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Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox

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Abstract. A modified peptidoglycan (PG) wall is required for maintenance of spore core dehydration and the accompanying metabolic dormancy and heat resistance. Production of the spore PG depends on the cooperative expression of gene products within both the mother cell and forespore compartments of the sporangium. Structural elements that differentiate spore PG from vegetative cell PG include the presence of the modified sugar muramic-δ-lactam and a low level of peptide cross-links between the glycan strands. Detailed analyses of PG structure in dormant spores and in developing forespores of wild-type and mutant strains are providing data on factors required for introduction of these modifications and the importance of these structural elements in determining spore properties. Muramic-δ-lactam is not required for spore core dehydration but serves as a specificity factor for spore germination lytic enzymes. Cross-linking of spore PG can vary over a relatively wide range without significantly effecting spore core dehydration but does have an influence on the rate of spore germination and outgrowth. Future studies will examine how the two cells within the sporangium coordinate the production of this unique structure between themselves and how specific spore PG structural modifications are produced and participate in determining spore resistance properties.

Key words. Spore; endospore; peptidoglycan; cortex; penicillin-binding proteins.

Introduction

During the recent US presidential election process there was repeated discussion of placing the funds of the US Social Security system in a 'lockbox', a situation that would prevent use of these funds for any other government program, thereby insuring the viability of the Social Security system for many years. Maintaining long-term viability through the use of perhaps the ultimate biological lockbox is a specialty of endospore-producing bacteria. A bacterial endospore is a single cell in which the cell's cytoplasm or 'core' is locked in a state of complete dormancy. The long-term viability of the cell is dependent on a lockbox with two types of walls, an inner specialized peptidoglycan (PG) wall, the spore cortex, and an outer protein wall, the spore coats. The spore core is held in a relatively dehydrated state, which is essential for spore heat resistance, long-term dormancy and full chemical resistance [1–4]. The spore coats are required for full resistance to several chemical and enzymatic treatments, but normal core dehydration and heat resistance can be attained in the presence of some significant alterations of coat structure and can be maintained during stripping of many coat proteins from mature spores [5, 6]. Maintenance of spore core dehydration is dependent on two structures, the inner forespore membrane and the subject of this review, the spore cortex [6, 7]. Degradation of the spore PG results in rapid rehydration of the spore core with a consequent resumption of metabolic activity and loss of resistance properties.

Spore PG consists of two layers that can be visualized due to their differential staining in thin-section electron micrographs (fig. 1). The thinner, more densely stained, inner layer, adjacent to the inner forespore membrane, is the germ cell wall (GCW). The GCW has a structure similar to that of the PG of a vegetative cell, is not degraded during spore germination and serves as the initial cell wall of the germinating spore [8, 9]. The much thicker, lightly staining outer PG layer is the cortex. Cortex PG has several structural modifications relative to vegetative cell

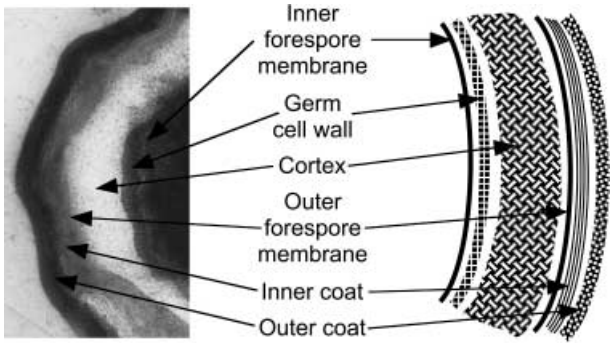

Figure 1. Thin section electron micrograph and schematic representation of bacterial endospore integument layers. On the inner, concave side of each image is the cytoplasm or core of the developing endospore and on the outer, convex side is the external milieu.

PG and is rapidly degraded during spore germination [8–13].

The spore PG is synthesized in the space between the two membranes that surround the developing forespore following engulfment. During the time frame of spore PG synthesis, the forespore enters the dormant state and becomes surrounded by the spore coat layers. Based on the coordinate timing of spore PG synthesis and the development of heat resistance and on a knowledge of the novel structure of the spore cortex PG, it has been theorized that the cortex may play an active role in the attainment of spore dehydration in addition to its role in maintenance of dehydration [14–16]. Recent studies of spore PG produced in mutant strains shed light on the proteins involved in spore PG synthesis and the roles played by specific PG structural modifications in attainment of spore resistance properties, maintenance of resistance and long term dormancy and germination.

