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A Method Revealing Bacterial Cell-wall Architecture by Time-dependent Isotope Labeling and Quantitative Liquid Chromatography/Mass Spectrometry

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Abstract

The molecular details of the biosynthesis and resulting architecture of the bacterial cell wall remain unclear but are essential to understanding the activity of glycopeptide antibiotics, the recognition of pathogens by hosts, and the processes of bacterial growth and division. Here we report a new strategy to elucidate bacterial cell-wall architecture based on time-dependent isotope labeling of bacterial cells quantified by liquid chromatography/accurate mass measurement mass spectrometry. The results allow us to track the fate of cell-wall precursors (which contain the vancomycin-binding site) in *Enterococcus faecium*, a leading antibiotic-resistant pathogen. By comparing isotopic enrichments of post-insertionally modified cell-wall precursors, we find that tripeptides and species without Asx bridges are specific to mature cell wall. Additionally, we find that the sequence of cell-wall maturation varies throughout a cell cycle. We suggest that actively dividing *E. faecium* cells have three zones of unique peptidoglycan processing. Our results reveal new organizational characteristics of the bacterial cell wall that are important to understanding tertiary structure and designing novel drugs for antibiotic-resistant pathogens.

Keywords

pulse labeling; pulse chase; LC/MS; MS; *Enterococcus faecium*; VSE; VRE

Enterococcus faecium is a prevalent nosocomial pathogen that continues to present treatment challenges in clinics around the world given the increasing frequency of isolates resistant to vancomycin.[1–3] Vancomycin, the so-called drug of last resort for antibiotic-resistant infections, targets bacterial peptidoglycan (also known as murein), the main structural component of the cell wall. Specifically, vancomycin forms complexes with the *D*-Ala-*D*-Ala carboxyl termini of peptidoglycan repeat units (Figure 1, right) outside of the cytoplasmic membrane, thereby interfering with the enzymatic processes involved in cell-wall biosynthesis.[4] The molecular details regarding the topological location, organization, and post-insertional metabolism of the vancomycin-binding sites within *E. faecium* peptidoglycan are important to drug design and understanding the architecture of the cell wall, but they remain unclear.

Interestingly, recent solid-state NMR[5] and LC/MS[6] experiments show that very few peptidoglycan stems in *E. faecium* have *D*-Ala-*D*-Ala vancomycin-binding sites, even in

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susceptible organisms. It was speculated that the peptidoglycan stems that are not cross-linked near the membrane exoface are cleaved into tripeptides in mature enterococcal peptidoglycan.[5, 6] In addition to tripeptide stems, other peptidoglycan structural variations are found in the *E. faecium* cell wall and are associated with murein maturation.[6] Among these are the formation of tetrapeptides, *O*-acetylation at C6 of *N*-acetylmuramic acid, succinimidation of cross-bridges, and amidation of aspartic acid, all of which ultimately comprise the complex three-dimensional structure of the bacterial cell wall.

The tertiary structure of the cell wall is a product of a sophisticated biosynthesis intimately involved with bacterial cell growth and division. Fluorescence studies provided insights into these processes and showed that a conserved set of proteins is recruited to the septum during cell division.[7, 8] Given the multitude of proteins involved and their likely redundant functions, however, understanding the organization and development of the peptidoglycan by protein localization methods alone is challenging. Here, we describe an analytical method based on mass spectrometric quantitation of isotopically labeled cells that is complementary to direct imaging for elucidating bacterial cell-wall morphogenesis.

Similar to bacterial labeling strategies successfully used by others before,[9–11] in our experiments growing bacterial cells are extracted from media containing only natural-abundance isotopes and reintroduced to defined media with a unique, isotopically enriched amino acid. Although this method is sometimes described as a pulse-labeling approach, we would like to emphasize that some of our data is from cells harvested after relatively long periods of growth in labeled media. Moreover, for most of the data presented, we did not reintroduce labeled cells back into media containing only natural-abundance isotopes. Thus, the labeling approach in our experiments may be better classified as a “step” function.

After the cells are introduced to enriched media, they continue to grow and the number of newly synthesized structures containing the heavy isotope is quantifiable by mass spectrometry. Similar analyses of time-dependent isotope incorporation with mass spectrometry were used previously in kinetics studies.[12–15] In this work we applied mass spectrometry-based quantitation of isotopically labeled cells, with an on-line LC/MS analysis that we recently reported for the identification of intact structural variations,[6] to investigate the sequence in which *E. faecium* peptidoglycan subunits containing vancomycin-binding sites are post-insertionally modified in cell-wall growth and maturation. Muropeptide profiles taken with respect to the length of heavy-isotope exposure provide snapshots of the progression of peptidoglycan species throughout the cell cycle.

Mass-spectrometry based quantitation of cells isotopically labeled as a function of time is particularly attractive for investigating the *E. faecium* cell wall given the significant number of peptidoglycan structural variations and the low frequency of cross-linked species larger than dimers. Furthermore, the biosynthesis of *E. faecium* peptidoglycan is of great clinical interest as it is likely to be an important target in the development and understanding of novel antibiotics. New glycopeptides effective against vancomycin-resistant *E. faecium*, for example, are suspected to interfere with the orientation of newly synthesized peptidoglycan such that nascent murein chains are not aligned to form the cross-links that are required for structural integrity.[16, 17] Using post-insertional peptidoglycan modifications as an intrinsic probe to track murein growth and assembly, we explore opposing models of glycan architecture and propose a molecular-level schematic for peptidoglycan morphogenesis throughout an enterococcal cell cycle that is consistent with current views of cell division.

Experimental Section

Starting cultures of *E. faecium* (ATTC 49624) were prepared by inoculating brain-heart infusion media with a single colony. Cultures were incubated overnight at 37 °C, but not

aerated. Experimental samples were prepared by inoculating brain-heart infusion (BHI) media with the overnight starter culture (1% final volume).

Enterococcal standard media (ESM) was made as described before,[5] with the pH adjusted to 7.0 prior to sterile filtration and natural abundance *L*-lysine replaced with uniformly labeled *L*-[$^{13}C_6$, $^{15}N_2$]lysine. The cells were extracted from BHI media when the absorbance at 660 nm was approximately 0.4 by centrifugation at 10,000 × g. Cells were then resuspended in the prewarmed ESM containing *L*-[$^{13}C_6$, $^{15}N_2$]lysine and incubated (Figure 1, pulse). *L*-lysine is a good choice because it has high enrichment efficiency in *E. faecium* and is not readily metabolized.[5] We use the expression "pulse labeling" in this text to be consistent with established convention,[18] but the term "step" may an equally appropriate description of the labeling strategy.

