ABSTRACT The complex cell envelope is a hallmark of mycobacteria and is anchored by the peptidoglycan layer, which is similar to that of Escherichia coli and a number of other bacteria but with modifications to the monomeric units and other structural complexities that are likely related to a role for the peptidoglycan in stabilizing the mycolyl-arabinogalactan-peptidoglycan complex (MAPc). In this article, we will review the genetics of several aspects of peptidoglycan biosynthesis in mycobacteria, including the production of monomeric precursors in the cytoplasm, assembly of the monomers into the mature wall, cell wall turnover, and cell division. Finally, we will touch upon the resistance of mycobacteria to β-lactam antibiotics, an important class of drugs that, until recently, have not been extensively investigated as potential antimycobacterial agents. We will also note areas of research where there are still unanswered questions.

The central core of the mycobacterial cell envelope consists of the mycolyl-arabinogalactan-peptidoglycan complex, also known as the MAPc. Anchoring the entire MAPc is the peptidoglycan (PG), which is composed of a glycan chain with alternating N-acylated glucosamine (GlcNAc) and muramic acid (MurNAcyl) residues bearing peptide chains, which may be cross-linked (Fig. 1) (4, 5). The glycan chain of the mycobacterial PG consists of alternating GlcNAc and MurNAcyl residues in a β-(1,4) linkage as seen in other bacteria, except that the MurNAcyl residues can be either N-glycolylated (MurNGlyc) or N-acetylated (MurNAc) (Fig. 1) (4, 5). The peptide chain attached to the lactyl moiety of MurNAcyl is similar to that of E. coli and many rod-shaped Gram-positive bacteria, typically consisting of L-alanyl-D-glutamine-meso-diaminopimelylc-D-alanyl-D-alanine (Fig. 1) (3). Variations in the peptide include the replacement of L-Ala with Gly in Mycobacterium leprae, amidation of the free carboxyl group of D-Glu (generating d-iso-Glu), and the amidation of the free carboxyl of the side chain of meso-diaminopimelate (DAP) (Fig. 2A and 2B) (2, 6, 7). The N-glycolylation of...
MurNAcyl is uncommon, only present in mycobacteria (an exception is *M. leprae*) and other closely related actinobacteria (4); this modification contributes to the lysozyme-resistant phenotype of mycobacteria (8) and potentiates the recognition of PG by the innate immunity protein Nod2 (9). The significance of the other modifications to mycobacterial PG biology is unknown.

The mature PG architecture is also marked by a high degree of direct peptide cross-links. Overall, 70 to 80% of the peptides are cross-linked, in two kinds of linkages. One type is between the d-Ala at position 4 of one peptide and the meso-DAP at position 3 of an adjacent peptide (Fig. 2A). These “4-3” linkages are catalyzed by typical D,D-transpeptidases, also known as penicillin-binding proteins (PBPs), which can be inhibited by various classes of β-lactam antibiotics (10). The other type of linkage is between two DAP residues, also known as a “3-3” linkage (Fig. 2B), that are catalyzed by the concerted activity of D,D-carboxypeptidases and L,D-transpeptidases, the latter of which have been found to be resistant to most β-lactam antibiotics, except for the carbapenem class (11–15). The role of 3-3 linkages is chiefly unknown (16), but they were first discovered in mycobacteria and they exist in a high proportion compared to most other bacteria, suggesting a correlation between the degree and type of cross-linking with the complexity of the mycobacterial cell envelope. It has subsequently been shown that 3-3 linkages are present in many other species of bacteria and that the relative percentage of linkage type can shift depending upon growth conditions or growth phase (17, 18). Early studies examining mycobacterial PG showed that about one-third of the cross-links are 3-3 (19), but more recent work suggests that the percentage is much higher, in the 60 to 80% range. The percentage of 3-3 to 4-3 linkages appears to be constant, regardless of growth stage, in *M. tuberculosis* (20) and *Mycobacterium abscessus* (21).

Mature mycobacterial PG is covalently connected to the galactan portion of the arabinogalactan of the MAPc via a disaccharide linker unit consisting of a rhamnose residue and N-acetylglucosamine 1-phosphate attached to carbon 6 of some of the MurNAcyl moieties (Fig. 2A and 2B) (22).
GENETICS OF PG PRECURSOR SYNTHESIS

Most of the cytoplasmic steps for the synthesis of PG precursors in mycobacteria are shared with those of other bacteria (see Fig. 3 for the full cytoplasmic precursor pathway). The mycobacterial genes involved in this pathway have been found primarily by homology to known genes in other bacteria (23, 24). By virtue of the critical nature of the PG, the genes encoding the enzymes for precursor synthesis are essential for mycobacterial survival since there are no redundancies. In a few instances, the function of a mycobacterial gene was confirmed by complementation of E. coli mutants, either auxotrophs (asd, dapB) (25, 26) or a temperature-sensitive mutant (murG) (27). In addition, there have been some direct biochemical and genetic experiments in mycobacteria to examine the enzymes involved in this stage of PG biosynthesis, as described below.

Cytosolic precursor (UDP-N-acetylmuramyl-l-alanyl-d-glutamyl-meso-diaminopimelyl-d-alanyl-d-alanine, or Park’s nucleotide; Fig. 1) synthesis begins with the conversion of UDP-GlcNAc to UDP-MurNAc via the products of the murA and murB genes (28). Subsequent steps in this part of the pathway add each amino acid to the growing peptide chain attached to the MurNAcyl. This requires the generation of d-amino acids at several steps to produce d-Glu, meso-DAP, and d-Ala (Fig. 3) (28). Mutation of genes involved with the biosynthesis of these amino acids is generally permitted in bacteria, given that the appropriate isomers of the amino acids are supplied to the mutants. The MurI racemase that produces d-Glu has a moonlighting function in many bacteria (including mycobacteria) in that it can bind to DNA gyrase and prevent its inactivation by ciprofloxacin (29, 30). Consistent with this view, overexpression of murI in M. tuberculosis and M. smegmatis can confer resistance to ciprofloxacin (30).

DAP is produced from l-aspartate in a series of reactions beginning with aspartokinase, encoded by the ask gene (Fig. 3) (25, 31). Deletion of this gene leads to DAP auxotrophy in M. smegmatis, and cell cultures of an ask mutant that are deprived of DAP will lyse within one generation after starvation (“DAP-less death”) (32). Surprisingly, spontaneous mutation of the ribosome-binding site of the cbs gene, encoding cystathionine β-synthase, can suppress DAP-less death in an M. smegmatis ask mutant (33, 34). This occurs by subsequent overexpression of cystathionine β-synthase activity, resulting in the biosynthesis of lanthionine, an analog of DAP that can be inserted into PG precursors in place of DAP (34, 35). However, such suppressor strains with a lanthionine-containing PG are hypersusceptible to β-lactam antibiotics.

