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Characterization of Novel *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* Mutants Hypersusceptible to β-Lactam Antibiotics

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Our laboratory previously constructed mutants of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* with deletions in the genes for their major β-lactamases, BlaC and BlaS, respectively, and showed that the mutants have increased susceptibilities to most β-lactam antibiotics, particularly the penicillins. However, there is still a basal level of resistance in the mutants to certain penicillins, and the susceptibilities of the mutants to some cephalosporin-based β-lactams are essentially the same as those of the wild types. We hypothesized that characterizing additional mutants (derived from β-lactamase deletion mutants) that are hypersusceptible to β-lactam antibiotics might reveal novel genes involved with other mechanisms of β-lactam resistance, peptidoglycan assembly, and cell envelope physiology. We report here the isolation and characterization of nine β-lactam antibiotic-hypersusceptible transposon mutants, two of which have insertions in genes known to be involved with peptidoglycan biosynthesis (*ponA2* and *dapB*); the other seven mutants have insertions which affect novel genes. These genes can be classified into three groups: those involved with peptidoglycan biosynthesis, cell division, and other cell envelope processes. Two of the peptidoglycan-biosynthetic genes (*ponA2* and *pbpX*) may encode β-lactam antibiotic-resistant enzymes proposed to be involved with the synthesis of the unusual diaminopimelyl linkages within the mycobacterial peptidoglycan.

The mycobacteria consist of a diverse group of organisms representing saprophytic bacteria as well as important human pathogens. Organisms such as *Mycobacterium chelonae*, *Mycobacterium smegmatis*, and *Mycobacterium fortuitum* are environmental bacteria and rarely cause disease. On the other hand, *Mycobacterium tuberculosis*, the causative agent of the disease tuberculosis, has successfully infected one-third of the world's population and is responsible for over 2 million deaths annually (15). Interest in mycobacterial pathogenesis and physiology has been stimulated by the emergence of multidrug-resistant strains of *M. tuberculosis* as well as the observation that the immunocompromised (e.g., those with AIDS) are at a higher risk for *M. tuberculosis* infection (20, 21).

The biosynthesis of the mycobacterial cell envelope is an area of considerable research interest, as several of the antibiotics used to treat mycobacterial infections target the synthesis pathways of several envelope components. The main feature of the mycobacterial cell envelope is a single, covalently linked structure composed of peptidoglycan, arabinogalactan, and mycolic acids (the mAGP complex) (5). The peptidoglycan is the innermost layer of the complex and is attached to the arabinogalactan layer via a rhamnose-*N*-acetylglucosamine linker. The arabinose residues are in turn esterified to mycolic acids, which are long-chain (60 to 90 carbon) α-alkyl, β-hydroxy fatty acids oriented perpendicularly to the cell membrane (36). Extractable lipids in association with the mycolic acids serve to form an outer membrane-like structure at the cell surface.

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Our laboratory studies mycobacterial peptidoglycan biosynthesis and the role of the peptidoglycan in the physiology of the mycobacterial cell envelope. We are interested in using β-lactam antibiotics, which target peptidoglycan assembly enzymes, as tools to study peptidoglycan biosynthesis. However, mycobacteria are intrinsically resistant to β-lactam antibiotics, primarily due to the production of β-lactamases, although the permeability of the cell envelope, drug efflux pumps, low-affinity penicillin-binding proteins (PBPs), and β-lactam-insensitive peptidoglycan-biosynthetic enzymes may also play a role in resistance to this class of antibiotics (8, 22, 29, 35). The intrinsic resistance of these organisms has hampered efforts to use these antibiotics to study cell wall biosynthesis.

To eliminate this problem, our laboratory has constructed mutants of *M. tuberculosis* and *M. smegmatis* with deletions of the major β-lactamase genes, *blaC* and *blaS*, respectively (17). We previously showed that the mutants have an increased susceptibility to most β-lactam antibiotics, particularly the penicillins. However, there is still a basal level of resistance in the mutants to certain penicillins, and the susceptibilities of the mutants to some cephalosporin-based β-lactams are essentially the same as those of the wild types (17). We found a minor cephalosporinase, BlaE, in *M. smegmatis* but showed that its contribution to β-lactam resistance is minimal (17). Our data are consistent with the view that additional mechanisms contribute to β-lactam resistance in these two organisms.

We hypothesized that characterizing mutants derived from β-lactamase deletion mutants that are hypersusceptible to β-lactam antibiotics might reveal novel genes involved with other mechanisms of β-lactam resistance, peptidoglycan assembly, and cell envelope physiology. To this end, we performed transposon mutagenesis of the β-lactamase mutants

TABLE 1. Strains used in this study

Strain	Description	Source or reference
E. coli		
DH10B	F⁻ *mcrA* Δ(*mrr-hsdRMSmcrBC*) ϕ80d*lacZ* ΔM15 Δ*lacX74 deoR recAI ara*Δ*139* (*ara leu*)7697 *galU galK* λ⁻ *rpsL endAI nupG*	Gibco-BRL
M. smegmatis		
PM759	*ept-1* Δ*lysA4 rpsL6* Δ*blaS1*	17
PM1078	PM759 *sdrA*::[EZ::Tn<R6Kγ*ori*/KAN-2>]	This study
PM1079	PM759 *nabA*::[EZ::Tn<R6Kγ*ori*/KAN-2>]	This study
PM1081	PM759 *dapB*::[EZ::Tn<R6Kγ*ori*/KAN-2>]	This study
PM1082	PM759 *enoA-cdpAB*::[EZ::Tn<R6Kγ*ori*/KAN-2>]	This study
PM1083	PM759 *ump*::[EZ::Tn<R6Kγ*ori*/KAN-2>]	This study
PM1084	PM759 *expA*::[EZ::Tn<R6Kγ*ori*/KAN-2>]	This study
M. tuberculosis		
PM638	H37Rv (virulent) Δ*blaC1*	17
PM1066	PM638 *Rv2199c-mmpS3*::Tn*5370*	This study
PM1067	PM638 *Rv2844*::Tn*5370*	This study
PM1068	PM638 *ponA2*::Tn*5370*	This study