Cells were harvested at various time points (see Figure 1, harvest points) by centrifugation at 10,000 × g and boiled for 5 min in 40 mM triethanolamine hydrochloride (pH 7.0) to quench biological activity before being frozen and lyophilized. Isotopic enrichment is time independent after cells are harvested and boiled. Enrichment measurements made immediately after sample preparation were within ± 0.5% of the values determined from the same sample 20 days after harvest. We acquired 10 time points to investigate cell-wall development over approximately one enterococcal cell cycle (estimated as 3 h after the pulse for these conditions).

For pulse-chase samples, the cells were removed from the *L*-[$^{13}C_6$, $^{15}N_2$]lysine enriched ESM after a defined time duration (Figure 1, *i*) by centrifugation at 10,000 × g, resuspended in prewarmed BHI, incubated, and harvested and boiled at a later time (Figure 1, *f*). The initial and final time points were chosen such that the cells were chased over the latter half of exponential growth.

Cell-wall isolates were prepared from lyophilized whole cells as detailed before.[19] The isolated cell walls were digested into muropeptides with lysozyme and mutanolysin.[20, 21] All manipulations were performed at neutral pH.

Liquid chromatography/MS was performed by using a PicoView PV-500 (New Objective, Woburn, MA) nanospray stage attached to an LTQ-Orbitrap mass spectrometer (ThermoFisher, San Jose, CA). The high-mass accuracy of the Orbitrap mass specterometer is important for identifying structures of chemically modified species with the least ambiguity. By using accurate-mass measurements, approximately 90% of the peaks in the mass spectra occurring within the range of expected muropeptide retention times were identified and isotopic enrichments were calculated for each.

Muropeptide samples were loaded into an uncoated 75 μm i.d. fused-silica capillary column with a 15 μm picofrit tip (New Objective, Woburn, MA), packed with C18 reverse-phase material (3 μm, 100 A; Phenomenex, Torrance, CA). The column was eluted with a flow rate of 250 nL/min for 10 min with 0.1% (vol/vol) formic acid in water and subsequently with a 60-min linear acetonitrile gradient (0%–40%) with 0.1% formic acid. The components, as they emerged from the column, were sprayed into an LTQ-Orbitrap mass spectrometer. The spray voltage was 2.0 kV. The capillary voltage and temperature were 27 V and 200 °C, respectively. Full mass spectra were recorded in the FT component of the instrument at 100,000 resolving power (at m/z = 400).

Results and Discussion

Muropeptide identification and cross-linking

Unmodified *E. faecium* peptidoglycan-repeat units (Figure 1, right) are characterized by a pentapeptide stem that contains the *D*-Ala-*D*-Ala vancomycin-binding site. The peptidoglycan-repeat units are incorporated into existing cell wall via the polymerization of their disaccharides (transglycosylation) and through cross-linked *D*-aspartic acid or *D*-asparagine bridges (transpeptidation, see Figure S1). Using *N*-acetylmuramidase enzymes, we selectively hydrolyzed the β-1,4 linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine, leaving the cross-links intact. We identified the majority of resulting digested peptidoglycan fragments, generally referred to as muropeptides, with LC/MS accurate-mass measurements as variations of monomers, dimers, and a few trimers. Muropeptides containing the vancomycin-binding site are referred to as pentapeptides. The accurate mass assignments for all the muropeptides identified and evaluated throughout this text are consistent with the proposed structures (Figure S2) and with those reported previously.[6] The percentage of cross-linked enterococcal peptidoglycan was determined from the distribution of monomer, dimer, and trimer species.

The biochemical composition of some cell walls is known to vary with the physiological state of the bacterial organism.[22–24] Most notably, the density of higher cross-linked oligomers increases for some Gram-positive cells in the viable but nonculturable state.[25] Previously, we reported that ~40% of *E. faecium* peptidoglycan stems are cross-linked in a vancomycin-susceptible organism in late-exponential growth.[6] As a first step in understanding the biosynthesis and maturation of the *E. faecium* peptidoglycan, we monitored the distribution of muropeptides in different phases of growth.

Consistent with the results obtained for *Enterococcus faecalis*,[25] we detected no substantive differences in *E. faecium* cell-wall composition as cultures progressed from exponential- to stationary-growth phase. Specifically, the cross-linking remained relatively constant (i.e., variation was less than ±2% with growth time) as illustrated by the proportions of monomers, dimers, and trimers (Figure S3, top). The quantification of each muropeptide species was determined by integrating extracted-ion chromatograms for singly and multiply charged ions. This approach of estimating cross-linking yields results in good agreement with those obtained from solid-state NMR, as we established previously.[6]

To investigate the dynamics of peptidoglycan processing and track the development of specific muropeptides, we removed *E. faecium* cells growing in natural-abundance media and resuspended them in defined media enriched with *L*-[$^{13}C_6$, $^{15}N_2$]lysine, which one may refer to as “the pulse” of our experiments, before harvesting the cells for mass spectrometric analysis. Although the proportion of monomers, dimers, and trimers did not significantly change throughout the *E. faecium* cell cycle, the percentage of these species incorporating one or more *L*-[$^{13}C_6$, $^{15}N_2$]lysine isotopes after the introduction of the label was time-dependent. We calculated the enrichment percentages by considering the proportion of muropeptide structures incorporating *L*-[$^{13}C_6$, $^{15}N_2$]lysine with respect to the sum of those same structures with and without *L*-[$^{13}C_6$, $^{15}N_2$]lysine incorporation. Although quantitative comparisons of peaks in ESI mass spectra are generally unreliable between different species because there is variability in efficiencies for ionization, the approach developed here avoids this problem given that molecular species carrying isotopes do not have inherently different ionization efficiencies.[26, 27]

Our experiments show that monomers are the first muropeptides to incorporate *L*-[$^{13}C_6$, $^{15}N_2$]lysine, followed by dimers and trimers (Figure S3, bottom). After growing cells for 38 min in labeled media, for example, monomers and dimers have 19 and 14% enrichments,

respectively. Moreover, the amount of each labeled species increases at a relatively constant rate over time, after an initial lag phase for dimers and trimers during which muropeptides are cross-linked. Yet, monomers always have the highest percentage of their muropeptides labeled, albeit the difference between monomers and dimers is small because transpeptidation happens early in cell-wall biosynthesis, highlighting an important characteristic of our experiments. At any point after the cells are introduced to isotopically labeled media, muropeptides produced earlier in cell-wall biosynthesis have higher enrichment percentages than those species made later. The error in isotopic enrichment percentages for all values reported in this text was determined to be ± 0.5% from three different trials (see Table 1). For example, after growing for 53 min in isotopically labeled media, the muropeptide with an *m/z* of 938.432 showed isotopic enrichments of 33.0, 33.1, and 33.2% in three independent measurements. Although it is impractical to repeat sample preparations with identical time and growth conditions, multiple sample preparations showed the same isotopic enrichment trends for all species. Specifically, the orders of muropeptides sorted on the basis of increasing enrichments were unchanged.