Spore PG structure

Bacterial PG is composed of glycan strands of alternating *N*-acetyl-glucosamine and *N*-acetyl-muramic acid residues. The *N*-acetyl-muramic acid residues initially carry pentapeptide side chains that are used to cross-link the glycan strands with concomitant cleavage to tetrapeptides (reviewed in [17]). Detailed analyses of the *Bacillus subtilis* spore PG and vegetative PG structures were first carried out by Warth and Strominger [12, 13, 18]. More detailed analyses have recently been carried out using reversed-phase high-pressure liquid chromatography (HPLC) and mass spectrometry methods [10, 11, 19]. *B. subtilis* PG is of the A1γ type [20] with peptide side chains of L-Ala-γ-D-Glu-meso-diaminopimelic acid (Dpm)-D-Ala-D-Ala. In vegetative cells of *B. subtilis* 84% of uncross-linked peptides are cleaved to tripeptides, ~ 40% of the muramic acid residues carry peptides in which the Dpm residues are involved in cross-linking via their free ε-amino groups and 99% of the Dpm residues are amidated on their free ω-carboxyl groups [18, 19]. In *B. subtilis* vegetative cell PG the average glycan chain length has been estimated to be 200 sugar residues [21], and teichoic acids are attached to ~ 2% of the muramic acid residues [18, 19]. *B. subtilis* spore PG differs from the vegetative PG most dramatically in the absence of teichoic acids and in the removal of many peptide side chains with a concomitant decrease in cross-linking of the glycan strands. Fifty percent of the muramic acid residues have their side chains entirely removed and are converted to muramic-δ-lactam (fig. 2). Another 24% of the muramic acid residues have their peptide side chains cleaved to single L-Ala residues, precluding their participation in cross-linking. As a result of this loss of peptide side chains, only 3% of the spore muramic acid residues carry peptides in which the Dpm is cross-linked. The average glycan length in *B. sphaericus* spore PG has been estimated to be $\geq$ 80 sugar residues [22]. Spore PG structure has been analyzed in some detail in *B. cereus*, *B. megaterium*, *B. sphearicus*, *B. stearothermophilus*, *Clostridium sporogenes*, and *C. perfringens* [13, 22, 23] [D. L. Popham et al., unpublished observation]. In all cases the general structure was similar to that of *B. subtilis*, with only slight modifications. Interestingly, the peptide side chain composition, specifically the presence of Dpm, is maintained in spore PG of all these species, despite the replacement of Dpm with lysine in the vegetative PG of *B. sphearicus* [24]. Detailed analyses have revealed the presence of small numbers of peptide side chains containing glycine, especially in strains carrying mutations that alter PG synthesis and in specific media [7, 19]. However, no significance has been attributed to this modification.

Recently, the progression of spore PG synthesis has been followed by obtaining samples of immature forespore PG from an actively sporulating culture [9]. Analysis of forespore PG structure throughout late sporulation revealed that the GCW is synthesized first and contains 5–10% of the total spore PG (fig. 3). This PG layer contains high levels of tripeptide side chains, low levels of muramic-δ-lactam, and > 6% of the muramic acid residues carry peptides with cross-linked Dpm, reminiscent of vegetative PG. The cortex PG is then synthesized on the outside of the GCW. In the first layers of cortex PG, the muramic-δ-lactam level rises rapidly, the cross-linking drops several-fold and most non-cross-linked peptides remain as tetrapeptides. During synthesis of succeeding cortex layers, the cross-linking rises slowly to > 4% of muramic acid residues, so that the average cross-linking over the entire spore PG structure is ~ 3.5% of muramic acid residues. Based upon analyses of phenotypic properties and PG structures of dormant spores produced by a number of mutant strains, a gradient of cross-linking within