of *M. tuberculosis* and *M. smegmatis* described above and screened for mutants hypersusceptible to the cephalosporin ceftriaxone. We report here the characterization (i) of seven β-lactam antibiotic-hypersusceptible mutants in which the transposon affects genes with no known function and (ii) of two mutants in which the insertions are in known peptidoglycan-biosynthetic genes. Our analyses suggest that the seven unknown proteins are involved with peptidoglycan biosynthesis, cell division, or other cell envelope processes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are shown in Table 1. *M. tuberculosis* PM638 (Δ*blaC1)* and *M. smegmatis* PM759 (Δ*blaS1)* are β-lactamase-deficient mutants of the common laboratory strains *M. tuberculosis* H37Rv and *M. smegmatis* mc²155 (17). *Escherichia coli* cultures were grown in Luria-Bertani (LB) broth (Difco, BD Biosciences, San Jose, Calif.) or on LB agar. *M. tuberculosis* cultures were grown in Middlebrook 7H9 broth (Difco, BD Biosciences) or on Middlebrook 7H11 medium (Difco, BD Biosciences). *M. smegmatis* was grown in either LB broth supplemented with 0.05% (vol/vol) Tween 80 (LBT) or Middlebrook 7H9 to which L-lysine was added at 40 μg/ml. All Middlebrook media were supplemented with 0.05% (vol/vol) Tween 80, 0.2% (wt/vol) glycerol, and ADS (0.5% [wt/vol] bovine serum albumin, fraction V; 0.2% [wt/vol] dextrose; and 0.85% [wt/vol] NaCl). Sucrose was added to media at a concentration of 2% (wt/vol). When necessary, ampicillin (50 μg/ml; Sigma-Aldrich Chemical, St. Louis, Mo.), hygromycin (50 μg/ml; Roche Applied Science, Indianapolis, Ind.), kanamycin (10 μg/ml for *M. smegmatis* or 25 μg/ml for *E. coli*; Sigma-Aldrich) or diaminopimelic acid (DAP; 200 μg/ml; Sigma-Aldrich) was added to media. *M. smegmatis* plates were incubated for 3 to 5 days, while *M. tuberculosis* plates were incubated for 3 to 4 weeks at 37°C. Inoculation and growth conditions for allelic exchange in *M. smegmatis* using the *sacB* suicide plasmid pYUB657 were as previously described (38).

DNA manipulation. Basic DNA methods were essentially as described previously (32). Detailed descriptions of the plasmids used in this study can be obtained from the corresponding author. Plasmids were constructed in *E. coli* DH10B and were prepared by an alkaline lysis protocol or with QIAGEN (Valencia, Calif.) columns, if used for allelic exchange. DNA fragments were isolated using QIAquick spin columns (QIAGEN). Genomic DNA was prepared as described previously from *M. tuberculosis* (38) and *M. smegmatis* (24). Oligonucleotides were synthesized by Invitrogen Life Technologies (Carlsbad, Calif.). A Perkin-Elmer GeneAmp 2400 temperature cycler was used for all PCRs. All restriction and DNA-modifying enzymes were from Fermentas (Hanover, Md.) or New England Biolabs (Beverly, Mass.). Electroporation of *M. smegmatis* and *M. tuberculosis* was as previously described (37, 38). DNA sequencing was performed by ACGT, Inc. (Wheeling, Ill.).

Transposon mutagenesis. Preparations of the temperature-sensitive phage phAE87, which harbors the transposon Tn*5370*, were generated from *M. smegmatis* and used to mutagenize *M. tuberculosis* PM638 as previously described (3, 34) but with a few modifications. *M. tuberculosis* cultures were grown to saturation in 50 ml of Middlebrook 7H9, washed two times in MP buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 2 mM CaCl), and resuspended in 2 ml of the same buffer. One milliliter of a high-titer phAE87 lysate (10^{11} PFU/ml) was added to the washed cells (multiplicity of infection of approximately 10), and the phage were allowed to adsorb to the cells for 2 h at 37°C. Cells were then plated onto Middlebrook 7H10 medium with hygromcyin and incubated at 37°C for 3 to 4 weeks. A total of 1,996 colonies were picked and patched onto media with or without ceftriaxone at 7.5 μg/ml.

For transposon mutagenesis in *M. smegmatis* PM759, we utilized the EZ::TN <R6Kγ*ori*/KAN-2> mutagenesis system (Epicentre Technologies, Madison, Wis.). In vivo transposition was carried out with in vitro-generated transposomes as previously described for *M. smegmatis* (14). Following electroporation, cells were plated on LBT medium containing kanamycin and incubated at 37°C for 5 to 7 days. A total of 550 mutants were picked and patched onto media with or without ceftriaxone at 7.5 μg/ml.

Susceptibility testing. Antibiotic disk diffusion tests were done as previously described for *M. tuberculosis* and *M. smegmatis* (17) with the following disks (Sensi-Disks; BBL, BD Biosciences): disks containing ampicillin (10 μg), cefoxitin (30 μg), ceftriaxone (30 μg), ethambutol (50 μg), isoniazid (5 and 1 μg), oxacillin (10 μg), and rifampin (5 μg).

MICs were determined in LBT by the broth macrodilution method as previously described for *M. smegmatis* (17). MICs for *M. tuberculosis* were determined at The Wadsworth Center (New York State Department of Health, Albany, N.Y.) by the radiometric (BACTEC) method. All antibiotics used in MIC determinations were obtained from Sigma-Aldrich. Lysozyme MICs were determined with freshly prepared hen egg white lysozyme (ICN Biomedicals, Aurora, Ohio) solutions.