O-Acetylation is characteristic of mature peptidoglycan

E. faecium has a large number of peptidoglycan structural variations that occur in monomers, dimers, and trimers.[6] A main objective of this work is to determine the order in which these modifications occur. Although the modification sequence could be determined in principle by sorting isotopic enrichments from a single data point by intensity, we analyzed bacterial cells grown in enriched media for 10 different time intervals to elucidate general trends in muropeptide variations and assess activity fluctuations throughout an entire enterococcal cell cycle. Asynchronous cell growth does not pose a problem for the interpretation of our experiments because the sequence of muropeptide structural maturation is the same for all cells, and, thus, the average we detect is representative.

Previous studies suggest that *O*-acetylation of peptidoglycan-repeat units at C6 of *N*-acetylmuramic acid, a post-insertional modification corresponding to a mass increase of 42.011, occurs in mature cell wall.[28, 29] To test further this hypothesis, we determined the enrichment of *O*-acetylated peptidoglycan after an *L*-[$^{13}C_6$, $^{15}N_2$]lysine pulse by considering the percentage of labeling in all muropeptide species with the structural variation. The *O*-acetylation enrichment trends were comparable for all species. Consistent with earlier results from cells in late-exponential growth,[6] some muropeptide species were rarely *O*-acetylated. We observe that the overall enrichment of *O*-acetylated muropeptides is low at early time points after the introduction of the pulse (<15% 1 h after the introduction of the isotopic label, see Figure S4). Only after cells have sufficient time to produce mature cell wall from the *L*-[$^{13}C_6$, $^{15}N_2$]lysine isotopes does the *O*-acetylation enrichment percentage increase. For all time points considered the isotopic enrichment of *O*-acetylated species is less than that of all other muropeptides, excluding only those structures without bridges, consistent with *O*-acetylation being unique to mature cell wall.

O-Acetylation and cross-linking

Although it was proposed that *O*-acetylation is linked to the cross-linking reaction,[30] our results suggest that the two processes are not directly coupled to one another in *E. faecium*. The rate at which dimers incorporate *L*-[$^{13}C_6$, $^{15}N_2$]lysine is nearly as fast as the enrichment rate of the monomers (Figure S3, bottom), indicating that transpeptidation occurs quickly in cell-wall biosynthesis. This interpretation is consistent with the previously described template model of cell-wall assembly where cross-linking occurs between the newest and most recently synthesized glycan chains.[16, 17] It should be noted that a greater difference between the enrichment of monomers and dimers does develop over the time course of the last two data points, introducing the possibility that some secondary cross-linking may occur in regions of

mature cell wall remote from the cytoplasmic membrane. We suggest that *O*-acetylation, having a low-enrichment percentage, is more likely to affect this secondary transpeptidation in *E. faecium*, representing a small fraction of overall cross-links.

Modified penta, tetra, and tripeptides

Other modifications of interest are associated with the peptide stem of the repeat units, more specifically, the three amino-acid residues involved in transpeptidation. When a cross-link forms, the penultimate *D*-Ala serves as a donor forming an amide bond to a *D*-Asp/*D*-Asn (*D*-Asx) bridge and a *D*,*D*-carboxypeptidase removes the terminal *D*-Ala. Studying the isotopic enrichment of monomers and dimers, as described above, provides insight into the dynamics of cross-linking during cell-wall biosynthesis. Here, we want to address modifications removing the terminal *D*-Ala, the penultimate *D*-Ala, or the *D*-Asx bridge that occur independent of transpeptidation. To simplify the analysis, we present results only for monomer species. Modifications on repeat units of dimers and trimers not cross-linked follow similar trends and are not discussed.

When the percentage of isotopic enrichment is considered for all identifiable peptidoglycan species at the last time point (Table 1), the species of highest abundance is that with a *D*-Ala-*D*-Ala terminus and a *D*-Asp bridge (Figure 2, of *m/z* = 1081.491). The result indicates that this muropeptide is the first to be incorporated into the cell wall, in accord with observations made by others.[31]

Interestingly, at the first time point only, the isotopic enrichment of this species is significantly lower than the same species with a *D*-Asn bridge (Figure 2, of *m/z* = 1080.507). Moreover, a similar trend occurs for every muropeptide structure with a bridge. That is, at the first time point, species with amidated bridges (*D*-Asn) have higher enrichments than their hydroxylated analogs (*D*-Asp). The difference is significant and may be due to a difference in cell-wall processing. We infer that at the beginning of cell division, the pentapeptide with a hydroxylated bridge is amidated rapidly; that is, the amidation of *D*-Asp has already occurred before the harvest of our first sample at 11 min. It should be noted that aside from this change between species with amidated and hydroxylated bridges after the first time point, no significant changes in the isotopic enrichment trends of muropeptides occur for increasing times of growth in labeled media.

The muropeptide that is the slowest to become enriched after the introduction of the *L*-[$^{13}C_6$, $^{15}N_2$]lysine pulse is the tripeptide with no bridge (Figure 2, of *m/z* = 824.388). The tetrapeptide with no bridge (of *m/z* = 895.426) is the second lowest muropeptide enriched (Figure 3, bottom), suggesting a new type of structural modification that was not previously reported to our knowledge. These muropeptides without bridges show no detectable isotopic enrichment at the first time point, ruling out the possibility that the species originate as cytoplasmic precursors. The data suggest that in certain regions of the cell wall, a final step in the maturation of the peptidoglycan is removal of the bridge in species that are not cross-linked.

The presence of tetra and tripeptides suggests *D*,*D*-carboxypeptidase and *L*,*D*-carboxypeptidase activity in the cell wall of *E. faecium*. Although it may have been expected that *D*,*D*-carboxypeptidase creates the substrate for *L*,*D*-carboxypeptidase, the data suggest that these two enzymes function independently of one another, at least in species with bridges. The tetrapeptide represented by the *m/z* of 1009.470 ion is enriched less than the tripeptide represented by the ion of *m/z* of 938.432 (Figure 3, middle), both of which are less enriched than the pentapeptide. Although the enrichment differences between the tetra and tripeptides are not large, there is a statistically significant difference between them (see Table 1 for error estimation).

The most likely rationale for the observation is that pentapeptides produce both tripeptides and tetrapeptides independently in separate reactions. The isotopic enrichments of other peptides, the tripeptide represented by the *m/z* 939.416 ion and tetrapeptide of *m/z* 1010.454, are consistent with this hypothesis. They are also enriched in nearly equal abundance (Figure 3, top), suggesting that both *D,D*-carboxypeptidase and *L,D*-carboxypeptidase are targeting the same pentapeptide substrate. For muropeptides without bridges (Figure 3, bottom), however, the tetrapeptide has a higher percentage of species incorporating *L*-[$^{13}C_6$, $^{15}N_2$]lysine than does the tripeptide. This may reflect a different sequence of processing in mature cell wall or be the consequence of greater *D,D*-carboxypeptidase activity in murein without bridges.

