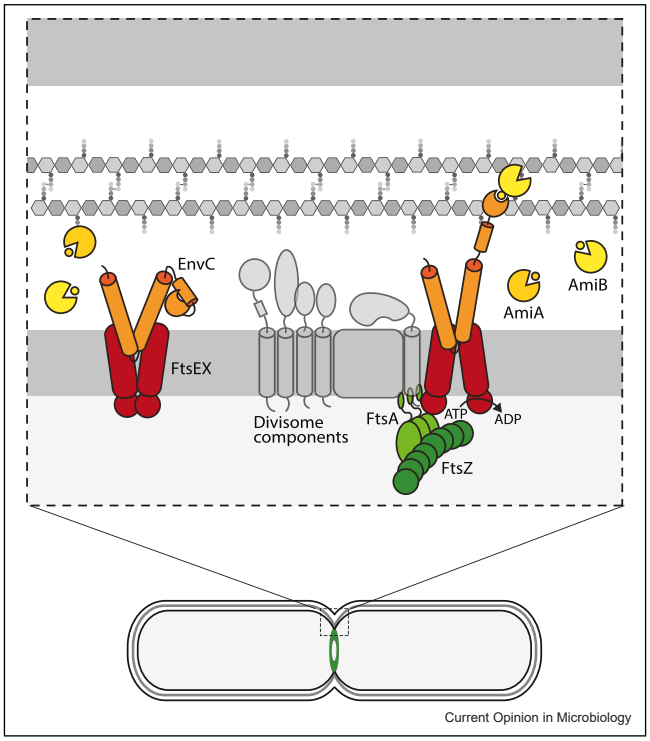
FtsEX: a broadly conserved regulatory module of diverse peptidoglycan hydrolases

The enzymes that synthesize the cell wall and many of the proteins that regulate them are broadly conserved among bacteria. By contrast, PG hydrolases are significantly more diverse, as are their regulators. The standout exception is the regulatory complex composed of FtsE and FtsX (FtsEX). This substrate-less ABC transporter has been found to regulate distinct PG hydrolases in diverse bacterial species, including *Escherichia coli* [6], *Streptococcus pneumoniae* [7], *Bacillus subtilis* [8,9], *Mycobacterium tuberculosis* [10], *Corynebacterium glutamicum* [11], *Caulobacter crescentus* [12], *and Vibrio cholerae* [13]. The FtsEX membrane complex provides one of the best examples of direct control of PG hydrolase activity combined with localization to a distinct subcellular site.

FtsEX is a member of the type-VII ABC transporter superfamily, members of which function in mechanotransmission rather than transport [14]. FtsE is the ATPase and FtsX is the transmembrane domain subunit of the complex [15]. The extracytoplasmic loops of FtsX interact with species-specific PG hydrolases [7] or regulators of PG hydrolases [6]. In both cases, the protein that directly contacts FtsX contains a regulatory coiled-coil domain that is the target of mechanotransmission.

The most well-studied example of this complex comes from *E. coli*, where FtsEX functions during cytokinesis to promote cleavage of the newly synthesized septal PG and thus enables outer membrane constriction [16] (Figure 1). The ABC transporter complex functions to localize and control the activity of two functionally redundant amidases, AmiA and AmiB [6]. A regulatory protein with a coiled-coil domain called EnvC links FtsEX to these enzymes [17,18]. Structural studies of EnvC indicate that its coiled-coils resemble molecular tweezers that hold its amidase-activating domain in an 'off' state. Recent structural work of the FtsEX•EnvC complex [19] and previous studies on AmiA and AmiB [20] provide a working model for how amidase activation is controlled by cycles of ATP hydrolysis (Figure 1). AmiA and AmiB contain autoinhibitory helices that bind to their active site clefts, rendering these enzymes inactive in the periplasm [20]. Relief of inhibition is triggered by EnvC binding, which must first escape from the clutches of its own coiled-coils. In its basal state, FtsEX maintains EnvC in its autoinhibited state. ATP hydrolysis by FtsE results in a conformational change in FtsX that is transmitted to EnvC's coiled-coils, resulting in release of its amidase-binding domain [19]. Interaction with the autoinhibitory helices of AmiA and AmiB transiently activates these enzymes [21], presumably until EnvC's amidase-activating domain is rebound by its coiled-coils.

