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A Role for the Class A Penicillin-Binding Protein PonA2 in the Survival of *Mycobacterium smegmatis* under Conditions of Nonreplication[▽]

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Class A penicillin-binding proteins (PBPs) are large, bifunctional proteins that are responsible for glycan chain assembly and peptide cross-linking of bacterial peptidoglycan. Bacteria in the genus *Mycobacterium* have been reported to have only two class A PBPs, PonA1 and PonA2, that are encoded in their genomes. We report here that the genomes of *Mycobacterium smegmatis* and other soil mycobacteria contain an additional gene encoding a third class A penicillin-binding protein, PonA3, which is a paralog of PonA2. Both the PonA2 and PonA3 proteins contain a penicillin-binding protein and serine/threonine protein kinase-associated (PASTA) domain that we propose may be involved in sensing the cell cycle and a C-terminal proline-rich region (PRR) that may have a role in protein-protein or protein-carbohydrate interactions. We show here that an *M. smegmatis* Δ*ponA2* mutant has an unusual antibiotic susceptibility profile, exhibits a spherical morphology and an altered cell surface in stationary phase, and is defective for stationary-phase survival and recovery from anaerobic culture. In contrast, a Δ*ponA3* mutant has no discernible phenotype under laboratory conditions. We demonstrate that PonA2 and PonA3 can bind penicillin and that PonA3 can partially substitute for PonA2 when *ponA3* is expressed from a constitutive promoter on a multicopy plasmid. Our studies suggest that PonA2 is involved in adaptation to periods of nonreplication in response to starvation or anaerobiosis and that PonA3 may have a similar role. However, the regulation of PonA3 is likely different, suggesting that its importance could be related to stresses encountered in the environmental niches occupied by *M. smegmatis* and other soil-dwelling mycobacteria.

The cell envelope of mycobacteria is a complex carbohydrate- and lipid-rich entity and is a major factor contributing to the success of these organisms as saprophytic and pathogenic bacteria (7, 8, 29, 35). The innermost layer of the cell envelope is a peptidoglycan (PG) composed of *N*-acylmuramic acid and *N*-acetylglucosamine with L-alanyl (or glycyl in the case of *Mycobacterium leprae*)–D-isoglutaminyl–*meso*-diaminopimelyl–D-alanyl–D-alanine pentapeptides attached to the muramic acid residues (13, 16, 54). While some of the muramyl residues are N acetylated, as they are in most other bacteria, a majority of the muramyl residues are N glycolylated (2, 37, 48, 49), a modification that confers lysozyme resistance (53) and also influences the innate immune response to mycobacterial cell walls (10). The pentapeptide chains of the mycobacterial PG can be modified by amidation, glycylation, or methylation, but the functional significance of these modifications is unknown (28, 31, 32, 38, 54).

Approximately 80% of the pentapeptides in mycobacterial PG are cross-linked, and a majority of the links are between the carboxyl group of a penultimate D-Ala residue in a pentapeptide precursor and the amino group of the side chain D center of a *meso*-diaminopimelic acid (DAP) residue from an adjacent peptide (referred to as a 4-3 cross-link), while approximately one-third of the links are between the carboxyl group of the L center of a DAP residue of one peptide and the amino group of the side chain D center of the DAP residue in an adjacent peptide (referred to as a 3-3 cross-link) (17, 65). The 4-3 linkage is considered the "standard" linkage and is catalyzed by classical, penicillin-sensitive DD-transpeptidases, while the novel 3-3 linkage is thought to be catalyzed by the concerted action of DD-carboxypeptidases and novel LD-transpeptidases (31, 34, 39–41). The reasons why bacteria produce both 4-3 and 3-3 linkages are unknown. Some workers have suggested that the 3-3 linkages might reinforce the wall during times of stress and under nonreplicating conditions or stabilize complex cell envelopes (17, 50, 51, 55). In this regard, the high percentage of 3-3 linkages found in the PG of mycobacteria and their predominance in stationary-phase *M. tuberculosis* cells (31) suggest that these linkages may have an important role in maintaining cell envelope integrity during periods of growth and under nonreplicating conditions.

The enzymes involved in peptidoglycan assembly, the penicillin-binding proteins (PBPs), have a triad sequence motif that forms the transpeptidation active site ([SxxK]——[S/YxN/C]——[K/H][T/S]G), which is the target of the β-lactam class of antibiotics (for a review, see reference 18). The PBPs have been grouped into several classes based on this motif, surrounding sequences, and other structural features (18). Of interest here are the class A PBPs, which are high-molecular-weight (HMW) proteins with both a transglycosylase domain (also called a non-penicillin-binding module [n-PB]) and a transpeptidase domain (also called a penicillin-binding module [PB]) (18). These proteins are tethered to the cytoplasmic membrane by a transmembrane helix with the catalytic do-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
M. smegmatis strains		
mc^{2}155	*ept-1*	58
PM939	*ept-1* Δ*lysA4 rpsL6* Δ*blaS* Δ*blaE*::*res-aph-res*	15
PM1482	*ept-1* Δ*lysA4 rpsL6* Δ*blaS* Δ*blaE*	This study
PM1536	PM1482 Δ*ponA2*	This study
PM1576	PM1536/pMV261	This study
PM1578	PM1536/pMP560 (*ponA2*$^+$)	This study
PM1864	PM1482 Δ*ponA3*	This study
PM1865	PM1536 Δ*ponA3*	This study
PM2258	PM1536/pMP907 (His$_6$-*ponA2*)	This study
PM2124	PM1536/pMP712 (*ponA3*$^+$)	This study
PM2044	PM1482 *lysA*$^+$	This study
PM2045	PM1536 *lysA*$^+$	This study
E. coli DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS mcrBC*) ϕ80Δ*lacZ*ΔM15 Δ*lacX74 deoR resA1 ara*Δ*139*(*ara-leu*)*7697 galU galK* λ$^-$ *rpsL endA1 nupG*	Invitrogen
Plasmids		
pMV261	Km^r *E. coli*-mycobacterium shuttle vector, contains the *groEL* promoter, ColE1, pAL500 oriM	59
pMP308	*sacB*$^+$ Hyg^r Amp^r suicide allelic exchange vector bearing the Δ*blaE2* allele	A. R. Flores and M. S. Pavelka, Jr., unpublished data
pMP498	*rpsL*$^+$ Hyg^r suicide allelic exchange vector	J. M. Spence and M. S. Pavelka, Jr., unpublished data
pMP560	pMV261 *ponA2*$^+$	This study
pMP581	pMP498 Δ*ponA2*	This study
pMP712	pMV261 *ponA3*$^+$	This study
pMP724	pMP498 Δ*ponA3*	This study
pMP907	pMV261 His$_6$-*ponA2*	This study

mains facing the outside of the cell. Mycobacteria have been reported to have only two genes that encode class A PBPs, *ponA1* and *ponA2*, which are annotated Rv*0051* and Rv*3682* in the sequence genome of *M. tuberculosis* H37Rv (9, 17). Previous studies that analyzed collections of transposon mutants to obtain clones with various phenotypes identified strains with insertions in these two genes. The phenotypes of these mutants have clearly shown that these PBPs play a complex role in mycobacterial physiology. One group of workers found a *ponA1* mutant of *M. smegmatis* in a search for mutants with an altered dye-binding phenotype (an indicator of changes in the cell envelope) and showed that this slowly growing mutant was hypersusceptible to β-lactam antibiotics and had altered permeability (6). A *ponA2* mutant of *M. smegmatis* was discovered in a screen for mutants defective for survival during long-term culture (25), while other workers isolated an *M. tuberculosis ponA2* mutant in a screen for mutants sensitive to low pH (61). The same group of workers also showed that the *M. tuberculosis* mutant was more sensitive to heat, H_2O_2, and NO and was attenuated for persistence in the mouse model of inhalation tuberculosis (62). We previously identified an *M. tuberculosis ponA2* mutant in a screen for mutants hypersusceptible to β-lactam antibiotics (14). All of these studies identified transposon mutants in searches for mutants with specific phenotypes, but there have been no direct genetic studies that have specifically examined the function of these PBPs in peptidoglycan metabolism.