Pulse-Chase Experiments

Analysis of samples isotopically labeled as a function of time shows directly that the amount of pentapeptides in mature peptidoglycan is small. If we consider the addition of heavy label to be a pulse, then a pulse-chase experiment would be an experiment where bacterial cells are reintroduced to media containing only natural-abundance isotopes after a duration of growth in enriched media. We conducted such an experiment and it provides additional evidence for the conclusion that the amount of pentapeptides is small in mature cell wall. When the isotopic *L*-[$^{13}C_6$, $^{15}N_2$]lysine pulse is chased into mature cell wall with media containing natural-abundance *L*-lysine, the average percentage of isotopic enrichment for pentapeptides (of *m/z* = 1080.507 and 1081.491) is less than 2%, compared to the 30% value before the introduction of the chase and the 75% value that results from a control culture harvested at the same time as the chase but grown continuously in *L*-[$^{13}C_6$, $^{15}N_2$]lysine enriched media (Figure 4). The pentapeptides must be quickly modified into other peptidoglycan species, being replaced by new repeat units that are not enriched with *L*-[$^{13}C_6$, $^{15}N_2$]lysine. In contrast, there is little difference in the isotopic enrichment of the muropeptide represented by an ion of *m/z* of 824.388 before and after the introduction of the chase. The data support that tripeptides without bridges in mature isotopically labeled peptidoglycan are not yet diluted by species incorporating natural-abundance *L*-lysine over the course of the chase.

Architecture of the glycan chains

The orientation of the glycan chains in bacterial cell walls has been a longstanding controversial issue of investigation in microbiology.[32, 33] Of the three proposals regarding the directionality of the glycan chains, parallel,[23, 34] perpendicular,[35] or randomly aligned[36] with respect to the cytoplasmic membrane, the first model is predominantly accepted. Here we offer enrichment patterns in mass spectra of muropeptide dimers as new evidence for its support.

The constitution of dimers with respect to old and new peptidoglycan subunits is intrinsically different in each model because the variable geometry of the glycan chains differentially restricts feasible cross-links on a steric basis. In the scaffold model where the glycan chains are perpendicular to the cell membrane, new peptidoglycan subunits are added at the ends of the glycan chains in the same plane. Each muropeptide has the ability to form a cross-link with one of four other subunits in the same plane, all of which are newly inserted.[35] Thus, in a scaffold model, both *L*-lysine residues in all dimers would be labeled after the introduction of an isotopic pulse. Our findings are largely inconsistent with this requirement.

At the first time point after the pulse, there are ions representing dimer structures in which only one *L*-[$^{13}C_6$, $^{15}N_2$]lysine is incorporated (Figure 5, top). The number of doubly-labeled dimers increases at intermediate times, but the percentage of structures with only one *L*-[$^{13}C_6$, $^{15}N_2$] lysine remains relatively constant compared with earlier time points (Figure 5, middle). For the last time point analyzed, more than half of the dimers (~60%) contain two *L*-[$^{13}C_6$, $^{15}N_2$] lysine residues, but structures with only one *L*-[$^{13}C_6$, $^{15}N_2$]lysine are still present at the same levels (~10%) as detected previously (Figure 5, bottom).

The data suggest that glycan chains parallel to the membrane are incorporated into existing murein via single-strand addition. We imagine that the first glycan chain to incorporate *L*-[$^{13}C_6$, $^{15}N_2$]lysine forms cross-links with the previously synthesized parallel glycan chain containing only natural-abundance amino acids. Consequently, some dimers assemble with only one *L*-[$^{13}C_6$, $^{15}N_2$]lysine label. Because the level of these dimers containing old and new peptidoglycan subunits remains relatively constant throughout the biosynthesis of the cell wall, the random orientation of glycan chains seems implausible in that singly-labeled dimers would likely be formed after the first time point in this model.

Zones of peptidoglycan processing

Much has been learned in recent years about the processes of bacterial growth and division from fluorescent studies investigating the localization of proteins involved in cell-wall biosynthesis and maturation.[7, 8] Although these studies have undoubtedly provided many insights, the peptidoglycan structural modifications associated with bacterial morphogenesis are still not understood. As a complement to the spatial resolution offered by fluorescent and autoradiogram studies,[9, 37] we tracked the sequence of peptidoglycan modifications occurring throughout the life span of *E. faecium* by comparing the isotopic enrichments for various muropeptide structures. The data show that tri and tetrapeptides are produced from pentapeptide repeat units and that, eventually, the bridges of these species are subsequently cleaved. Combining our temporally resolved data with the spatial data from imaging experiments offers new insights about the types of peptidoglycan processing occurring in different regions of the cell wall.

The time sequence of muropeptide post-insertional modifications is well represented by the order of isotopic enrichments as determined from the last experimental time point (Table 1). First, pentapeptides with hydroxylated bridges (*D*-Asp) are incorporated into existing murein and are subsequently cleaved into either tri or tetrapeptides, without aspartic acid becoming amidated. Next, pentapeptide repeat-units with amidated bridges (*D*-Asn) are produced, presumably from pentapeptides with *D*-Asp bridges, and are then modified into tri and tetrapeptides. Lastly, the *D*-Asp or *D*-Asn of the tetra and tripeptides is cleaved.

It is unlikely that the tri and tetrapeptides first produced from pentapeptides with hydroxylated bridges are subsequently converted back into pentapeptides with amidated bridges. Thus, we suspect that the pentapeptides with amidated bridges represent one spatially distinct region of cell-wall growth in *E. faecium* whereas those with hydroxylated bridges represent another. In the region or zone characterized by pentapeptides with amidated bridges, new pentapeptide repeat units are amidated before being cleaved into tri and tetrapeptides.

Extending this logic, we propose that there are three unique zones of cell-wall maturation in *E. faecium* (Figure 6, top). In zone I, murein repeat units with aspartic-acid bridges are cleaved into tri and tetrapeptides. Bridges are amidated in zone II before being cleaved into tri and tetrapeptides, and in zone III the bridges are completely removed. On the basis of our experiments alone, we cannot spatially organize these zones a priori. Using fluorescent vancomycin, however, others recently demonstrated that peptidoglycan with *D*-Ala-*D*-Ala termini is localized to the septum in dividing *Lactococcus lactis* cells.[38] *L. lactis* is an appropriate model organism because, like *E. faecium*, it is ovoid in shape[39] and also has an active *L,D*-carboxypeptidase involved in peptidoglycan maturation.[40]

The fluorescent vancomycin results are consistent with other fluorescence[41] and ultrastructural[42] studies, substantiating that new cell-wall material is incorporated at the septum in dividing cells. It is widely accepted that cell division is initiated as peptidoglycan at an equatorial ring grows centrally to form a cross wall or septum that ultimately becomes the new halves of progeny cells. That is, progeny cells are composed of half new murein and half old.