Mapping of transposon insertions. Genomic cosmid libraries were generated from each mutant by using the cosmid vector pYUB328 (2) and kanamycin- or hygromycin-resistant cosmid clones isolated from *E. coli* and used for DNA sequencing with the EZ::TN-specific primers R6KAN-2 RP-1 and KAN-2 FP-1 or with primers specific for the *hyg* gene carried on Tn*5370*. A minimum of 600 bp of sequence was obtained from at least one end of the transposon in each mutant. The DNA sequences were used to search the *M. smegmatis* mc²155 genome at The Institute for Genomic Research (http://www.tigr.org/tdb/mdb/mdbinprogress.html) or the annotated *M. tuberculosis* H37Rv genome (http://www.sanger.ac.uk/Projects/M_tuberculosis/, and http://genolist.pasteur.fr/TubercuList/). Broader searches were performed at the National Center for Biotechnology website (http://www.ncbi.nlm.nih.gov) and utilized the basic local alignment search tool (BLAST) (1) for searching the nonredundant protein database and the conserved domain database with reverse-position-specific BLAST (33).

Phase-contrast microscopy. Differential interference contrast (DIC) microscopy was performed on mutants grown in LBT. Prior to microscopy, cultures were sonicated at low intensity in a water bath for 30 s. Five microliters of sonicated culture was placed on a slide, covered with a coverslip, and subjected

TABLE 2. MICs for *M. smegmatis* wild-type PM759 and transposon mutants[a]

Antibiotic	MIC(s) (μg/ml)						
	PM759	PM1078	PM1079	PM1081	PM1082	PM1083	PM1084
Amoxicillin	2	0.5	<0.125	0.5	0.5	<0.125	0.5
Ampicillin	16	2	1	2–4	2	<0.125	4
Carbenicillin	16–32	2–4	2–4	2	2–4	2–4	16
Oxacillin	8	<0.125	0.5–1	2	0.25	<0.125	2–4
Cefoxitin	4	1	1	0.5–1	1–2	0.5–1	2–4
Ceftriaxone	16	0.25	<0.125	0.25–0.5	0.25–0.5	<0.0625	2
Isoniazid	4	2	4	8	8	4	8
Ethambutol	0.5	0.5	0.5	0.5	0.5	0.25	0.5
Rifampin	2	0.25	2	2–4	1–2	0.5–1	2–4
Lysozyme	2,000	125	62.5	250	250	31.25–62.5	125

[a] Values are reported as a range or as a single number in cases of nonvariant results from determinations performed in duplicate with at least two cultures.

to DIC microscopy performed with a Leica TCS SP1 microscope; images were captured using Leica LCS 1347 imaging software.

RESULTS

Isolation of β-lactam-hypersusceptible mutants. Transposon mutants with increased susceptibility to ceftriaxone on patch plates were retested by streak plating, and then Southern blotting was performed to identify those mutants with a single transposon insertion in their chromosome (data not shown). Out of 11 mutants (8 *M. smegmatis* and 3 *M. tuberculosis* mutants) that were identified as hypersusceptible, only 6 of the *M. smegmatis* mutants and all 3 *M. tuberculosis* mutants contained a single transposon insertion.

Susceptibility testing of transposon mutants. We initially used disk diffusion assays to test the antibiotic susceptibilities of the *M. smegmatis* mutants and showed that all had increased susceptibility to different β-lactam antibiotics and had little or no increased susceptibility to other classes of antibiotics (data not shown). To further define the extent of this increased susceptibility, we determined the MICs of several antibiotics for the mutants (Table 2). Some of the antibiotics (oxacillin, ceftriaxone, and cefoxitin) used in these assays were chosen because they are antibiotics to which the parental β-lactamase mutant strains show no appreciable difference in susceptibility from that of the wild type (17). All the *M. smegmatis* mutants showed increased susceptibilities to ceftriaxone; the MICs for them were 8- to 256-fold lower than those for the parental strain PM759. There were comparable decreases in the MICs of most other β-lactam antibiotics for the mutants, with only a marginal change in susceptibility to cefoxitin. We observed no significant decreases in the MICs of the common antimycobacterial drugs isoniazid, ethambutol, or rifampin, which do not target peptidoglycan biosynthesis. The one exception was the rifampin MIC for strain PM1078, which exhibited an eightfold decrease.

We also tested the *M. smegmatis* mutants for susceptibility to growth inhibition by lysozyme, an endo-*N*-acetylmuramidase that hydrolyzes the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan (19). All the mutants showed significantly increased susceptibilities to the enzymatic action of lysozyme compared to that of the parental strain PM759; the MICs for them were 8- to 320-fold less (Table 2).

The changes in the susceptibilities of the *M. tuberculosis* transposon mutants as determined by disk diffusion tests are shown in Table 3. All three mutants showed significant increases in the sizes of the zones of inhibition caused by oxacillin, ceftriaxone, cefoxitin, and ampicillin and no change in susceptibility to isoniazid or rifampin.

We also determined MICs for the *M. tuberculosis* mutants by using the BACTEC radiometric system. The MIC of ceftriaxone for strain PM1066 (30 μg/ml) was twofold lower than that for wild-type PM638 (60 μg/ml), and the MIC of oxacillin was fourfold lower (60 μg/ml versus 240 μg/ml for PM638). The oxacillin MICS for strains PM1067 and PM1068 were twofold lower than that for the parental strain (120 μg/ml versus 240 μg/ml), while there were no changes in the MICs of cefoxitin. There were no changes in the MICs of rifampin, isoniazid, streptomycin, and ethambutol.