In this study we demonstrated that *M. smegmatis* has three class A PBPs. We show here that a newly recognized protein, which we designated PonA3, is a paralog of the PonA2 protein and is found only in certain environmental species of mycobacteria. We analyzed the phenotypes of *M. smegmatis* mutants with in-frame deletions of *ponA2* and *ponA3* singly and in combination to increase our understanding of the role that these PBPs play in mycobacterial peptidoglycan biology.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. PM1482, the designated wild-type strain, is a lysine-auxotrophic, double β-lactamase mutant derived from the common laboratory strain *M. smegmatis* mc^{2}155 (see construction details below). PM1482 has in-frame deletions of the *blaS* and *blaE* genes encoding the major and minor β-lactamases of *M. smegmatis*, respectively (15). All mutants discussed in this paper were constructed in this background. *Escherichia coli* cultures were grown at 37°C in Luria-Bertani (LB) broth (Difco, BD Bioscience, San Jose, CA) or on LB agar. Mycobacterial cultures were grown at 37°C in Middlebrook 7H9 broth and were plated on Middlebrook 7H10 agar (Difco, BD Biosciences). The Middlebrook media were supplemented with 0.05% Tween 80, 0.2% glycerol, and ADS (0.5% bovine serum albumin fraction V, 0.2% dextrose, and 0.85% NaCl). L-Lysine was added to all Middlebrook medium cultures at a concentration of 40 μg/ml. When required, antibiotics were used at the following concentrations: hygromycin, 100 μg/ml for *M. smegmatis* and 200 μg/ml for *E. coli*; ampicillin, 100 μg/ml for *E. coli*; apramycin and kanamycin, 50 μg/ml for *E. coli* and 25 μg/ml for *M. smegmatis*; and streptomycin, 1,000 μg/ml for *M. smegmatis*. Hygromycin and bovine serum albumin fraction V were obtained from Roche Applied Science (Indianapolis, IN); all other antibiotics and additives were obtained from Sigma-Aldrich Chemical (St. Louis, MO).

Plasmids and DNA manipulations. Plasmids used in this study are shown in Table 1. Basic molecular biology techniques were performed as previously described (1). Plasmids were constructed in *E. coli* DH10B and were prepared by using Qiagen columns (Qiagen, Valencia, CA) if they were used for recombination. DNA fragments were isolated using agarose electrophoresis and were purified with a QIAquick gel purification kit (Qiagen). Restriction and DNA modification enzymes were obtained from Fermentas (Hanover, MD) or New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by Invitrogen Life Technologies (Carlsbad, CA). Genomic DNA was prepared from *M. smegmatis* either by a cetyltrimethylammonium bromide (CTAB) protocol or by

a guanidine thiocyanate (GTC) protocol (46, 47). Electroporation of *M. smegmatis* and Southern blotting were done as previously described (46, 47). DNA sequencing was performed by ACGT, Inc. (Wheeling, IL).

Construction of *M. smegmatis* Δ*blaS* Δ*blaE* deletion mutant PM1482. *M. smegmatis* strain PM1482 is a double β-lactamase mutant derived from strain PM939 (Δ*lysA4* Δ*blaS* Δ*blaE*::*res-aph-res*) (15), in which the Δ*blaE*::*res-aph-res* allele was exchanged with an in-frame Δ*blaE* allele carried on a *sacB* suicide plasmid, pMP308 (A. R. Flores and M. S. Pavelka, Jr., unpublished data), using two-step allelic exchange as previously described (46). Southern blotting (not shown) confirmed the allelic exchange.

Construction of *M. smegmatis* Δ*ponA2* deletion mutant PM1536. Using *M. smegmatis* mc^{2}155 genomic DNA as the template, PCR was performed using primers Pv496 (5′-GAGCACGAAACGGTCATCGA-3′) and Pv497 (5′-ATCG ACGGATCCAGCGCGCGGACA-3′) with an XL-PCR and r*Tth* DNA polymerase (Applied Biosystems, Foster City, CA) to amplify the *ponA2* gene region (4,455 bp). After digestion with BamHI, the fragment was ligated to the pMV261 vector cut with BamHI to generate pMP560. An in-frame, unmarked 2,304-bp deletion of *ponA2* was generated by performing inverse PCR with pMP560 using primers Pv501 (5′-ATCATCACGATATCGCTGAGCAA-3′) and Pv502 (5′-G CATACAGATATCCGAGCTTGCTG-3′). The deletion allele with ~1 kb of flanking DNA on each side was cloned into the *rpsL* suicide vector pMP498 (J. M. Spence and M. S. Pavelka, Jr., unpublished data) to generate plasmid pMP581, which was used for standard allelic exchange with *M. smegmatis* strain PM1482 to generate the Δ*ponA2* mutant PM1536. Deletion of the gene was confirmed by Southern hybridization (data not shown).

Construction of *M. smegmatis* Δ*ponA3* and Δ*ponA2* Δ*ponA3* deletion mutants. Primers Pv647 (5′-ATGCGCACCATGATATCCACCATGCA-3′) and Pv648 (5′-ATGAACTTCCAGCTGATATCGTCGAGGTA-3′) containing engineered EcoRV sites were used to amplify the *ponA3* gene from mc^{2}155 genomic DNA. The 4,498-bp amplicon was digested with EcoRV and ligated into the PvuII site of the pMV261 vector to generate pMP709. An inverse PCR to delete 2,334 bp of the *ponA3* gene was performed using pMP709 as the template and primers Pv670 (5′-ATGCAGCGATATCTTGGCAACCGTGA-3′) and Pv671 (5′-ATG TCGTGCTGATATCCGGACTTCCG-3′) with engineered EcoRV sites, followed by EcoRV digestion and self-ligation to produce plasmid pMP718. The fragment containing the *ponA3* deletion with ~1,000 bp flanking the mutation was removed from pMP718 with DdeI, blunt ended, and cloned into the allelic exchange vector pMP498 to generate the suicide plasmid pMP724. Allelic exchanges were performed with pMP724 and PM1482 to generate the Δ*ponA3* single mutant (PM1864) and with PM1536 to generate the Δ*ponA2* Δ*ponA3* double mutant (PM1865). Deletion of *ponA3* in both strains was confirmed by Southern hybridization (data not shown).

Construction of *lysA*$^+$ strains. Cosmid pYUB601 (47) containing a wild-type copy of the *M. smegmatis lysA* gene was digested with PacI, which released the insert. The 50-kb insert was then used as a long linear recombination substrate to repair the Δ*lysA4* lesion (3). The substrate was purified and electroporated into PM1482 (wild type) and PM1536 (Δ*ponA2*), and clones were selected on Middlebrook 7H10 medium lacking lysine, resulting in *lysA*$^+$ strains PM2044 (wild type) and PM2045 (Δ*ponA2*).

Construction of complementing plasmids. All complementing plasmids (Table 1) were constructed in the *E. coli*-mycobacterium shuttle vector pMV261 (59) so that the genes were expressed from the constitutive *groEL* promoter on the plasmid. Detailed descriptions of construction of these plasmids can be obtained from one of us (M. S. Pavelka, Jr.).

Antimicrobial susceptibility testing. Zones of inhibition measured by the disk diffusion method with Sensi-discs (Becton, Dickinson and Company, Franklin Lakes, NJ) and MIC determined by the macrodilution method were used to assay the antibiotic susceptibility of *M. smegmatis* strains as previously described (15).

Microscopy. For light microscopy, samples from 8-day-old stationary-phase cultures (optical density at 600 nm [OD_{600}], 1.0 to 1.2) were stained with crystal violet and examined using an Olympus BX41 microscope with a ×100 oil immersion objective. Thin-section electron microscopy was performed using cells from 10 ml of an 8-day-old culture harvested by centrifugation and washed twice with 0.1 M HEPES buffer (pH 6.8). Cells were processed for electron microscopy using a previously described protocol (45). The microscopic analysis was performed using an Hitachi 7100 electron microscope and a MegaView III digital camera with AnalySIS software (Soft-imaging System, Lakewood, CO).