It is, therefore, not surprising that *E. faecium* cells that divide in the presence of an isotopic *L*-[$^{13}C_6$, $^{15}N_2$]lysine pulse have approximately half of their mature muropeptides labeled as determined by our experiments. The higher enrichment of other muropeptides suggests that these species are more likely to be found in the newer half of progeny-cell peptidoglycan. We should emphasize, for example, that pentapeptides with hydroxylated bridges have a 90% enrichment at the last experimental time point. Enrichment this large is only possible if the pentapeptides are readily modified into other muropeptides.

Murein biosynthesis and cell division

We hypothesize that cell-wall biosynthesis occurring at the septum is spatially characterized by zone I growth where the bridges are not yet amidated (Figure 6, bottom). Localization studies indicate that, as the cross-wall begins to separate into two halves, peptidoglycan assembly remains active even as the new murein goes from an environment of low to high internal osmotic pressure.[38] We speculate that under these unique conditions *D*-Asp bridges on pentapeptides are amidated before tri and tetrapeptides are formed, typifying zone II growth. Subsequently, as murein biosynthesis becomes decreasingly active, the cell wall goes through its final maturation in zone III.

Surprisingly, in samples analyzed after the shortest time of growth in labeled media, enrichment percentages are characteristic of zone II growth and not that of zone I. This may suggest peripheral cell-wall growth, in zone II where the new wall material is subjected to high internal osmotic pressure, prior to cross-wall formation. The observation is consistent with a recent proposal suggesting that peripheral cell-wall biosynthesis precedes formation of the septum by a small fraction of the doubling time.[43] Possibly related is the identification of isotopically enriched succinimidated muropeptides at the first time point after the pulse. In general, for all time points, the enrichment of succinimides lies in between the labeling percentages of species with aspartic acid and asparagine bridges, raising the possibility that succinimides are intermediate structures in the modification of the bridge as was proposed previously[44] or potentially even precursors for zone II cell-wall biosynthesis.

Conclusions

In summary, mass spectrometry-based quantitation of cells grown in isotopically labeled media as a function of time should become a widely applied technique for tracking chemical transformations in biological systems at the molecular level. Here we describe the approach and show an exciting application to bacterial peptidoglycan, providing new insights in the complexities and dynamics of murein processing. The data reveal the order in which peptidoglycan subunits are post-insertionally modified with varying sequences based on growth phase, and probably topological location. Although vancomycin-binding sites are limited to newly synthesized peptidoglycan in *E. faecium*, our results suggest that structurally modified subunits are important in the development of an osmotically stable and mature cell wall. These modifications may represent important antibiotic targets, even for vancomycin-resistant pathogens, and prove valuable for understanding the recognition of pathogens by host-immune systems and the processes by which bacterial cells grow and divide. Despite the significant changes in cell-wall structure with time and topological location, however, the template model for single-strand addition of glycan chains seems to be generally true for all types of murein assembly. Thus, interfering with template recognition in peptidoglycan biosynthesis continues to present a promising role for antibiotics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

D-Asx, *D*-asparagine or *D*-aspartic acid; ESM, enterococcal standard media; LC/MS, liquid chromatography-mass spectrometry.

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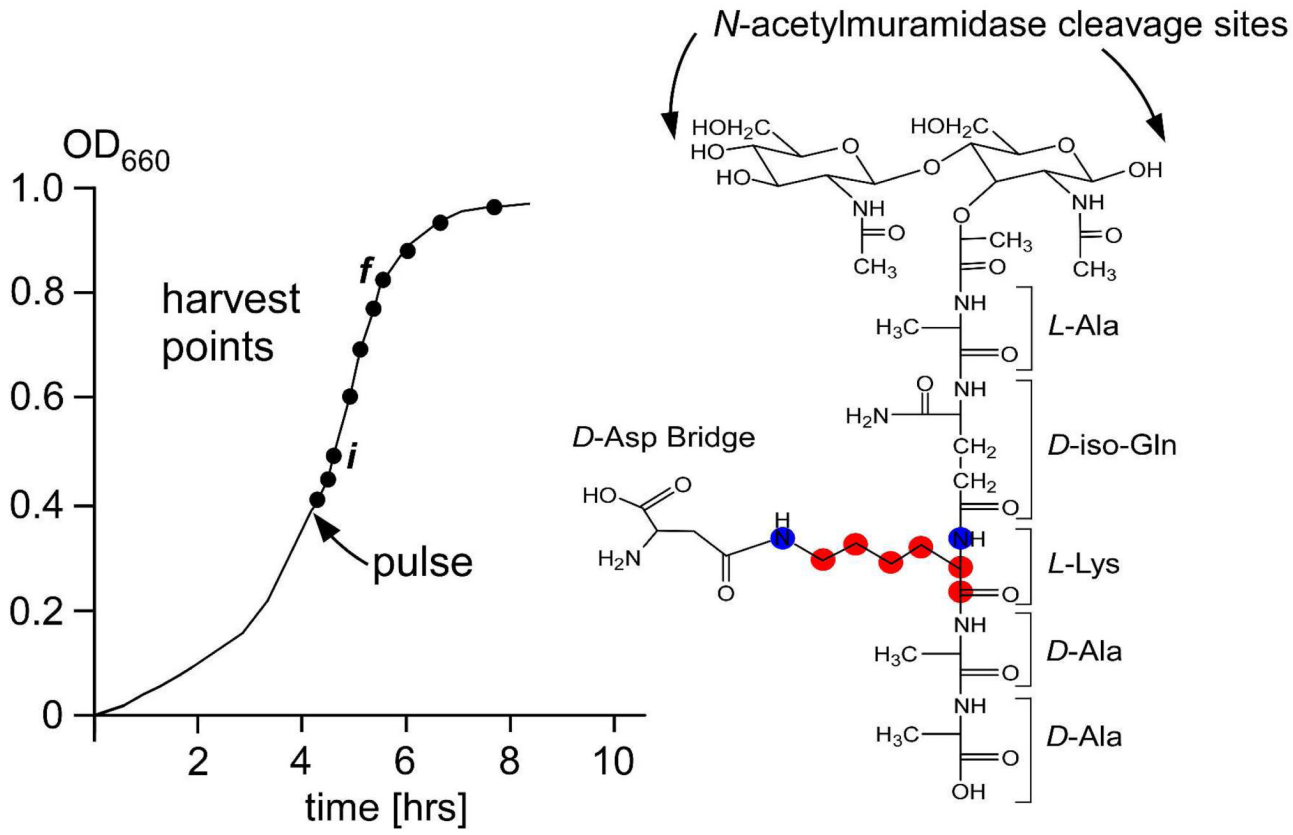

Figure 1.