Morphology of transposon mutants. The mutants did not have any noticeable change in colony morphology, but to determine if there were morphological changes at the cellular level, we examined the mutants, grown in broth cultures, by acid-fast staining of formalin- or heat-fixed smears (data not shown). Four mutants (all *M. smegmatis*) appeared to have morphological changes, and so we examined live cells of these mutants by DIC microscopy (Fig. 1). Compared to the wild-type strain PM759 (Fig. 1A), mutant PM1079 was elongated and had a slightly filamentous morphology (Fig. 1B). The mutants PM1082, PM1083, and PM1084 showed slightly elongated forms (Fig. 1C and D), but the difference was not as marked as that observed for PM1079. Cultures of strain PM1083 (Fig. 1D) and PM1084 (Fig. 1E) tended to have cells with swollen termini.

TABLE 3. *M. tuberculosis* Tn*5370* mutant disk diffusion values

Antibiotic (amt [μg])	Diam of zone of inhibition (mm)[a]			
	PM638	PM1066	PM1067	PM1068
Ampicillin (10)	35	80	65	80
Oxacillin (10)	0	35	40	40
Cefoxitin (30)	0	20	45	30
Ceftriaxone (30)	0	80	35	40
Isoniazid (1)	55	60	60	60
Rifampin (5)	75	80	65	80

[a] Zones were measured to the nearest 5 mm, and tests were performed in triplicate, with a variation of <10 mm.

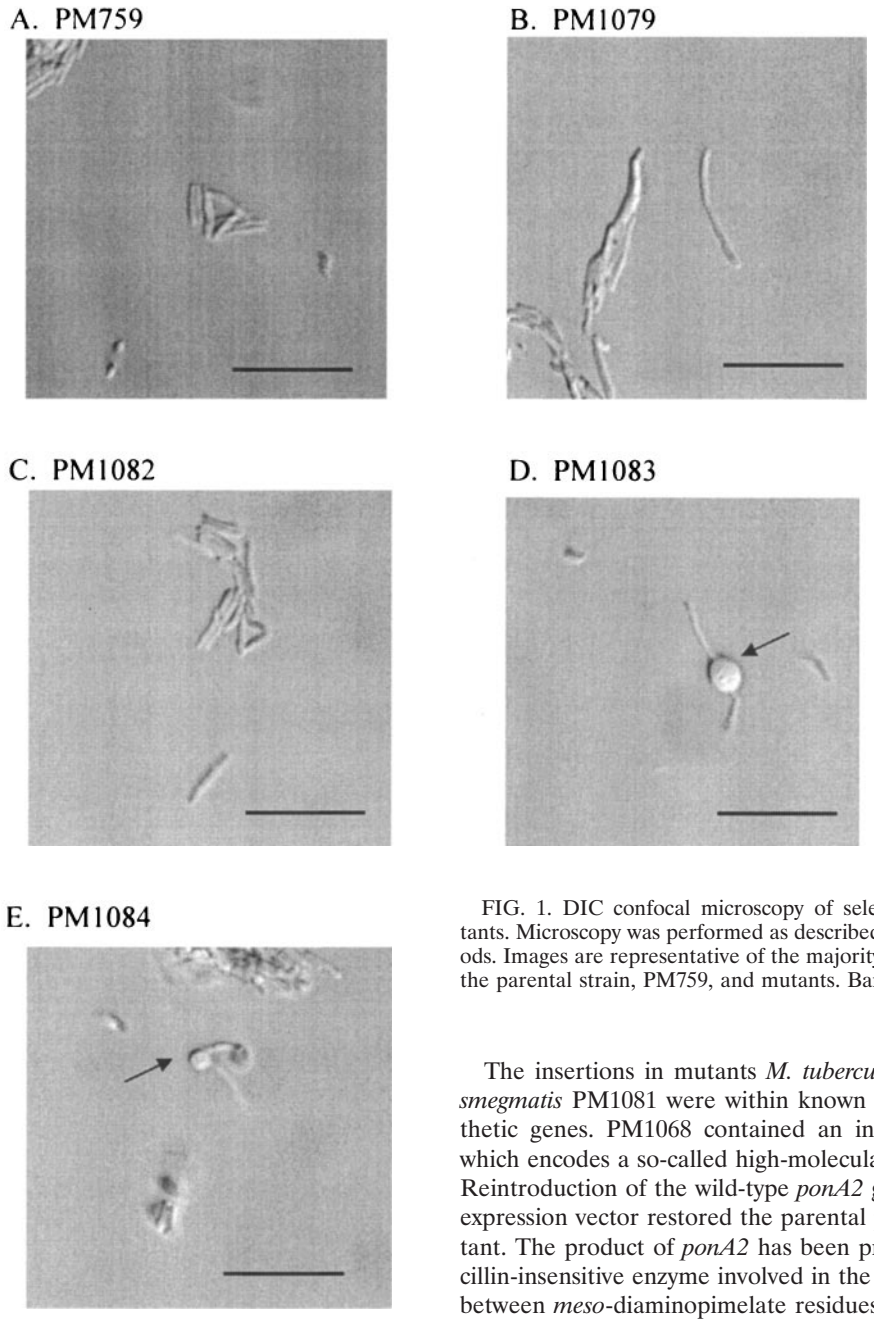

FIG. 1. DIC confocal microscopy of selected *M. smegmatis* mutants. Microscopy was performed as described in Materials and Methods. Images are representative of the majority of cell morphologies of the parental strain, PM759, and mutants. Bars represent 10 μm.

Mapping of transposon insertions and genetic analyses. Figure 2 shows the insertion site, the gene arrangement, and the orientation of the transposon in each mutant. Two mutants, *M. tuberculosis* PM1066 and *M. smegmatis* PM1082, each had a transposon inserted between open reading frames, while the other seven mutants contained insertions within predicted genes. The disrupted gene(s), their predicted functions, and their homologies to *M. tuberculosis* H37Rv genes, where applicable, are shown in Table 4. Complementation tests were done with the mutants by using wild-type genes cloned into replicating or single-copy integrating (*attB*/*int*) vectors as described below and assayed by streak plating onto ceftriaxone medium and by antibiotic disk diffusion assays.