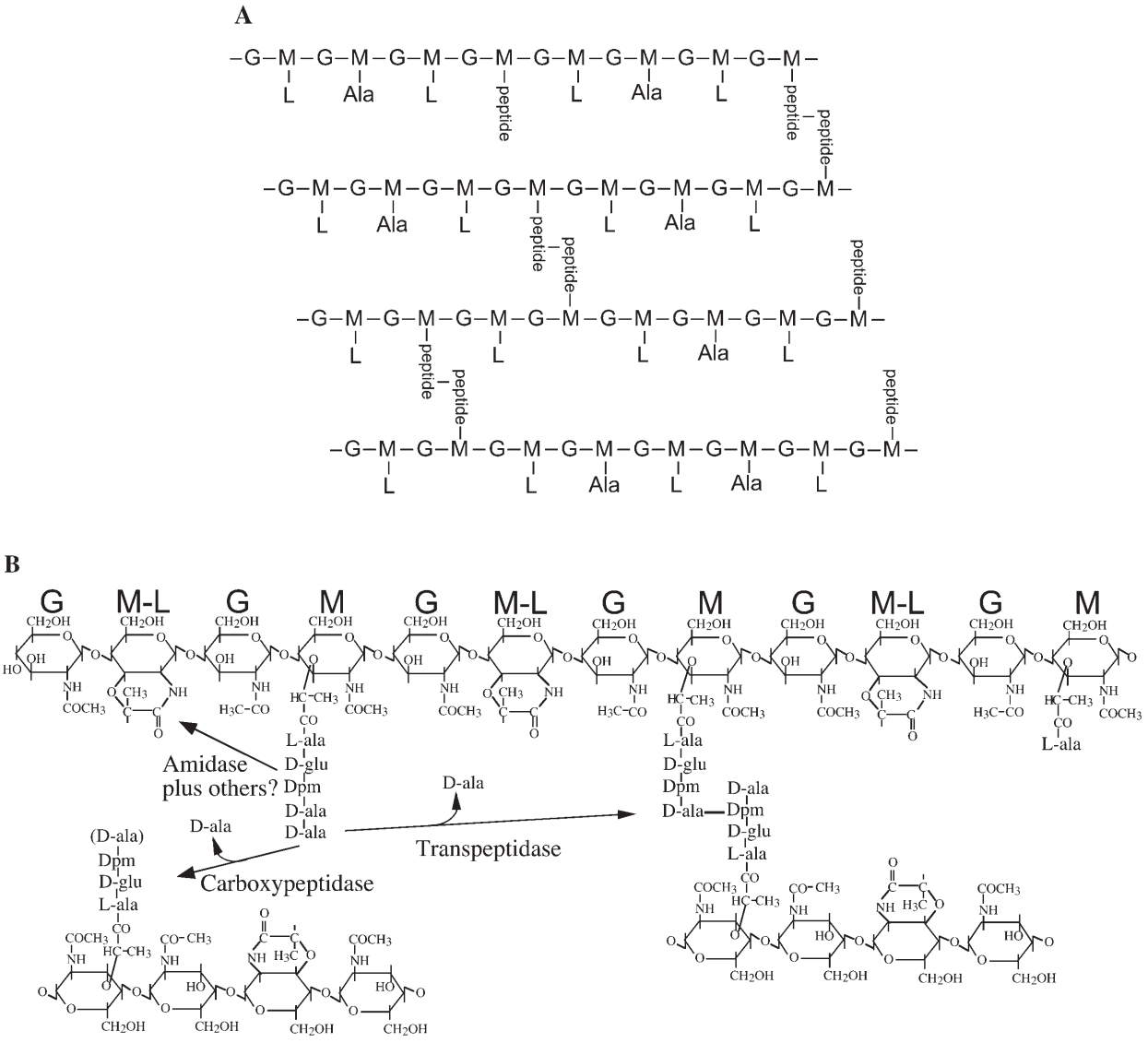

Figure 2. Schematic representations of spore PG structure. (*A*) The mesh structure of PG contains glycan strands of alternating *N*-acetylglucosamine (G) and *N*-acetyl-muramic acid (M) residues. Fifty percent of the muramic acid residues have their peptide side chains removed and are converted to muramic-*δ*-lactam (L). The remaining peptides are cleaved to single L-ala residues or to tri and tetrapeptides that can form cross-links between the glycan strands. (*B*) A more detailed diagram shows the structure of muramic-*δ*-lactam (M-L) and the reactions resulting in peptide removal, cleavage and cross-linking.

the spore PG had been previously hypothesized to be involved in attainment of spore core dehydration [15]. However, further studies (see below) have indicated that this gradient of cross-linking is not required for spore dehydration [9].

Enzymes involved in spore PG synthesis

Between the cessation of vegetative growth and the initiation of sporulation, very little PG synthesis takes place in the cell aside from synthesis of the asymmetric septum [9, 25]. Some of the enzymes required for spore PG synthesis are certainly shared with the vegetative PG synthesis machinery and may still be active at stage three of sporulation. Other PG synthesis and modification enzymes are specific for spore PG and are induced during sporulation.