In spite of the ability to construct a DAP auxotroph by deletion of the ask gene, it has not been possible to delete genes further down the pathway. We have made several attempts to delete the dapB gene in M. smegmatis as well as M. bovis BCG to no avail. However, a dapB mutant of M. smegmatis was isolated from a transposon library screened for mutants that are hypersusceptible to β-lactam antibiotics, but the mutant has an uncharacterized mutation, not in cbs, that suppresses death from DAP starvation (36). The reason why other dapB deletions have not been successful is unknown, but perhaps it may be due to suppression by an unrecognized dapB paralog residing in the genomes of these bacteria. Another potential barrier to the construction of DAP auxotrophs in other mycobacterial species is that DAP is the precursor to lysine, and mycobacteria exhibit differences in their ability to both utilize exogenous lysine and balance the DAP pool to support both lysine and PG requirements (32, 37). These factors may impact the viability of certain DAP auxotrophic strains.

The production of d-Ala in many bacteria is catalyzed by either alanine racemases or d-amino transaminases (38). Some bacteria have both types of enzymes, but mycobacterial genomes appear to encode only an alanine racemase (alr). Earlier work suggested that there is a redundant system for d-Ala production in M. smegmatis, since an alr insertion mutant did not exhibit a requirement for d-Ala (39). However, subsequent work showed that deletion of alr in M. smegmatis or M. tuberculosis resulted in mutants that required d-Ala supplementation for growth and, in the latter case, attenuated growth of the mutant in macrophages and mice (40, 41). The discrepancies between these studies likely resulted from partial activity of a truncated Alr protein being produced from the insertion mutation in the earlier work with M. smegmatis. Understanding the metabolism of d-alanine is important, since alanine racemase is the target of d-cycloserine, a potent second-line antitubercular drug. Overexpression of alr and the d-alanine ligase encoded by ddl confers increased resistance to d-cycloserine in both M. smegmatis and M. tuberculosis (42).

BIOSYNTHESIS OF PG CYTOSOLIC INTERMEDIATES

As noted above, the PG biosynthetic machinery bears a resemblance to the basic E. coli PG biosynthesis pathway. The PG biosynthesis in E. coli and likewise in mycobacteria occurs in three subcellular locations (16, 43, 44). The final cytosolic intermediate of PG biosynthesis is UDP-N-acetylmuramyl-l-alanyl-d-isoglutamyl-
meso-diaminopimelyl-β-alanyld-alanine (UDP-MurNAc pentapeptide) precursor, or Park’s nucleotide (Fig. 1). Synthesis of UDP-MurNAc pentapeptide initiates with the formation of UDP-MurNAc from UDP-GlcNAc in a two-step reaction catalyzed by MurA and MurB (45). MurA transfers the enolpyruvyl moiety of phosphoenol pyruvate to the 3′-hydroxyl group of UDP-GlcNAc, forming enolpyruvyl-UDP-GlcNAc, which is the first committed step of bacterial cell wall biosynthesis. The murA gene of M. tuberculosis has a point mutation that changes a canonical cysteine in the active site to an aspartate that confers resistance to the antibiotic fosfomycin (46). Eschenburg et al. proposed that the substitution of Cys115 with another amino acid has no effect on the catalytic activity of MurA (47). In the second step of the reaction, MurB (Rv0482) reduces the enolpyruvate residue of enolpyruvyl-UDP-GlcNAc to d-lactate, forming UDP-MurNAc (45). This reaction is NADH dependent. No direct biochemical study on M. tuberculosis MurA has been performed, but in silico studies to develop a three-dimensional structure and identify specific inhibitors have been performed (48). In mycobacteria the N-acetyl groups of the MurNAc residues can be oxidized to an N-glycolyl function by a UDP-MurNAc hydroxylase (NamH) encoded by Rv3818 (8). This gene is nonessential in M. tuberculosis H37Rv (49), and deletion of namH in M. smegmatis resulted in loss of N-glycolylated PG precursors (8). The namH gene in M. leprae is a pseudogene, which explains the absence of MurNGlyc from the PG of M. leprae (6). However, the formation of MurNGlyc has not been extensively studied, and UDP-N-acetyl-muramyl-tripeptide and lipid-linked intermediates are potential substrates of NamH (2, 5).

The biosynthesis of UDP-MurNAc-pentapeptide occurs via stepwise additions of l-Ala, d-Glu, meso-DAP, and d-Ala-d-Ala to the UDP-MurNAc. These reactions are catalyzed by the Mur family of ligases (45), which are nonribosomal peptide synthases requiring ATP and a divalent cation such as Mg2+ or Mn2+ for activity. Biosynthesis of the peptide side chain initiates with the addition of an l-Ala to the d-lactoyl group of MurNAc residue by MurC (50). MurC enzymes from M. tuberculosis (Rv2152c) and M. leprae (ML0915) have been overexpressed and partially characterized (51), showing similar substrate specificities and kinetics. Both enzymes demonstrated the ability to use l-Ala and Gln as substrates (51, 52). Despite the high degree of similarity between M. tuberculosis and M. leprae MurC enzymes in both sequence and specificity, the first amino acid in the M. leprae PG stem peptide is exclusively Gln; the reason for this substitution is not clear and may result from the intracellular growth of M. leprae (53). In a subsequent reaction, catalyzed by MurD, a d-Glu residue is added to the carboxy terminus of the l-Ala residue of the UDP-MurNAcyl- l-Ala. The MurD enzyme (Rv2155c) from M. tuberculosis has been overexpressed, and the crystal structure has been evaluated and compared with the structure of MurD ligases of other organisms (52, 54). MurE catalyzes the synthesis of UDP-MurNAcyl-l-Ala-d-Glu-m-DAP from UDP-MurNAcyl-l-Ala and meso-DAP. The M. tuberculosis MurE (Rv2158c) is highly specific for meso-DAP, like its E. coli counterpart (55). The last two amino acids are added by MurF as a dipeptide (d-Ala-d-Ala) to the UDP-MurNAcyl-l-Ala-d-Glu-m-DAP, yielding Park’s nucleotide. MurF (Rv2157c) from M. tuberculosis has been overexpressed and partially characterized; this enzyme has much lower KM values for UDP-MurNAcyl-l-Ala-d-Glu-m-DAP and ATP than E. coli and Staphylococcus aureus MurF enzymes (52).