Figure 1



FtsEX controls amidase localization and activity during cytokinesis in *E. coli*. Schematic model of FtsEX regulation of amidases required for cell division in *E. coli*. The periplasmic amidases AmiA and AmiB are autoinhibited by helices (circles) that occlude their active sites. Extracytoplasmic loops of FtsX dimers interact with EnvC, maintaining the autoinhibition of its amidase-activating domain. The FtsEX complex is recruited to the divisome by FtsZ and then interacts with FtsA. The onset of septal PG synthesis during cytokinesis is hypothesized to be the signal that stimulates FtsE ATPase activity. ATP hydrolysis is thought to catalyze a conformational change in FtsX that is transmitted to EnvC, triggering relief of inhibition. EnvC, in turn, activates AmiA and AmiB by binding to their autoinhibitory helices. Thus, autoinhibition and relief of inhibition by FtsEX–EnvC restrict amidase activity to the septum during division.

The FtsEX complex not only regulates AmiA and AmiB, it also spatially controls their activity. Studies in *E. coli* indicate that FtsEX is initially recruited to the cytokinetic ring by the tubulin-like protein FtsZ [22] but then interacts with the actin-like protein FtsA [23] (Figure 1). The signal(s) that stimulate cycles of ATP hydrolysis are currently unknown but are thought to be linked to the onset of PG synthesis during cytokinesis and could be triggered by FtsA. Furthermore, since septal PG synthesis is thought to stimulate PG hydrolysis [24], FtsEX serves to intimately coordinate these opposing processes during division.

Other Gram-negative bacteria have been found to encode one or more homologs of AmiA and AmiB and EnvC-like proteins [12,13,25,26]. Recent work on a few

of these factors highlights both similarities and differences in the regulatory control mechanism established in *E. coli*. In several Gram-positive bacteria, FtsEX complexes have been found to directly control cell wall hydrolases that contain regulatory coiled-coil domains [8,9,27]. In these cases, the coiled-coil tweezers hold the catalytic domain inactive and mechanotransmission is thought to transiently relieve this inhibition in a manner analogous to the control of EnvC [27]. Although most characterized FtsEX homologs function in cell division, in *B. subtilis*, FtsEX controls a hydrolase required for cell wall elongation [8,9]. The current thinking is that *B. subtilis* FtsEX coordinates PG hydrolysis with PG synthesis carried out by the cell wall elongation complex [8,9], perhaps using actin-like MreB proteins to stimulate FtsE's ATPase activity [9,28]. This interesting model requires further exploration but suggests that the broad conservation of the FtsEX regulatory module relates to its intimate association with conserved PG synthesis machineries. Future work will address the breadth of ways this complex functions to activate cell wall hydrolysis and will focus on the signals that stimulate FtsE's ATPase activity and the mechanisms by which FtsEX coordinates PG synthesis and hydrolysis.

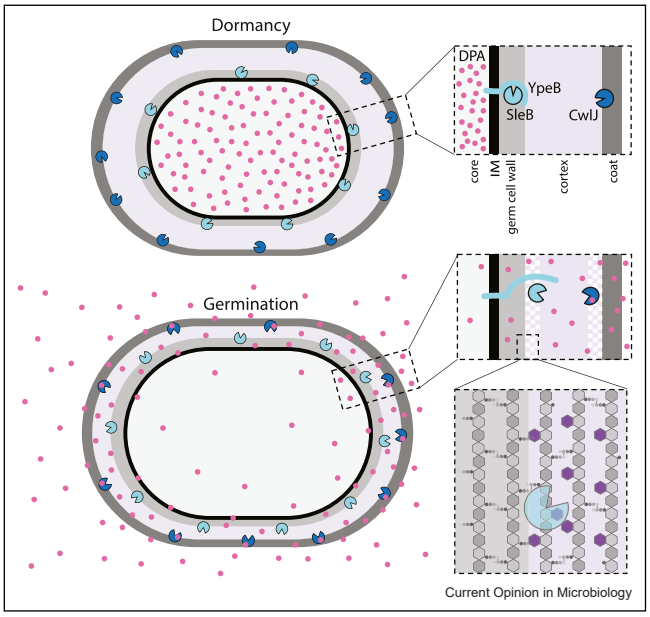
The regulation of autolytic enzymes during spore germination

Exit from dormancy requires the degradation of the specialized cell wall that encases and protects the spore. The enzymes that cleave this PG layer are regulated by small-molecule activation, relief of inhibition, and substrate modification.