PG is polymerized from disaccharide-pentapeptide precursors that are produced in the cytoplasm, linked to undecaprenylphosphate lipid, and then flipped across the membrane. The substrates for *B. subtilis* vegetative and spore PG synthesis are apparently identical, aside from amidation of Dpm. The presence of only one copy of the known genes required for PG precursor synthesis in the *B. subtilis* genome [26, 27] suggests that a single set of

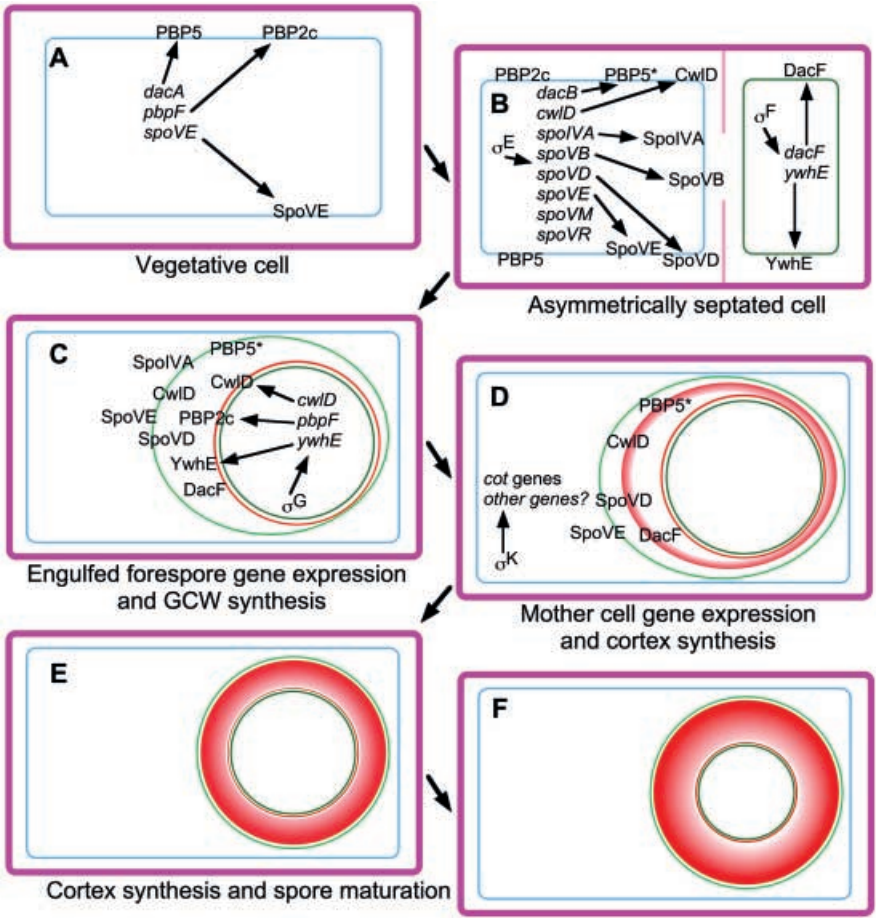

Figure 3. Compartmentalized expression of gene products required for spore PG synthesis and progression of spore PG synthesis. Schematic representations of cells progressing from vegetative growth to spore maturation steps of spore PG synthesis are shown. Genes and their products that are known to affect spore PG synthesis are shown. Demonstrated association of a protein with the membrane or the outer surface of a membrane is indicated by overlap of the protein name with the membrane. Distances between membranes and wall layers are exaggerated in order to allow placement of gene product names. (*A*) The membrane and PG wall surrounding the mother cell cytoplasm are shown in blue and dark purple, respectively. (*B*) A sporulating cell that has undergone asymmetric septation then activates expression of σ^F- and σ^E-dependent genes in the forespore and mother cell, respectively. The membrane (dark green) surrounding the forespore will become the inner forespore membrane. The asymmetric septum PG (shown in light purple) initially spans the entire cell and is shown in the process of being degraded from the center in order to allow engulfment. (*C*) Following the completion of engulfment the developing forespore is contained within the mother cell and is surrounded by the inner and outer forespore membranes (light green). The coat protein layers that begin to form around the developing forespore are not shown. Expression of σ^G-dependent genes is activated in the forespore, and synthesis of spore PG is initiated. The GCW PG (bright red), in which cross-linking is relatively high, is produced first, adjacent to the inner forespore membrane. (*D*) The first cortex PG layers (shaded pink to red) are produced outside the GCW and have very low cross-linking. σ^K-dependent gene expression is activated in the mother cell and is required for completion of cortex synthesis [56]. It is not yet clear which σ^E-, σ^F-, σ^G- and σ^K-dependent gene products are specifically required for germ cell wall synthesis and for cortex synthesis. (*E*) Successive layers of cortex PG with increasing cross-linking are laid down from the mother cell side of the intermembrane space. Increasing cross-linking is indicated by the gradient from light to dark red. (*F*) Cortex synthesis is completed as the spore reaches maturity, and the relative dehydration of the spore core is indicated by its reduced volume. References for cell-specific expression of specific genes are [32, 43, 46–52, 57–60] and cell-specific activation of sigma factors is reviewed in an accompanying article in this multi-author review, covering sigma factors and compartmentalized gene expression.

