Bacterial peptidoglycan (murein) hydrolases

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Abstract

Most bacteria have multiple peptidoglycan hydrolases capable of cleaving covalent bonds in peptidoglycan sacculi or its fragments. An overview of the different classes of peptidoglycan hydrolases and their cleavage sites is provided. The physiological functions of these enzymes include the regulation of cell wall growth, the turnover of peptidoglycan during growth, the separation of daughter cells during cell division and autolysis. Specialized hydrolases enlarge the pores in the peptidoglycan for the assembly of large trans-envelope complexes (pili, flagella, secretion systems), or they specifically cleave peptidoglycan during sporulation or spore germination. Moreover, peptidoglycan hydrolases are involved in lysis phenomena such as fratricide or developmental lysis occurring in bacterial populations. We will also review the current view on the regulation of autolysins and on the role of cytoplasm hydrolases in peptidoglycan recycling and induction of β-lactamase.

Introduction

Bacterial peptidoglycan hydrolases form a vast and highly diverse group of enzymes capable of cleaving bonds in polymeric peptidoglycan (sacculi) and/or its soluble fragments (Shockman & Hölterj, 1994; Shockman et al., 1996). They participate in bacterial cell wall growth and its regulation and also in different lysis phenomena. It is often difficult to assign a distinct function to a peptidoglycan hydrolase for several reasons. First, many bacteria possess a high number of hydrolases and they appear to have redundant roles (Hölterj & Tuomanen, 1991; Smith et al., 2000). Second, a hydrolase may have more than one function. For example, Escherichia coli has five N-acetylmuramyl-1-alanine amidases, six membrane-bound lytic transglycosylases and three peptidoglycan endopeptidases, all of which appear to contribute (at variable extent) to cleavage of the septum during cell division to allow separation of daughter cells (Hölterj et al., 2001, 2002). In addition, these hydrolases are responsible for the release of turnover products from peptidoglycan during cell growth (Goodell & Schwarz, 1985). Turnover products serve as signalling molecules for recognition of bacteria by other organisms and, in some bacteria, for the induction of β-lactamase (Jacobs et al., 1997). Moreover, these hydrolases are also responsible for autolysis induced under certain conditions (Tomasz, 1974, 1979a, b, 1980; Shockman, 1992; Shockman & Hölterj, 1994; Shockman et al., 1996). There are examples of hydrolases for every glycosidic and amide bond in peptidoglycan, although not every specificity is found in every species. It is impossible to cover all knowledge gathered on peptidoglycan hydrolases over the last decades in a single review. Here, the focus will be on bacterial peptidoglycan hydrolases. An overview on the different specificities will be presented, and the various functions of hydrolases and their regulation will then be described.

Specificities of peptidoglycan hydrolases

The cleavage sites of the different hydrolases are shown in Figs 1 and 2 (Hölterj, 1995). N-Acetylmuramyl-1-alanine amidases hydrolyse the amide bond between MurNAc and l-alanine separating the glycan strand from the peptide, whereas carboxy- and endopeptidases cleave the various...
LD- and DD-bonds in the stem peptides (Fig. 1). There are three types of glycan strand-cleaving enzymes (glycosidases), N-acetylglucosaminidases, lysozymes and lytic transglycosylases, the latter two cleaving the same glycosidic bond (Fig. 2). Lysozymes and lytic transglycosylases are also collectively known as N-acetyl-D-muramidases (muramidases). It should be noted that the activity of a hydrolase is often specific for a certain peptidoglycan type (Vollmer et al., 2008), for the presence or the absence of (a) secondary modification(s) (Vollmer, 2007), or for either high-molecular-weight (MW) peptidoglycan or small fragments.

**N-Acetylmuramyl-l-alanine amidases**

N-Acetylmuramyl-l-Ala amidases (MurNAc-LAAs) cleave the amide bond between MurNac and the N-terminal l-alanine residue of the stem peptide (Fig. 1). These enzymes, also referred to in the literature as peptidoglycan amidases or amidases, are found in bacterial and bacteriophage or prophage genomes (Lopez et al., 1981; Young, 1992; Shockman & Höltje, 1994). In most cases, bacterial MurNac-LAAs are members of the bacterial autolytic system and carry a signal peptide in their N-termini that allow their transport across the cytoplasmic membrane. However, the bacteriophage peptidoglycan amidases are endolysins. As opposed to autolysins, almost all endolysins have no signal peptides and their translocation through the cytoplasmic membrane is thought to proceed with the help of phage-encoded holin proteins (Young, 1992; Loessler, 2005).

Most often, several different MurNac-LAAs are present in a bacterial proteome. For example, five MurNac-LAAs are present in *E. coli*: AmiA, AmiB, AmiC (Heidrich et al., 2001) and AmiD (Uehara & Park, 2007; A. Pennartz, C. Génèreux, & B. Joris, unpublished data) have a periplasmic localization, whereas AmpD is cytoplasmic (Jacobs et al., 1995). After cleavage of their signal peptide, AmiA, AmiB and AmiC are soluble in the periplasm whereas AmiD is synthesized as prelipoprotein and matured to become a lipoprotein anchored in the external membrane (Uehara & Park, 2007; A. Pennartz, C. Généreux, & B. Joris, unpublished data). Amino acid sequence comparisons show that the five *E. coli* amidases can be subdivided into two structurally distinct clusters. The first group comprises the homologous AmiA, AmiB and AmiC enzymes, and the second comprises AmiD and AmpD. Among the three enzymes forming the first group, AmiA possesses the shortest sequence for the
amidase module. The sequence of this amidase module is conserved in AmiB and AmiC but it is fused at its N-terminal end to a module of unknown function different in the two amidases. AmiA, AmiB and AmiC play an important role in cleaving the septum to release daughter cells after cell division (see 'Separation of daughter cells'). Functional fusion proteins of AmiA or AmiC and GFP show different subcellular localization patterns in living cells. AmiA-GFP is dispersed throughout the periplasm whereas AmiC-GFP appears throughout the periplasm in small cells and is concentrated almost exclusively at the septal ring in dividing cells. AmiA is the first entirely periplasmic component of the divisome to be localized, and its recruitment to the division ring is mediated by its N-terminal nonamidase domain (Bernhardt & de Boer, 2003). Interestingly, AmiA and AmiC are exported by the Twin-arginine transport (Tat) system instead of using the Sec machinery, which presumably exports AmiB (Bernhardt & de Boer, 2003). The Tat system is dedicated to the translocation of folded proteins containing cofactor(s) across the bacterial cytoplasmic membrane. AmiA and C are not predicted to contain a cofactor. AmiB and AmiC are Zn\(^{2+}\)-containing metallo-enzymes. This could explain their secretion by the Tat pathway.

Unexpectedly, these structures are similar to those of the eukaryotic peptidoglycan-recognizing proteins (PGRPs). PGRPs are found in most animals, including insects, echinoderms, molluscs and vertebrates, but not in lower metazoa or plants and are a part of the innate immune system (Dziarski & Gupta, 2006). In insects, PGRPs activate antimicrobial pathways in the haemolymph and/or cells, or are lytic MurNAc-LAAs. In mammals, some PGRPs are MurNAc-LAAs and reduce the proinflammatory activity or kill bacteria. Others are noncatalytic, bind to peptidoglycan and are probably involved in signalling pathways.

The redundancy and the diversity of E. coli MurNAc-LAAs reflect well the plethora of peptidoglycan amidases described in the literature or predicted after bioinformatic analysis of known bacterial genomes. Even though it is impossible to describe all these enzymes in this review, it is, however, possible to extract some specific features that characterize this type of amidases. Generally, the amidase module...
catalytic module is fused, by its N- or C-terminal end, to another functional module that is responsible for binding of the protein to the cell wall. Based on their amino-acid sequences, two different types of catalytic modules can be identified. The first one corresponds to the AmiA/B/C catalytic domain and the second one to that of AmiD/AmpD. Six structures are known. The bacteriophage PSA PlyA endolysin (Korndorfer et al., 2006) and the Paenibacillus polymyxa CowV catalytic domain (T. Yamane, Y. Koyama, Y. Nojiri et al. unpublished data) are representative of the AmiA/B/C catalytic modules. On the other hand, the T7 lysozyme from T7 bacteriophage (Cheng et al., 1994), the C. freundii AmpD amidase (Liepinsh et al., 2003), the Bacillus anthracis prophage PlyL endolysin (Low et al., 2005) and the E. coli AmiD (S. Petrella, R. Herman, C. Genereux, A. Pennartz, E. Sauvage, B. Joris, P. Charlier et al., unpublished data) are members of the AmiD/AmpD catalytic domain. Greater diversity than in the catalytic domain lies in the noncatalytic domain fused to the amidase module. Usually, they are repeat domains named cell wall-binding domains (CBDs) that bind to peptidoglycan, lipoteichoic acid or pneumococcal teichoic acids containing choline (see ‘CBDs associated with peptidoglycan hydrolases’).

The presence of peptidoglycan amidase-sequence signatures in known translated genomes highlights that the catalytic and/or the repeated domains of the functional modules are present in several eukaryotic cells. These features are probably another example of horizontal gene transfer between prokaryotes and eukaryotes (Ponting et al., 1999).

