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More than just lysins: peptidoglycan hydrolases tailor the cell wall

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Abstract

Enzymes that degrade the peptidoglycan (PG) cell wall layer called PG hydrolases or autolysins are often thought of as destructive forces. Phages employ them to lyse their host for the release of virion particles and some bacteria secrete them to eliminate (lyse) their competition. However, bacteria also harness the activity of PG hydrolases for important aspects of growth, division, and development. Of course, using PG hydrolases in this capacity requires that they be tightly regulated. While this has been appreciated for some time, we are only just beginning to understand the mechanisms governing the activities of these ‘tailoring’ enzymes. This review will focus on recent advances in this area with an emphasis on the regulation of PG hydrolases involved in cell division.

Introduction

A key component of the bacterial envelope is the peptidoglycan (PG) cell wall, a normally essential structure that fortifies the cell membrane against osmotic rupture [1]. It is constructed from arrays of polysaccharide strands connected by covalent crosslinks between attached peptide moieties, ultimately forming a continuous network that surrounds the cell (Figure 1a). Because they are encased within this polymeric shell, bacterial growth is intimately tied to the expansion and division of the PG layer. Since lesions in the matrix can lead to cell lysis, these processes must occur without disrupting the integrity of the PG network.

During growth, new PG material is synthesized by the high molecular weight penicillin-binding proteins (HMW-PBPs) [2]. To properly incorporate nascent PG into the existing wall, however, a multitude of additional cellular factors are required, including cytoskeletal structures that organize the assembly process [3,4]. Counted among this cast of PG biogenesis characters is a diverse group of enzymes capable of breaking bonds in the PG layer called PG hydrolases or autolysins [5]. While these enzymes are commonly associated with cell wall destruction (e.g. lysozyme), bacteria also harness their activity for important aspects of growth and development [5]. Using PG hydrolases in this capacity, however, requires that their activities be tightly regulated. Although this has been appreciated for some time, we are only now beginning to understand the control mechanisms governing the activities of these ‘tailoring’ PG hydrolases. This review will focus on recent advances in PG hydrolase function and regulation with an emphasis on their roles in cell division. We point the reader to several excellent reviews for a more general discussion of PG biogenesis and cell shape [3,4], the different types and activities of PG hydrolases [5,6], and the many cellular processes in which PG hydrolases have been implicated [5].

PG hydrolases and cell division

PG hydrolases have long been known to be required for the completion of cell division [7,8]. During cytokinesis, the tubulin-like FtsZ protein organizes the formation of a ring-shaped division apparatus called the divisome or septal ring [9,10]. This machine promotes the highly localized synthesis of new (septal) PG that ultimately forms the poles of the developing daughter cells [3]. Septal PG is initially shared between the daughters and must be split by PG hydrolases to shape the cell poles and complete the division process (Figure 1b and c) [5]. Promoting the localized hydrolysis of the cell wall is obviously a dangerous operation that must be tightly controlled and coordinated with septal PG synthesis. A regulatory failure could easily lead to a breach in PG integrity and cell lysis. While the PG hydrolases responsible for septal PG splitting/cell separation have been identified in a large number of organisms, until recently, the mechanisms by which they are spatiotemporally regulated remained largely undetermined.

Regulation of cell separation in *E. coli*

Work from our lab has begun to uncover major control points governing septal PG splitting in the model gram-negative bacterium *Escherichia coli* (Figure 1c). The most important cell separation enzymes in this organism are the LytC-type PG amidases (Pfam: Amidase_3): AmiA, AmiB, and AmiC (Figure 1a) [11]. We also uncovered a crucial role for EnvC and NlpD in cell separation [12]. These divisome-associated proteins both contain LytM (Pfam: Peptidase_M23) domains, which typically possess endopeptidase activity directed against PG crosslinks [6]. In the case of EnvC and NlpD, however, genetic and biochemical analysis revealed that, rather than directly cleaving PG, these factors promote septal PG splitting via amidase activation [13*]. Both *in vivo* and *in vitro* experiments indicate that EnvC specifically activates AmiA and AmiB, while NlpD specifically activates AmiC [13*]. Thus, an important regulatory point in the initiation of septal PG splitting in *E. coli* is the activation of the amidases by the LytM factors.