enzymes function in both situations. In *B. sphearicus*, where the vegetative and spore PG precursors differ, there is evidence that the spore PG-specific enzymes are induced during sporulation [28]. The recent finding that Dpm residues are amidated in the spore PG of a particular *B. subtilis spoIVA* mutant strain suggests that specific inactivation of the enzyme required for this vegetative PG modification may take place during sporulation [29].

The glycosyl transferase and transpeptidase activities required for PG strand polymerization and cross-linking, respectively, are carried in the penicillin-binding proteins (PBPs) (reviewed in [30]). Significant functional redundancy among these enzymes makes it unclear which of the PBPs are responsible for spore PG synthesis [31]. However, some PBP requirements are clearly defined by studies of *B. subtilis* mutant strains. The Class A PBPs en-

coded by *pbpF* and *ywhE*, which should carry out both glycosyl transferase and transpeptidase activities, play a redundant role in synthesis of spore PG [31a]. The class B PBP encoded by *spoVD*, which should carry out transpeptidation, is required for spore PG synthesis. A null mutant appears to produce no cortex PG [32], whereas strain with the *spoVD156* mutation, apparently a partial loss-of-function mutation, produces a significant amount of spore PG but with an altered structure [33]. The *spoVE* gene, which encodes a class of protein believed to function with class B PBPs in determining PG architecture [34, 35], is also essential for spore PG synthesis. A known null mutant [36] as well as a strain carrying a presumed point mutation, *spoVE85* [37], produce little or no spore PG [33]. The ability to track PG synthesis in developing forespores throughout sporulation [9] will allow a very clear determination of precisely how much spore PG these strains produce and what structural alterations may be present.

At least three low MW PBPs that carry carboxypeptidase activity are involved in spore PG synthesis. The *dacA* product, PBP5, is the most abundant PBP in vegetative cells [38, 39] and is required for cleavage of peptide side chains to tripeptides [19]. Significant amounts of PBP5 remain in sporulating cells [40, 41]. A *dacA* mutant produces spore PG with an increased number of tetrapeptide and pentapeptide side chains [10, 11, 15], but with no alteration of spore dehydration or heat resistance [3, 42]. The *dacB* and *dacF* products, PBP5* and DacF, respectively, play partially redundant roles in regulating spore PG cross-linking. Presumably, they regulate cross-linking by cleaving pentapeptide side chains to tetrapeptides, removing them from the pool available for transpeptidation (fig. 2). A *dacF* mutant produces apparently normal spore PG [10, 11], whereas a *dacB* mutant produces spore PG with a fivefold increase in cross-linking [10, 11]. A *dacB dacF* double mutant produces spore PG with a >8-fold increase in cross-linking, relative to the wild type, revealing the role played by *dacF* in the absence of *dacB* [15].