**Endopeptidases and carboxypeptidases**

These enzymes cleave amide bonds between amino acids in peptidoglycan or its soluble fragments (Shockman & Höltje, 1994; Höltje, 1995). A large number of peptidases are known for the cleavage of the different amide bonds in the various types of peptidoglycan. According to the strict definition for a peptide bond as being a bond between the α-carboxylic group of one amino acid and the α-amino group of another, some bonds in the peptides of peptidoglycan could not be designated as a peptide bond. For example, the amide bond between D-Glu and meso-A2pm involves the γ-carboxylic group of D-Glu, and the DD-cross-bridge involves the ε-amino group of meso-A2pm (meso-2,6-diaminopimelic acid) or l-Lys. For the sake of clarity and consistency with the previous literature (e.g. Shockman & Höltje, 1994; Smith et al., 2000), in this review the terms ‘endopeptidase’ and ‘carboxypeptidase’ are used for enzymes hydrolysing the amide bond between two amino acids in peptidoglycan, and the term ‘amidase’ is reserved for enzymes cleaving between MurNAc and l-Ala. Depending on their specificity, peptidoglycan peptidases are classified as carboxypeptidases (removal of a C-terminal amino acid) or endopeptidases (cleavage within the peptide). DD-Peptidases cleave between two D-amino acids, whereas LD- or DL-peptidases cleave between an L- and a D-amino acid (Shockman & Höltje, 1995; Smith et al., 2000).

DD-Endopeptidases hydrolyse the D-Ala-meso-A2pm cross-bridges, which had been formed by DD-transpeptidation in nascent peptidoglycan by the class A and B high-MW penicillin-binding proteins (HMW-PBPs) (Sauvage et al., 2008). The side chain ammonium-carboxylate group at the peptidoglycan transpeptidation reaction (Goffin & Ghuysen, 1998; Sauvage et al., 2008). The second one is a mono-modular enzyme devoid of a C-terminal domain, but with an amphipathic helix that anchors the protein to the cytoplasmic membrane. These enzymes belong to the type-7 PBPs (Sauvage et al., 2008).

Three X-ray structures of type-4 PBPs are available: the Actinomadura R39 PBP, the E. coli PBP4 (Fig. 3) and the Bacillus subtilis PBP4a (Sauvage et al., 2005, 2007; Kishida et al., 2006). They share the same overall fold that is composed of three domains, one of which (domain I) possesses the typical structure and active site of the PBPs. The penicillin-binding/endopeptidase domain is associated with two other domains (II and III), which are not in N- or C-terminal positions but are inserted into the penicillin-binding/endopeptidase domain between the conserved motif I (with the SXXX signature, with S being the active site) and motif 2 (SNN). In all type-4 PBPs, the positioning of the active site residues is nearly identical, with only minor differences affecting the very fine structure of the binding cavity. The bottom of the active site is characterized by the presence of a hydrophobic residue at the C-terminus of the β3 strand and several residues belonging to the additional domain II. These residues form a pocket that accommodates the terminal H2N-C–CH–COO– group of the diaminopimelic acid, the antepenultimate amino acid of the peptidoglycan stem peptide, as observed in the complex of B. subtilis PBP4a with ε-aminopimelyl-S-D-alanyl-D-alanine (Sauvage et al., 2007). Unlike in some other PBPs,
the binding of a β-lactam does not result in a significant movement of the active site residues. Different X-ray structures show that the antibiotics covalently linked to the active site serine adopt the common standard positioning as described for the acyl-enzyme PBP4a-β-aminopimelyl-D-β-alanyl (Sauvage et al., 2005, 2007; Kishida et al., 2006). Type-4 PBPs are very loosely associated with the cytoplasmic membrane. A positively charged surface appears in domain II of the DD-peptidase of Actinomadura R39 and B. subtilis PBP4a but not in E. coli PBP4. It has been suggested that this positive surface could interact with the teichoic acid present in Bacillus and Actinomycetales (Sauvage et al., 2007). Type-4 PBPs are generally considered as being indirectly involved in cell morphology (Meberg et al., 2004), in daughter cell separation (Priyadarshini et al., 2006) and could be implicated in biofilm formation (Gallant et al., 2005) (see ‘Physiological functions of extracytoplasmic peptidoglycan hydrolases’).

PBP7 is the second penicillin-sensitive DD-endopeptidase in E. coli and is encoded by the pbpG gene (Henderson et al., 1995). PBP7 lacks DD-carboxypeptidase activity and is membrane-associated (Romeis & Hölte, 1994a). It hydrolyses the D-Ala-meso-A2pm cross-bridge in high-molecular mass sacculi and not in water-soluble low-molecular mass muropeptides. In vitro, PBP7 as well as its proteolytic degradation product PBP8 stabilizes and stimulates the activity of another peptidoglycan hydrolase, the soluble lytic transglycosylase Slt70, by direct protein–protein interaction (Romeis & Hölte, 1994b). The crystal structure of Mycobacterium tuberculosis PBP7 has been determined (Krieger, unpublished data, Fig. 3). Its global fold is similar to the penicillin-binding domain of the E. coli DD-carboxypeptidase PBP5 (Fig. 3). PBP7 lacks the N-terminal helix and exhibits on the top of the active site a β-hairpin protuberance, which, in other type-7 PBPs, was hypothesized to anchor the protein to the plasma membrane (Fonze et al., 1999).

There are also DD-endopeptidases unrelated to PBPs, which are not inhibited by β-lactams. The penicillin-insensitive endopeptidase MepA from E. coli hydrolyses both DD-(meso-A2pm–D-Ala) and LD-(meso-A2pm–meso-A2pm) peptide cross-links (Keck & Schwarz, 1979; Keck et al., 1990; Engel et al., 1992). MepA has crystallized as a dimer and its structure has been solved (Marcyjaniak et al., 2004) (Fig. 4). The enzyme is metal-dependent and contains a conserved His(113)-Asp(120)-His(211) Zn²⁺-binding triad essential for its activity. Two other histidine residues (at positions 206 and 209) were implicated in catalysis although the precise mechanism is not yet clear (Firczuk & Bochtler, 2007). MepA shares the active-site architecture with a large group of metallopeptidases including, for example, the peptidoglycan glycyglycyl endopeptidases LytM (Odintsov et al., 2004; Firczuk et al., 2005), lysostaphin (Heinrich et al., 1987) and ALE-1 (Lu et al., 2006), all of which cleave in the pentaglycine interpeptide bridge in the peptidoglycan of Staphylococcus aureus, the D-Ala-D-Ala carboxypeptidase...
which presumably cleaves the same peptide bond (between \(\text{L-Ala} \) and \(\text{D-Glu} \)), and that is responsible for the high extent of MurNAc residues carrying a single \(\text{L-Ala} \) in the spore peptidoglycan of this species (Horsburgh et al., 2003a).

\textit{Bacillus subtilis} has various \(\text{DL}\)-endopeptidases cleaving the bond between \(\text{D-Glu} \) (position 2) and meso-\(\text{A2pm} \) (position 3) of the peptide. These enzymes are members of two different families of \(\text{DL}\)-endopeptidases. CwlS (Fukushima et al., 2006), CwlO (Yamaguchi et al., 2004), LytE (Ohnishi et al., 1999) and LytF (Margot et al., 1999; Ohnishi et al., 1999) are related to the family II \(\text{DL}\)-endopeptidase from \textit{Bacillus sphaericus} (Hourdou et al., 1992) and the p60 autolysin from \textit{L. monocytogenes} (Wuensch et al., 1993).

The homologous LytE, CwlS and LytF proteins differ mainly in the number of their LysM peptidoglycan-binding repeats (three in LytE, four in CwlS and five in LytF) (see ‘CBDs associated with peptidoglycan hydrolases’) (Fukushima et al., 2006). \textit{Bacillus subtilis} also has a hypothetical member (\(\text{YqgT} \)) of the family I \(\text{DL}\)-endopeptidases (Smith et al., 2000) that are \(\text{Zn}^{2+} \)-dependent enzymes with sequence similarity to endopeptidase I from \textit{B. sphaericus} (Hourdou et al., 1993). This enzyme has both \(\text{DL}\)-endopeptidase and \(\text{DL}\)-carboxypeptidase activities and cleaves the peptide bond between \(\text{D-Glu} \) and \(\text{m-A2pm} \) in tripeptides \((\text{L-Ala-D-Glu(}\gamma\text{-meso-A2pm}) \) or tetrapeptides \((\text{L-Ala-D-Glu(}\gamma\text{-meso-A2pm-D-Ala}) \) with or without attached GlcNAc-MurNAc moiety (at \(\text{L-Ala} \)). Another member of this family is the cytoplasmic \(\text{DL}\)-endopeptidase \(\text{MpaA} \) from \textit{E. coli} (Uehara & Park, 2003).