**FIGURE 2** PG cross-links. (A) The direct 4-3 cross-link between d-Ala and meso-DAP. (B) The direct 3-3 cross-link between two meso-DAP residues. Also shown are various modifications of the PG: R1 = H or disaccharide linker connecting the PG to the arabinan of the arabinogalactan; R2 = N-acetyl or N-glycolyl on the muramic acid residue; R3 = OH, NH2, or glycine; R4 = OH or NH2, l-Ala, d-Glu, meso-DAP, and d-Ala are depicted in gold, blue, green, and red, respectively. doi:10.1128/microbiolspec.MGM2-0034-2013.12
the MurNAcy1 residues (7). This results in a pool of differentially modified Lipid II molecules, but it is not clear if all these modifications are found in mature PG. With the exception of the *namH* gene, the genes responsible for these modifications have not been experimentally identified in mycobacteria (8). However, genes homologous to those encoding enzymes involved in the amidation of DAP in *Lactobacillus plantarum*, *asnB1* (56), and the amidation of D-Glu in *S. aureus* (*murT*, *gatD*) (57, 58) are present in mycobacteria, represented by Rv2201, Rv3712, and Rv3713, respectively.

**TRANSLOCATION OF LIPID-LINKED INTERMEDIATES**

The formation, and likely the modification, of Lipid II occurs at the cytoplasmic face of the plasma membrane, and thus, the sugar peptide moiety of the mature Lipid II must be transported to the outer leaflet of the plasma membrane. The transfer of Lipid II across the membrane is not well understood but requires a protein transporter, or “flippase.” The protein-facilitated transmembrane diffusion of lipid-linked biosynthetic intermediates and the lipids themselves is an essential and ubiquitous feature in the synthesis and assembly of a wide variety of extracellular glycoconjugates in both prokaryotes and eukaryotes (59–61). The highly hydrophilic disaccharide-pentapeptide (PG monomer) moiety of Lipid II prevents spontaneous flipping across the membrane (62), and a dedicated flippase is essential for this process (63).

In addition, the relatively low amount of decaprenyl diphosphate (Dec-PP) that is released by the transglycosylation reaction must be recycled back to the cytoplasmic face of the plasma membrane (64) in order to generate more Lipid II. Inhibition of the recycling reaction by the antibiotic bacitracin is lethal in mycobacteria (65). Like Lipid II flipping, the recycling of phosphorylated carrier-lipids (Dec-P or Dec-PP) is likely...

---

**FIGURE 3** Pathways for cytoplasmic steps of PG precursor synthesis.
to require a flippase-mediated transport of this amphipathic molecule across the bilayer. Thus, the sustained export of PG precursors requires at least two distinct membrane translocation events: the eversion of Lipid II to the outside of the plasma membrane and the inversion of the carrier-lipid to allow reutilization. The structure of the recycled lipid carrier molecule has not been elucidated, leading to several possible scenarios for converting periplasmically localized Dec-PP into cytoplasmically oriented Dec-P. Each of these scenarios would require distinct accessory kinases and/or phosphatases.

The proteins that mediate the transbilayer movement of Lipid II and the reutilization of Dec-PP remain unidentified. However, both genetic and bioinformatic evidence suggests that the FtsW and MviN proteins (66–68) could be flippases involved in PG synthesis in several bacteria; in *M. tuberculosis*, the Rv3910 gene encodes a protein that has an N-terminal MviN-like domain, which is essential, and a C-terminal pseudokinase domain (69). Although not universally essential, homologs of the large MviN integral membrane protein are found in the genomes of PG-producing organisms and in several cases are located in operons that also encode the Lipid I-generating MraY enzyme (70). While direct sequence identity between flippases is generally very weak, MviN proteins are members of the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily (68, 71), which includes RfbX, a protein proposed to flip prenylphosphate-linked O-antigen units.

**FIGURE 4** Structure of the mycobacterial Lipid II PG precursor. $R_1 = \text{N-acetyl or N-glycolyl on the muramic acid residue.}$ doi:10.1128/microbiolspec.MGM2-0034-2013.f4
MviN orthologs are required for PG synthesis in *E. coli* (66, 68) and mycobacteria (69), and the inhibition of MviN causes an accumulation of the mature lipid- and nucleotide-linked PG precursors that would be expected from a block in export. The pseudokinase domain of Rv3910 can be phosphorylated by the essential cell division kinase, PknB, which results in the recruitment of the regulatory protein FhaA to MviN and subsequent inhibition of PG biosynthesis (69). Although it has been shown that MviN is essential for mycobacterial PG synthesis and growth, its specific role in PG synthesis remains elusive.

**PG ASSEMBLY**

Incorporation of the Lipid II precursors into the mature PG in the MAPc has essentially three steps: polymerization of the glycan chains, peptide cross-linking, and coupling of the PG to the galactan portion of the arabinogalactan via the N-acetylglucosamine-rhamnose linker. The last step is not clearly understood, but the genes involved with synthesis of the linker are essential (74). The enzyme involved with the actual coupling of the PG to the linker has not been identified, but it is known that the linkage of the PG to the arabinogalactan requires concomitant cross-linking of the PG, implying that the incorporation of newly synthesized precursors is coordinated with the assembly of the overall MAPc structure (75, 76).

The polymerization of the sugar backbone and cross-linking of the peptides of the PG are carried out by a variety of transglycosylases, transpeptidases, and carboxypeptidases that are schematically depicted in Fig. 5. *M. tuberculosis* has eight PBPs likely involved with transglycosylation, D,D-transpeptidation, and carboxypeptidation reactions, as well as five L,D-transpeptidases, but the number of these enzymes can vary between species (see the Fig. 5 legend) (10, 23, 24). The mycobacterial PBPs involved with transglycosylation and D,D-transpeptidation fall into different classes (10, 77). The PonA1 and PonA2 proteins are large, class A PBPs that have both transglycosylase and transpeptidase domains (10, 78, 79). An *M. smegmatis* ponA1 transposon mutant was isolated in a screen for mutants defective for long-term starvation (83), while ponA2 transposon mutants of *M. tuberculosis* were isolated from independent screens for β-lactam hypersusceptibility (36) or sensitivity to low pH (84). In the latter case, the mutant was also found to be hypersensitive to reactive nitrogen and oxygen species and attenuated in the mouse model (85). An unmarked, in-frame ponA2 deletion mutant of *M. smegmatis* exhibits decreased susceptibility to β-lactam antibiotics and increased susceptibility to rifampin and has morphological and survival defects under conditions of nonreplication (86). These data suggest that PonA2 has a regulatory role for maintaining cell wall integrity. Some soil mycobacteria contain an additional copy of PonA2, called PonA3, which is missing from pathogenic mycobacteria. An *M. smegmatis* mutant lacking ponA3 has no phenotype, but when expressed from a constitutive promoter on a multicopy plasmid, the ponA3 gene can partially complement a ΔponA2 mutant, suggesting some overlap of function (86). All soil mycobacteria that bear the ponA3 gene were isolated from contaminated with polycyclic aromatic hydrocarbons, and it is possible that the PonA3 protein may have a specific role in responding to cell envelope stresses in this particular niche.