Several other proteins are clearly required for spore PG-specific structural modifications. A *cwlD* mutant produces spores that completely lack muramic-δ-lactam [7, 10]. The *cwlD* product has sequence similarity to known muramoyl-L-alanine amidases [43]. It is not yet clear whether CwlD simply cleaves the peptide side chain from the muramic acid and other proteins are required for lactam cyclization or whether CwlD can carry out an additional step(s) in the process (fig. 2). However, when CwlD was expressed in *Escherichia coli*, simple amidase products, but no lactam, were produced [M. E. Gilmore and D. L. Popham, unpublisched observation]. An L-ala-D-glu peptidase is presumed to be required for production of the single L-ala side chains, but such an activity has not been demonstrated. A search of the *B. subtilis* genome [26] revealed that the *ycdD* gene product was similar to a demonstrated L-alanoyl-D-glutamate peptidase encoded by a *Listeria monocytogenes* phage [44]. However, a *ycdD* null mutant strain produces spore PG containing a normal amount of single L-ala side chains [J. Meador-Parton and D. L. Popham, unpublished observation]. The products of many other sporulation genes are required for synthesis of normal spore PG. Thin-section electron microscopy suggests that strains with null mutations in the *spoIIIG* or *spoIVB* genes are completely unable to initiate spore PG synthesis [45]. Mutations in several other *spoIV* and *spoV* genes – for example *spoIVA* [33], *spoVB* [33, 37, 46], *spoVM* [47], and *spoVR* [48] – appear to make altered and/or insufficient amounts of spore PG. Determination of the amount and structure of spore PG these strains produce may clarify their roles.

An unresolved question concerns which side of the intermembrane space surrounding the forespore the GCW is made from. Analysis of spore PG synthesis throughout sporulation suggested that the GCW was made first, followed by the cortex [9]. The position of the cortex on the outside of the GCW means that cortex synthesis certainly has to take place on the surface of the outer forespore membrane using precursors made in the mother cell. Early studies on the production of PG precursor synthetic enzymes required for spore PG synthesis in *B. sphearicus* demonstrated that the Dpm-adding enzyme was produced only in the mother cell, whereas the Lysine-adding enzyme was present in both the mother cell and forespore [28]. This led to the hypothesis that cortex PG, which contains Dpm, was made from the mother cell side, whereas GCW PG, which was presumed to resemble vegetative PG in containing lysine, was made from the forespore side. However, amino acid analysis of *B. sphearicus* spore PG revealed no lysine [D. L. Popham, B. Illades-Aguiar and P. Setlow, unpublished observation] raising the possibility that all spore PG is made from the mother cell side.

Examination of the sites of expression of other spore PG synthetic enzymes (fig. 3) and the effects of their loss on spore PG structure has not yet clearly revealed the site of GCW synthesis. SpoVD is expressed only in the mother cell, and whereas a *spoVD* null mutant produces no detectable cortex [32], it does appear to produce some GCW PG [unpublished observation, D. L. Popham]. The expression and effects of the *pbpF* and *ywhE* products, class A PBPs, is more complex. The *pbpF* product, PBP2c, is made at low levels during vegetative growth and thus should be present on the membranes of both cell compartments of the sporangium [49]. This gene is then also induced within the forespore during sporulation [49]. Expression of *ywhE* takes place only during sporulation and only within the forespore [50]. Both proteins would be expected to cross a membrane and remain associated with the outer surface of the membrane, as has

been demonstrated for a number of class A PBPs (reviewed in [30]). Induction of these genes within the forespore led us to believe that they might function in synthesis of the GCW. However, the effect exerted by *pbpF* and *ywhE* on spore PG synthesis appears to extend beyond GCW synthesis [31a]. It is possible that the GCW is altered in a way that is undetectable by our current assays (perhaps in terms of its precise three-dimensional structure) and which prevents normal cortex synthesis. Alternatively, it is possible that the more important mode of *pbpF* expression is that which leads to the presence of PBP2c in the mother cell and on the outer forespore membrane. YwhE expressed within the forespore might compensate for loss of PBP2c if YwhE does not remain associated with the inner forespore membrane but is able to traverse the inner membrane space and then function in cortex synthesis at the surface of the outer forespore membrane. A similar situation occurs with the low MW PBPs PBP5* and DacF. PBP5* is expressed only within the mother cell [51] and is associated with the outer forespore membrane [41], whereas DacF is produced only within the forespore and is expected to be associated with the inner forespore membrane [52]. Loss of PBP5* results in synthesis of an altered cortex, as expected [10, 11]. The further loss of DacF in the *dacB dacF* double mutant results in alteration of not only the GCW but in the entire cortex [15]. Again, a possible explanation is that DacF is not remaining associated with the inner forespore membrane and thus is able to effect spore PG layers outside of the GCW. It is noteworthy that neither DacF nor YwhE have been detected in traditional penicillin-binding assays in which labeled penicillin is incubated with isolated membrane preparations [15, 50]. A possible explanation for these results is the failure of these proteins to remain membrane associated. Studies of the requirements for compartment-specific expression of these PBPs will shed light on whether they can act from either side of the intermembrane space and may provide further clues as to the site of GCW synthesis.