\(\text{DL}\)-carboxypeptidases cleave between \(\text{m-A2pm} \) (position 3) and \(\text{D-Ala} \) (position 4) in a tetrapeptide to remove the terminal \(\text{D-Ala} \) residue. Although such an activity has been detected in many Gram-negative and Gram-positive species, there are only a few enzymes known to have this specificity. One of them might be encoded by the \(\text{dacB} \) gene of \textit{Lactococcus lactis} as, unlike the wild type, a \(\text{dacB} \) mutant lacks tripeptides in its peptidoglycan and has an increased percentage of tetrapeptides (Courtin et al., 2006). Like several of the \(\text{LD}\)-specific endopeptidases, \(\text{DacB} \) has sequence similarity to the \(\text{VanY} \text{D-Ala-D-Ala carboxypeptidase} \) but the enzyme has not yet been characterized. LdcA from \textit{E. coli} is a cytoplasmic \(\text{LD}\)-carboxypeptidase involved in peptidoglycan recycling and is essential at the onset of the stationary phase of growth (Templin et al., 1999).

The substrates of LdcA are free tetrapeptides \((\text{L-Ala-D-Glu(}\gamma\text{-meso-A2pm-D-Ala}) \) or tetrapeptides linked to MurNAc-GlcNAc-MurNAc or UDP-MurNAc, but not peptidoglycan sacculi or cross-linked fragments. The crystal structure of the homologous \(\text{LD}\)-carboxypeptidase from \textit{Pseudomonas aeruginosa} has been solved (Korza & Bochtler, 2005) (Fig. 4). The enzyme has a two-domain architecture with a conserved catalytic Ser–\(\text{His}–\text{Asp} \) triad. This triad is very rare among the known serine peptidases, most of which contain a Ser–\(\text{His}–\text{Asp} \) catalytic triad Ser115–His285–Glu217. (b) \(\text{LD}\)-Endopeptidase MepA

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Structures of penicillin-insensitive \(\omega\)-peptidases. (a) \(\omega\)-carboxypeptidase LdcA (\textit{Pseudomonas aeruginosa}), PDB code 1ZRS, with the catalytic triad Ser115–His285–Glu217. (b) \(\omega\)-Endopeptidase MepA (\textit{E. coli}), PDB code 1U10, with the catalytic zinc ion represented as a blue sphere and its three ligands (His113–Asp120–His211) with the two putative general base residues His206 and His209 represented as sticks.}
\end{figure}
tridy and are evolutionarily unrelated to LdcA (Korza & Bochtler, 2005). Usually, α-carboxypeptidases belonging to type-5 PBPs are the most abundant low-MW PBPs. Three crystal structures of type-5 PBPs are available, PBP5 from *E. coli* (Davies et al., 2001; Nicholas et al., 2003) (Fig. 3), PBP3 from *Streptococcus pneumoniae* (Morlot et al., 2005) and PBP4 from *S. aureus* (http://www.pdb.org/pdb/explore.do?structureId=1TVF). They are bimodular enzymes with a penicillin-binding domain of classical topology and a β-strand-rich C-terminal domain that was shown in *E. coli* to be essential for the correct functioning of PBP5 (Nelson & Young, 2001). This terminal domain is characterized by an amphipathic helix at its end, which is responsible for anchoring the enzyme to the cytoplasmic membrane. Truncated PBP5 lacking the C-terminal domain is soluble (like PBP7) and its overproduction leads to cell lysis (Nelson & Young, 2001). Structures of α-carboxypeptidases are reviewed in more detail in another article in this issue (Sauvage et al., 2008).

### N-Acetyl-β-d-muramidas

*N*-acyl-β-β-d-muramidas (termed *N*-acetylmuramidas in this review) are ubiquitous enzymes cleaving the β1,4-glycosidic bond between MurNAc and GlcNAc residues in peptidoglycan. This bond can be cleaved in two different ways (Fig. 2): lysozymes hydrolyse the glycosidic bond, resulting in a product with a terminal reducing MurNAc residue. In contrast, lytic transglycosylases cleave the glycosidic bond with a concomitant intramolecular transglycosylation reaction, resulting in the formation of the 1,6-anhydro ring at the MurNAc residue of the product.

### Lysozymes

Lysozymes are produced in phages, bacteria, fungi, vertebrates and invertebrates, and they are among the most and best-studied enzymes in biology and biochemistry (Jolles, 1996). There are nine classes of proteins with a proven or a predicted ‘lysozyme-like’ fold (Pei & Grishin, 2005). Although all of them cleave β1,4-glycosidic bonds, some of these enzymes hydrolyse chitin [β1,4(GlcNAc)-polymer] or chitosan (partly deacetylated chitin), or are lytic transglycosylases. Four classes contain members with proven hydrolytic activity against peptidoglycan. The prototypes of these four classes are hen egg-white lysozyme (HEWL), goose egg-white lysozyme (GEWL), bacteriophage T4 lysozyme (T4L) and *Chalaropsis* lysozyme. Possibly HEWL, GEWL and phage T4L are the result of divergent evolution from a common ancestor. Despite the fact that they do not share any statistically significant sequence identity, their three-dimensional structures show some intriguing, albeit distant, similarities (Strynadka & James, 1996). The active site is located in a crevice between two domains, which are connected by a long α-helix. A glutamic acid residue, proposed to be essential for general acid catalysis, is located at the C-terminal end of an α-helix in the N-terminal half of each of the proteins. In contrast, the structure of cellosyl, which belongs to the *Chalaropsis* type of lysozymes, comprises a single domain, with the shape of a flattened ellipsoid. Its β/α-barrel fold is unrelated to the tertiary structures of HEWL, GEWL and T4L (Rau et al., 2001).

As compared with other peptidoglycan hydrolases, there are relatively few well-characterized bacterial lysozymes with an experimentally proven specificity (Höltje, 1996b). Two autolytic lysozymes have been identified in *Enterococcus faecium* ATCC9790 (now *Enterococcus hirae* ATCC9790) (Kawamura & Shockman, 1983; Barrett et al., 1984). One of them, termed SF muramidase, has been shown to be an exoenzyme processively degrading glycan strands from their GlcNAc end. In contrast, pesticin is an endo-specific *N*-acytelmuramidase like HEWL (Vollmer et al., 1997). Pesticin is encoded by the *pst* gene, which is tandemly arranged with its immunity gene *pin* on a small *Yersinia pestis* virulence plasmid pPCP1. Such a gene arrangement is typical for bacteriocin (colicin)-encoding plasmids. Another bacterial lysozyme, cellosyl, is produced by *Streptomyces coelicolor* and belongs to the class of *Chalaropsis* lysozymes. Unlike HEWL and T4L, cellosyl exhibits a β1,4-N,6-O-diacytelmuramidase activity and is able to degrade O-acetylated peptidoglycan present in *S. aureus* and other pathogens. Cellosyl is also active on peptidoglycan-carrying (*N*-deacytelated) glucosamine and/or muramic acid residues in their glycan strands (Vollmer, 2007). Next to cellosyl, the autolysin LytC from *S. pneumoniae* is another bacterial *N*-acetylmuramidase belonging to the class of *Chalaropsis* lysozymes (Garcia et al., 1999a). Like other pneumococcal peptidoglycan hydrolases, LytC contains a choline-binding module mediating its attachment to cell wall teichoic acid, which is essential for its activity. The structures of the catalytic and choline-binding domains of LytC have been modelled (Monterroso et al., 2005).

### Lytic transglycosylases

Lytic transglycosylases cleave the β1,4-glycosidic bond between MurNAc and GlcNAc with the concomitant formation of a 1,6-anhydro ring at the MurNAc residue (Höltje et al., 1975). The proposed intramolecular transglycosylation reaction involves a single catalytic acidic residue, often...
glutamate, which donates its proton to the glycosidic oxygen between MurNac and GlcNac (Thunnissen et al., 1994). The resulting oxocarbonium presumably is stabilized by the N-acetamido group at position 2 of MurNac under formation of an intermediate oxazoline ring (Scheurwater et al., 2007). Deprotonation of the hydroxyl group at C-6 of MurNac by the catalytic glutamate allows nucleophilic attack at C1 to form the 1,6-anhydro ring and resolve the oxazoline intermediate. This mechanism is in accordance with the observed inhibition of the membrane-bound lytic transglycosylase MltB from P. aeruginosa by N-acetylglucosamine thiazoline, which is a structural analogue of the oxazoline intermediate (Reid et al., 2004).

Many catalytic domains of lytic transglycosylases, for example that of the soluble lytic transglycosylase Slt70 from E. coli, possess a similar fold as GEWL, which also has an acid glutamate residue in the active site (Thunnissen et al., 1995a). However, there are prominent structural differences between GEWL and lytic transglycosylases. The environment of the catalytic glutamate residue is much more hydrophobic in lytic transglycosylases, which also lack the second catalytic residue (an aspartate) present in lysozymes, explaining the different reaction mechanisms (hydrolysis vs. intramolecular ring formation) of both types of enzymes. Also, the substrate specificities of lysozymes and lytic transglycosylases differ. Lysozymes are endo-N-acetylmuramidases whereas most of the characterized lytic transglycosylases exhibit exo-lytic activity releasing 1,6-anhydroMurNac containing disaccharide peptide units from one of the glycan strand ends. This is consistent with crystal structures showing differences in the arrangements of the substrate-binding pockets. Whereas lysozymes accommodate a hexasaccharide in the substrate-binding site, different lytic transglycosylases have binding sites for three to six N-acetylamino sugar residues. In the case of Slt70, exoenzymatic activity is also determined by the overall doughnut shape of the enzyme (Thunnissen et al., 1995a). Furthermore, N-acetylmuramidases differ in their substrate specificities with respect to the requirement of peptidoglycan substitution for activity. For example, Slt70 and bacteriophage T4 lysozymes only cleave peptidoglycan containing the peptides linked to the glycan strands, and they are inactive against unsubstituted glycan strands (lacking the peptides). On the other hand, the membrane-bound lytic transglycosylase MltA from E. coli and HEWL can cleave equally well peptide-substituted and unsubstituted glycan strands (Romeis et al., 1993; Ursinus & Holtje, 1994). These differences in substrate requirement indicate the presence of binding sites for peptides in some but not all N-acetylmuramidases.