Stationary-phase survival. Starter cultures containing 10 ml of Middlebrook 7H9 medium that were grown overnight were diluted 1:100 in roller bottles and incubated on a roller apparatus. The first samples used for optical density analysis, viable counting, and microscopy were taken after the initial dilution. The experiment was performed in duplicate with two individual cultures for each strain. The plates were incubated for 4 days in a 37°C incubator before the numbers of CFU were recorded.

Lysozyme aggregation assays. Eight-day-old stationary-phase cell cultures were transferred to a cuvette in a temperature-controlled Beckman DU series 500 spectrophotometer at 37°C. For each determination, hen egg white lysozyme (MP Biochemicals, Solon, OH) was added to the cuvette at a final concentration of 200 μg/ml in buffer (1× Tris-EDTA TE buffer, 9.5), and the optical density of the suspension was automatically recorded every 30 s for 15 min. Control suspensions received only buffer.

Anaerobiosis recovery. Duplicate cultures of *M. smegmatis* strains were grown in Middlebrook 7H9 medium to mid-log phase (OD_{600}, 0.5 to 0.6) and then diluted in duplicate and plated on 13 sets of plates for determination of viable counts. One set of plates was incubated aerobically at 37°C for 4 days as a control. The remaining plates were incubated at 37°C in a Coy anaerobic chamber (Coy Laboratories Inc., Ann Arbor, MI) with an atmosphere consisting of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide for 1 to 12 days. Each day, one set of plates was moved to an aerobic incubator and then incubated for 4 days at 37°C.

Bocillin FL labeling assay. *M. smegmatis* membranes were prepared as described previously (42) from mid-log-phase (OD_{600}, 0.5 to 0.6) and 2-day-old stationary-phase cultures. The protein concentration was determined using the Bio-Rad protein assay (Hercules, CA). One hundred micrograms of total membrane protein was incubated with 30 μM Bocillin FL (Invitrogen, Carlsbad, CA) in 1× phosphate-buffered saline (PBS) in a 100-μl mixture for 30 min at 37°C. An equal volume of SDS-PAGE denaturing buffer was added, and the samples were boiled for 3 min and then chilled on ice. Then 150 μl of a sample was loaded in each well on a 10% SDS-PAGE gel. After electrophoresis, the gel was rinsed in distilled, deionized water for 2 h, and the fluorescence of the labeled proteins was detected using a FluorImager 575 (with excitation at 488 nm and emission at 530 nm). The gel was then stained with Coomassie brilliant blue for visualization of total protein.

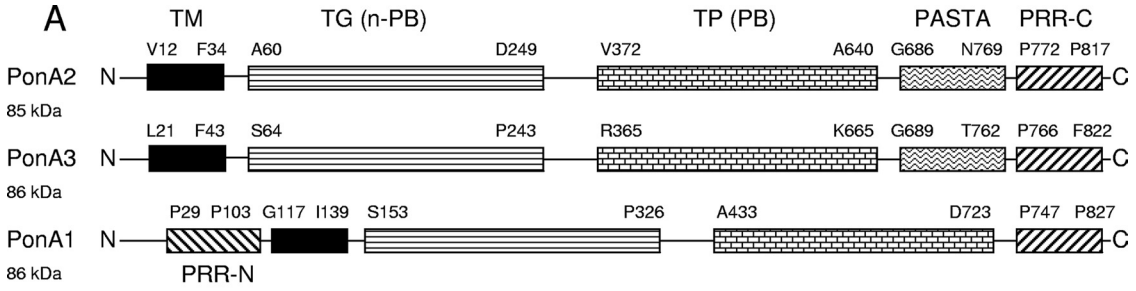
Statistical analysis. Data were analyzed with an unpaired Student *t* test using GraphPad software (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Three HMW class A PBPs are encoded in the genome of *M. smegmatis*. Only two HMW class A PBPs, PonA1 (also designated PonA) and PonA2 (also designated Pon1), have been identified previously in mycobacteria (4, 5, 17, 36). However, the last compilation of PBPs in these organisms was published prior to the recent expansion of the number of sequenced mycobacterial genomes deposited in the GenBank database (17). We found that the genome of *M. smegmatis* contains three genes encoding class A PBPs, MSMEG6900 (*ponA1*), MSMEG6201 (*ponA2*), and MSMEG4388, which we designated *ponA3*. Furthermore, of the 15 mycobacterial genomes in the GenBank database, only 6 contain the *ponA3* gene (*M. smegmatis*, *M. gilvum* PYR-GCK, *M. vinbaalenii*, *Mycobacterium* sp. MCS, *Mycobacterium* sp. JLS, and *Mycobacterium* sp. KMS), and all of these organisms are soil organisms (26, 27, 43). The nine species that lack *ponA3* are the pathogenic mycobacteria *M. tuberculosis*, *M. bovis*, *M. abscessus*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. leprae*, *M. marinum*, and *M. ulcerans*.

In addition to common transmembrane, transglycosylase, and transpeptidase domains (Fig. 1A), all three proteins have extensive C-terminal proline-rich regions (PRR-C) (Fig. 1A and Fig. 2B). The PonA1 protein is distinct from the other two proteins as it has an additional proline-rich region at the N terminus (PRR-N), as shown in Fig. 1A and Fig. 2C. It is noteworthy that a PxxP motif is frequently found in both types of PRR domains (Fig. 2B and 2C).

Both PonA2 and PonA3 have a penicillin-binding protein and serine/threonine kinase-associated (PASTA) domain that is located just before the proline-rich domain (Fig. 1A). The



B

	PonA3									
	FL		TG		TP		PASTA		PRR-C	
	ID	SIM	ID	SIM	ID	SIM	ID	SIM	ID	SIM
PonA1	31	49	33	69	22	55	N/A	N/A	28	49
PonA2	72	82	78	93	68	84	62	89	80	86

FIG. 1. Comparison of the class A PBPs of *M. smegmatis.* (A) Domain organization. The calculated molecular mass of each of the three proteins is indicated below the designation. The amino acids at the beginning and the end of each domain are shown above the domain. TM, transmembrane (as determined by TMHMM); TG (n-PB), transglycosylase; TP (PB), transpeptidase; PASTA, PBP and serine/threonine kinase (STK)-associated domain; PRR-C, C-terminal proline-rich region; PRR-N, N-terminal proline-rich region. (B) Levels of identity (ID) and similarity (SIM) (identical and conserved) for the *M. smegmatis* PonA3 protein and the PonA1 and PonA2 proteins. FL, full length. The various annotations of *ponA1* of *M. tuberculosis* and *M. smegmatis* in the GenBank database do not agree with each other. The original annotation for the *M. smegmatis* gene (GenBank accession no. AAG13121) is too short, and the translation product starts in the middle of the transmembrane domain. This annotation was apparently used for subsequent assignment of the open reading frame (ORF) in *M. tuberculosis*. The ORF was changed in the *M. smegmatis* genome project (accession no. ABK70639), and a new start codon was located 81 codons upstream of the start site in the original annotation, which allowed identification of the entire transmembrane region. We reannotated the *M. smegmatis ponA1* gene and placed the start site of the ORF 42 codons upstream of the revised annotation in the *M. smegmatis* genome project. This revealed all of the C-terminal proline-rich regions in the PonA1 proteins of both mycobacterial species and showed that the sizes of all of the class A PBPs are similar.

PASTA domain has been found in several protein kinases and PBPs of Gram-positive bacteria, and it is thought to interact with the D-Ala–D-Ala termini of uncross-linked PG pentapeptides (23, 67). An alignment of the PASTA domains of the PonA2 and PonA3 proteins (Fig. 2A) revealed the alpha helix and the three beta strands that comprise the domain along with the residues that are conserved in a large number of PASTA family members (67). All three of the class A PBPs of *M. smegmatis* are approximately the same size and share several key features, but the domain organization of PonA3 is more similar to that of PonA2 (Fig. 1A), the levels of amino acid similarity and identity for PonA3 and PonA2 are higher than those for PonA3 and PonA1 (Fig. 1B), and the conserved active site residues identified in the transglycosylase and transpeptidase domains (17) of PonA2 are the same as those of PonA3 (data not shown). We propose that PonA3 is a paralog of PonA2. In this study, we focused on exploring the role of PonA2 and PonA3 in *M. smegmatis* PG physiology.