Roles of PG structural modifications

Detailed studies of spore PG structures have been carried out for a variety of mutant strains with the hope of determining which spore PG structural modifications are important for determining spore resistance properties. The most dramatic and unique structural modification present in spore peptidoglycan, the presence of muramic-δ-lactam, was long believed to play an important role in spore dormancy and/or resistance properties. It was thus quite surprising to find that a *cwlD* mutant strain [43] that produced spores with normal dehydration and resistance properties [7] had no muramic-δ-lactam in its spore PG [7, 10]. The observation that this strain was able to initiate germination, taking up water and releasing the spore solute dipicolinic acid, but was unable to initiate spore PG degradation [7, 8, 43] suggested that muramic-δ-lactam was a recognition element for germination lytic enzymes [7, 10]. This specificity has now been demonstrated for several germination lytic enzymes [53, 54]. The presence of muramic-δ-lactam in the spore cortex but not in the GCW allows rapid degradation of the cortex without loss of the GCW, an event which could result in cell lysis.

The significance of single L-ala side chains in the spore PG is unknown. It is possible that cleavage of these side chains is a mechanism for reducing the cross-linking of the PG or that it allows recycling of unneeded peptides within the nutrient-limited sporulating cell. It is clear from analyses of mutant strains that neither a twofold reduction nor a 50% increase in L-ala side chains prevented formation of dormant spores [7–11]. Demonstration of a role for this structural modification may await identification and genetic analysis of the gene(s) required for its production.

The early observation of low spore PG cross-linking [12, 13] gave rise to the idea that the spore PG could have a flexibility that was important in attaining or maintaining spore dehydration [14, 16]. Analyses of mutant strains has indicated that changes in spore PG cross-linking over a relatively wide range does not greatly effect spore dehydration. The 2-, 6- and 10-fold increases in cross-linking in *cwlD*, *dacB and cwlD dacB* double mutants, respectively, have no effect on attainment of spore core dehydration [3, 10, 11, 55]. However, the *dacB* mutants are unable to maintain full dehydration upon heating [3]. Only when much higher levels of cross-linking are produced, in a *dacB dacF* double mutant, is attainment of spore dehydration and dormancy prevented [9, 15]. As mentioned above, cross-linking is not uniform across the span of the spore PG. Cross-linking is relatively high in the innermost PG layers (the GCW), decreases dramatically in the first layers of cortex PG and then increases gradually over the span of the cortex [9]. We had earlier predicted that such a gradient of cross-linking might be the factor that would allow the spore PG to exercise a mechanical activity specifically inwards against the core [15]. However, this gradient of cross-linking is not present in *cwlD* and *dacB* mutant spores that are able to achieve full spore core dehydration [9]. Increased cross-linking of spore PG does seem to effect the rate of spore outgrowth. Spores produced by *dacB* mutants have a decreased rate of outgrowth [15]. This may simply be due to the greater time required for full dissolution of the more highly cross-linked cortex PG in order to allow expansion of the outgrowing cells.

Future directions

Several interesting questions remain with regard to the synthesis of spore PG and the roles its plays in determining spore properties. A number of the enzymes that must be directly involved in polymerization and modification (production of muramic-δ-lactam and L-ala side chains) remain to be identified. In addition, a variety of mutant strains that are blocked at a late stage of sporulation have been suggested to be defective in spore PG synthesis. The proteins affected by these mutations may play direct roles in spore PG synthesis or may function in targeting PG synthetic enzymes to the forespore, inhibiting vegetative PG synthesis, or assembling specific protein complexes required for synthesis of GCW and cortex. The ability to now examine the structure of PG produced in immature forespores will allow the determination of just how much spore PG is made in these mutants and the structure of this PG. The question of which side of the intermembrane space the GCW PG is made from may be resolved by studying requirements for cell-specific expression and localization of PBPs. Finally, mutant strains with a variety of types of changes in spore PG structure are able to achieve full spore core dehydration, arguing against an active role for this structure in the attainment of dehydration. The possibility that such a role exists is difficult to disprove due to the difficulty of measuring intracellular water content during sporulation. Perhaps further studies of PG synthesis in mutant strains will reveal a particular aspect of the spore PG wall, aside from simply its volume, that is essential for locking away the spore contents from lurking agents that would steal its viability.

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