Lytic transglycosylases have been grouped into four families according to their amino acid sequences and the presence of conserved sequence motifs (Blackburn & Clarke, 2001). Family 1 was divided into five subfamilies (termed 1A–1E). Family 4 contains primarily lytic transglycosylases from bacteriophages. With the exception of a family 4 enzyme, E. coli has one member of each (sub-)family of lytic transglycosylases: Slt70 (family 1A), MltC (family 1B), EmTA (family 1C), MltD (family 1D), YfHD (family 1E), MltA (family 2) and MltB (family 3). All these enzymes either have a peri- plasmic localization (Slh70) or are lipoproteins attached to the inner leaflet of the outer membrane (all Mlts and EmTA). Proteolytic degradation of membrane-bound MltB yields the soluble Slh35. One of the lytic transglycosylases of E. coli, EmTA, shows endo-specific activity (Kraft et al., 1998). The crystal structures of the following lytic transglycosylases have been solved: E. coli Slh70 alone (Thunnissen et al., 1994) and with bound 1,6-anhydromuropeptide (van Asselt et al., 1999a) or bound inhibitor bulgecin A (Thunnissen et al., 1995b) (Fig. 5); E. coli MltA alone (van Straaten et al., 2005) or with bound chitohexaose (van Straaten et al., 2007) (Fig. 5); Neisseria gonorrhoeae MltA (Powell et al., 2006); E. coli Slh35 alone (van Asselt et al., 1999b) and in complex with two different peptidoglycan fragments or bulgecin A (van Asselt et al., 2000) (Fig. 5); and the phage λ lytic transglycosylase with bound hexa-2N-acetylchitohexaose (Leung et al., 2001).

N-Acetyl-β-D-glucosaminidases

Endo-N-acetyl-β-D-glucosaminidases (termed ‘N-acetylglucosaminidases’ in this review) are widespread in bacteria. They hydrolyse the glycosidic bond between N-acetyl-β-D-glucosamine residues and adjacent monosaccharides in different oligosaccharide substrates including peptidoglycan, chitin and N-glycans (Karamanos, 1997). Most of these enzymes have a catalytic domain belonging to the protein family (Pfam) 01832. N-acetylglucosaminidases cleaving in peptidoglycan often have one or several CBDs (e.g. the LysM domain, see ‘CBDs associated with peptidoglycan hydro- lases’). Lactococcus lactis has three known (AcmA, AcmB and AcmC) and one hypothetical (AcmD) N-acetylglucosaminidase, two of which (AcmA and AcmD) having three LysM peptidoglycan-binding domains and one (AcmB) having another, putative CBD (Huard et al., 2003, 2004; Steen et al., 2005). Interestingly, the presence of three LysM domains was shown to be optimal for the activity of AcmA because protein variants with less or more LysM domains were less active (Steen et al., 2005). Similarly, the N-acetylglucosaminidase AtlA from Enterococcus faecalis has six LysM domains required for optimal enzymatic activity (Eckert et al., 2006), whereas LyrT8 from S. pneumoniae has 18 imperfect repeats mediating its binding to teichoic acid phosphocholine residues (De Las Rivas et al., 2002). Other recently identified and characterized peptidoglycan N-acetylglucosaminidases are AtlA from S. aureus (a bifunctional enzyme also containing an amidase domain) (Oshida et al., 1995;
Biswa et al., 2006), Acd from Clostridium difficile (Dhalluin et al., 2005) and LytD and LytG from B. subtilis (Margot et al., 1994; Rashid et al., 1995; Horsburgh et al., 2003b).

In contrast to the endo-N-acetylglucosaminidases mentioned above, which hydrolyse glycosidic bonds in high-MW peptidoglycan, the cytoplasmic N-acetylglucosaminidase NagZ from E. coli utilizes a disaccharide substrate, GlcNAc-MurNAc-L-Ala-D-iGlu, which is an intracellular intermediate in the peptidoglycan-recycling pathway (see 'Cytoplasmic hydrolases for peptidoglycan recycling and induction of β-lactamases') (Vötsc & Templin, 2000). The crystal structure of NagZ from Vibrio cholerae has been solved recently with a bound small-molecule inhibitor mimicking the putative transition state of the catalytic reaction (Stubbs et al., 2007).

**CBDs associated with peptidoglycan hydrolases**

Hydrolytic enzymes degrading a high-MW substrate (such as cellulose or chitin) often have a substrate-binding domain, whose presence greatly enhances enzymatic activity (Gilbert, 2007). The same is true for many peptidoglycan hydrolases. At least seven different proven or hypothetical CBDs are reported in the literature: (1) the cysteine and histidine-dependent amidohydrolases/peptidases (CHAP)
domain (Bateman & Rawlings, 2003); (2) the GW domain for noncovalent attachment to lipoteichoic acid (Scott & Barnett, 2006); (3) the SH3 domain (Whisstock & Lesk, 1999); (4) the PlyPSA CBD (Korn dorfer et al., 2006); (5) the FtsN-peptidoglycan-binding domain (Ursinus et al., 2004; Yang et al., 2004); (6) the choline-binding domain (Fernandez-Torner o et al., 2002); and (7) the LysM CBD (Bateman & Bycroft, 2000). The structures of the last four CBDS are known and they can be fused to cell wall hydrolase domains of different specificity.

For example, the autolytic amidase (Ami) in L. monocytogenes is bound to cell wall lipoteichoic acid via a domain containing GW modules (Milohanic et al., 2001). The GW domain also has a role in interaction with host cells and is present in peptidoglycan hydrolases from other species.

The LysM–CBD has been described initially for the E. hirae extracellular muramidase 2 and consists of about 40 amino acid residues (Joris et al., 1992). This domain has been found in over 1500 proteins including many peptidoglycan hydrolases from all types of bacteria (Desvaux et al., 2006). LysM motifs are located generally at one end of a protein and vary in number from one to six. They bind directly to peptidoglycan and the exact number of motifs affects the efficiency of substrate binding as well as function (Steen et al., 2005). It is also present in many other bacterial proteins involved in pathogenicity such as staphylococcal IgG-binding proteins and E. coli intimin (Bateman & Bycroft, 2000). LysM-CBDS are also found in eukaryotic proteins. For example, in Medicago truncatula, a small legume, specific recognition of Sinorhizobium meliloti and its nodulation (Nod) factors requires the Nod factor perception gene (NFP), which encodes a putative receptor in which the extracellular sensor domain contains three tandem LysM CBDS (Mulder et al., 2006). The structure of the LysM–CBD of E. coli membrane-bound lytic peptidoglycan transglycosylase D (MltD) has been elucidated. The LysM-CBD consists of a ββαβ secondary structure with the two helices packing on the same side (Bateman & Bycroft, 2000).

A choline-binding domain is present in several proteins from a range of species to anchor proteins to the choline residues present in the wall-associated polymers lipoteichoic and teichoic acid (Desvaux et al., 2006). The structure of the choline-binding domain of S. pneumoniae LytA has been solved and the molecular basis for interaction has been determined (Fernandez-Tornero et al., 2001).

Several peptidoglycan hydrolases contain direct repeat domains (often three) unrelated to the above-mentioned CBDS and required for cell wall binding (Margot & Karamata, 1992; Baba & Schneewind, 1998). In the case of Atl from S. aureus, the repeats target the various processed forms of the enzyme to the septum by binding to an unknown receptor (Baba & Schneewind, 1998; Biswas et al., 2006).

### Physiological functions of extracytoplasmic peptidoglycan hydrolases

Extracytoplasmic bacterial peptidoglycan hydrolases have a variety of physiological functions for the producing cell and for the cell population (Table 1). In the following sections, the various functions of peptidoglycan hydrolases are listed and examples for each function are included.