Labeling Strategy. (Left) Growth of *E. faecium* as measured by optical density (660 nm) as a function of time. The cells were pulsed and harvested at the points indicated. For the pulse-chase experiment, cells were resuspended in media containing natural-abundance isotopes at point *i* and harvested at point *f*. (Right) Chemical structure of *E. faecium* peptidoglycan before modification, highlighting *L*-[$^{13}C_6$, $^{15}N_2$]lysine. The unmodified subunit contains a pentapeptide stem and a *D*-Asp bridge.

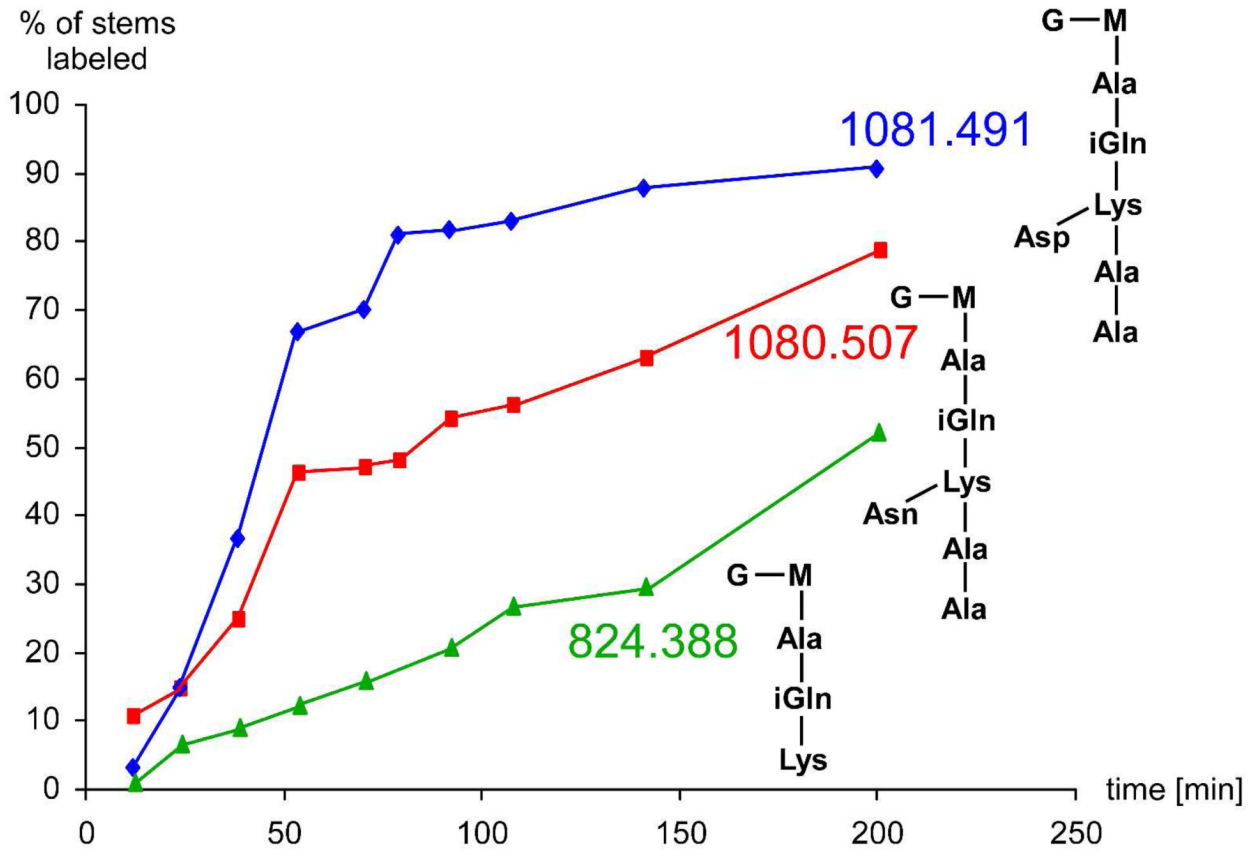

Figure 2.
Plot of the Percentage of Isotopically Enriched Ions with respect to Time After Pulse. (Blue) Ions with an *m/z* of 1081.491. (Red) Ions with an *m/z* of 1080.507. (Green) Ions with an *m/z* of 824.388.

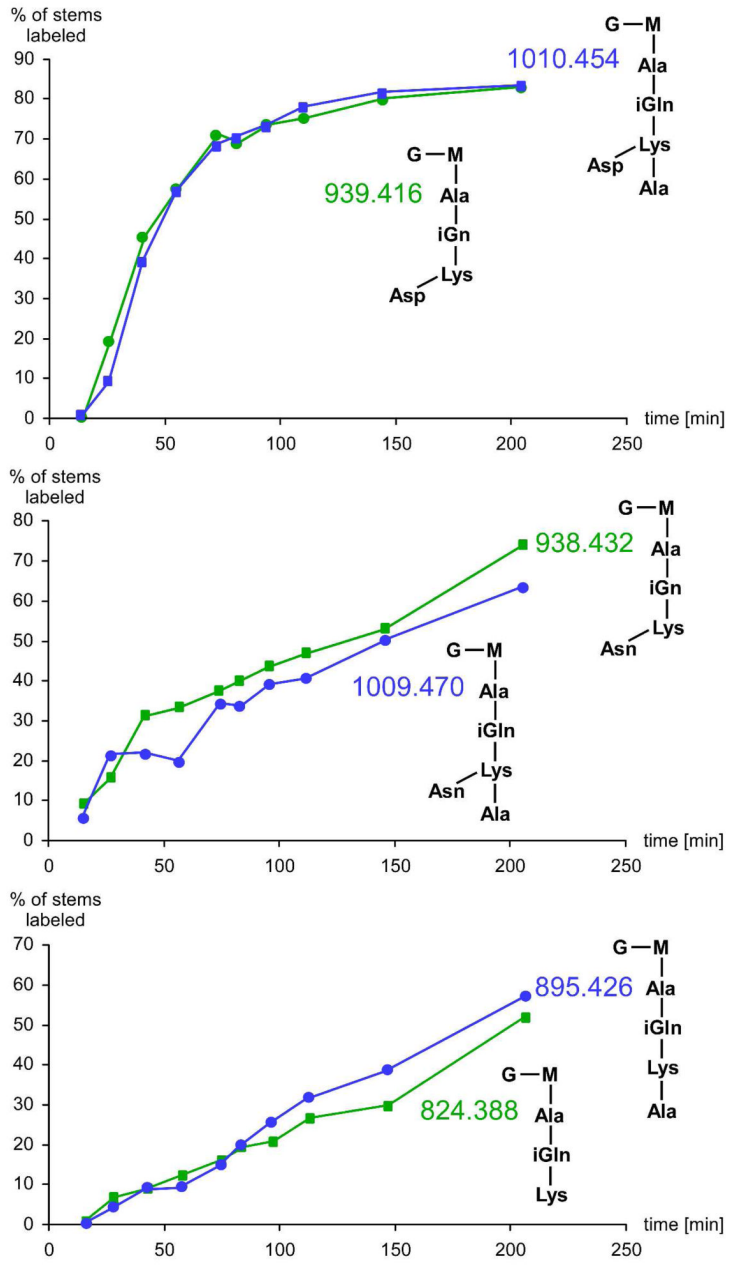

Figure 3.