Class B PBPs are generally involved with cell division/morphology and typically have only a transpeptidase domain. Mycobacteria have three class B proteins (see Fig. 5); two of them, PbpA and PbpB (FtsI), will be described in more detail in the cell division section below (10, 24). Note that PbpB is essential (49) and belongs to the B3 subgroup, owing to its additional transglycosylase domain (10). The remaining class B PBP is encoded by the Rv3627c gene in *M. tuberculosis*. 

(72), and WxzE, which has been shown to flip the Lipid III intermediate of the enterobacterial common antigen pathway (73). MviN orthologs are required for PG synthesis in *E. coli* (66, 68) and mycobacteria (69), and the inhibition of MviN causes an accumulation of the mature lipid- and nucleotide-linked PG precursors that would be expected from a block in export. The pseudokinase domain of Rv3910 can be phosphorylated by the essential cell division kinase, PknB, which results in the recruitment of the regulatory protein FhaA to MviN and subsequent inhibition of PG biosynthesis (69). Although it has been shown that MviN is essential for mycobacterial PG synthesis and growth, its specific role in PG synthesis remains elusive.
or the MSMEG_2584 gene in *M. smegmatis* (10). It is annotated as a potential lipoprotein that is not essential to *M. tuberculosis* (24, 49), but little else is known about this particular PBP.

The remaining PBPs are the carboxypeptidases DacB (PBP4), DacB1, and DacB2 (10, 24). *M. smegmatis* has an additional copy of DacB2, encoded by MSMEG_2433 (dacB2b), which is immediately downstream of the DacB2 (aka the dacB2a gene, MSMEG_2432). The role of these Dac proteins in mycobacterial PG biosynthesis is not entirely clear. An attenuated dacB1 transposon mutant of *M. tuberculosis* was identified in a nonhuman primate signature-tagged mutagenesis (STM) screen (87). A dacB2 mutant of *M. tuberculosis* has reduced growth in Sauton’s medium under acidic and microaerobic conditions but increased survival in host cells (88). Overexpression of the *M. tuberculosis* dacB2 gene in *M. smegmatis* results in reduced growth and altered colony morphology with concomitant changes in biofilm formation and sliding motility (88).

The last set of PG assembly enzymes are the L,D-transpeptidase (Ldt) enzymes. These proteins belong to
the YkuD superfamily and break L,D PG peptide bonds in order to form 3-3 cross-links in PG or link the PG peptides to other proteins (15, 17, 89). The latter reaction has only been seen in E. coli, in which a subset of L,D-transpeptidases couple Braun’s lipoprotein to the PG (90). Overall, these proteins are distinct from classical PBPs and are evolutionarily related to sortase enzymes that are found in Gram-positive bacteria (91). As mentioned earlier, the presence of 3-3 linkages was first discovered in mycobacteria, but since then these linkages have been found in many other bacteria. A specific role for these linkages has not been discovered, but they may function to stabilize the cell envelope (16).

The Ldt enzymes are encoded by several genes in mycobacteria, but the number varies according to species (24, 92). M. tuberculosis has five noncontiguous ldt genes (Fig. 5), while M. smegmatis has an additional variant (MSMEG_1322, ldtF) of the ldtB gene (MSMEG_47457), for a total of six. M. leprae has the same genes as M. tuberculosis, but the ldtE gene is a pseudogene. The LdtA, LdtB, and LdtD proteins are very similar to each other, while the LdtC protein appears to be a lipoprotein and has a C-terminal proline-rich region like that of the class A PBPs. The LdtE protein is notable because it has an N-terminal proline-rich region. The function of these proline-rich regions is unknown, but they may be involved with protein-protein interactions. All five Ldts of M. tuberculosis have been expressed in E. coli, but only the LdtA and LdtB proteins exhibit L,D-transpeptidase activity (12, 92). An M. tuberculosis ldtB transposon mutant was isolated in a screen for mutants with altered colony morphology (92); this mutant exhibited increased susceptibility to amoxicillin and was attenuated in the mouse model, while an M. smegmatis ldtB transposon mutant was isolated in a screen for resistance to ubiquinated peptides (93); this latter mutant had a reduced envelope permeability phenotype. The ldt genes are not individually essential, since transposon mutants of M. tuberculosis have been isolated with insertions in each of the genes, although only the ldtB mutant appears to have a phenotype (92). There may be issues of redundancy that prevent the identification of additional phenotypes, so further analysis of these genes is warranted.

The formation of 3-3 linkages requires the generation of tetrapeptide PG precursors from the pentapeptide precursors via the action of D,D-carboxypeptidases (11, 13). The tetrapeptides are the substrate for the L,D-transpeptidases. The role of the DacB, DacB1, and DacB2 carboxypeptidase enzymes described above has not been thoroughly investigated in the formation of 3-3 linkages, although it was recently shown that recombinant M. tuberculosis DacB2, purified from E. coli, has D,D-carboxypeptidase activity (20).

**PG TURNOVER**

The disruption of preexisting bonds is required for the insertion of newly synthesized PG into the maturing cell wall. These two opposing activities of breakdown and assembly must be balanced in order to maintain cellular integrity. Furthermore, because PG synthesis is energetically costly, bacteria have evolved mechanisms to recycle cell wall material turned over as a result of growth and cell division. Gram-negative bacteria such as E. coli have a complex series of enzymes that comprise a sophisticated PG recycling pathway capable of recovering and reusing the sugars and peptides of the PG and, in some cases, regulating the expression of transcriptionally repressed β-lactamases in response to PG damage (94). Not as much is known about the PG turnover systems in Gram-positive bacteria, and even less is known about such systems in mycobacteria. However, the mist is beginning to clear with some recent work that has examined various PG glycan hydrolases and endopeptidases (Table 1) that have been identified in mycobacteria.