A *ponA2* deletion mutant, but not a *ponA3* deletion mutant, has altered susceptibility to various antibiotics. We constructed in-frame deletions of the *ponA2* and *ponA3* genes both singly and in combination in an *M. smegmatis* Δ*blaS* Δ*blaE* double-deletion mutant lacking the genes responsible for the β-lactamase activity and primary β-lactam resistance in this organism (15). The use of a β-lactamase null mutant facilitated screening for changes in the susceptibility of the PBP mutants to β-lactam antibiotics.

As shown in Table 2, Δ*ponA2* strains have decreased susceptibility to the β-lactams ampicillin and ceftriaxone, as shown by the decrease in the ampicillin disk diffusion zone diameter from 50 to 36 mm and the increase in the ceftriaxone MIC from 8 to 16 to 64 to 128 μg/ml. The Δ*ponA2* strains have increased susceptibility to rifampin, an inhibitor of transcription, as shown by the increase in the disk diffusion zone diameter from 14 to 26 mm (Table 2), but there were no differences in susceptibility to isoniazid, ethambutol, vancomycin, or imipenem (data not shown). Complementation of the *ponA2* deletion with the *ponA2*$^+$ gene on a multicopy vector expressed from the *groEL* promoter restored the wild-type phenotype (Table 2). However, complementation with *ponA3* carried on a multicopy plasmid expressed from the same promoter restored only the wild-type rifampin susceptibility phenotype (Table 2).

In contrast to the Δ*ponA2* mutant, the Δ*ponA3* mutant showed no change in antibiotic susceptibility compared to the wild type (Table 2). In addition, the Δ*ponA2* Δ*ponA3* double mutant had the same susceptibility profile as the Δ*ponA2* strain, indicating that loss of *ponA3* does not affect the antibiotic resistance phenotype of this organism.

PonA2 and PonA3 can be derivatized with penicillin. To determine if loss of PonA2 or PonA3 affected the PBP profile of the mutants, we performed penicillin-binding protein assays with purified membranes using a fluorescently labeled β-lactam, Bocillin FL (68). As shown in Fig. 3A, the Δ*ponA2* mutant (lanes 3 and 4) lacked a ~80-kDa protein that was present in membranes purified from both mid-log-phase (lanes 1 and 2) and stationary-phase (lanes 5 and 6) wild-type cells. Complementation of the Δ*ponA2* deletion with *ponA2*$^+$ on a plasmid resulted in reappearance of the ~80-kDa protein (Fig. 3B,

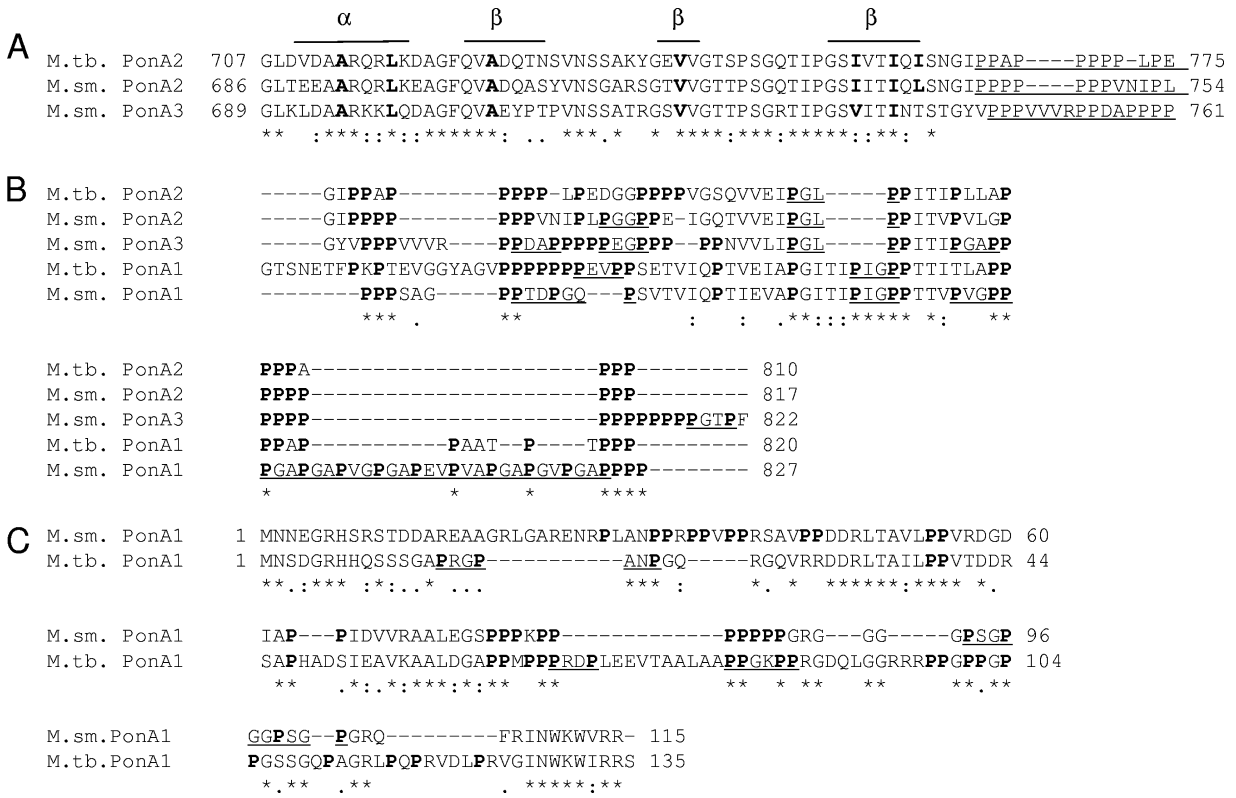


FIG. 2. Clustal W alignments of domains in the class A PBPs of *M. smegmatis* and *M. tuberculosis*. (A) PASTA domains of PonA2 and PonA3, showing the three β-strands and the single α-helix that make up the PASTA fold. Bold type indicates residues that are highly conserved in many PBPs and serine/threonine kinases. Note the proximity of the PRR-C domain, which is underlined. (B) PRR-C domains of all three class A proteins. Bold type indicates the proline residues, and the PxxP core motifs are underlined. (C) PRR-N domains of PonA1 proteins. Bold type indicates the proline residues, and the PxxP motifs are underlined. Asterisks indicate identical residues, while colons and periods indicate conserved and semiconserved residues, respectively.M.tb., *M. tuberculosis*; M.sm., *M. smegmatis*.

compare lanes 1 and 2 to lanes 3 and 4 and to lanes 5 and 6). To confirm that the protein missing in the Δ*ponA2* mutant is PonA2, we constructed a N-terminal His-tagged version of PonA2 and expressed it from a multicopy vector in the Δ*ponA2* strain. A PBP assay performed with purified membranes of this strain (Fig. 3C) revealed a novel protein that migrated slightly higher than the ~80-kDa protein observed for wild-type strain and the Δ*ponA2 ponA2*$^+$ complemented strain that was missing in the Δ*ponA2* mutant. Considering that the His-tagged PonA2 protein is 2.4 kDa larger than the wild-type protein, we concluded that the ~80-kDa band observed for wild-type cells that was not present for the Δ*ponA2* mutant was the PonA2 protein.