Plots of the Percentage of Isotopically Enriched Tri (green) and Tetrapeptides (blue) with respect to Time After Pulse. (Top) Ions with an *m/z* of 1010.454 and 939.416, corresponding to muropeptides with an aspartic-acid bridge. (Middle) Ions with an *m/z* of 938.432 and 1009.470, corresponding to muropeptides with an asparagine bridge. (Bottom) Ions with an *m/z* of 895.426 and 824.388, corresponding to muropeptides without bridges.

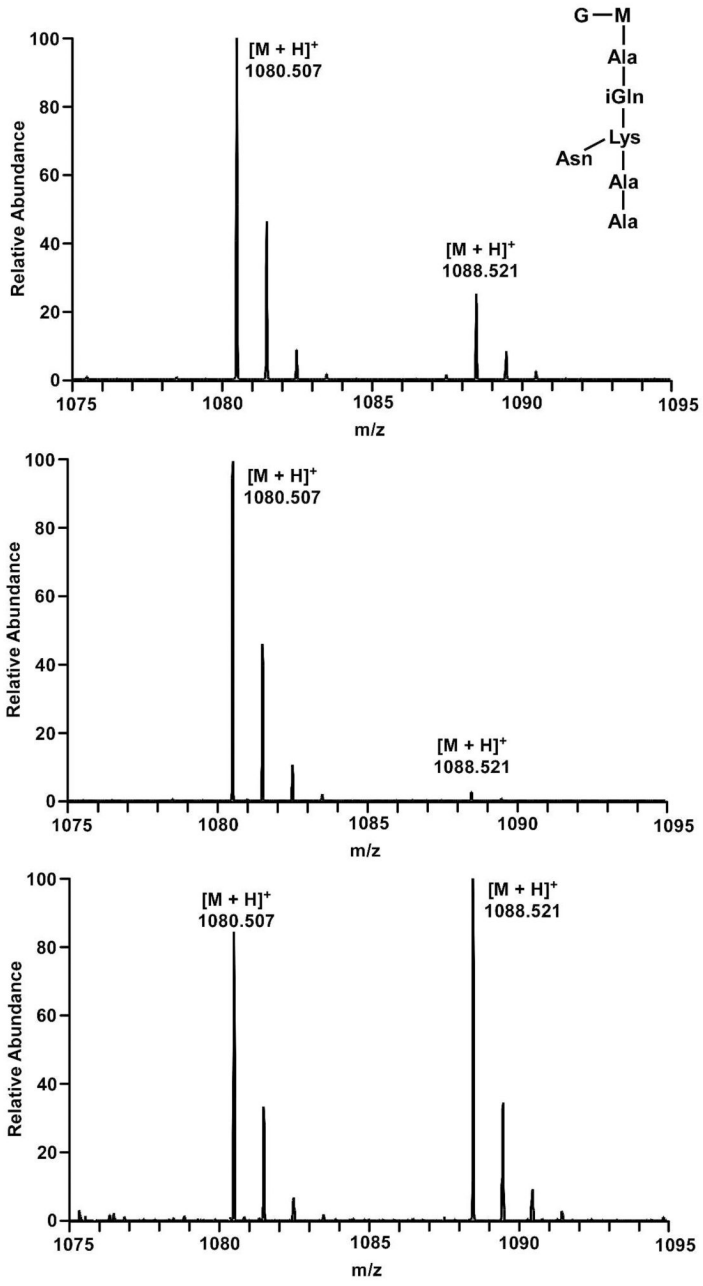

Figure 4.

Mass Spectra for an Ion with an *m/z* of 1080.507 Corresponding to a Pentapeptide with an Asp Bridge. (Top) Cells harvested and analyzed after a 38 min pulse. (Middle) Cells chased for 54 min with media containing only natural-abundance isotopes after an initial 38 min pulse. (Bottom) Cells labeled continuously for 92 min.

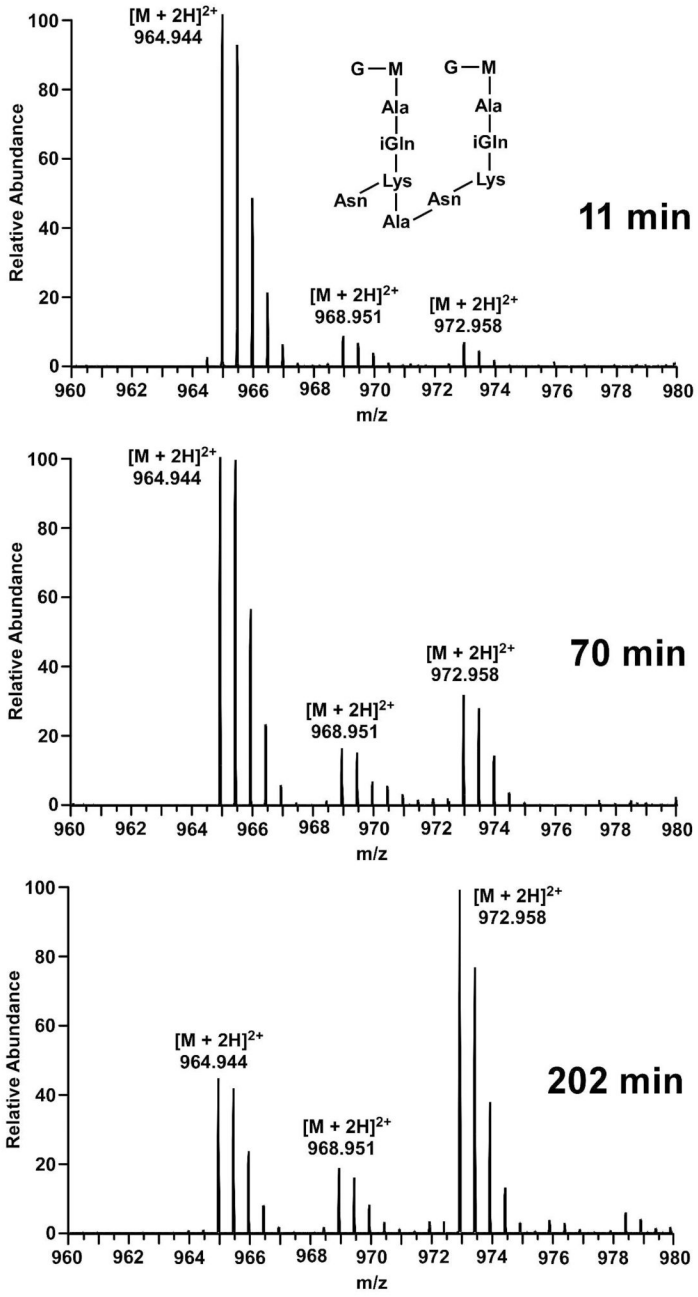

Figure 5.