### Regulation of cell wall growth by Dd-carboxypeptidases

Dd-carboxypeptidases remove terminal D-alanine residues at position 5 of pentapeptides in the peptidoglycan to form tetrapeptides; this alters the substrate properties of the peptide. While pentapeptides can act as donors and acceptors in transpeptidation (cross-linking) reactions, the tetrapeptides can only serve as acceptors. Apparently, the controlled enlargement of the sacculus requires the trimming of an excess of pentapeptides to tetrapeptides by Dd-carboxypeptidases in some but not all bacteria. For example, S. pneumoniae mutants lacking the Dd-carboxypeptidase PBP3 form aberrant septa and have a thickened cell wall (Schuster et al., 1990; Morlot et al., 2004), and E. coli mutants lacking the Dd-carboxypeptidase PBP5 and additional PBPs may have bended or even branched cell shapes (Denome et al., 1999; Nelson & Young, 2001), indicating that these enzymes are required for correct positioning of the division plane and for correct cell shape. PBP5 (dacA) is the major Dd-carboxypeptidase found in vegetative cells of B subtilis (Lawrence & Strominger, 1970). PBP5 has no role in spore peptidoglycan synthesis but two other Dd-carboxypeptidases, PBP5* (dacB) and DacF (dacF), function in regulating the degree of cross-linking of spore peptidoglycan (Popham et al., 1999). DacB is expressed only in the mother cell compartment of the developing sporangium whereas dacF is expressed in the forespore compartment and they can act differently on the nascent spore peptidoglycan (Popham et al., 1999). The proportion of pentapeptides was largely increased in a dacA (PBP5) mutant of B. subtilis (Atrih et al., 1999). In L. monocytogenes, cells lacking PBP5 displayed an irregular morphology and shape (Guinane et al., 2006) and a thicker cell wall (Korsak et al., 2005a). Moreover, the ratio of pentapeptides to tripeptides was increased in cells lacking PBP5. Other species such as Caulobacter crescentus appear to lack Dd-carboxypeptidase activity and have a high content of pentapeptides in their peptidoglycan (Markiewicz et al., 1983).

### Enlargement of the peptidoglycan sacculus

The peptidoglycan sacculus is a giant bag-shaped and net-like molecule. In order to expand such a network, new...
subunits have to be inserted by peptidoglycan synthases. It is impossible to envision a mechanism of expansion of such a net that does not include the rupture of covalent bonds by hydrolases (Weidel & Pelzer, 1964; Shockman & Höltje, 1994). Thus, deletion of peptidoglycan hydrolases genes required for this process should result in growth arrest due to failure to expand the sacculus. So far, there is no example of such a phenotype of a single or a multiple deletion (or depletion) mutant of any species. This is probably due to the occurrence of multiple hydrolases in most species examined, making it difficult if not impossible to delete all corresponding genes. For example, E. coli has as many as 12 known peptidoglycan hydrolases with a periplasmic location (Völlmer & Bertsche, 2007). The following observations provide indirect evidence for the participation of hydrolases in cell wall growth and for a link between peptidoglycan synthesis and hydrolysis during growth.

There is no doubt that hydrolases are active during wall growth in cell elongation because there is a release of peptidoglycan fragments from the wall during growth, a process termed peptidoglycan turnover in both Gram-negative (Goodell & Schwarz, 1983, 1985; Goodell, 1985) and Gram-positive species (Pooley, 1976; de Boer et al., 1998, 1999). Escherichia coli loses about 40% of the existing peptidoglycan in one generation, and the structure of the turnover products implies that they are products of lytic transglycosylases, endopeptidases and amidases. Furthermore, the amount of turnover products is reduced in a peptidoglycan hydrolase mutant of E. coli (Kraft et al., 1999). It has been proposed by Höltje that synthetic and hydrolytic reactions

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*Only examples are given, see text for references.*
are coordinated temporarily and spatially for a safe enlargement of the sacculus by the formation of multi-enzyme complexes combining different murein synthases and hydrolases. This would ensure that the hydrolases are active only at sites of new synthesis (Höltje, 1996a,c, 1998). A block in peptidoglycan biosynthesis, either at early steps of precursor synthesis or the late periplasmic steps (e.g., inhibition of the PBPs), results in an uncontrolled activity of hydrolases (autolysins) and cell lysis. This observation is consistent with multi-enzyme complexes, which are fully active in the hydrolytic subcomplex but are (partly) blocked in their synthetic subcomplex. This view has been backed up by indirect genetic evidence. Cells lyse if catalytically inactive variants of the synthase PBP1B (harbouring single amino acid exchanges) are overproduced in wild-type cells, but not in mutants lacking the activities of the lytic transglycosylases (Meisel et al., 2003). It was proposed that PBP1B is active in a complex with lytic transglycosylase(s), and that the overproduced inactive enzyme replaced active PBP1B from the complexes, resulting in lysis due to the activity of lytic transglycosylases. Indeed, affinity chromatography experiments with immobilized murein enzymes identified several direct and/or indirect interactions between lytic transglycosylases and peptidoglycan synthases. Immobilized MltA retains the murein synthases PBP1B, PBP1C, PBP2 and PBP3 as well as non-PBP proteins (Vollmer et al., 1999). One of these non-PBP-proteins, MipA, was identified as a structural protein mediating the interaction between MltA and PBP1B. Immobilized Slt70 retains the synthases PBP1B, PBP1C, PBP2, PBP3 as well as the hydrolase PBP7/8 from crude membrane extract, whereas immobilized MltB specifically binds PBP1B, PBP1C and PBP3 (Romeis & Höltje, 1994b; von Rechenberg et al., 1996).

Rod-shaped Gram-positive bacteria grow according to an inside-to-outside growth mechanism (Pooley & Shockman, 1970; Archibald, 1976; Koch & Doyle, 1985). Accordingly, new peptidoglycan is attached underneath the existing wall. Autolysins cleave in the stress-bearing layer(s), which results in stretching the new material that will eventually bear the stress. The outermost wall layers are degraded and released into the growth medium as turnover material. Cell wall turnover has been studied extensively in B. subtilis (Hughes et al., 1970; Pooley, 1976). Importantly, there is a correlation between the growth rate and the rate of cell wall turnover (de Boer et al., 1981, 1982; Cheung et al., 1983), indicating that the hydrolases are more active at high growth rates. Because of the requirement to cleave covalent bonds to allow cell wall enlargement, it has been speculated that hydrolases are pacemakers for cell growth (Höltje, 1995). A direct interaction between peptidoglycan synthases and hydrolases, as has been observed in E. coli, appears to be impossible in Gram-positive species, because they are physically separated. The synthases form the inner, new layers whereas the hydrolases act, well apart from the synthases, on the outer layers. In most rod-shaped species, including E. coli and B. subtilis, peptidoglycan synthesis is controlled by the actin-like, cytoplasmic MreB proteins. These cytoskeletal elements form helical filaments at the cytoplasmic membrane and direct peptidoglycan synthesis presumably by localizing the cell wall synthesis complexes (Carballido-Lopez & Errington, 2003; Daniel & Errington, 2003). In B. subtilis, the peptidoglycan hydrolase LytE has been shown to interact with the MreB-homologue MreBH, which is required for correct localization of LytE on the cylindrical part of the cell wall (Carballido-Lopez et al., 2006). Consequently, lytE and mreBH mutants show similar morphological, cell wall-related defects. Thus, peptidoglycan synthases and hydrolases required for cell elongation might be colocalized in rod-shaped, Gram-positive bacteria by the intracellular cytoskeleton.

**Separation of daughter cells**

It is well established that peptidoglycan hydrolases are required to cleave the septum to allow separation of daughter cells. In most Gram-positive species, a septum is formed and then cleaved along a middle line. In contrast, in most Gram-negative species, cleavage of the septum occurs almost simultaneously with cell division, leading to the typical V-shaped constriction rather than septation. There are many examples of mutants lacking one or several hydrolases and having a defect in cell separation. For example, a coccoid S. aureus atl mutant forms clusters of nonseparated cells (Takahashi et al., 2002). A lytB mutant of S. pneumoniae forms long chains of non-separated cells that could be dispersed by the addition of purified LytB enzyme (Garcia et al., 1999b). As expected, both hydrolases, Atl and LytB, localized at the site of cell division (Yamada et al., 1996; Garcia et al., 1999b).

Multiple hydrolases cleave the septum during cell division in E. coli. Cells of the amiC deletion mutant separate poorly during division, and a fraction of 20–30% of the cell population exists as a chain of three to six unseparated cells. In these chains, the cells are separated by a septum made of peptidoglycan sandwiched between the cytoplasmic membranes of neighbouring cells. If amiA is inactivated, only 5–10% of the population grows as chains of three to four cells. Deletion of amiB produces no chaining phenotype at all. Deletion of three amidase genes (encoding for AmiA, AmiB and AmiC) or of six lytic transglycosylase genes (Slt70, MltA, MltB, MltC, MltD and MepA) results in the formation of chains with up to 20 cells per chain (Heidrich et al., 2001, 2002). Also the endopeptidases PBP4, PBP7 and MepA contribute to cleavage of the septum. If deletions in amidases are combined with deletions in lytic
transglycosylases or endopeptidases, the chains become very long and may contain up to 100 cells (Heidrich et al., 2002; Priyadarshini et al., 2006). These cells have additional defects including the presence of aberrant intracellular membrane structures and increased permeability of the outer membrane, making them susceptible to antibiotics that do not kill wild-type *E. coli* cells (e.g. vancomycin) (Heidrich et al., 2002; Korsak et al., 2005b). In *N. gonorrhoeae*, deletion of a single amidase gene, *amiC*, results in a defect in cell separation and in the formation of cell clumps (Garcia & Dillard, 2006).