In response to starvation, bacteria in the orders *Bacillales* and *Clostridiales* differentiate into metabolically inactive spores that are resistant to a myriad of environmental insults, including heat, desiccation, radiation, and toxic chemicals [29]. Spores can remain dormant for decades yet rapidly resume growth in response to nutrients [30,31]. A key step in the exit from dormancy is the degradation of the spore-specific PG called the cortex that encases the dormant spore (Figure 2). This envelope layer maintains the spore core in a highly desiccated state by physically restricting its expansion and thereby preventing hydration [32]. In *Bacillus* species, two functionally redundant lytic transglycosylases (CwlJ and SleB) that can degrade the cortex are deposited in the spore's envelope layers during spore formation [33]. These enzymes remain inactive for decades yet rapidly activate during germination [30].

The regulation of CwlJ and SleB is incompletely understood but involves two distinct mechanisms of control. CwlJ is activated by a small molecule called dipicolinic acid (DPA) [34]. The mother cell that nurtures the developing spore synthesizes and transports large quantities of this molecule into the spore cytoplasm during sporulation. The accumulation of DPA displaces water, contributing to core dehydration [35]. One of the earliest events in germination is the expulsion of DPA from the core and genetic evidence suggests that CwlJ requires DPA as a cofactor for PG hydrolase activity [34]. Thus, this enzyme, which resides in the envelope layers, is inactive during dormancy but can be rapidly activated when DPA is expelled during germination (Figure 2). This simple on–off switch for PG cleavage activity awaits biochemical confirmation. Unlike CwlJ, SleB does not require DPA for its activity. Instead, this enzyme is encoded in an operon with a membrane protein (YpeB) that is thought to hold SleB inactive [36] (Figure 2). The mechanism by which SleB escapes inhibition during germination is currently unknown but is certain to be distinct from the relief of inhibition described above for the FtsEX module.

Figure 2



Regulation of autolytic enzymes during spore germination. Schematic models of dormant and germinating spores, highlighting the regulation of the cortex-degrading enzymes CwlJ and SleB. Both proteins are deposited in the developing spore before dormancy and remain inactive until germination. SleB is hypothesized to be held inactive by YpeB at the spore's IM. CwlJ localizes in the spore coat adjacent to the cortex PG. Evidence suggests that CwlJ activity requires DPA as a cofactor and is therefore inactive until the large stores of DPA (pink circles) are released from the spore core. In response to nutrients (not shown), DPA is expelled from the core and activates CwlJ, while SleB is activated by an unknown mechanism. These two enzymes rapidly degrade the cortex working from opposite faces of this envelope layer. CwlJ and SleB specifically recognize the muramic-δ-lactam sugars (purple and gray hexagons) in the cortex, ensuring its degradation while sparing the germ cell wall that serves as the foundational PG layer of the outgrowing vegetative cell.

In addition to control of enzymatic activity, CwlJ and SleB are subject to spatial control at the levels of subcellular localization and substrate modification. CwlJ localizes adjacent to the distal edge of the cortex [37], while YpeB and likely SleB are located adjacent to the spore inner membrane (IM) [38]. Thus, upon activation, these enzymes act on opposing faces of the cortex (Figure 2). Importantly, both lytic transglycosylases specifically cleave the glycan strands of the cortex PG that are synthesized during sporulation but must avoid a thinner layer of cell wall that lies adjacent to the spore membrane. This layer, called the germ cell wall, is similar in structure to vegetative PG and functions as the foundational layer of PG during spore outgrowth. The cortex PG differs from the germ cell wall and vegetative PG through a cyclic modification on the MurNAc sugar that converts it to muramic-δ-lactam [32] (Figure 2). CwlJ and SleB specifically recognize glycan strands with these modified sugars and are incapable of cleaving the germ cell wall that lacks them [39]. Thus, spore germination features direct regulatory control of the cortex lytic enzymes combined with subcellular localization and substrate modification to ensure efficient degradation of the spore's protective PG layer while sparing the cell wall template required for outgrowth.