TABLE 2. Antibiotic susceptibilities of the Δ*ponA2* and Δ*ponA3* mutants

Strain	Genotype	Disk diffusion diam (mm)[a]		Ceftriaxone MIC (μg/ml)[b]
		Ampicillin	Rifampin	
PM1482	*ponA2*$^+$ *ponA3*$^+$	50 ± 2	14 ± 1	8–16
PM1536	Δ*ponA2*	36 ± 2	26 ± 2	64–128
PM1576	Δ*ponA2 ponA2*$^+$	50 ± 2	14 ± 2	8–16
PM1578	Δ*ponA2*/pMV261	35 ± 2	25 ± 2	64–128
PM1864	Δ*ponA3*	52 ± 2	16 ± 2	8–16
PM1865	Δ*ponA2* Δ*ponA3*	34 ± 2	25 ± 2	64–128
PM2124	Δ*ponA2 ponA3*$^+$	37 ± 2	17 ± 1	ND

[a] For disk diffusion tests, the zones of inhibition are averages ± standard deviations of duplicate determinations for two independent cultures of each strain. Ampicillin was used at a concentration of 50 μg/disk, and rifampin was used at a concentration of 5 μg/disk.

[b] MICs were determined in duplicate for two independent cultures, and the variation was ±1 dilution. ND, not determined.

In contrast to the results that we obtained with the Δ*ponA2* mutant, the PBP profile of the Δ*ponA3* mutant is the same as that of the wild-type strain (Fig. 4, lanes 1 and 3). However, overexpression of the *ponA3* gene on a multicopy plasmid in the Δ*ponA2* mutant resulted in the appearance of a new band at approximately the size of PonA3 (86 kDa) (Fig. 4, compare lane 6 to lanes 2, 4, and 5 [Δ*ponA2* strains]), indicating that the *ponA3* gene encodes a functional polypeptide.

PonA2, but not PonA3, has a role in stationary-phase survival. PonA2 has been implicated in the survival of *M. smegmatis* during long-term culture or under carbon limitation conditions (25), suggesting that PonA2 has a role in adaptation to starvation. We examined this possibility by testing the survival of the Δ*ponA2* mutant in stationary phase. As shown in Fig. 5A, the viable counts of the Δ*ponA2* mutant and the mutant bearing the vector control began to drop by day 2 and continued to drop so that by the end of the experiment there was a >1-log$_{10}$ difference compared to the wild type and the complemented control. We also found that *ponA3* carried on a

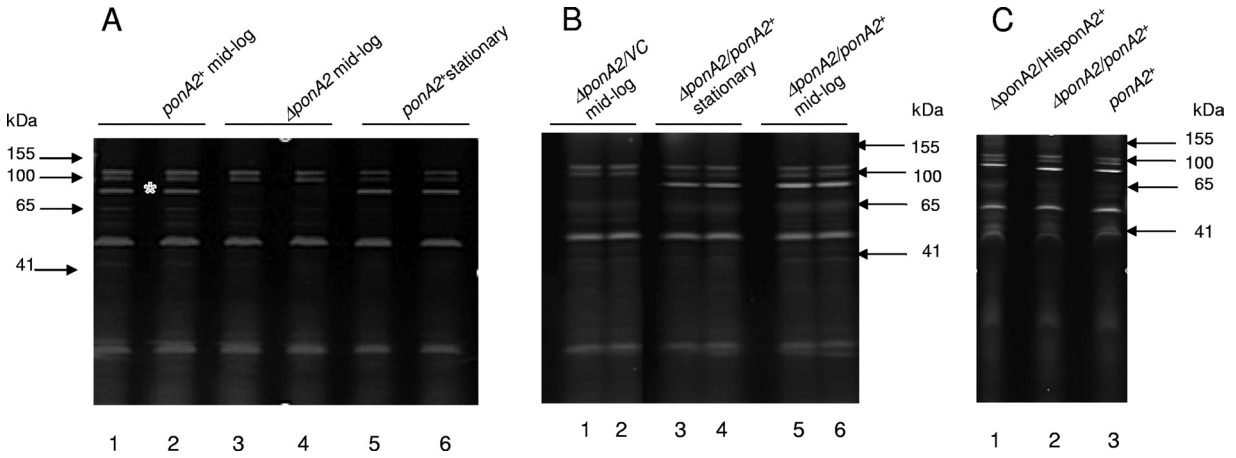


FIG. 3. Penicillin (Bocillin FL) binding assays with purified *M. smegmatis* membranes showing PonA2 labeling. (A) Assay results for membranes prepared from wild-type strain PM1482 (*ponA2*$^+$) mid-log-phase cultures (lanes 1 and 2) and stationary-phase cultures (lanes 5 and 6) or prepared from PM1536 Δ*ponA2* mid-log-phase cultures (lanes 3 and 4). Each strain was assayed using duplicate cultures. The ~80-kDa protein present in all wild-type samples is indicated by an asterisk. (B) Membrane assays for two independent mid-log-phase cultures of the Δ*ponA2*/pMV261 vector control strain (lanes 1 and 2) and stationary-phase (lanes 3 and 4) and mid-log-phase (lanes 5 and 6) cultures of the Δ*ponA2* mutant complemented with the wild-type *ponA2* gene carried on a multicopy plasmid (PM1576 Δ*ponA2*/*ponA2*$^+$). The ~80-kDa protein not present in the membranes prepared from the mutant is present in the membranes prepared from the complemented strain. (C) Results for membranes from the Δ*ponA2* mutant with the plasmid bearing the His_6-PonA2 gene (lane 1), the positive complementation strain PM1576 Δ*ponA2*/*ponA2*$^+$ (lane 2), and the wild-type strain PM1482 (lane 3).

multicopy plasmid could restore the wild-type stationary-phase survival phenotype in the Δ*ponA2* mutant (data not shown).

We performed the same experiment with the Δ*ponA3* and Δ*ponA2* Δ*ponA3* mutants and compared these mutants to the parental controls. We found that the Δ*ponA3* strain had the same viability as the wild-type strain, and no difference was observed between the viability of the Δ*ponA2* Δ*ponA3* mutant and the viability of the Δ*ponA2* parent (Fig. 5B). Throughout these experiments, the optical densities remained stable after they reached a plateau at day 2 (data not shown), and the cultures remained free of aggregates that could have complicated interpretation of the results. Determinations of the growth curves were terminated when the cells in the cultures began to clump.

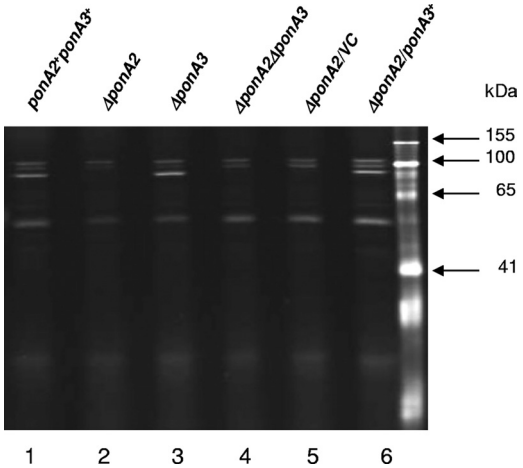


FIG. 4. Bocillin FL binding assays with purified *M. smegmatis* membranes, showing PonA3 labeling. The membrane profile of the PM1864 (Δ*ponA3*) strain (lane 3) is the same as that of wild-type strain PM1482 (*ponA2*$^+$ *ponA3*$^+$) (lane 1). Deletion of *ponA3* in the Δ*ponA2* background did not change the PBP profile compared to that of the Δ*ponA2* parent (compare lanes 2 and 4). However, overexpression of *ponA3*$^+$ on a multicopy vector in the Δ*ponA2* mutant (lane 6) resulted in appearance of a new ~80-kDa protein that was not present in the vector control strain (Δ*ponA2*VC) (lane 5).

Starvation could be a factor that contributes to the stationary-phase phenotype of the Δ*ponA2* mutant, since this mutant is a lysine auxotroph (Δ*lysA4*). To examine this possibility, the Δ*ponA2* mutant and the wild-type parent were made *lysA*$^+$ by allelic exchange as described in Materials and Methods, and the stationary-phase survival of the prototrophic strains was examined. We found that the Lys$^+$ Δ*ponA2* derivative PM2044 lost viability in stationary phase to the same degree as the Lys$^-$ parent, although it took approximately seven times longer to do so. As expected, the Lys$^+$ *ponA2*$^+$ control strain PM2045 did not have a viability defect (data not shown). These results suggest that the stationary-phase defect of the Δ*ponA2* mutant was due to the loss of PonA2 and not to the inability to synthesize L-lysine, although the auxotrophic defect decreased the time needed to see a viability defect.