The insertions in mutants *M. tuberculosis* PM1068 and *M. smegmatis* PM1081 were within known peptidoglycan-biosynthetic genes. PM1068 contained an insertion within *ponA2* which encodes a so-called high-molecular-weight PBP (9, 22). Reintroduction of the wild-type *ponA2* gene in an integrating expression vector restored the parental phenotype to the mutant. The product of *ponA2* has been predicted to be a penicillin-insensitive enzyme involved in the formation of linkages between *meso*-diaminopimelate residues in the mycobacterial peptidoglycan (22).

Mutant PM1081 has an insertion in the *dapB* gene, whose product, dihydropicolinate reductase, is involved in the synthesis of DAP and L-lysine (16). DAP is the third amino acid in the pentapeptide of mycobacterial peptidoglycan and is involved in forming DAP-DAP and DAP-alanine cross-links (44). DAP has been shown to be essential in *M. smegmatis*, and strains with mutations in DAP biosynthesis require the addition of DAP to the growth medium (37). The $dapB^+$ gene restored the parental phenotype to mutant PM1081, whereas the vector control did not. Also, addition of DAP to the medium returned the β-lactam antibiotic susceptibility of PM1081 to that of the parent (data not shown).

We did not add DAP to the transposon mutagenesis selection medium; thus, a *dapB* mutant should not have been ob-

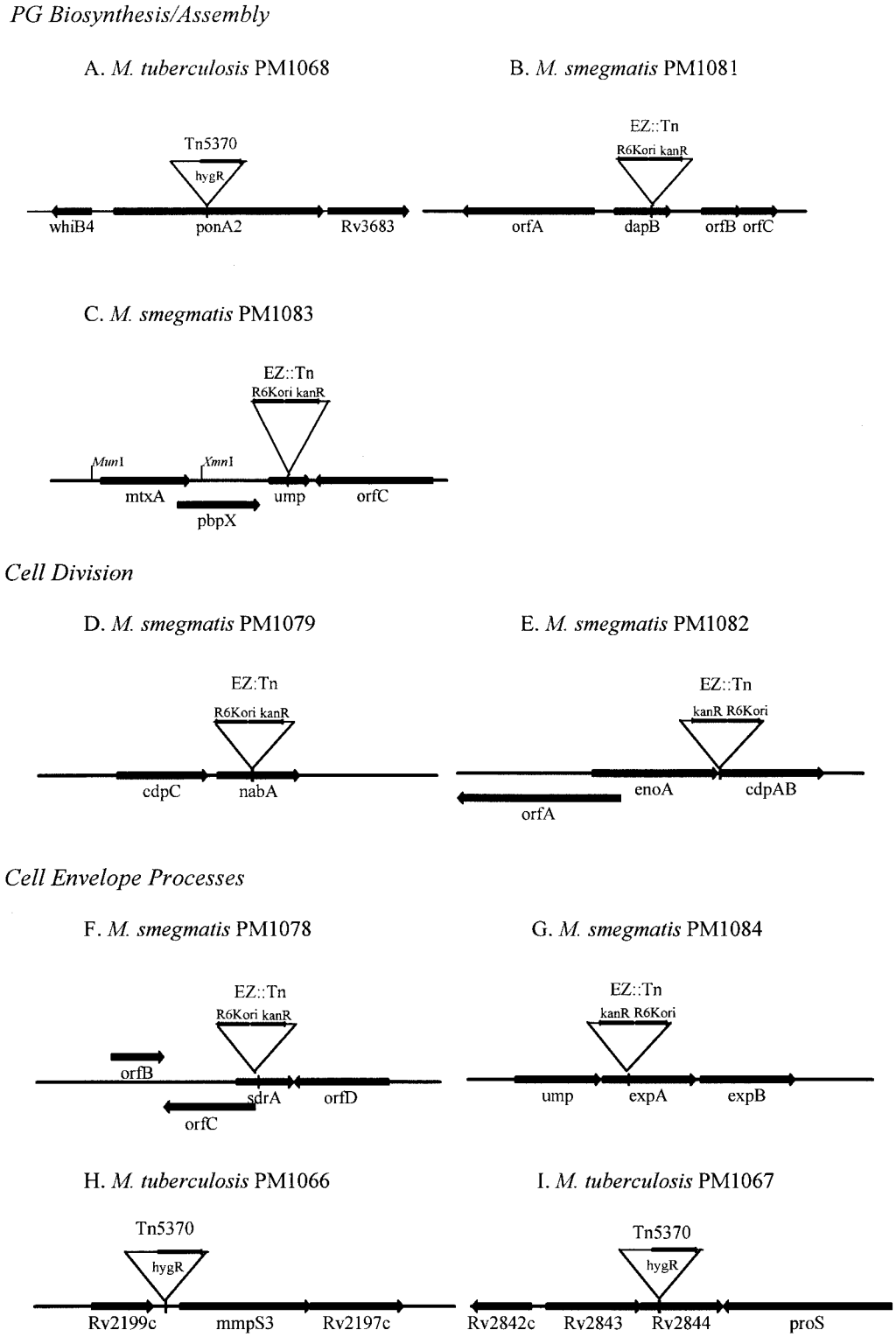

FIG. 2. Mapping of transposon insertions in *M. tuberculosis* and *M. smegmatis* mutants. Transposon insertion sites are shown with orientations and gene annotations. Maps and gene structures are not necessarily drawn to scale.

tained in our screen. However, spontaneous, extragenic mutations with the ability to suppress the requirement for DAP in DAP auxotrophs of *M. smegmatis* have been previously described (10). A phenotype of these mutants is a hypersusceptibility to β-lactam antibiotics when the mutants are grown in the absence of DAP. We previously identified one such suppressor mutation in the cystathionine-β-synthase gene, *cbs*, of *M. smegmatis* that results in the utilization of lanthionine in