**Peptidoglycan hydrolases active in sporulation and germination**

Under conditions of nutrient deprivation, *Bacillus* and *Clostridium* species undergo a differentiation process that leads to the production of highly resistant endospores (Fig. 6) (Errington, 2003; Piggot & Hilbert, 2004). Endospores have two juxtaposed peptidoglycan layers of different structure: an inner primordial cell wall, which will become the basis of the new vegetative cell wall after germination, and an outer spore-specific cortex with a unique structure (Atrih et al., 1996). The spore retains an alert sensory mechanism that can respond to specific germinants, which eventually leads to outgrowth to form a new vegetative cell (Atrih et al., 1998). The morphological changes during differentiation indicate a number of stages where peptidoglycan hydrolases are involved (Fig. 6; Foster & Popham, 2002).

**Digestion of the asymmetric septum**

The first stage at which autolysin activity appears necessary for sporulation is hydrolysis of the asymmetric septum to allow prespore engulfment. In *B. subtilis*, this involves a complex of two autolysins, SpoIID and SpoIIP, both of which are required for septal hydrolysis and have peptidoglycan hydrolase activity (Abanes-De Mello et al., 2002; Chastanet & Losick, 2007). SpoIID is homologous to LytB, the modifier protein that enhances the activity of the major vegetative cell amidase LytC (Abanes-De Mello et al., 2002). SpoIIP has similarity to the catalytic domain of CwlV (an amidase of *P. polymyxa*) (Chastanet & Losick, 2007). SpoIID and SpoIIP form a complex with SpoIIM (an integral membrane protein). SpoIIM localizes the two autolysins at the septal membrane. Digestion of the asymmetric septal peptidoglycan requires both the activities (Chastanet & Losick, 2007).

**Cortex maturation**

The spore cortex, which surrounds the spore core, has a unique and highly conserved structure, which is essential for the maintenance of heat resistance and dormancy. The basic structure of cortex peptidoglycan is similar to that of *B. subtilis* vegetative peptidoglycan. The cortical peptidoglycan is subject to sporulation-specific modifications either during or after its assembly. About 50% of the disaccharide subunits in the cortex have the muramic acid δ-lactam...
structure, which is found only in bacterial endospores. The \(\beta\)-lactams are not randomly distributed, but occur predominantly at every alternate disaccharide. The formation of \(\beta\)-lactam residues requires two proteins acting in concert (Gilmore et al., 2004). CwlD is a sporation-specific amidase, which cleaves off the peptide side-chain (Atrih et al., 1996; Popham et al., 1996). Then, PdaA deacetylates the N-acetyl muramic acid product and forms the lactam ring via a cyclization event (Fukushima et al., 2002; Gilmore et al., 2004).

The spore cortex is the least cross-linked of any peptidoglycan, at only 2.9% of muramic acid residues (Atrih et al., 1996), compared with that seen in vegetative cell walls, glycan, at only 2.9% of muramic acid residues (Atrih et al., 2004). The cortex is expressed only late in sporulation (Foster, 1992; Kuroda et al., 1999). As the cortex is essential for the maintenance of spore dormancy, its specific hydrolysis is a requirement for dormancy, its specific hydrolysis is a requirement for spore water content or cortex cross-linking.

Mother cell lysis

The final morphologically observable event during sporulation is hydrolysis of the mother cell wall peptidoglycan to release the mature endospore. Two autolysins are present in large amounts at the time of mother cell lysis: the major vegetative amidase, LytC, and a 30 kDa amidase, CwlC, which is expressed only late in sporulation (Foster, 1992; Kuroda et al., 1993; Smith & Foster, 1995). Single inactivations of either lytC or cwlC have no effect on mother cell lysis. However, a mutant inactivated in both genes is blocked in mother cell lysis, and so the two amidases have a mutually compensatory role in the hydrolysis of the mother cell wall (Smith & Foster, 1995). A third sporulation-specific amidase (CwlH) has also been shown to be required for mother cell lysis in which it acts in a compensatory fashion with CwlC (Nugroho et al., 1999). It is possible that, as for asymmetric septum hydrolysis, a complex of autolytic amidases act in concert to degrade the mother cell wall and allow endospore release.

Germination

As the cortex is essential for the maintenance of spore dormancy, its specific hydrolysis is a requirement for germination, allowing subsequent uptake of water, core expansion and outgrowth (Foster & Johnstone, 1990). Biochemical analysis of spore peptidoglycan dynamics during germination of B. subtilis shows that a probable stereochemical change, a likely epimerase, occurs at or near the muramic \(\beta\)-lactam (Atrih et al., 1998). Accompanying this, hydrolytic events, primarily lytic transglycosylase and to a lesser extent glucosaminidase, cause release of the cortical peptidoglycan as muropeptide fragments (Atrih et al., 1998). The muramic \(\beta\)-lactam is crucial for substrate recognition by the germination-specific lytic enzymes (GSLEs), as a cwlD mutant that lacks the \(\beta\)-lactam, produces resistant endospores, which cannot outgrow as the cortex is refractile to GSLE activity (Sekiguchi et al., 1995; Atrih et al., 1996, 1998; Popham et al., 1996). The primordial cell wall, which does not have \(\beta\)-lactam, is not hydrolysed and remains to develop into the cell wall of the outgrowing spore.

Bacillus subtilis has 2 GSLEs (SleB and CwlJ) required for the later stages of germination, as a double mutant is totally blocked in cortex hydrolysis and outgrowth (Ishikawa et al., 1998). Loss of SleB results in slow germination and coincides with a total lack of lytic transglycosylase activity during germination (Boland et al., 2000). SleB is present in the spore integument and inner membrane and its presence in the dormant spore depends on a cotranscribed gene, ypeB (Moriyama et al., 1999; Chirakkal et al., 2002). YpeB may act as an anchor to give correct SleB localization. How SleB is activated as part of the germination process is unknown. CwlJ is present in the spore coats and can be activated during germination by the release of dipicolinate, as an early event during spore germination (Paidhungat et al., 2001; Bagyan & Setlow, 2002). The rationale for the possession of apparently functionally redundant GSLEs is justified by their different activity optima and resistance to deleterious treatments, thus allowing the germination mechanism to be active under a broad range of conditions and after environmental assault (Atrih & Foster, 2001).

Assembly of large trans-envelope structures

The pores in the stretched peptidoglycan of E. coli are quite uniform in size and they allow penetration of globular proteins of up to 50–100 kDa (Demchick & Koch, 1996; Vazquez-Laslop et al., 2001). However, these pores appear to be too small for the assembly of large trans-envelope complexes such as secretion systems of types II, III and IV, type IV pili and flagella. The large gene clusters encoding for proteins for the regulation and assembly of these structures typically contain a peptidoglycan hydrolase gene, most often encoding for a lytic transglycosylase (Koraimann, 2003). These are specialized hydrolases required to enlarge gaps locally in the peptidoglycan to allow penetration of trans-envelope structures. These hydrolases include, for example, the VirB1 enzyme encoded from the plasmid Ti of Agrobacterium tumefaciens, which is required for a type IV secretion system (DNA transport into plant cells) (Zahrl et al., 2005),

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the PilT enzyme involved in type IV pilus synthesis in E. coli (Koraimann, 2003) or the Flg enzyme for flagellum synthesis in E. coli (Nambu et al., 1999).

**Resuscitation of dormant cells**

More than 60 bacterial species including many pathogens were reported to be able to enter a viable-but-nonculturable (VBNC) state in which they do not divide and have very low metabolic activity (Oliver, 2005). For example, Micrococcus luteus can enter such a dormant state by prolonged stationary-phase culturing. Resting cells of M. luteus can be stimulated to divide (resuscitated) by addition of the RpfA protein (rfp, resuscitation-promoting factor) to the culture medium (Mukamolova et al., 1998). Mycobacterium tuberculosis has five RpfA-like proteins, which presumably have redundant growth-promoting activities on resting cells. Single and double deletions of Rpf proteins have no effect but different combinations of triple deletions generated mutants that cannot resuscitate spontaneously in culture (Downing et al., 2005). Strikingly, Rpf proteins have weak similarity in amino acid sequence to lysozymes and lytic transglycosylases. This has been confirmed by analysing the crystal structure of RpfB from M. tuberculosis and by demonstration of a muramidase activity of Rpf from M. luteus (Mukamolova et al., 2006). Either these peptidoglycan hydrolases are required for daughter cell separation during the first cell division following the resting period, or the released peptidoglycan fragments serve as signalling molecules initiating regrowth of the cells (Keep et al., 2006).

**Lysis of prey cells and nonimmune cells**

The Gram-negative myxobacteria are predatory bacteria living in the soil (Reichenbach, 1999). They have several unusual features making them unique among prokaryotes living in the soil (Reichenbach, 1999). They have several peculiarities that make them unique among prokaryotes. The myxobacteria are predatory bacteria that feed on other microorganisms. They have a unique method of lysis that is different from the Gram-negative bacteria. The myxobacteria feed on other microorganisms and use a lytic enzyme to kill them. This lytic enzyme is called lysozyme and it is produced by the myxobacteria. The lysozyme is released into the environment and is able to break down the cell wall of the prey cell, allowing the myxobacteria to feed on the contents of the cell.