Stationary-phase Δ*ponA2* cells have a spherical morphology. There were not any changes in the colony morphologies of the Δ*ponA2* and Δ*ponA3* mutants, so we examined the strains microscopically to determine if there were any changes at the cellular level. During the exponential phase of culture growth, all strains maintained a bacillary shape (data not shown). However, light microscopy and transmission electron microscopy (TEM) indicated that during stationary phase, cells in the Δ*ponA2* cultures became swollen spheres (Fig. 6D, E, and F). This change was not seen in cultures of the wild type or the complemented strain, in which the morphology was bacillary (Fig. 6A, B, and C). Consistent with our observation that PonA3 does not play a role in stationary-phase survival, the Δ*ponA3* single mutant and the Δ*ponA2* Δ*ponA3* double mutant

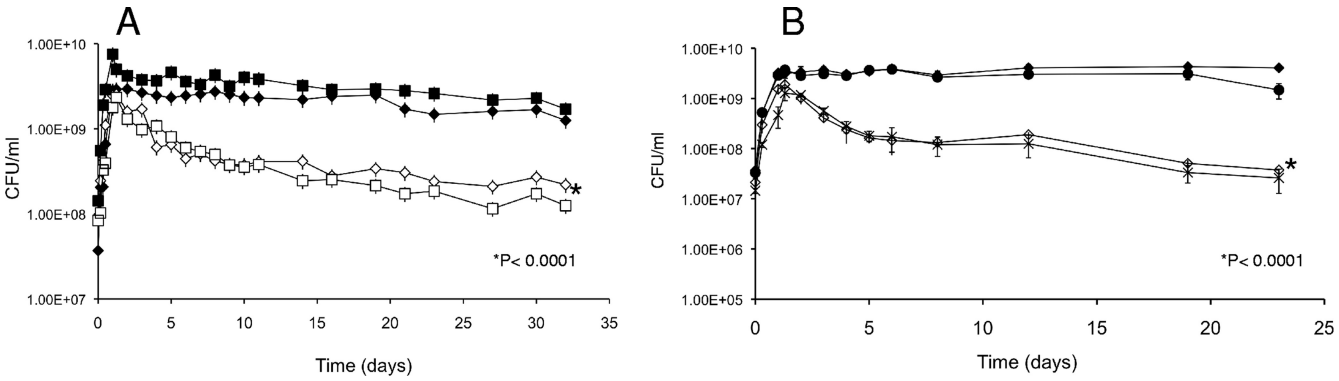


FIG. 5. Survival of the *M. smegmatis* mutants in stationary phase. The results are expressed in CFU/ml. The values are the averages and standard deviations of duplicate determinations using two independent cultures. (A) Δ*ponA2* strains. Open diamonds, PM1536 (Δ*ponA2*); open squares, PM1578 (Δ*ponA2* vector control); filled diamonds, PM1482 (*ponA2*$^+$); filled squares, PM1576 (Δ*ponA2 ponA2*$^+$). *, *P* value for statistical analysis of the Δ*ponA2* mutant compared to the wild type and the Δ*ponA2* vector control and the Δ*ponA2 ponA2*$^+$ complemented strain. (B) Δ*ponA3* and Δ*ponA2* Δ*ponA3* strains. Filled diamonds, PM1482 (*ponA2*$^+$*ponA3*$^+$); open diamonds, PM1536 (Δ*ponA2*); filled circles, PM1864 (Δ*ponA3*); multiplication signs, PM1865 (Δ*ponA2* Δ*ponA3*). *, *P* value for statistical analysis of the Δ*ponA2* mutant compared to the wild type and the Δ*ponA2* Δ*ponA3* mutant compared to the Δ*ponA3* mutant.

showed no change in cell morphology during mid-log or stationary phase compared to their parental strains. However, *ponA3* expressed from plasmid pMP712 could restore the wild-type morphology to the Δ*ponA2* mutant in stationary phase (data not shown).

Alteration of the cell surface of the Δ*ponA2* mutant in stationary phase. The experiments described above suggested that the Δ*ponA2* mutant has diminished cell wall integrity during stationary phase. To examine the surface of the cell envelope of the mutant, we developed a cellular aggregation assay that exploits the electrostatic properties of lysozyme, a very basic protein. In this assay, the positive charges distributed throughout lysozyme interact with the negative charges on the surface of the bacteria, resulting in aggregation of cells in suspension (22).

We took cell suspensions prepared from 8-day-old stationary-phase cultures, incubated them with hen egg white lysozyme at a sublethal concentration (200 μg/ml), and monitored their optical densities for 15 min. Control suspensions received buffer alone. We found that the optical density of the wild-type cell suspension decreased but the optical density of the mutant cell suspension remained essentially unchanged (Fig. 7). The decrease in the turbidity of the wild-type suspension did not result from lysis, as cellular debris was not apparent in the suspension; instead, the cells aggregated and sank to the bottom of the cuvette (Fig. 7). The Δ*ponA2* mutant did not display this behavior, even after incubation for several hours, while the wild type completely settled out of suspension and formed a dense mat of aggregates. In contrast, the Δ*ponA3* mutant aggregates in stationary phase, like the wild-type strain (data not shown). The same aggregation phenomenon observed for the wild type was observed when the Δ*ponA2* mutant was complemented in *trans* with either *ponA2* (Fig. 7) or *ponA3* (data not shown). The control suspensions that received only buffer did not aggregate; thus, this phenomenon was lysozyme dependent and not the result of autoaggregation. We also tested exponential-phase cultures of the strains in the same way and found that all of the mutants and the wild type remained in suspension after exposure to lysozyme (data not shown).

We think that the aggregation phenomenon was due to the physicochemical nature of lysozyme and its charge interactions with the cell surface of wild-type cells, and our data suggest that the net negative charge on the cell surface increased as the cells entered the stationary phase. This hypothesis is supported by the results of our control experiments (not shown), which showed that the aggregation effect was blocked by addition of 10 mM NaCl to the stationary-phase cell suspensions prior to lysozyme addition. The addition of salt did not affect the enzymatic activity of the lysozyme, as another control experiment using a suspension of highly lysozyme-sensitive *Micrococcus luteus* cells showed that there was rapid and complete lysis with or without additional NaCl (data not shown). Taken together, these results support the idea that the net negative charge density of the surface of *M. smegmatis* cells increases during stationary phase, but this does not happen in the Δ*ponA2* mutant.

PonA2, but not PonA3, is important for survival under anaerobic conditions. Mycobacteria are obligate aerobes, but they can survive prolonged periods of anaerobiosis, and in this state they do not replicate (63). This characteristic is thought to be relevant to the survival of "nonreplicating persistors" in the pathogenesis of latent *M. tuberculosis* infection (19). Since we found that the Δ*ponA2* mutant is defective for survival in stationary phase, a situation in which many of the cells no longer replicate, we decided to determine if the mutant was also defective for survival under anaerobic conditions. As shown in Fig. 8, the recovery of the Δ*ponA2* strain from anaerobiosis was reduced compared to the recovery of the wild type beginning on day 4. In addition, we noted that the Δ*ponA2* mutant formed smaller colonies than the wild type formed after recovery. We also tested the Δ*ponA3* and Δ*ponA2* Δ*ponA3* mutants and found no significant difference in recovery after anaerobiosis between the two mutants (Fig. 8). The difference between the Δ*ponA2* Δ*ponA3* double mutant and the Δ*ponA3* single mutant was significantly greater than the