What coordinates amidase activation with septal PG synthesis? One mechanism appears to involve the order in which the amidases and their activators are recruited to the division site [14]. While all of the cell separation factors except for AmiA assemble at the septal ring [12,14,15], they are recruited at different times [14]. Importantly, experiments with the septal PG synthesis inhibitor cephalixin indicate that the amidases cannot be recruited to midcell if septal PG synthesis is blocked [14]. The LytM factors, on the contrary, localize before the onset of septal PG synthesis [14]. Thus, a fail-safe appears to be 'built-in' to the septal ring assembly pathway ensuring that the activators and amidases are only concentrated in the same subcellular location after septal PG synthesis is initiated. This presumably helps prevent premature activation of the amidases and the generation of fatal PG lesions at the division site.

An ABC transporter-like complex regulates cell separation

Control of protein localization is not the only means of regulating the septal PG splitting process. We recently discovered that EnvC is recruited to the septal ring through a direct interaction with the division factor FtsX [16**]. This polytopic membrane protein forms the transmembrane domain (TMD) component of a conditionally essential ABC transporter-like complex that also includes the nucleotide binding domain (NBD) component FtsE [17,18]. ABC transporters are ubiquitous membrane protein complexes that undergo remarkable conformational changes in their TMDs in response to ATP hydrolysis by their NBDs [19]. Interestingly, variants of the FtsEX complex predicted to be defective in the ATPase activity of FtsE still recruit EnvC to the septum but fail to promote cell separation [16**,20*]. Our results thus suggest the attractive possibility that amidase activation via EnvC in the

periplasm is regulated by conformational changes in the FtsEX complex driven by ATP hydrolysis in the cytoplasm. Such a model is appealing because it provides a means for converting septal PG hydrolysis into a discrete process whereby a fixed number of PG bonds are broken per ATP hydrolyzed. The modulation of the ATPase activity of FtsEX by components of the septal ring would then allow exquisite control over septal PG hydrolysis, a highly desirable feature given the inherent risks involved. Additionally, since FtsE has been shown to interact with FtsZ in the cytoplasm [21], FtsEX may serve as a bridge to directly couple septal PG hydrolysis to the contraction of the FtsZ ring (Figure 1c).

Importantly, a role for FtsEX in the regulation of cell separation is conserved. Working in *Streptococcus pneumoniae* Winkler and co-workers have also directly connected an essential cell separation factor, PcsB [22], with FtsEX [23^{**}]. Interestingly, while their sequences are largely unrelated, the domain structures of PcsB and EnvC are strikingly similar. Like EnvC, PcsB has an N-terminal region predicted to form coiled-coils and a C-terminal PG hydrolase-like domain (CHAP as opposed to LytM) [22]. However, it is currently not known whether PcsB directly degrades PG or if, analogous to EnvC, it is an activator of other PG hydrolases. Nevertheless, the results of Sham *et al.* (2011) [23^{**}] indicate that PcsB activity is probably governed by FtsEX to properly control the process of septal PG splitting in *S. pneumoniae*. The major challenge now is to determine the mechanism by which FtsEX modulates the activity of EnvC and PcsB and how FtsEX activity might be linked to septal PG synthesis and the contraction of the septal ring.