**Developmental lysis**

Upon starvation, myxobacteria aggregate to form a fruiting body with myxospores in a highly ordered developmental process. Developmental lysis in M. xanthus describes the autolysis of most of the cells – 80–90% of the initial population – within the first 72 h of development of the fruiting body. It is assumed that lysis of these cells is a regulated phenomenon of ‘programmed cell death’ and is of benefit for the subset of cells determined to form the myxospores. According to this view, the nutrients released by the lysed cells would feed the sporulating cells (Rosenbluh & Rosenberg, 1993; Lewis, 2000). Developmental lysis is induced by auticides, a mixture of at least five lipid compounds and by glucosamine. The mechanism leading from induction to lysis as well as the mechanism for regulation and activation of the autolysins are as yet unknown.

A similar lysis phenomenon has been observed in the early sporulation stage of B. subtilis although this species does not form a fruiting body. A fraction of starved cells secrete factors to kill and lyse sibling cells that have not developed immunity to these compounds. This behaviour has been termed ‘cannibalism’ and it has been speculated that its biological function for the producing cell is to get nutrients to delay or avoid sporulation (Gonzalez-Pastor et al., 2003; Ellermeier et al., 2006).

**Autolysis in genetic transformation (alloysis)**

In S. pneumoniae, competence for genetic transformation is induced in the early exponential phase by a quorum-sensing mechanism involving a peptide hormone, the competence-stimulating factor. Cells in the competent state are capable of taking up exogenous DNA, which might then be integrated into the chromosome. Competent cells are more
prone to autolysed (Seto & Tomasz, 1975), and the two autolysins of this bacterium, LytA and LytC, contribute to the release of DNA in a culture of competent cells (Moscoco & Claverys, 2004). Results from micro-array experiments indicate that there is a connection between competence, stress responses and autolysis (Dagkessamanskaia et al., 2004), and competent cells were shown to be able to lyse competence-deficient cells during cocultivation (Steinmoen et al., 2003). A careful examination of the genetic requirements for the lysis of noncompetent cells revealed a new phenomenon that was termed ‘alloysis’ (Guiral et al., 2005). Accordingly, competent cells are able to trigger lysis of cocultivated noncompetent cells, resulting in the release of DNA from the lysed cells. The mechanism involves the two-peptide bacteriocin CibAB, its immunity factor CibC, the two autolysins LytA and LytC and the putative murein hydrolase CbpD. A model was proposed, according to which the bacteriocin (and the immunity factor) is produced by competent cells. It is active and kills only noncompetent cells, because they do not produce the immunity factor. The bacteriocin activates the autolysins, which disrupt the cell wall of the predated, noncompetent cells resulting in lysis. Induced lysis of genetically identical cells has been termed ‘fratricide’. The biological role of fratricide for S. pneumoniae in the context of its particular ecological niche (the nasopharynx of humans) and its population dynamics is yet to be defined. Cannibalism (see ‘Developmental lysis’) and fratricide in bacterial populations are discussed in a recent review (Claverys & Havarstein, 2007).

**Biofilm formation**

Most bacteria in their natural environment are associated with solid surfaces, be this simply attached or in more complex bacterial community structures as biofilms (Costerton et al., 1987). Biofilms are of particular importance for pathogens when associated with host tissues or indwelling medical devices as the bacteria are often refractory to antibiotic treatment. *Staphylococcus epidermidis* is an opportunistic pathogen very frequently found on infected catheters as a biofilm. The major autolytic Atl is required for biofilm formation on polymer surfaces as an atlE mutant is biofilm negative and cannot mediate the initial attachment process (Heilmann et al., 1997). AtlE is also able to bind human ligands and so may be involved in host–pathogen interaction. AtlE is the equivalent of At from *S. aureus*, and loss of either results in a cell division defect and cluster formation (Foster, 1995; Oshida et al., 1995; Heilmann et al., 1997).

In *L. lactis*, the major autolysin AcmA is also required for biofilm formation whereby an acmA mutant forms non-adherent long chains of cells (Mercier et al., 2002). Addition of lysozyme disrupts the chains and results in a simultaneous increase in adherence. The role of the peptidoglycan hydrolase activity may be to alter surface charge or to expose other cellular adhesins. Also in *Streptococcus mutans*, the autolysin AtlA is necessary for the production of effective biofilms (Ahn & Burne, 2006).

**Host–pathogen interaction**

Recently, autolysins have been implicated in the interaction between several pathogens and their hosts. This occurs due to their ability to release bioactive products, act as adhesins, affect the mammalian immune system, be required for biofilm formation, allowing pathogen separation and dispersal and likely many other mechanisms. Such functions are covered in detail in recent review articles (Dziarski, 2003; Dziarski & Gupta, 2005, 2006; Mengin-Lecreulx & Lemaitre, 2005).

**Regulation of extracytoplasmic hydrolase activity**

Peptidoglycan hydrolases are potentially lethal enzymes able, in many cases, to degrade the cell wall of producing organisms. Thus, they must be highly regulated to prevent adventitious cell lysis. This regulation occurs at multiple levels from the transcriptional to the posttranslational. For many bacteria, cell lysis is a natural part of the lifecycle and in some scenarios it is in fact advantageous to remove damaged cells from the population (Rice & Bayles, 2003).

**Genetic regulation**

Control of production of peptidoglycan hydrolases is important but in many cases is overridden by biochemical regulation as many of the enzymes are found to be associated with their substrate but controlled at the level of activity. Most of the enzymes involved in sporulation and germination are controlled at the transcriptional level by the sporulation-specific cascade of sigma factors that allows their production only in particular sporangial compartments and at particular times for their exact functions. During vegetative growth, the control of peptidoglycan hydrolase expression has been particularly illuminating in the determination of the role of some enzymes (with a few particular examples discussed below).

**Coregulation with flagellation and motility**

In *B. subtilis*, most autolysin activity is produced as the cells enter the stationary phase (Foster, 1992). The two major enzymes are LytC and LytD, an amidase and N-acetylglucosaminidase, respectively (Margot & Karamata, 1992; Margot et al., 1994). Their production coincides with major
morphological and physiological changes to the cells. The short chains break up into single cells, they become motile, secrete large amounts of extracellular enzymes and develop natural competence. Many of these processes are coregulated by the alternative sigma factor SigD (Serizawa et al., 2004). SigD controls expression of the ‘flagellar, chemotaxis and motility regulon’.

Transcription of the lytABC operon proceeds from two promoters. One is controlled by σD, which accounts for 70–90% of the transcription during growth (Lazarevic et al., 1992; Kuroda & Sekiguchi, 1993). Ninety-five percent of lytD transcription is controlled by σD (Margot et al., 1994). The physiological rationale for the coregulation of the autolysins with motility lies in the phenotype of their respective mutants. The enzymes are required for dechaining as cells linked together are unable to chemotax effectively, the so-called pushmi-pullyu effect (Blackman et al., 1998).

In *S. aureus*, perturbation of cell wall synthesis, either by the addition of a cell wall-targeting antibiotic at sub-inhibitory concentration or by lowering the transcription of a cell wall synthesis gene, results in the formation of a weaker cell wall (with less cross-links) that is more rapidly digested *in vitro* by peptidoglycan hydrolases. Surprisingly, cells grown under this condition show reduced autolysis rates, which was found to be due to reduced expression of autolysin genes. Thus, there appears to be an as yet unknown regulatory mechanism by which expression of autolysin genes is down-regulated when cell wall synthesis is disturbed (Antignac et al., 2007).

Elusive essential enzyme

For many years, it has been proposed that hydrolysis of existing bonds within peptidoglycan is necessary to allow wall expansion, cell growth and division (Shockman & Höltje, 1994). In a number of Gram-positive species, a homologous essential sensor regulator has been characterized (originally called YycGF) (Fabret & Hoch, 1998; Martin et al., 1999; Clausen et al., 2003). Analysis of its regulon has revealed the control of a number of putative and proven peptidoglycan hydrolases (Howell et al., 2003; Dubrac & Msadek, 2004). This alluded to their individual or combined essentiality for growth. Only in *S. pneumoniae* has an essential enzyme been identified. PcsB is essential and controlled by YycGF (VikSR) (Ng et al., 2003, 2004). The hydrolytic bond specificity of PcsB is unknown but it contains a CHAP domain present in several hydrolases that may have amidase function (Kajimura et al., 2005). Depletion of PcsB results in deregulated cell wall synthesis and bacteriostasis (Ng et al., 2004). Whether the essentiality of PcsB is due directly to its hydrolytic properties, and the molecular basis for its role, are currently unknown.

Programmed cell death

The LytSR sensor regulator of *S. aureus* has been proposed to respond to a decline in proton motive force across the cell membrane (Patton et al., 2006). LytSR controls the expression of LrgAB, which encode a postulated antiholin system (Brunskill & Bayles, 1996). LrgAB may interact with their cognate holins (CidAB) and prevent cell lysis (Rice & Bayles, 2003). Under conditions of stress (such as the presence of penicillin), CidAB may collapse the PMF and allow access of autolysins to their substrate, or deregulation of activity, resulting in cellular lysis (Rice & Bayles, 2003).