Mass Spectra for the Ion with an *m/z* of 964.944 Corresponding to a Doubly-Charged Dimer with Asn Bridges. (Top) After an 11 min pulse. (Middle) After a 70 min pulse. (Bottom) After a 202 min pulse. The number of singly-labeled dimers incorporating one *L*-[$^{13}C_6$, $^{15}N_2$]lysine remains relatively constant at all three time points.

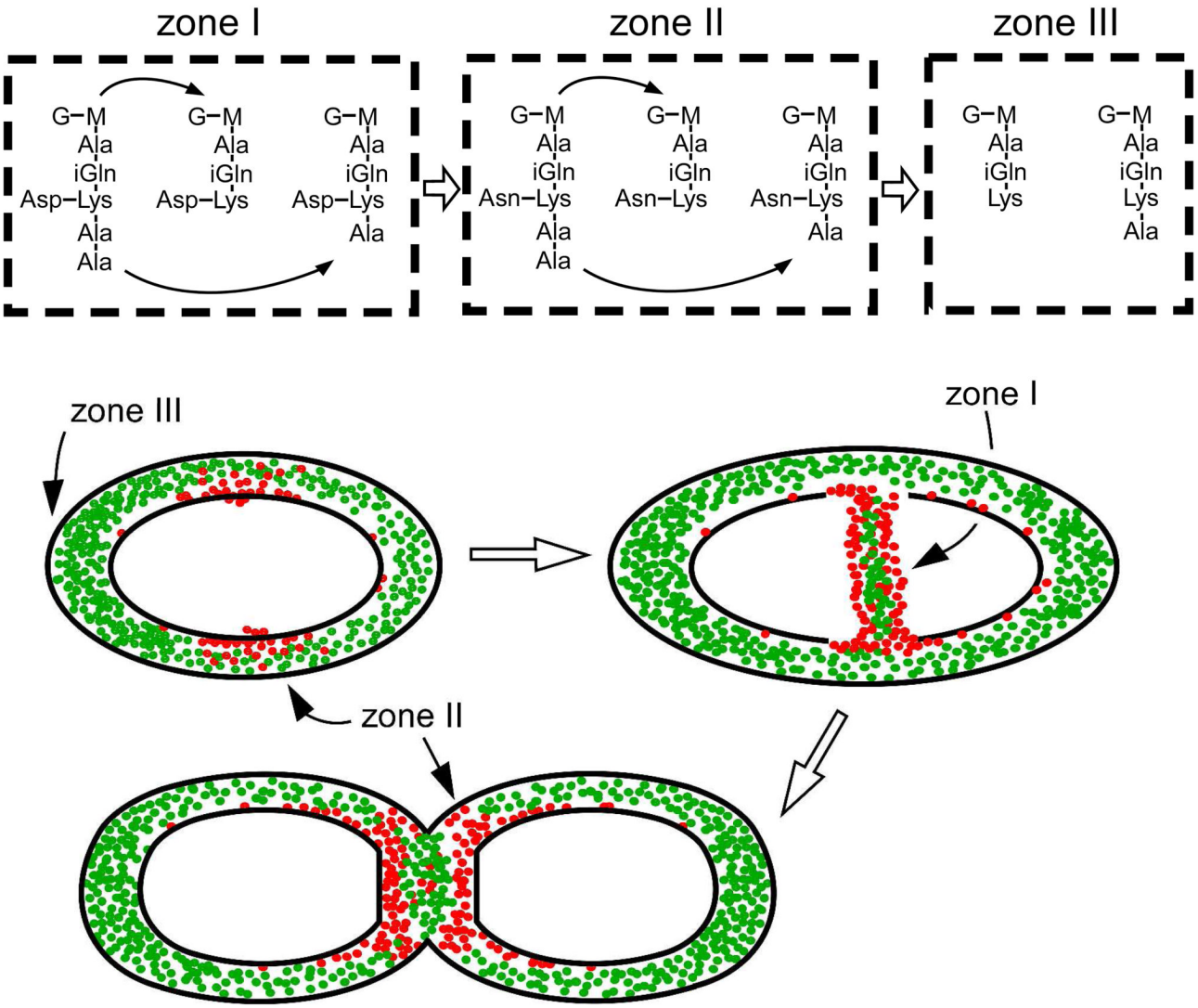

Figure 6.

Zones of Peptidoglycan Processing. (Top) Muropeptide processing characteristic of each of three proposed zones of growth. In zone I, pentapeptides with Asp bridges are cleaved into tri and tetrapeptides. In zone II, the Asp bridge is amidated to Asn first. Subsequently, tri and tetrapeptides are produced with Asn bridges. In mature peptidoglycan, zone III, the bridges of the structures are removed. (Bottom) Schematic of peptidoglycan processing during different phases of cell division. Cell-wall biosynthesis produces pentapeptides with *D*-Ala-*D*-Ala vancomycin-binding sites as indicated by red dots. In subsequent processing, the pentapeptides are cleaved into tripeptides that are represented by green dots. Cell-wall biosynthesis is most active during peripheral growth and in the formation and division of the cross wall (septum). We propose that zone I processing characterizes the septum, while zone II processing is unique to peripheral growth and cross-wall division.

Table 1

Isotopic Enrichments After 202 min Growth in Enriched Media

m/z	Species	final % enriched[a]
1081.491	G-M-Ala-iGln-Lys(Asp)-Ala-Ala	90
1010.454	G-M-Ala-iGln-Lys(Asp)-Ala	83
939.416	G-M-Ala-iGln-Lys(Asp)	81
1080.507	G-M-Ala-iGln-Lys(Asn)-Ala-Ala	75
938.432	G-M-Ala-iGln-Lys(Asn)	73
1009.470	G-M-Ala-iGln-Lys(Asn)-Ala	61
895.426	G-M-Ala-iGln-Lys-Ala	55
824.388	G-M-Ala-iGln-Lys	52

[a]For three different trials, the isotopic enrichments for all species described in this text were determined as +/− 0.5% of the values provided (e.g., 90 +/− 0.5%, 83 +/−0.5%, etc…).