Regulation of cell wall cleavage by surface polymers

A terrific example of spatial control of autolytic enzymes by surface polymers comes from recent work on the Gram-positive pathogen *S. pneumoniae* (*Sp*). Like most Firmicutes, *Sp*'s envelope is studded with anionic polymers called teichoic acids [40]. These polymers are both lipid-linked (lipoteichoic acids (LTAs)) and attached to the PG (wall teichoic acids (WTAs)). However, unlike most Firmicutes, in *Sp*, these polymers are assembled from the same precursor and are decorated with phosphocholine moieties [41]. Also distinct from other bacteria, several PG hydrolases in *Sp* are fused to choline-binding domains (CBDs) that target these factors to the LTAs proximal to the membrane or to the WTAs within the PG [42]. *Sp* uses the phosphocholine moieties in two ways to spatially regulate its autolysins (Figure 3). In the first, it controls the levels of LTAs throughout growth by regulating the abundance of the LTA synthase, TacL [43]. By controlling the levels of TacL, the cell can modulate flux into LTA or WTA synthesis since they use a common precursor. Increasing the levels of LTAs during exponential growth sequesters the CBD-containing PG hydrolases, LytA, LytB, and LytC, away from the wall and thus reduces overall hydrolytic activity during growth. However, in stationary phase or in response to cell wall-targeting antibiotics, TacL levels drop, causing a reduction in LTAs and an increase in WTAs. This switch in surface polymer synthesis relocates PG hydrolases to the cell wall where LytA triggers autolysis [43] (Figure 3).

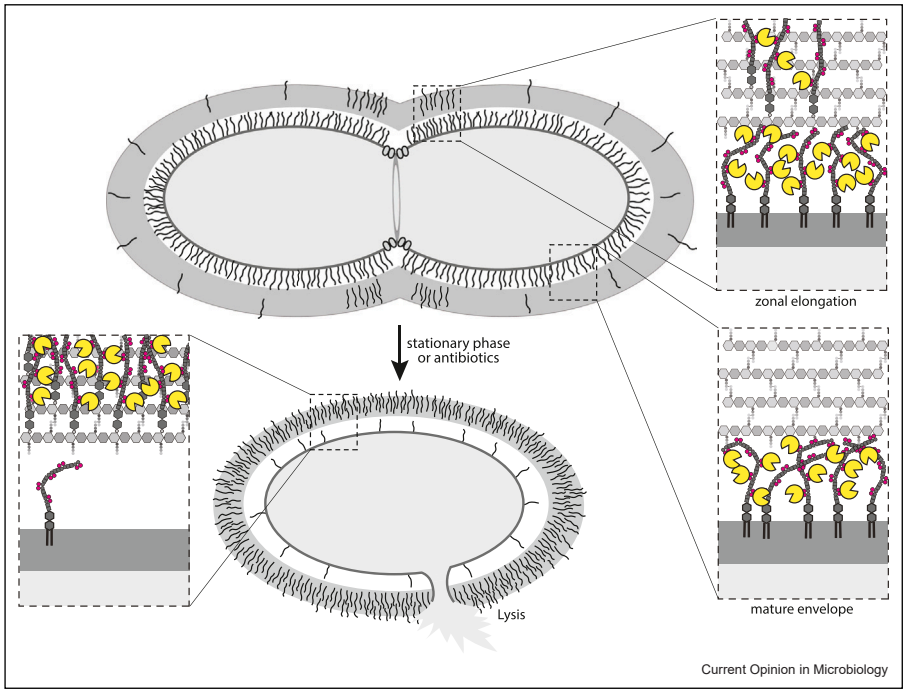
The second mechanism of spatial control of PG cleavage involves a WTA hydrolase called WhyD. Recent work indicates that during exponential growth, WhyD actively removes most of the WTA from the PG, helping to prevent autolysis [44,45]. However, WhyD also functions to regulate the spatial localization of the CBD-containing hydrolases by sparing some of the WTAs from removal. During growth, WTAs are enriched in regions adjacent to the divisome where the majority of cell elongation occurs (Figure 3). Directing the CBD-containing PG hydrolases to this region is thought to promote expansion of the meshwork at these sites [44]. Thus, *Sp* controls PG hydrolase activity by sequestering these enzymes away from the wall and by tailoring the WTAs in the PG meshwork to spatially control what regions of the wall get cleaved. How TacL abundance is regulated and the mechanism by which WhyD avoids specific WTAs are outstanding questions for future investigation.

In a variation on this theme, several Gram-positive bacteria spatially regulate PG hydrolases by controlling the localization of WTAs or LTAs on their surface. The presence or absence of these polymers have been found to recruit or exclude specific hydrolases [46–48]. The molecular bases for this spatial control remain to be discovered. The regulation of PG hydrolases by teichoic acids naturally raises the question whether modifications of these polymers could provide an additional layer of control [49]. WTAs and LTAs are modified by D-alanylation and glycosylation and the enzymes responsible for these decorations are often induced during envelope stress [50]. Whether and how these modifications affect hydrolase control is an exciting question for the future.