TABLE 4. Summary of transposon mutants of *M. smegmatis* and *M. tuberculosis*

Class	Mutant	Organism	Affected gene(s)[a]	*M. tuberculosis* homolog (%)[b]	Conserved domain(s)[c] (E value)[d]	Putative function[g]	Cell morphology
Peptidoglycan biosynthesis or assembly	PM1068	*M. tuberculosis*	*ponA2* (*Rv3682*)		PASTA[e] (10^{-6}), transglycosylase (10^{-49}), transpeptidase (10^{-11})	HMW PBP (known)	Normal
	PM1081	*M. smegmatis*	*dapB*	*dapB* (84) (*Rv2773c*)	Dihydropicolinate reductase (10^{-64})	DAP biosynthesis (known)	Normal
	PM1083	*M. smegmatis*	*mtxA*	*Rv2258c* (69)	*O*-Methyltransferase (10^{-4})	SAM-dependent methyltransferase	Elongated, swollen termini
			pbpX	*Rv2257c* (69)	β-Lactamase (10^{-18})[f]	PBP involved in PG assembly	
			*ump**	*Rv2256c* (83)	None	Unknown	
Cell division	PM1079	*M. smegmatis*	*cdpC*	*Rv2927c* (59)	DivIVA, cell division initiation protein (10^{-18})	Cell division initiation	Filamentous
			*nabA**	*Rv2926c* (71)	Predicted metal-binding, nucleic acid-binding protein (10^{-26})	Regulator	
	PM1082	*M. smegmatis*	*cdpAB*	*Rv1024/Rv1025* (57/76)	DivIC, septum formation initiator (10^{-10})	Cell division initiation	Elongated
Cell envelope processes	PM1066	*M. tuberculosis*	*mmpS3* (*Rv2198c*)		None	Unknown	Normal
	PM1067	*M. tuberculosis*	*Rv2844*	*Rv2509* (78)	None	Unknown	Normal
	PM1078	*M. smegmatis*	*sdrA*	*Rv2224c/Rv2223c* (81/68)	Short-chain dehydrogenase (10^{-66})	Fatty acid metabolism (?)	Normal
	PM1084	*M. smegmatis*	*espA*		α/β hydrolase fold (10^{-13}), acyltransferase (10^{-6})	Exported protease	Elongated, swollen termini

[a] Genes marked with an asterisk indicate genes disrupted by transposon insertion within a given operon.
[b] Values in parentheses are percentages of identity to the *M. tuberculosis* gene.
[c] Determined by searching the Conserved Domain Database (33) at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).
[d] E values are the probability that the domain would be found in the given translated gene by chance alone.
[e] PASTA, PBP and serine/threonine kinase-associated domain. This domain binds the β-lactam stem and acts as a D-alanyl–D-alanine sensor.
[f] Includes distantly related D,D-carboxypeptidases.
[g] HMW, high-molecular-weight; SAM, *S*-adenosylmethionine; PG, peptidoglycan.

place of DAP for peptidoglycan biosynthesis (10). We hypothesized that the *dapB* mutant PM1081 had an additional mutation that can suppress the requirement for exogenous DAP in a similar fashion. To assess the possibility that PM1081 harbored a suppressor mutation in the *cbs* gene, we disrupted the gene via allelic exchange with the suicide plasmid pMP244 (11). The resulting derivative could still grow without DAP, suggesting the presence of a different suppressor mutation in strain PM1081 (data not shown).

Two other mutants appear to have transposons affecting genes adjacent to the insertion site. The *M. smegmatis* mutant PM1083 has an insertion within an open reading frame of unknown function with homology to the product of the *M. tuberculosis* H37Rv gene *Rv2256c*, which we named *ump* (stands for unknown mycobacterial protein). However, attempts to complement PM1081 by using a plasmid bearing a wild-type copy of *ump* were unsuccessful. Next, we repaired the lesion via allelic exchange using a *sacB* suicide vector bearing wild-type DNA that spanned the insertion site. We verified the repair by Southern blotting and found that the resultant derivative had the β-lactam susceptibility pattern of the wild-type strain (data not shown). Thus, the transposon insertion was responsible for the phenotype of the mutant. Two putative genes (named *mtxA* and *pbpX* by us) were identified upstream of *ump* (Fig. 2), and one of these, *pbpX*, has homology to β-lactamases and D,D-carboxypeptidases (Table 4). Introduction of integrating plasmids bearing $mtxA^+$ or $pbpX^+$ into PM1081 did not restore the wild-type phenotype, but introduction of an integrating plasmid carrying all three genes (Fig. 2) complemented the insertion mutation (data not shown). We propose that the relevant gene is *pbpX* and that expression of this gene is optimally achieved only from an intact polycistronic message, as complementation required the entire region encompassing *pbpX*.

M. smegmatis strain PM1079 has an insertion within a gene that we termed *nabA* with a predicted nucleic acid-binding protein product (Fig. 2D; Table 4). Complementation tests with $nabA^+$ were unsuccessful. Further analysis of the region revealed a hypothetical unknown gene (which we call *cdpC* for cell division protein C) 69 bp upstream of *nabA*, whose product has limited homology to the *Bacillus subtilis* cell division initiation protein DivIVA (Table 4). Introduction of an integrating plasmid bearing $cdpC^+$ into PM1079 did not restore the parental susceptibility phenotype. However, repair of the transposon lesion through allelic exchange using wild-type DNA encompassing the insertion site resulted in a wild-type phenotype (data not shown). In a fashion similar to that of the *pbpX* strain above, this *nabA* insertion mutant could be complemented to the wild type by a plasmid containing a DNA fragment bearing both the wild-type *cdpC* and *nabA* genes (data not shown).