The culm gene is essential to M. tuberculosis and encodes an amidase that cleaves between the muramyl acid residues and the L-alanine at the first position in the PG peptide (95). This is a typical autolysin activity that is often also found encoded in bacteriophage genomes. A similar enzyme, encoded by the lysA gene of mycobacteriophage Ms6, has found utility as a reagent in the analysis of mycobacterial PG structure (96). A proteomic analysis of phosphoproteins in M. tuberculosis

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Genes involved with PG turnover</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv#</td>
<td>Gene</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>Rv3915</td>
<td>cwiM</td>
</tr>
<tr>
<td>Rv0867c</td>
<td>rpfA</td>
</tr>
<tr>
<td>Rv1009</td>
<td>rpfB</td>
</tr>
<tr>
<td>Rv1884c</td>
<td>rpfC</td>
</tr>
<tr>
<td>Rv2389c</td>
<td>rpfD</td>
</tr>
<tr>
<td>Rv2450c</td>
<td>rpfE</td>
</tr>
<tr>
<td>Rv1477</td>
<td>ripA</td>
</tr>
<tr>
<td>Rv1478</td>
<td>ripB</td>
</tr>
<tr>
<td>Rv0024</td>
<td>Homologous to RipA/B</td>
</tr>
<tr>
<td>Rv2190c</td>
<td>Homologous to RipA/B</td>
</tr>
<tr>
<td>Rv1566c</td>
<td>Homologous to RipA/B, but lacks active site residue</td>
</tr>
<tr>
<td>Rv1728c</td>
<td>NLP/P60 family member, M. smegmatis protein has PG hydrolytic activity</td>
</tr>
<tr>
<td>Rv0320</td>
<td>NLP/P60 family member, M. smegmatis protein has PG hydrolytic activity</td>
</tr>
</tbody>
</table>
showed that CwlM is phosphorylated at several positions, but the significance of this is unknown (82).

A group of enzymes that also act on the glycan chain, known as “resuscitation-promoting factors,” encoded by the rpf genes (Table 1), play an enigmatic role in mycobacterial PG biology (97–100). The first Rpf protein was discovered in Micrococcus luteus and shown to resuscitate dormant cultures (97, 101). Rpf proteins were then found in a variety of Gram-positive bacteria and shown to have a lysozyme-like structure that could cleave the glycan chain of the PG (99, 102). The Rpf protein of M. luteus is essential, does cleave the PG, and has been shown to resuscitate dormant, nonculturable mycobacterial cultures (102–105). There are two hypotheses about the function of the Rpf proteins: they are responsible for the release of PG fragments that act as signaling messengers on other bacterial cells, or the cleavage of the PG is required to initiate replication after a period of dormancy (106). These functions may not be mutually exclusive in bacteria with multiple rpf genes. In either case, the action of the Rpf proteins is thought to be on PG, resulting in activation from dormancy. There are five rpf genes in M. tuberculosis, but only three of the genes found in M. tuberculosis (rpfA, rpfB, and rpfC) are present in M. leprae (24, 107, 108). M. tuberculosis mutants with single or multiple rpf mutations are viable (106, 108), and although the genes seem to have a certain degree of redundancy, some mutants do have altered phenotypes. Global transcriptional analysis of single unmarked deletion mutants of H37Rv showed that an rpfC mutant had the largest number of genes with altered expression, with a large transcriptional overlap with the rpfB, rpfD, and rpfE mutants and less of an overlap with an rpfA mutant (109).

Comparisons between different rpf studies are complicated by variations in mouse strains, route of infection, and background M. tuberculosis strains. Some studies used M. tuberculosis Erdman (108, 110), while others used an H37Rv derivative that is deficient in phthiocerol dimycocerosate (PDIM) production (111, 112). These studies have significantly increased our understanding of the influence of these proteins on mycobacterial physiology and pathogenesis, although the mechanism(s) by which these proteins function has remained elusive.

Single rpf marked deletion mutants of strain Erdman have no in vitro phenotype, except for a small colony phenotype for a ΔrpfB mutant that is the result of a polar effect on the downstream ksgA gene, encoding a 16S rRNA methyltransferase (108). The single mutants had no phenotype in an aerosol challenge using C57Bl/6 mice. Subsequently, the same group showed that the ΔrpfB mutant was defective for reactivation in the mouse model following immune suppression in the chronic stage of infection using aminoguanidine (110). Further work (113) using double deletion mutants showed that a ΔrpfA ΔrpfB mutant had a colony morphology change in vitro and was more deficient for resuscitation in vivo than the ΔrpfB mutant. A similar, if less pronounced, in vivo phenotype was seen for a ΔrpfA ΔrpfD mutant. The double ΔrpfA ΔrpfB mutant grew less well in murine bone marrow-derived macrophages and was more proinflammatory than wild type. With the exception of the macrophage survival defect, all phenotypes could be rescued by complementation with both the rpfA and rpfB genes.

Another study used mutants of H37Rv with multiple, unmarked deletion alleles of the rpf genes and the mouse B6 background using an intravenous administration route (111, 112). In this system, a ΔrpfA ΔrpfD ΔrpfE triple mutant had no phenotype, while a ΔrpfA ΔrpfC ΔrpfB and a ΔrpfA ΔrpfC ΔrpfD triple mutant were both attenuated in the mouse and were defective for spontaneous resuscitation in vitro. Another study, by Biketov et al. (111), using the same H37Rv strain background and mutants but using C57Bl/6 mice and an intraperitoneal route of entry, showed that a double mutant (ΔrpfA ΔrpfC) was less able to establish an infection in the lungs compared to wild type or a (ΔrpfA/ΔrpfB) double mutant. Both mutants persisted in the lungs at lower amounts than wild type and were defective in reactivation via aminoguanine treatment. These results for the ΔrpfA ΔrpfB mutant are in agreement with those of Russell-Goldman et al. (113), but the latter group did not see a phenotype with a ΔrpfA ΔrpfC mutant; this may be due to differences in bacterial strain background and route of entry. Biketov et al. (111) also showed that the ΔrpfA ΔrpfC ΔrpfB and ΔrpfA ΔrpfC ΔrpfD strains that were previously shown to have an in vitro resuscitation defect also have difficulty establishing a lung infection after intraperitoneal injection, with the ΔrpfA ΔrpfC ΔrpfD mutant exhibiting a greater defect. Furthermore, both strains were unable to reactivate in the mouse model after treatment with either aminoguanine or anti-TNFα antibody.