Cell separation in other gram-negative bacteria

Much less is known about cell separation in gram-negative bacteria other than *E. coli*. LytC-type amidases have been implicated in cell separation in *Neisseria gonorrhoeae* [24], *Helicobacter pylori* [25], and the filamentous cyanobacterium *Nostoc punctiforme* [26], suggesting that mechanisms similar to *E. coli* may be at work in these organisms. Intriguingly, the LytM factor DipM was recently shown by several groups to be an important cell division/separation factor in *Caulobacter crescentus* [27^{*},28^{*},29^{*}]. Interestingly, however, inactivation of the lone LytC-type amidase apparently had no effect on division [28^{*}]. This suggests that either DipM directly cleaves PG to promote cell separation or that it has a differential specificity from EnvC and NlpD and can activate a different class of PG hydrolases to stimulate septal PG splitting. One important difference between *C. crescentus* and *E. coli* is in their septal geometry. Dividing *C. crescentus* cells display very shallow constrictions relative to *E. coli* and this probably necessitates at least some variations in PG processing at the division site. It will be interesting to see if common mechanistic or regulatory themes emerge as more is learned about cell separation in different proteobacterial systems.

Cell separation in gram-positive bacteria

Unlike *S. pneumoniae* and other constricting ovococci, most gram-positive bacteria construct a (true) flat septum during cell division, the splitting of which is temporally uncoupled from membrane constriction and fission (Figure 1b) [30]. Septal PG splitting in these cells is therefore likely to have distinct regulatory requirements from those of constricting cells. While many different families of PG hydrolases have been identified as cell separases in gram-positive bacteria [5,6], the only post-translational regulator of PG hydrolase activity identified in flat-septum forming organisms is IseA, an inhibitor of the *B. subtilis* cell separation endopeptidases [31]. This factor does not appear to function as a cell cycle regulator of PG hydrolase activity, however, because it is mainly expressed as cells enter stationary phase [32,33].

Although little is still known about how the enzymatic activity of PG hydrolases is controlled in gram-positive bacteria, a great deal has been learned about the regulation of PG hydrolase gene expression. One of the key regulators is the essential WalK/WalR two-component system [34,35], which plays a major role in maintaining cell wall homeostasis [35]. In several different firmicutes, the phosphorylated form of the WalR response regulator (WalR-P) has been shown to activate the expression of genes that code for cell separation PG hydrolases and to repress the expression of their inhibitors [35]. Interestingly, in *B. subtilis*, the WalK kinase is recruited to, and directly interacts with, components of the septal ring [36^{**},37^{**}]. Several lines of evidence indicate that WalK also requires a functional division apparatus to phosphorylate (activate) WalR [36^{**},37^{**},38^{*}]. Thus, the sensor kinase appears capable of effectively tuning the level of cellular PG hydrolase activity to match the rate of growth and division. In addition to expression regulation, the subcellular localization and activity of gram-positive PG hydrolases appears to be controlled by teichoic acid components of the cell wall layer [39–41] and the acetylation status of the PG sugar backbone [42,43], respectively. Also, several gram-positive PG hydrolases are proteolytically processed and, at least *in vitro*, this processing can stimulate their activity [44–46]. Going forward, it will be important to determine exactly how all of these control strategies might work together to spatiotemporally control PG hydrolase activity needed for proper growth and division in the firmicutes and whether or not additional regulatory mechanisms exist.

Mycobacterial cell separation

While technically gram-positive, mycobacteria have a unique envelope structure with additional layers of arabinogalactan and mycolic acids [47]. Cell separation in these organisms is mediated by the essential (NlpC/P60) endopeptidase RipA [48]. Interestingly, the *in vitro* PG hydrolase activity of RipA synergizes with the activity of another hydrolase, called RpfB [48], and this synergy can be inhibited by the interaction of RipA with the PG synthase PBP1 [49^{*}]. Thus, while the mechanistic details are currently unclear, the interplay between RipA, RpfB, and PBP1 is probably providing a means for coordinating the processes of PG synthesis and PG hydrolase activity during cell division in mycobacteria.