The insertion in *M. smegmatis* strain PM1082 was 4 nucleotides upstream of a gene encoding a protein with partial homology to DivIC, a septum formation initiator in *B. subtilis* (Table 4). The mutant was complemented when a wild-type copy of this gene, designated by us as the cell division protein AB gene (*cdpAB*), was introduced (data not shown). The *cdpAB* gene of *M. smegmatis* is represented by two separate genes, *Rv1024* (*cdpA*) and *Rv1025* (*cdpB*), in the genome of *M. tuberculosis*. Hydropathy plots of CdpAB and the *M. tuberculosis* proteins revealed that Rv1024 and Rv1025 constitute

CdpAB of *M. smegmatis* (data not shown). A putative transmembrane domain that corresponds to Rv1024 exists in the N-terminal region of CdpAB, while the C terminus of CdpAB and most of Rv1025 (CdpB) contain a conserved domain of unknown function that is restricted to bacteria in the class *Actinomycetales*.

M. tuberculosis strain PM1066 had an insertion between genes *Rv2199c* and *mmpS3* (Fig. 2) and could be complemented by the *mmpS3*$^+$ gene (data not shown). The remaining mutants, *M. tuberculosis* PM1067, *M. smegmatis* PM1078, and *M. smegmatis* PM1084, contained insertions within unknown genes. The disrupted gene in PM1067 corresponds to *Rv2844*, whose hypothetical product is an alanine-rich protein. The insertion site in PM1078 was within a gene predicted to be a short-chain dehydrogenase or reductase that we named *srdA*. Furthermore, strain PM1078 had a generation time of 6 h versus 3 h for the wild-type strain PM759 (data not shown). Strain PM1084 had an insertion in a gene encoding a putative exported protease that we called *expA*, directly upstream of a similar gene that we termed *expB*. All three of these insertion mutants reverted to the parental phenotype upon introduction of the respective wild-type genes (data not shown).

DISCUSSION

We propose that the increased susceptibility of these mutants to β-lactam antibiotics or lysozyme is either directly due to defects in peptidoglycan biosynthesis or indirectly due to perturbations of processes that involve the peptidoglycan or changes in cell envelope composition. We believe that our experimental approach was validated by the isolation of two mutants with insertions in known peptidoglycan-biosynthetic genes (*ponA2* and *dapB*), which allows us to propose potential functions for the products of the genes examined in this study.

Peptidoglycan-biosynthetic genes (*ponA2* and *pbpX*). We propose that the *ponA2* and *pbpX* genes encode enzymes used for the formation of a peculiar type of peptidoglycan cross-link whose pathway is not well understood but may be a new drug target. Mycobacteria produce two kinds of peptide cross-links in the cell wall; one is the common DAP-Ala linkage, also called a 4-3 linkage, catalyzed by β-lactam-sensitive D,D-transpeptidase enzymes, and the other is an unusual DAP-DAP linkage, also called a 3-3 linkage, believed to be catalyzed by β-lactam-insensitive L,D-transpeptidase enzymes (25, 56). The role of the 3-3 linkages is unknown, although up to 25% of the peptides are linked in this fashion in the wall of *M. smegmatis* (56). *E. coli* produces predominantly 4-3 linkages; however, as the generation time of *E. coli* increases from 0.8 to 6 h, the amount of 3-3 linkages increases proportionately over the 4-3 linkages (43), suggesting that 3-3 linkages are important in stationary phase or periods of starvation. Very little is known about the enzymes responsible for these unusual 3-3 linkages in any bacterial species. However, through extensive in silico genomic analyses, Goffin and Ghuysen (22) predicted that there are several penicillin-resistant acyltransferases in mycobacteria that might be responsible for the formation of the 3-3 peptide linkages and suggested that the *ponA2* gene of *M. tuberculosis* encodes one such enzyme. Furthermore, Pon1, a peptidoglycan-biosynthetic enzyme with low affinity to β-lactam antibiotics that has been described to occur in *Mycobacterium leprae* is 27% homologous to the *ponA2* product of *M. tuberculosis* (4). In addition, a mutant of *M. smegmatis* isolated from a screen for mutants defective in long-term stationary-phase survival had a mutation in a gene whose product is 88% homologous to the *M. tuberculosis* PonA2 protein (27). These data, taken together with the results of this study, suggest that the PonA2 protein may be a β-lactam-insensitive L,D-transpeptidase involved in synthesizing the 3-3 linkages in the mycobacterial cell wall. Loss of this protein may result in increased susceptibility to β-lactam antibiotics. The effects of this mutation are more pronounced when cells grow on solid media than when they grow in liquid media, as we saw a greater change in the susceptibility of the *ponA2* mutant in the disk diffusion tests than was seen from the MIC determinations. We have previously observed differences between these two techniques and believe that they might indicate differences in wall architecture resulting from the different growth conditions (17).

We also propose that the *pbpX* gene encodes a D,D-carboxypeptidase important for the production of 3-3 (DAP-DAP) linkages. Consistent with this idea, the PbpX protein is predicted to have a single N-terminal transmembrane domain and an extracellular C-terminal domain. Alternatively, PbpX may be a new β-lactamase since the PbpX protein has a conserved β-lactamase domain which is distantly related to that of D,D-carboxypeptidases (18). Distinguishing these enzymes based on sequence homology alone is difficult; however, we have previously shown that all detectable β-lactamase activity in *M. smegmatis* derives from two other genes, *blaS* and *blaE* (17). Thus, we believe that *pbpX* is unlikely to encode a β-lactamase; rather, we think that it encodes a D,D-carboxypeptidase. Our model concerning PbpX function is based upon a novel mechanism of β-lactam antibiotic resistance that has been described for *Enterococcus faecium*. Mainardi and coworkers (30) showed that a spontaneous ampicillin-resistant mutant of *E. faecium* produces a peptidoglycan entirely composed of 3-3 peptidoglycan interpeptide linkages, unlike what occurs with the wild type, where almost all of the linkages are of the 4-3 type. Subsequent studies showed that overproduction of a β-lactam-insensitive D,D-carboxypeptidase in the mutant leads to an accumulation of muramyl-tetrapeptides, the preferred donor peptides in L,D-transpeptidation reactions, and this was responsible for its increased resistance (31). In a similar fashion, the loss of PbpX in the mutant may reduce the number of muramyl-tetrapeptides available for L,D-transpeptidation reactions, resulting in an increase in antibiotic susceptibility.