Further work with this strain set and the construction of quadruple ΔrpfA ΔrpfC ΔrpfB ΔrpfE and quintuple ΔrpfA ΔrpfC ΔrpfB ΔrpfE ΔrpfD mutants showed that these mutants had delayed growth on solid media, with a greater delay seen on Middlebrook 7H11 than 7H10 (106). These mutants were also hypersusceptible to detergent, and it was shown that this was due to loss of
both \( \text{rpfB} \) and \( \text{rpfE} \). Multiple deletion mutants \( \Delta \text{rpfA} \Delta \text{rpfC} \Delta \text{rpfD} \Delta \text{rpfA} \Delta \text{rpfC} \Delta \text{rpfB} \Delta \text{rpfE} \) were defective for in vitro resuscitation as well as persistence in B6 mice following aerosol challenge, with the latter mutant exhibiting a more pronounced defect. These mutants differed little in their ability to grow in human peripheral blood mononuclear cells, in contrast to the intracellular growth defect observed for a \( \Delta \text{rpfA} \Delta \text{rpfB} \) mutant in murine bone marrow-derived macrophages as described above. Additional studies showed that the \( \Delta \text{rpfA} \Delta \text{rpfC} \Delta \text{rpfD} \Delta \text{rpfA} \Delta \text{rpfC} \Delta \text{rpfD} \Delta \text{rpfE} \) mutants are attenuated after aerosol infection of mice and that they cannot reactivate after aminoguanidine treatment. Interestingly, the mutants can persist in mice after subcutaneous injection and can be as protective as BCG in vaccination experiments (\(114\)).

It is clear from these studies that the Rpf proteins have important roles in the physiology of dormancy and in pathogenesis. There is some overlap in function, but it seems that RpfB and RpfE have the most significant roles, with RpfD and RpfC in smaller roles that have redundancies with RpfA. The lack of \( \text{rpfD} \) and \( \text{rpfE} \) genes in \( \text{M. leprae} \) and the lack of \( \text{rpfD} \) in \( \text{M. smegmatis} \) may suggest adaptation to different niches, or variations in PG physiology for these bacteria, compared to \( \text{M. tuberculosis} \).

Several mycobacterial genes encode enzymes belonging to the NPL/P60 protein family (\(115\)). This family, an early member of which was identified in \( \text{Listeria monocytogenes} \) as an invasin protein, commonly share a PG degradation domain that includes a conserved cayltic cysteine residue (\(115, 116\)). There are seven such genes in the \( \text{M. tuberculosis} \) genome (Table 1); all but two (\( \text{Rv0024}, \text{Rv1728c} \)) are present in \( \text{M. leprae} \) (\(24, 107\)). The best studied are the \( \text{Rv1477} \) and \( \text{Rv1478} \) genes, also known as \( \text{ripA} \) and \( \text{ripB} \), or alternatively, \( \text{iipA} \) and \( \text{iipB} \). A transposon mutant with an insertion in this operon of genes was first identified in \( \text{Mycobacterium marinum} \), and the genes were named \( \text{iip} \) because the mutant was defective for entry into, and survival in, host cells (\(117\)). A constructed \( \text{iipA}::\text{kan} \) mutant had an invasion and intracellular survival defect; was attenuated in zebrafish; was hyper-susceptible to rifampin, ciprofloxacin, erythromycin, and lysozyme; and had a septation defect. These phenotypes could be complemented by the \( \text{M. marinum} \) or \( \text{M. tuberculosis} \) genes, but some phenotypes could only be partially complemented by \( \text{iipB} \) (\( \text{ripB} \)) and some required both genes. This suggests that both genes are not entirely redundant. Both RipA and RipB from \( \text{M. tuberculosis} \) have been crystallized and their structures determined (\(118\)). While they share a common core, there are substantial changes in their N-termini. Both enzymes are \( \text{L-D} \) endopeptidases, cleaving PG fragments between the \( \text{D-iso}-\text{glutamine} \) and \( \text{meso-DAP} \), with RipA being capable of degrading intact PG while RipB cannot (\(118\)).

RipA has been shown to interact with RpfE and RpfB, and RipA/RpfB localize to the septa where RipA is responsible for digesting the PG in the process of septation (\(119\)). Furthermore, RipA depletion results in a severe septation defect, which is \( \text{ripA} \) specific because \( \text{ripB} \) cannot complement this defect. In addition, the degradative activity of RipA on the PG appears to synergize with that of RpfB (\(120\)). RipA also interacts with the class A PBP PonA1, which regulates the activity of the protein during vegetative growth (\(81\)). More recent work has shown that RipA is produced as a zymogen and that a complex series of protein-protein interactions serves to keep the protein tightly regulated (\(121\)). Consistent with this view, the dysregulation of this control circuit results in morphological changes and defects in cell division (\(121\)).

The remaining five endopeptidases are not as well studied as RipA/RipB and Ibp/IbpB. Three of them, \( \text{Rv0024}, \text{Rv2190c}, \text{and Rv1566c} \), are homologous to RipA/B, but the \( \text{Rv1566c} \) protein lacks the active site cysteine and may have a different function. \( \text{Rv2109c} \) has a role in cell envelope physiology and pathogenesis. An \( \text{M. tuberculosis} \) \( \text{Rv2109c} \) transposon mutant has a growth and colony morphology defect, has altered levels of phtihioconid dimyocercosate, is slightly more susceptible to lysozyme, and is attenuated in the mouse model (\(122\)). The endopeptidases \( \text{Rv1728c} \) and \( \text{Rv0320} \) have not been studied in \( \text{M. tuberculosis} \), but recombinant versions of the \( \text{M. smegmatis} \) proteins have been shown by zymographic analysis to degrade PG (\(123\)). Additional work is needed to define the substrate specificities of these enzymes and to determine what role they play in PG metabolism.