Cell separation and biofilm development

In addition to the spatiotemporal regulation of PG hydrolases during the cell cycle, the expression of these enzymes is also regulated to control cell separation in response to environmental cues [5]. Notably, the expression of cell separation hydrolases is repressed in *B. subtilis* cells under conditions that favor biofilm formation [50]. The regulatory circuitry that controls cell chaining during *B. subtilis* biofilm development was recently uncovered [51^{*}]. Interestingly, stochastic changes in the status of the Slr-SinR circuit as well as in the expression of the PG hydrolase activator gene, *sigD*, also generate the mixed populations of motile and chaining cells observed in exponentially growing cultures of *B. subtilis* [51^{*},52^{*}]. Cell chains are also observed in biofilms of some gram-negative bacteria, but the underlying regulatory mechanism controlling chain formation remain to be elucidated [53,54].

Cell elongation and shape determination

Because of the continuous nature of the PG matrix, bonds in the existing structure must be broken allow for the insertion of new material [5]. Thus, in addition to cell division, PG hydrolases have long been thought to be essential for cell wall expansion [5]. Experimental support for this notion has only recently been uncovered. In *B. subtilis*, inactivation of the hydrolases LytE and CwlO was found to block cell elongation [32]. LytE was also found to interact with MreBH, which forms part of the actin cytoskeleton required for the elongation of many rod-shaped cells [55]. Recent theoretical modeling of the PG network has also

indicated a potential role for PG hydrolases in generating complex cell shapes via the formation of appropriately positioned lesions in the PG network [56^{**}]. Accordingly, LytM endopeptidases were recently shown to be required for the helical shape of *H. pylori* [57^{**}, 58^{**}]. These exciting findings should pave the way towards a mechanistic understanding of how PG hydrolase activity is controlled to safely expand and shape the PG network.

Conclusions

Progress in our understanding of PG hydrolase function and regulation has clearly been accelerating. Nevertheless, much remains to be learned. While clues are accumulating, we still only have a vague picture about how PG hydrolysis is coordinated with its synthesis, both during cell division and elongation. Determining the structure of the septal PG and how the cell separation hydrolases precisely identify and cleave the adjoining PG layers while leaving the developing polar layers intact also remains a major challenge. Another exciting area going forward will be investigations into the mechanisms coordinating septal PG splitting with the constriction of the outer membrane layer (Figure 1c). Finally, since PG hydrolases involved in cell separation and cell shape are essential in some cases [32,48,59] and have been shown to be important for virulence and envelope integrity in others [25,46,57^{**},58^{**},60–62], it will be important to explore PG hydrolases as potential targets for therapeutic development. With so many open questions, the coming years hold great promise for continuing the rapid pace of discovery.

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Figure 1.

Overview of PG structure and modes of division. **(a)** Diagram of the chemical structure of PG: M, *N*-acetylmuramic acid; G, *N*-acetylglucosamine. Colored dots represent the attached peptides. The PG structure continues in all directions to envelop the cell (green arrows). Red arrows indicate cleavage locations for PG hydrolases: 1) *N*-acetylglucosaminidases, 2) *N*-acetylmuramidases and lytic transglycosylases, 3) endopeptidases, and 4) amidases. Classic designations for bacterial envelope types are shown below the PG structure. Gram-negative bacteria have a thin PG layer surrounded by an outer membrane. Gram-positive bacteria have a single membrane surrounded by a thick PG layer. **(b)** Basic modes of cell division. In bacteria that construct a flat septum, PG splitting is initiated some time after the completion of membrane fission. In bacteria that constrict, splitting is coordinated with membrane invagination. **(c)** Close-up diagram of the coordinated constriction of cell envelope layers during division of *E. coli*. Septal PG splitting is coordinated with inner membrane (IM) constriction and allows constriction of the outer membrane (OM). Proteins involved in the septal PG splitting process are indicated in the diagram. The FtsEX ABC system controls the activity of the EnvC protein, which serves as an activator of the PG amidases AmiA and AmiB. See text for details.