Cell division genes (*cdpAB* and *cdpC*). The *M. smegmatis* mutant PM1079 contained an insertion within a predicted nucleic acid-binding protein, NabA, but we propose that the phenotype of the mutant results from decreased expression of the upstream gene, *cdpC*. The *cdpC* homolog in *M. tuberculosis*, *Rv2927c*, is annotated as a conserved hypothetical protein and is essential, as determined by a saturation mutagenesis study (40). We believe that the expression of the gene is only decreased in the mutant, as *cdpC* is probably essential in *M. smegmatis*; a conditionally lethal strain will be needed to study this gene further. The CdpC protein has some regional homology to the *B. subtilis* cell division protein DivIVA. Null mutants of DivIVA are viable and show a minicell phenotype, while overexpression leads to filamentous forms (7). DivIVA is also

involved in chromosome partitioning during spore formation in *B. subtilis* (42). More recently, DivIVA was shown to be important for the maintenance of the rod shape of *Brevibacterium lactofermentum* (39).

The affected gene in *M. smegmatis* PM1082 encodes a putative cell division gene, CdpAB, which has some homology to DivIC, a cell division protein in *B. subtilis*. Like *cdpC* discussed above, the *cdpA* and *cdpB* genes of *M. tuberculosis* are annotated as essential genes (40). Again, we surmise that the transposon insertion only decreases expression of the *cdpAB* gene in the *M. smegmatis* mutant. The susceptibility phenotype and the slightly filamentous nature of the *cdpAB* and *cdpC* mutants, the essential nature of the genes, and the limited homology of the gene products to division proteins in *B. subtilis* suggest that these proteins may be involved with some unique aspects of mycobacterial cell division.

Genes involved with other cell envelope processes (*mmpS3*, *Rv2844*, *srdA*, and *expA*). The functions of the other genes identified in this study are more enigmatic. The gene affected in PM1066 is *mmpS3*, which encodes a member of the *M. tuberculosis* Mmp (stands for mycobacterial membrane protein) family of proteins, of which there are 14 long (MmpL1 to -L13b) and 5 short (MmpS1 to -S5) members. MmpL7 and MmpL8 are involved with the synthesis and localization of lipids in the cell envelope and appear to be important for virulence (6, 12, 13). The roles of the MmpS proteins in the physiology and pathogenesis of *M. tuberculosis* are undefined. Perhaps MmpS3 is involved with transporting a lipid or other cell envelope component to the surface, the loss of which affects the β-lactam antibiotic resistance phenotype in the mutant.

Rv2844 shows no significant homology to proteins outside of the mycobacteria. The gene for a predicted integral membrane protein, *Rv2843*, precedes *Rv2844*; the two genes overlap by 4 bp, and both proteins are alanine rich, suggesting perhaps that the proteins interact. A possible membrane location for the proteins might indicate some transport function for Rv2844/Rv2843 similar to that of MmpS3, described above.

M. smegmatis PM1078, with an insertion in *sdrA*, a gene for a putative short-chain dehydrogenase or reductase, was the only mutant identified with a reduced growth rate. A mutant of *M. tuberculosis* with a transposon insertion in the *sdrA* homolog, *Rv2509*, has also been shown to have a lower growth rate (41). SdrA may be involved with fatty acid metabolism, and its loss may affect cell envelope lipids, either in the outer portion of the envelope or within the cytoplasmic membrane. This idea is supported by our observation that this mutant is more susceptible to the lipophilic antibiotic rifampin when it is grown in liquid medium.

The *M. smegmatis expA* mutant PM1084 showed the smallest increase in susceptibility to β-lactam antibiotics; however, its susceptibility to lysozyme increased markedly (16-fold), and we observed swollen termini in cells grown in liquid culture. Immediately downstream of *expA* is another putative exported protease, *expB*, with 51% identity to *expA* (Fig. 2). Each gene product contains an N-terminal secretion signal and a single transmembrane domain and belongs to the α/β hydrolase fold superfamily, which includes various peptidases and acyltransferases. The same gene architecture is found in the *M. tuberculosis* homologues *Rv2224c* and *Rv2223c*, which have been described as probable exported proteases.

We propose that these exported proteases may be involved in either the breakdown of cell wall components themselves or the control of the enzymes involved in biosynthesis and the degradation of cell wall components. Mycobacteria can be autolytic, but very little is known about autolysis mechanisms in these organisms. Several murein hydrolases have been identified in *E. coli* and are necessary for proper elongation and division; they are also involved in autolysis (reviewed in reference 23). However, the mechanism of regulation of such lytic enzymes is unknown. One proposed mechanism was derived from the observation that rates of peptidoglycan turnover in *B. subtilis* are greater in extracellular-protease-deficient mutants (26). Furthermore, a secreted-protease-hyperproducing strain of *B. subtilis* is resistant to nafcillin (25), suggesting a protease-dependent downregulation of autolytic enzymes. In this view, ExpA might be a protease that specifically degrades an autolytic enzyme, and the loss of ExpA may result in increased autolysin activity. This increased activity may result in defects at the pole since the greatest stress-bearing forces of the cell wall are found at the poles of a rod-shaped bacterium (28). The swollen-terminus phenotype observed in the *expA* mutant is consistent with this mechanism and may be more pronounced in an *expA expB* double mutant, since ExpB might partially compensate for the loss of ExpA.

The genes described in this study will be further investigated by the construction of defined deletion mutants and conditionally lethal mutants, in the case of essential genes, to allow us to address some of the proposed functions of the encoded proteins. We are particularly interested in studying the role of the PonA2 and PbpX proteins in the formation of 3-3 cross-links to see if these proteins can be evaluated as potential drug targets.

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