**PG GENES AND CELL DIVISION**

Mycobacteria have most of the cell division genes commonly seen in other rod-shaped bacteria, with the notable lack of the Mre proteins for coordinating lateral cell wall growth. Most of the cell division genes have been identified by homology; the complete list, thus far, is shown in Table 2 (\(24\)). The \( \text{fts} \) genes, involved with development of the septal FtsZ ring and DNA partitioning, are present, along with the essential class B3 PbpB (aka FstI or PBP3) and the class B PbpA. PbpB is essential (\(49\)), as it is in other bacteria, and it has been shown to interact with FtsW and form a trimolecular
Several other cell division genes unique to mycobacteria have been identified. One of them, crgA (Rv0011c), which encodes a nonessential homolog of a protein involved with hyphal wall regulation in *Streptomyces*, was shown to interact with FtsZ and FtsQ, as well as PbpA and PpbB (134, 135). CrgA appears to have a role in septum production, as a deletion mutant of *M. smegmatis* grew as elongated cells with polar bulges, and overexpression of CrgA seems to stabilize the localization of PpbB at the septum. CrgA also interacts with another nonessential protein called CwsA (Rv0008c), which is involved with cell shape and cell wall biosynthesis (135). A cwsA deletion mutant of *M. smegmatis* exhibits rounding, bulges, and a decrease in PG synthesis. Loss of both crgA and cwsA in a double mutant leads to a synergistic cell wall defect with a loss of viability and increased autolysis with detergent. Another protein, ChiZ (Rv2719c), is an unspecified cell wall hydrolase that has a role in regulating division indirectly by influencing the stability of FtsZ ring formation, possibly by its interactions with FtsQ and PpbB (136). Another protein that influences cell division is the Ssd, or septum site-determining protein, encoded by the Rv3660c gene (137). Overexpression of this gene in *M. smegmatis* and *M. tuberculosis* results in longer, smoother cells, while an *M. tuberculosis* ssd transposon insertion mutant exhibits shorter cells (137). Interestingly, expression of ssd was linked to a transcriptional program related to dormancy and stress, suggesting a connection with cell division regulation and an adaptive environmental response.

### OTHER GENES INVOLVED WITH ANTIBIOTIC RESISTANCE AND CELL WALL METABOLISM

Mycobacteria, and *M. tuberculosis* in particular, are inherently resistant to β-lactam antibiotics such as penicillins, cephalosporins, and carbapenems, and thus these drugs have not been used to treat infections. A notable exception is the use of cefoxitin or imipenem in conjunction with clarithromycin or amikacin in the treatment of *M. abscessus* infections (138). In general, resistance to the β-lactams has been thought to result from (i) reduced permeability of the cell envelope to these drugs, (ii) differences in the susceptibility of PG assembly enzymes, and (iii) enzymatic degradation of the antibiotics by β-lactamases. Efflux pumps may also play a smaller role in resistance (139). Several groups have shown that the use of a β-lactam inhibitor such as clavulanic acid in conjunction with a β-lactam antibiotic is lethal to mycobacteria, suggesting that the primary

### TABLE 2 Genes involved with cell division

<table>
<thead>
<tr>
<th>H37Rv#</th>
<th>Gene</th>
<th>Description of gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv0008c</td>
<td>cwsA</td>
<td>Cell wall synthesis and cell shape protein</td>
</tr>
<tr>
<td>Rv0011c</td>
<td>crgA</td>
<td>Facilitates cell septation</td>
</tr>
<tr>
<td>Rv0017c</td>
<td>rodA (ftsW)</td>
<td>Facilitates cell septation</td>
</tr>
<tr>
<td>Rv0016c</td>
<td>pbpA</td>
<td>Class B PBP</td>
</tr>
<tr>
<td>Rv2163c</td>
<td>ppbB (ftsI)</td>
<td>Class B PBP</td>
</tr>
<tr>
<td>Rv2150c</td>
<td>ftsZ</td>
<td>Cell septation ring initiator/scaffold</td>
</tr>
<tr>
<td>Rv2151c</td>
<td>ftsQ</td>
<td>Involved with coordinating septation/PG synthesis</td>
</tr>
<tr>
<td>Rv2748c</td>
<td>ftsK</td>
<td>DNA motor involved with chromosome partitioning</td>
</tr>
<tr>
<td>Rv2921c</td>
<td>ftsY</td>
<td>Signal recognition particle receptor</td>
</tr>
<tr>
<td>Rv3101c</td>
<td>ftsX</td>
<td>ABC transporter component</td>
</tr>
<tr>
<td>Rv3102c</td>
<td>ftsE</td>
<td>ABC transporter component</td>
</tr>
<tr>
<td>Rv3610c</td>
<td>ftsH</td>
<td>Quality control membrane protease</td>
</tr>
<tr>
<td>Rv2154c</td>
<td>ftsW-like</td>
<td>Facilitates cell septation?</td>
</tr>
<tr>
<td>Rv2145c</td>
<td>wag31</td>
<td>DivIVA family member, mediates cell division</td>
</tr>
<tr>
<td>Rv3660c</td>
<td>ssd</td>
<td>Septum site determining protein</td>
</tr>
<tr>
<td>Rv2719c</td>
<td>chiZ</td>
<td>Hydrolase involved with regulation of cell division</td>
</tr>
</tbody>
</table>
mechanism of resistance is enzymatic degradation (140–145). In *M. tuberculosis*, the BlaC enzyme, encoded by the blaC gene, or RV2068c, has been shown to have a broad spectrum of activity (147), and a blaC deletion mutant of *M. tuberculosis* is hypersusceptible to penicillins (148). However, the mutant has little or no change in susceptibility to cephalosporins. A similar situation was observed in an *M. smegmatis* mutant lacking the major β-lactamase, BlaS. *M. smegmatis* has another β-lactamase, encoded by blaE, but this enzyme makes a very minor contribution to the resistance profile of this organism (148).

The expression of β-lactamase activity in *M. tuberculosis* is constitutive, but the blaC gene can be induced in response to β-lactam antibiotics via a regulatory system encoded by the blaR-blaI operon (Rv1845c-Rv1846c), which is similar to the mecI-mecR system of *S. aureus* (149). The system is remarkable in that it also regulates the ATP synthase operon, suggesting a control circuit between synthesis of the cell wall and ATP generation. The blaC gene is absent in *M. leprae*, but the regulatory blaR-blaI genes appear to be intact, suggesting that the β-lactam response may be more important for linking cell wall damage to ATP synthesis control rather than direct protection of the cell from β-lactam damage by the β-lactamase (149). This raises the general question, Why does *M. tuberculosis* still have a β-lactamase? The presence of β-lactamase genes in environmental mycobacteria can easily be explained by their ecological niches being replete with β-lactam-producing organisms, but for a pathogen such as *M. tuberculosis* with no environmental niche, one can only speculate. Perhaps the blaC gene hasn’t yet had sufficient time to degenerate as it has in *M. leprae*, or perhaps the protein has some other function in PG metabolism such that there is pressure for its continued existence in the *M. tuberculosis* genome.