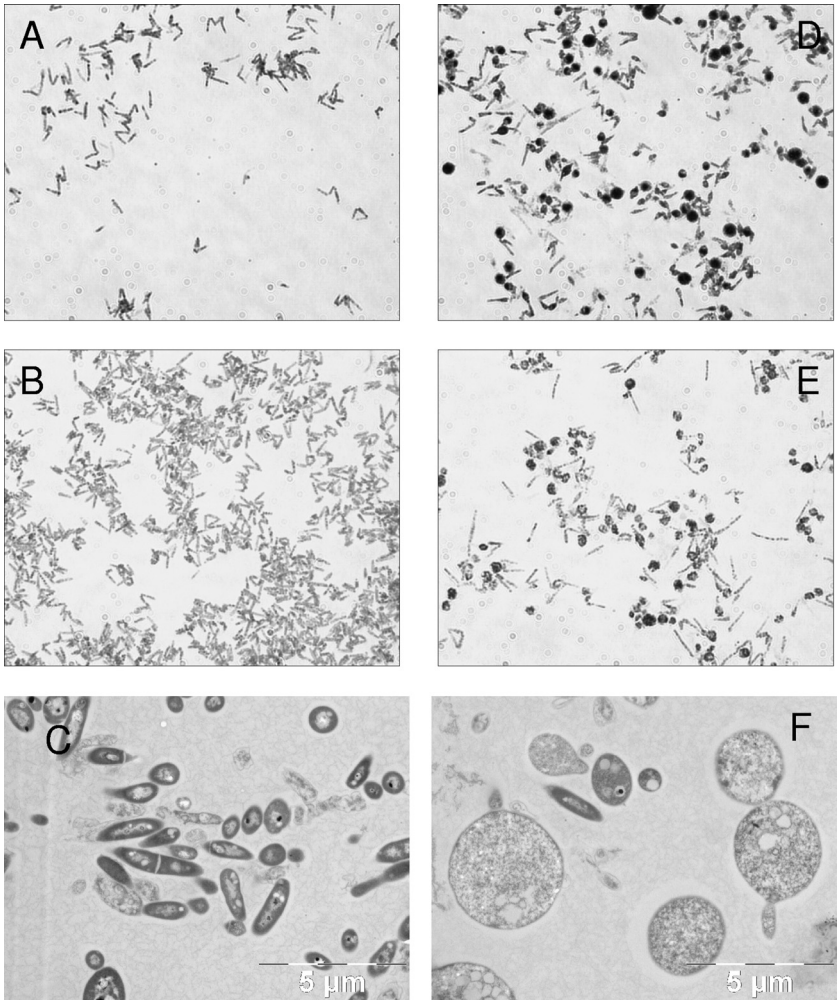


FIG. 6. Light microscopy (A, B, D, and E) and TEM (C and F) of 8-day-old stationary-phase cultures demonstrating the loss of the characteristic bacillary shape of the Δ*ponA2* mutants. (A and C) PM1482 (*ponA2*$^+$); (D and F) PM1536 (Δ*ponA2*); (B) PM1576 (Δ*ponA2 ponA2*$^+$); (E) PM1578 (Δ*ponA2* vector control).

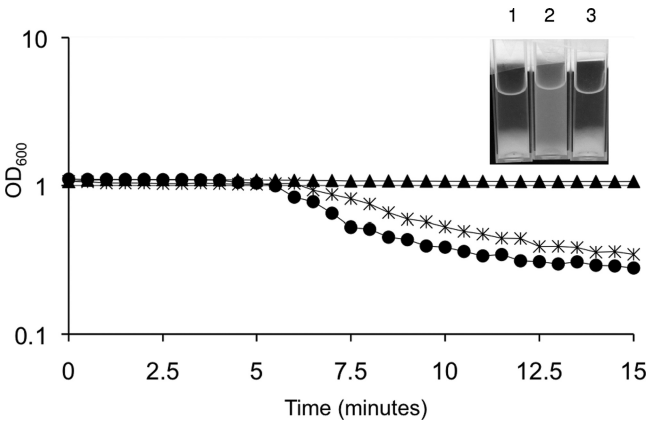


FIG. 7. Lysozyme cellular aggregation assay: optical density scans over time for assays with wild-type strain PM1482 (asterisks), the complemented Δ*ponA2* mutant PM1576 (filled circles), and the Δ*ponA2* mutant PM1536 (filled triangles). (Inset) Suspensions after incubation at room temperature for 1 h. Tube 1, wild-type strain PM1482; tube 2, Δ*ponA2* mutant PM1536; tube 3, complemented Δ*ponA2* mutant strain PM1576.

difference between the Δ*ponA2* mutant and the wild type, but the difference between the Δ*ponA2* single mutant and the Δ*ponA2* Δ*ponA3* double mutant was not significant. Complementation of the Δ*ponA2* mutation with the *ponA2*$^+$ plasmid, but not complementation with the *ponA3*$^+$ plasmid, resulted in a wild-type anaerobic survival phenotype (data not shown).

DISCUSSION

In this study we demonstrated that the class A PBP PonA2 is essential for the survival of *M. smegmatis* under nonreplicating conditions. We also discovered a previously unrecognized class A PBP, PonA3, which is present in only a subset of environmental mycobacteria. Our results show that the PonA3 function is dispensable under the conditions that we used to identify phenotypes for the Δ*ponA2* mutant. The *ponA3* gene is probably not expressed from the chromosome at a high level since we could detect the PonA3 protein in PBP assays only when the gene was expressed from a constitutive promoter on a multicopy plasmid. Under these conditions, the *ponA3* gene was able to complement most (but not all) of the phenotypes of

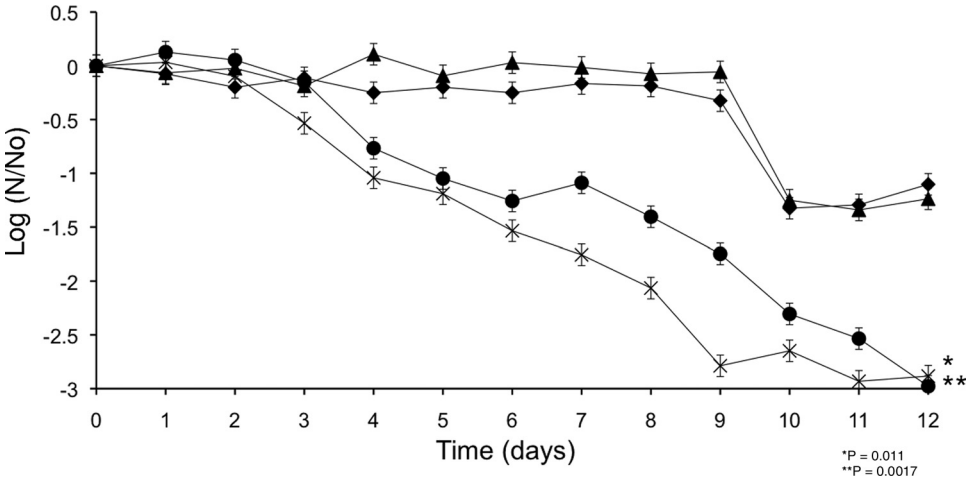


FIG. 8. Anaerobic survival of the Δ*ponA2*, Δ*ponA3*, and Δ*ponA2* Δ*ponA3* strains. The viable counts recovered after anaerobic incubation are expressed as $\log(N/N_0)$, where N_0 is the initial number of CFU and N is the number of CFU after 1 to 12 days of anaerobic incubation followed by 4 days of aerobic incubation. Filled diamonds, PM1482 (wild type); filled triangles, PM1864 (Δ*ponA3*); filled circles, PM1536 (Δ*ponA2*); multiplication signs, PM1865 (Δ*ponA2* Δ*ponA3*). *, *P* value for statistical analysis of the Δ*ponA2* mutant compared to the wild type; **, *P* value for the comparison between the Δ*ponA2* Δ*ponA3* mutant and the Δ*ponA3* mutant.

the Δ*ponA2* mutant. We postulate that PonA3 is a paralog of PonA2 that provides adaptive functions in the environmental saprophytic mycobacteria.

Our analysis revealed a proline-rich domain located at the C termini of all three class A PBPs and another PRR located at the N terminus of PonA1. These domains contain a PxxP core motif that is found primarily in proteins from eukaryotic organisms but have been found to be present in a large number of *M. tuberculosis* proteins (52). This motif is commonly embedded in polyproline tracts that constitute a helix with a hydrophobic surface for protein-protein interactions (24, 66). We performed comparative searches with the GenBank database and found that the class A PBPs of other actinobacteria belonging to the same family as *Mycobacterium*, such as *Nocardia farcinica*, *Gordonia bronchialis*, and *Rhodococcus erythropolis*, also have termini with similar proline-rich regions. This phylogenetic association suggests that there is a functional relationship between these domains and the cell envelope structure shared by these bacteria.