Posttranslational regulation

Subcellular localization of peptidoglycan hydrolases

In many species, defects in autolysin production lead to aberrant cell separation after septation. Thus, hydrolysis of the nascent septa is required for cell division. In *S. aureus*, loss of the major autolysin, Atl, results in cell cluster formation and microscopy studies have revealed a ring of Atl at the septum (Yamada et al., 1996). Amidase activity is required for septal hydrolysis in *E. coli* and the periplasmic AmiC has been found to localize specifically at the septum (Heidrich et al., 2001; Bernhardt & de Boer, 2003). In contrast, AmiA is found throughout the periplasm. More recently, the putative periplasmic endopeptidase EnvC has also been found to be septum associated (Bernhardt & De Boer, 2004). The Mre proteins of *B. subtilis* are required for maintenance of rod morphology and form a helical pattern along the cylindrical axis (Jones et al., 2001). MreBH interacts with the autolysin LytE (a putative endopeptidase), leading to its localization in a helical pattern and so coordinates cell wall hydrolysis with cylinder elongation (Carballido-Lopez et al., 2006). The subcellular localization of autolysins must reflect specific targeting mechanisms.

Peptidoglycan hydrolase targeting

Secretion of peptidoglycan hydrolases occurs primarily by the well-established Sec dependent pathway but can also use the TAT system and in some cases there is no signal sequence (Smith & Foster, 1995; Bernhardt & de Boer, 2003). The interaction between the enzymes and the cell wall is crucial for their activity. Several conserved mechanisms have been found for a number of enzymes. Covalent binding of autolysins to their substrate, which may render the enzymes inactive until they are further processed, is rare but has been reported for the GSLE of *B. megaterium* (Foster & Johnstone, 1988) and a sortase-linked enzyme from *S. mutans* (Catt & Gregory, 2005). Much more common are
the use of conserved, often repeated, motifs or domains for ionic attachment to peptidoglycan or other cell wall components (see ‘CBDs associated with peptidoglycan hydrolases’). Many enzymes have an overall basic charge at neutral pH, which may enhance their binding to negatively charged cell wall components.

In *E. coli*, five lytic transglycosylases are made as lipoproteins and they are targeted to the outer membrane (Lommatzsch *et al.*, 1997). It has been postulated that this specific orientation in respect of the peptidoglycan opposite to the biosynthetic machinery would allow cell wall expansion and growth (Lommatzsch *et al.*, 1997).

**Peptidoglycan hydrolase processing**

Proteolytic processing of enzymes is common not only in their activation but also in their stability in response to a changing environment. The major autolysin of *S. aureus* called Atl is produced as a 138 kDa proenzyme that undergoes proteolytic processing to generate the two extracellular lytic enzymes found in the supernatant of *S. aureus* cultures identified as the 51 kDa endo-β-acetylglucosaminidase and a 62 kDa *N*-acetylMuramyl-1-alanine amidase (Foster, 1995; Oshida *et al.*, 1995). Pro-Atl, amidase and glucosaminidase display peptidoglycan hydrolase activity in zymogram analysis. The signal peptide of 138 kDa pro-Atl is first cleaved, followed by further processing through intermediate forms of 115 and 85 kDa to result in the mature active 51 and 62 kDa cell surface-associated, and secreted, glucosaminidase and amidase (Komatsuzawa *et al.*, 1997). Proteases have also been postulated to maintain a balance between production and degradation of peptidoglycan hydrolases during growth of *B. subtilis* to maintain appropriate activity levels (Yamamoto *et al.*, 2003). Proteolytic cleavage of the lipoprotein precursor of a membrane-bound lytic transglycosylase of *E. coli*, MltB, results in the formation of a soluble form, the soluble lytic transglycosylase (Slt35). It is not known whether this ‘periplasmic solubilization’ of MltB has a particular physiological role (Ehlert *et al.*, 1995).

**Control of activity**

The activity of peptidoglycan hydrolases must be highly regulated and a number of mechanisms have been proposed. These include physical or chemical alterations to the peptidoglycan substrate including conformational changes (Koch *et al.*, 1985) and covalent modification (Clarke & Dupont, 1992; Atrih *et al.*, 1998; Bera *et al.*, 2005). The presence, absence or exact chemical nature of other wall polymers has also been shown to affect activity. Teichoic acids modify the activity of the amidase of *B. subtilis* (Herbold & Glaser, 1975a, b), and other amphiphiles such as cardiolipin and lipoteichoic acids, etc. are known to inhibit peptidoglycan hydrolase activities of many Gram-positive species (Höltje & Tomasz, 1975; Cleveland *et al.*, 1976; Fischer *et al.*, 1981). Growth at a low pH has also been shown to inhibit bacteriolysis (Goodell *et al.*, 1976). This may be due to a change in the wall ionic environment affecting autolysin activity (Cheung & Freese, 1985) as the energized state of the membrane of *B. subtilis* is also important in regulation (Jolliffe *et al.*, 1981; Calamita *et al.*, 2001). It has also been proposed that bonds near the surface of Gram-positive cell walls, and the bonds at the middle of a division septum, are under stress and may be recognized by ‘smart autolysins’ (Koch *et al.*, 1985; Koch, 1990).

For the enlargement of the thin sacculus of Gram-negative bacteria, Höltje has proposed that different peptidoglycan synthases and hydrolases form multi-enzyme complexes. This would allow the enlargement of the peptidoglycan layer by a defined mechanism and would restrict the activities of the potentially dangerous hydrolases to the sites of synthesis. As indicated above (‘Enlargement of the peptidoglycan sacculus’), there is evidence for the existence of protein–protein interactions between lytic transglycosylases and peptidoglycan synthases.

**Cytoplasmic hydrolases for peptidoglycan recycling and induction of β-lactamase**

The activity of exocytoplasmic peptidoglycan results in a massive peptidoglycan turnover in *E. coli* (Vollmer & Höltje, 2001). In one generation, about 40–50% of the peptidoglycan is released from the sacculus by lytic transglycosylases, *ββ*-endopeptidases and amidases (Goodell & Schwarz, 1983, 1985; Goodell, 1985). The muropeptides released contain a 1,6-anhydroMurNAc residue and are subject to an efficient recycling pathway (Park, 1995, 1996). In *E. coli* and presumably in most other Gram-negative bacteria, the 1,6-anhydro-disaccharide-peptides are transported into the cytoplasm by the AmpG permease. Several cytoplasmic enzymes are involved in recycling of these peptidoglycan fragments. The AmpD amidase liberates the tripeptide 1-Ala-γ-D-Glu-*meso*-A2pm (Höltje *et al.*, 1994; Jacobs *et al.*, 1995, 1997), which can then be attached to UDP-MurNAc by the Mpl ligase (Mengin-Lecreulx *et al.*, 1996). Interestingly, AmpD has a strict specificity for 1,6-anhydroMurNAc-containing muropeptides and therefore does not hydrolyse the murein precursor molecules such as UDP-MurNAc-pentapeptide also present in the cytoplasm. The tripeptide can be further processed by the γ-D-carboxypeptidase MpaA, which releases the terminal *meso*-A2pm residue (Uehara & Park, 2003). The resulting 1-Ala-γ-D-Glu dipeptide can be degraded to the γ-amino acids. For this, the epimerase YcjI forms 1-Ala-γ-L-Glu, which is then hydrolysed by the peptidase PepD (Schmidt *et al.*, 2001; Uehara *et al.*, 2005).
Peptidoglycan hydrolases

The GlcNAc-1,6-anhydroMurNAc disaccharide is hydrolysed by the N-acetylglicosaminidase NagZ to GlcNAc and 1,6-anhydroMurNAc (Vötsch & Templin, 2000). GlcNAc is converted in two steps by NagK and NagA to GlcN-6-phosphate, which is the substrate for both the peptidoglycan precursor UDP-GlcNAc and the central metabolite fructose-6-phosphate (Uehara & Park, 2004). 1,6-AnhydroMurNAc is converted by AnmK to MurNAc-6-phosphate, which is hydrolysed by the etherase MurQ to GlcNAc-6-phosphate (Schmidt et al., 2001; Uehara et al., 2005, 2006).

Interestingly, in Gram-negative bacteria having an inducible AmpC β-lactamase, the induction mechanism is directly linked to the relative cytoplasmic levels of 1,6-anhydromuramylpeptides and UDP-MurNAc-pentapeptide, both of which can bind to the transcriptional activator AmpR (Jacobs et al., 1997). Inactivation of the ampD gene leads to accumulation of 1,6-anhydromuramylpeptides and therefore to constitutive β-lactamase overexpression in C. freundii (Jacobs et al., 1997).

Concluding remarks

Since the discovery of lysozyme by Alexander Fleming, enormous knowledge has been gathered on the vast group of peptidoglycan hydrolases identified so far. Today, a comprehensive collection of all relevant literature on peptidoglycan hydrolases would fill many bookshelves, and it is impossible to summarize all the data in a single article. In this review, an overview has been presented on the known specificities of these hydrolases and a few examples of structures has been shown that have been solved recently. The knowledge is still incomplete, and it is likely that new enzyme families of peptidoglycan hydrolases will be discovered in the future. We have also exemplified the different roles of these enzymes in the physiology of a bacterial cell as well as at the bacterial population level. Perhaps one of the most important aspects is the regulation of the activity of peptidoglycan hydrolases. In many cases, knowledge of this is very limited. For example, all 12 known periplasmic peptidoglycan hydrolases from E. coli have the potential to degrade the sacculus and cause lysis of the cell. The question as to why this does not occur under normal growth conditions still remains to be answered. We will understand the roles of peptidoglycan hydrolases only if we know how their activities are regulated in the cell.

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