Searching the *M. tuberculosis* H37Rv genome for the term “lactamase” results in several genes that are annotated as β-lactamases or esterases or carboxypeptidases (see Table 3; Rv0339c, Rv0406c, Rv3677c, Rv0907, Rv1703c, Rv1923, Rv1497, and Rv3762), but very little is known about the function of the corresponding proteins (24). The Rv0406c and Rv3677c genes have been expressed in *E. coli*, purified, and shown to be able to degrade antibiotic (150). β-lactamases and carboxypeptidases are related to each other, and thus, some carboxypeptidases that are involved with PG metabolism may have weak β-lactamase activity, so such results should be interpreted with caution (151). A mutant of *M. smegmatis* with a deletion of the gene corresponding to Rv0907 is less susceptible to vancomycin, although the significance of this is not known (123). The Rv1923 and Rv1497 proteins are annotated as putative lipases and thus may not have any role in PG metabolism. Further investigation into these genes is warranted to achieve a better understanding of the interaction between β-lactam antibiotics and mycobacteria, particularly since the idea of using β-lactamase inhibitors with these antibiotics is gaining traction in the treatment of tuberculosis.

Several other genes have been identified in a transposon screen of β-lactamase-deficient mutants of *M. tuberculosis* and *M. smegmatis* looking for mutants with increased susceptibility to cephalosporins (36). Several mutants were found, all with increased susceptibility to penicillins and cephalosporins, as well as lysozyme. Some of the affected genes were already known to be involved with PG biosynthesis (ponA2, namH, dapB), but a few other genes are worthy of note. Two mutants of *M. smegmatis* had cell division defects, with insertions that affected genes encoding proteins with homology to proteins involved with cell division. One gene, MSMEG_5414 (cdpA), homologous to Rv1024, is similar to DivIVC, which is

<table>
<thead>
<tr>
<th>H37Rv#</th>
<th>Gene</th>
<th>Description of gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv0268c</td>
<td>blaC</td>
<td>Class A β-lactamase</td>
</tr>
<tr>
<td>Rv0399c</td>
<td>lipK</td>
<td>Homologous to β-lactamases/esterases/carboxypeptidases</td>
</tr>
<tr>
<td>Rv0406c</td>
<td>Homologous to β-lactamases/esterases/carboxypeptidases</td>
<td></td>
</tr>
<tr>
<td>Rv3677c</td>
<td>Homologous to β-lactamases/esterases/carboxypeptidases</td>
<td></td>
</tr>
<tr>
<td>Rv0907</td>
<td>Homologous to β-lactamases/esterases/carboxypeptidases</td>
<td></td>
</tr>
<tr>
<td>Rv1703c</td>
<td>Homologous to β-lactamases/esterases/carboxypeptidases</td>
<td></td>
</tr>
<tr>
<td>Rv1923</td>
<td>lipD</td>
<td>Homologous to β-lactamases/esterases/carboxypeptidases (may be a lipase)</td>
</tr>
<tr>
<td>Rv1497</td>
<td>lipL</td>
<td>Homologous to β-lactamases/esterases/carboxypeptidases (may be a lipase)</td>
</tr>
<tr>
<td>Rv3762</td>
<td>Homologous to β-lactamases/esterases/carboxypeptidases</td>
<td></td>
</tr>
<tr>
<td>Rv1024</td>
<td>cdpA</td>
<td>DivIC family member, septum formation initiator</td>
</tr>
<tr>
<td>Rv2927c</td>
<td>cdpC</td>
<td>DivIVA family member, cell division inhibitor protein</td>
</tr>
<tr>
<td>Rv2198c</td>
<td>mmpS3</td>
<td>Mycobacterial membrane protein, small</td>
</tr>
<tr>
<td>Rv2224c-Rv2225c</td>
<td>Secreted proteases</td>
<td></td>
</tr>
</tbody>
</table>
involved with the initiation of septum formation in Gram-positive bacteria, while the other gene, MSMEG_2416 (cdpC), homologous to Rv2927c, is similar to the DivIVA protein, which is important for the initiation of division in Gram-positive bacteria. Another M. smegmatis mutant with an insertion in the first gene in a two-gene operon (MSMEG_4296-MSMEG_4295), homologous to Rv2224c-Rv2223c, has swollen poles and is highly susceptible to lysozyme. The proteins encoded by these genes appear to be secreted proteases that may have a role in regulating the levels of proteins involved with PG metabolism. Transposon mutants of M. tuberculosis with an insertion in Rv2224c were isolated in two independent screens for hypersensitivity to low pH (84) and defective intracellular survival (152); a defined Rv2224c mutant was attenuated in the mouse, promoted a stronger innate immune response, and was hypersusceptible to lysozyme (153). The Rv2224c protein was also shown to process the GroEL2 chaperon (153). The link between protease activity, PG metabolism, and pathogenesis is not entirely clear, but this protein (and Rv2225c) should be further investigated. Lastly, an M. tuberculosis mutant with an insertion in the putative promoter region of the mmpS3 gene had normal cell morphology with increased β-lactam susceptibility (36). Little is known about the MmpS3 protein, but it belongs to a family of small membrane proteins that are involved with transport functions (154). This protein may play a role in assembly or some other aspect of the cell envelope that may allow better penetration of the β-lactam, but the mutant was not hypersusceptible to other cell envelope-specific antibiotics such as isoniazid or ethambutol.

**FUTURE RESEARCH**

While much is known about the basic pathways involved with mycobacterial PG monomer synthesis, there are several areas in which additional work is needed. What are the enzymes involved with PG modifications such as amidation, and what role do these modifications play in mycobacterial PG biology? How does the cell move PG monomers across the cell membrane? Why are there so many L,D-transpeptidases in mycobacteria? How are the 3-3 linkages synthesized, and what is their function? How is assembly of the PG coupled to that of the arabinogalactan? How is the division machinery linked to PG wall metabolism and the pathways involved with assembly of the MAPC? With regard to turnover of the PG: What is the role of Rpf proteins in PG metabolism, and how does this influence the ability of M. tuberculosis to persist and reactivate in an infection? How do the cell wall hydrolases interact with PBPs and Ldts to balance PG biosynthesis with degradation? Many proteins involved with PG metabolism appear to be phosphorylated, and this is likely to be a global mechanism of control that may be important in coordinating all these aspects of cell wall biosynthesis. Investigating the interactions with mycobacteria and β-lactam antibiotics, the role accessory proteins may play in this, and the significance of β-lactamases in mycobacteria will not only provide a clearer picture of PG metabolism, but also help in the rational development of potential new drug therapies to treat intractable mycobacterial infections.

**ACKNOWLEDGMENT**

We thank Fabio L. Fontes for the preparation of Fig. 1, 2, and 4.

**REFERENCES**