Transcriptional control of peptidoglycan hydrolases and their regulators

Transcriptional control of PG hydrolases is among the most common strategies to regulate enzyme abundance. Given the need to rapidly adjust hydrolase activity throughout the cell cycle, this mechanism of control is often combined with additional layers of regulation, including direct inhibition, substrate modification, and intrinsic instability. Bacteria employ sensory proteins, usually two-component signaling systems (TCSS) or sigma/anti-sigma modules, that determine when to adjust cell wall cleavage activity. In most cases, the signals that these sensory proteins monitor are unknown. The WalK–WalR TCSS from *B. subtilis* provides a useful example as it is well studied and encompasses multiple modes of regulation (Figure 4). The WalR response regulator is a transcriptional activator of two cell wall hydrolases (LytE and CwlO) required for cell growth [51]. Both enzymes are D,L-endopeptidases that cleave peptide cross-bridges enabling expansion of the PG meshwork [52]. Recent studies indicate that when the

Figure 3



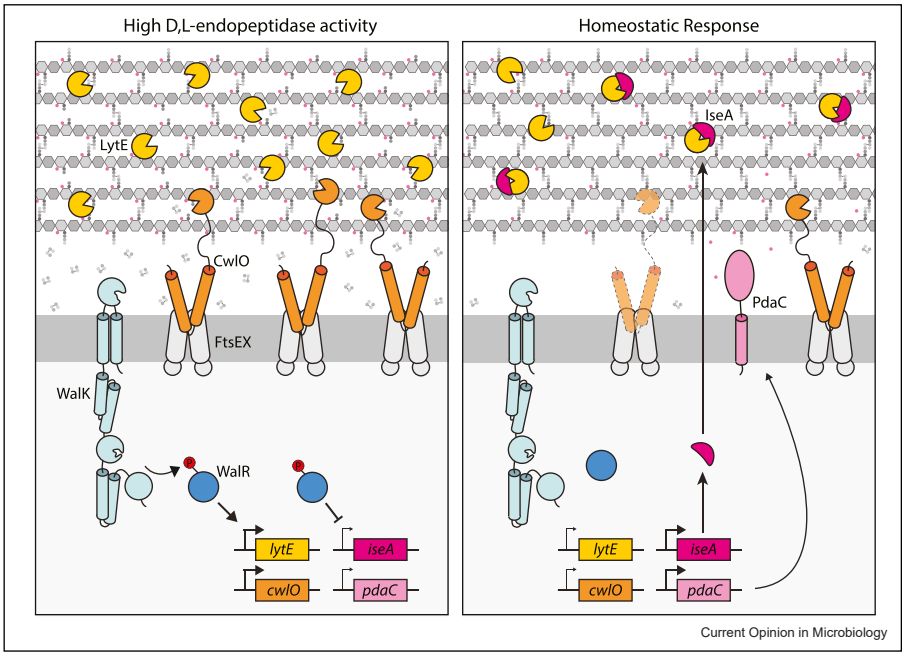
Regulation of cell wall cleavage by surface polymers in *S. pneumoniae.* Schematic models of *Sp* during growth and stationary phase, highlighting the regulation of cell wall hydrolases by LTAs and WTAs. During exponential growth, both LTAs and WTAs are produced but a WTA hydrolase WhyD (not shown) removes most WTAs from the cell wall. Only WTAs adjacent to the septum are spared. The PG hydrolases LytA, LytB, and LytC (yellow) contain phosphocholine-binding domains that bind these moieties on teichoic acids (pink circles). During growth, these enzymes are largely sequestered away from the cell wall by the LTAs. However, some of these PG hydrolases are recruited to the WTAs adjacent to the septum to promote cell wall elongation during zonal PG synthesis. The enzymes that synthesize the PG are shown schematically as gray ovals. During stationary phase or in response to cell wall-targeting antibiotics, the LTA synthase TacL is degraded (not shown), leading to a dramatic reduction in LTAs. The TA precursors are instead attached to the cell wall increasing WTA levels. It is unknown whether WhyD activity is also reduced under these conditions. In response to this switch in surface polymer localization, the PG hydrolases relocate to the wall and cause autolysis. LytA is the primary autolysin under these conditions.

levels of these enzymes are low, WalR activity increases, presumably as a consequence of phosphorylation by WalK, and the levels of the PG hydrolases rise [53]. Reciprocally, when D,L-endopeptidase activity is too high, WalR activity decreases reducing expression of these enzymes. Interestingly, the signal that modulates changes in WalRK signaling is directly linked to the activity of the enzymes. Evidence suggests that the sensor kinase WalK senses a cleavage product generated by D,L-endopeptidases and in turn modulates the phosphorylation status of WalR [53]. Thus, this TCSS functions to maintain a goldilocks level of these autolytic enzymes (Figure 4).