Proline-rich domains with PxxP motifs are found in many eukaryotic proteins and have been shown to interact with Src homology 3 (SH3) domains (24). Proteins with SH3-like domains (SH3b) are present in bacteria and are involved in peptidoglycan degradation (33, 60, 64), This suggests that the PRR domains of the PonA proteins might interact with PG disassembly proteins. We have examined the sequences of various mycobacterial proteins involved in PG metabolism (the hydrolase CwlM [12], the peptidase RipA [21], and the resuscitation promoting factor [Rpf] proteins [44, 69]) and found no apparent SH3b domains in any of them. In fact, there do not appear to be any mycobacterial proteins with recognizable SH3b domains. However, the mycobacterial SH3b-like sequences may have diverged significantly from the corresponding sequences of other bacteria because of the high G+C content of their genomes, so the possibility that SH3b-bearing proteins are present in mycobacteria cannot be excluded. We should also consider the possibility that the hydrophobic PRR domains might interact with each other. Thus, we propose that the proline-rich domains could be involved in modulating the interaction between the PBPs and proteins involved in PG turnover. Alternatively, since polyproline tracts can function as hydrogen bond acceptors (24), these domains could allow the PBPs to interact with cell envelope precursors to coordinate assembly of the PG with assembly of other components of the mycolyl-arabinogalactan-peptidoglycan (mAGP) cell wall skeleton (35). These functions do not have to be mutually exclusive.

The PonA2 and PonA3 proteins also share a PASTA domain, which was originally discovered in an analysis of the crystal structure of the PBP2x protein of *Streptococcus pneumoniae*, and it is now known that single or multiple PASTA domains are present in a variety of proteins in Gram-positive bacteria (20, 67). The PASTA domain is thought to interact with the terminal D-Ala–D-Ala of unlinked PG peptides (23, 67). This domain occurs four times in the C terminus of PknB, a mycobacterial serine/threonine kinase that is required for the phosphorylation of PbpA, resulting in movement of PbpA to the cell septum for division (11). It has been proposed that the function of a PASTA domain is to guide proteins to the site of cell division where the concentration of PG pentapeptide precursors is highest. Alternatively, duplicated PASTA domains in the same protein may sense different types of PG peptides, allowing the protein to react to different modifications of the cell wall precursors (23). Since PonA2 and PonA3 each have a single PASTA domain, we propose that this domain may allow these proteins to detect PG pentapeptide precursors as a mechanism to sense whether the cell is dividing. This would be consistent with results presented here that show that PonA2 is involved in survival of the cell under nonreplicating conditions and that PonA3 can substitute for PonA2 during stationary-phase adaptation. The idea that the PonA2 function is related to cell cycle progression is supported by the observation that the number of *ponA2* transcripts decreases in *M. tuberculosis* cells treated with antibiotics that dysregulate cell division (56,

57). It is unclear why PonA3 cannot rescue the anaerobic phenotype, but this finding might indicate that cessation of cell growth as a result of anaerobic conditions involves more complicated signals. Mutagenesis, deletion, and domain-swapping experiments with the PonA2 and PonA3 PRR and PASTA domains should provide additional information about the roles of these domains.

Our results show that PonA2 functions in adaptation of cultures to stationary phase, during which many of the cells do not replicate, and to anaerobic conditions, during which none of the cells replicate. These results are in agreement with previous work that identified an *M. smegmatis ponA2* transposon mutant in a screen for mutants unable to survive starvation conditions (25). In that study, the survival defect manifested over several months, as opposed to the few weeks observed in our study. The difference is probably the result of our mutant having a lysine auxotrophic phenotype, since we showed that a prototrophic derivative took approximately seven times longer to exhibit a survival defect in stationary phase than the auxotrophic strain. We surmise that the prototrophic strain probably experienced a different type of starvation, perhaps carbon limitation. The results of our comparison of the auxotrophic and prototrophic strains suggest that amino acid starvation plays a role in this adaptation, indicating that the stringent response potentially contributes to this phenomenon.

The mechanism of PonA2 in adaptation to stationary phase and anaerobiosis is not entirely clear. A recent report demonstrated that a *Vibrio cholerae* PBP1A mutant quickly became spherical in stationary phase as a result of D-amino acid efflux into the medium (30). It was shown that PG metabolism is regulated by the endogenous production of specific D-amino acids by a novel D-amino acid racemase during stationary phase (30). We do not know if the same phenomenon can occur in mycobacteria, but given the widespread alterations in the phenotype of the Δ*ponA2* mutant, the length of time required for the phenotype to occur, and the lack of any additional amino acid racemases in the *M. smegmatis* genome, we think that D-amino acid sensitivity is probably not a factor that contributes to the stationary-phase phenotype of this mutant. Other workers have proposed that PonA2 may be one of the LD-transpeptidases responsible for the synthesis of novel DAP-DAP (3-3) PG cross-links that have been implicated in adaptation to stationary phase (17). However, more recent work on a class of novel LD-transpeptidases involved in PG assembly and modification that were first discovered in *Enterococcus faecium* and are now known to be present in a variety of bacteria, including mycobacteria, has challenged this idea (31, 34, 39–41). Determining the transpeptidase activity of the PonA2 protein and the cross-link composition of the PG of the Δ*ponA2* mutant should provide important information to help us understand how this PBP functions in PG assembly.

In addition to the stationary-phase survival defect of the Δ*ponA2* mutant, we also showed that the surface of the mutant is different than that of the wild type in stationary phase. Our data from the lysozyme aggregation experiments suggest that the net negative charge density of *M. smegmatis* cells increases in stationary phase but that this does not happen in the Δ*ponA2* mutant. To our knowledge, this is the first indication that the electrostatic nature of the surface of a mycobacterial species differs depending on the growth phase. The increase in the net negative charge density could result from the shedding of positively charged molecules, such as basic proteins, or from an increase in the expression of negatively charged glycolipids in the outer cell envelope as the wild-type cells transition to stationary phase. A decrease in the negative charge density of the Δ*ponA2* mutant could result from an increase in the number of positively charged molecules that might shield the surface or from shedding of the negatively charged species in the outer envelope as a result of the dysregulation of envelope assembly. The latter explanation is consistent with the substantial disorganization of the cell envelope structure indicated by the swollen cells seen in the TEM images of Δ*ponA2* cells taken from stationary-phase cultures.

The only phenotype that we examined that was not related to nonreplicative conditions was the antibiotic susceptibility of exponentially growing cultures. The loss of a PBP usually results in an increase in the susceptibility to β-lactam antibiotics. However, we found that the Δ*ponA2* mutant has decreased susceptibility to both penicillin- and cephalosporin-type β-lactams. This suggests that this mutant may somehow compensate for the loss of PonA2 by producing additional cross-links in the PG so that a higher concentration of antibiotics is required to affect the cell. Structural analysis of PG purified from the mutant should help us interpret the β-lactam antibiotic phenotype. The susceptibility of the mutant to hydrophilic antibiotics, such as isonizaid and ethambutol, is not changed, but its susceptibility to the hydrophobic antibiotic rifampin is increased, suggesting that loss of PonA2 may somehow affect the lipid-rich regions of the cell envelope. Our previously identified *M. tuberculosis ponA2* transposon mutant displayed increased susceptibility to β-lactam antibiotics and no change in susceptibility to rifampin, suggesting that loss of PonA2 in *M. tuberculosis* has different effects (14). However, the *M. tuberculosis* mutant was isolated from a library specifically screened for increased susceptibility to β-lactam antibiotics, so we cannot rule out the possibility that there is a suppressor mutation in this strain. We are currently reconstructing the *M. tuberculosis ponA2* mutant by using allelic exchange to resolve this issue and to test the ability of the mutant to survive under stationary-phase and anaerobic conditions.

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