Transcriptional regulation alone would not be sufficient to alter PG hydrolase activity on the timeframe of a cell cycle. Accordingly, other regulatory mechanisms are built into this circuit. CwlO has a half-life of less than 5 min [53] and its transcript is highly unstable [54]. Thus, changes in transcription quickly translate into changes in protein abundance. Similarly, WalR is not only a transcriptional activator of *cwlO* and *lytE*, it is also a repressor of *iseA*, a secreted inhibitor of LytE [51,55]. IseA was given its name (inhibitor of cell separation A) because of its ability to inhibit D,L-endopeptidases required for cell separation when overexpressed on a multicopy plasmid [56]. However, recent work suggests its primary target may be LytE in its role in cell elongation [53,57]. IseA's solution structure resembles a hacksaw, the 'blade' of which (a flexible loop) has been modeled in the active site of D,L-endopeptidase family members [58]. This model has led to the proposal that IseA sterically blocks these PG hydrolases by substrate mimicry. The reciprocal transcriptional regulation of hydrolase and inhibitor provides an efficient mechanism to rapidly control D,L-endopeptidase activity (Figure 4).

As an additional measure of control, when D,L-endopeptidase cleavage is high, WalR also derepresses *pdaC* encoding a PG deacetylase [59]. PdaC is a membrane-anchored polysaccharide deacetylase that acts on the MurNAc sugars [60] such that LytE, CwlO, and

Figure 4



Transcriptional control of PG hydrolases and their regulators. The TCSS WalK–WalR homeostatically controls the levels and activities of CwlO and LytE during growth. The WalK sensor kinase is thought to monitor the D,L-endopeptidase activity of CwlO and LytE by sensing cleavage products generated by these enzymes. When D,L-endopeptidase activity gets too high (left panel), WalK responds by reducing phosphorylation of WalR (right panel), resulting in decreased transcription of *cwlO* and *lytE* and derepression of *iseA* and *pdaC*. CwlO has a very short half-life and therefore its levels are rapidly reduced when transcription decreases. IseA is a secreted inhibitor of LytE. Finally, PdaC deacetylates the muramic acids (small pink circles) in the PG reducing cleavage by both CwlO and LytE. As D,L-endopeptidase activity decreases, WalK-dependent phosphorylation of WalR rises (left panel), leading to increase expression of *cwlO* and *lytE* and repression of *iseA* and *pdaC*.

likely other autolytic enzymes cannot bind and/or cleave the nascent deacylated PG [57] (Figure 4). This provides a mechanism to rapidly reduce cell wall cleavage during growth. Taken together, substrate modification, direct inhibition, and intrinsic instability of hydrolases coupled with transcriptional regulation of hydrolases and their regulators allows *B. subtilis* to tightly control the autolytic activity required for growth.

The WalRK system is broadly conserved throughout Firmicutes, and in all known cases, the WalR regulon contains PG hydrolase genes [51,61–63]. However, it is not yet known if the regulatory circuit described here is conserved among these organisms. In fact, recent studies suggest that WalRK signaling in *Clostridioides difficile* is not linked to the PG hydrolases under WalR control, arguing that distinct signals modulate WalK activity [62]. Nevertheless, the modes of regulation exhibited by components of the *B. subtilis* WalRK circuit are present throughout bacteria. Regulation of PG hydrolase abundance by sigma/anti-sigma systems [64], TCSS [65], and other transcription factors [66] is very common and regulation by PG hydrolase instability has been suggested for endopeptidases in both *E. coli* [67] and *Pseudomonas aeruginosa* [68].

Outlook

Bacteria employ a variety of mechanisms to control the levels, activity, and localization of their PG hydrolases. Here, we have described a few of the most highly conserved and well-characterized examples of how cells integrate these control mechanisms to finely tune cell wall cleavage. Owing to space constraints, several emerging examples [69–73] were not discussed. Although work in recent years has provided a framework for hydrolase control, future studies in this area will be required to provide mechanistic understanding and to elucidate the extent to which cells combine control mechanisms to more precisely regulate cell wall cleavage during growth, division, and morphogenesis.

Data Availability

No data were used for the research described in the article.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- • of special interest
- •• of outstanding interest

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