Rv2744c Is a PspA Ortholog That Regulates Lipid Droplet Homeostasis and Nonreplicating Persistence in *Mycobacterium tuberculosis*

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**ABSTRACT**

*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), remains a significant cause of morbidity and mortality worldwide, despite the availability of a live attenuated vaccine and anti-TB antibiotics. The vast majority of individuals infected with *M. tuberculosis* develop an asymptomatic latent infection in which the bacterium survives within host-generated granulomatous lesions in a physiologically altered metabolic state of nonreplicating persistence. The granuloma represents an adverse environment, as *M. tuberculosis* is exposed to various stressors capable of disrupting the essential constituents of the bacterium. In Gram-negative and Gram-positive bacteria, resistance to cell envelope stressors that perturb the plasma membrane is mediated in part by proteins comprising the phage shock protein (Psp) system. PspA is an important component of the Psp system; in the presence of envelope stress, PspA localizes to the inner face of the plasma membrane, homo-oligomerizes to form a large scaffold-like complex, and helps maintain plasma membrane integrity to prevent a loss of proton motive force. *M. tuberculosis* and other members of the *Mycobacterium* genus are thought to encode a minimal functional unit of the Psp system, including an ortholog of PspA. Here, we show that Rv2744c possesses structural and physical characteristics that are consistent with its designation as a PspA family member. However, although Rv2744c is upregulated under conditions of cell envelope stress, loss of Rv2744c does not alter resistance to cell envelope stressors. Furthermore, Rv2744c localizes to the surface of lipid droplets in *Mycobacterium* spp. and regulates lipid droplet number, size, and *M. tuberculosis* persistence during anaerobically induced dormancy. Collectively, our results indicate that Rv2744c is a bona fide ortholog of PspA that may function in a novel role to regulate lipid droplet homeostasis and nonreplicating persistence (NRP) in *M. tuberculosis*.

**IMPORTANCE**

*Mycobacterium tuberculosis* is the causative agent of tuberculosis, a disease associated with significant morbidity and mortality worldwide. *M. tuberculosis* is capable of establishing lifelong asymptomatic infections in susceptible individuals and reactivating during periods of immune suppression to cause active disease. The determinants that are important for persistent infection of *M. tuberculosis* or for reactivation of this organism from latency are poorly understood. In this study, we describe our initial characterizations of Rv2744c, an ortholog of phage shock protein A (PspA) that regulates the homeostasis of lipid bodies and nonreplicating persistence in *M. tuberculosis*. This function of PspA in *M. tuberculosis* is novel and suggests that PspA may represent a unique bacterial target upon which to base therapeutic interventions against this organism.

*Mycobacterium tuberculosis* is the causative agent of the respiratory disease tuberculosis (TB) and is a human-specific pathogen of global importance. This bacterium is responsible for >9.6 million new cases of TB and 1.5 million deaths annually (1), ranking TB as the leading cause of death in the world due to an infectious agent, alongside HIV/AIDS. A key aspect of the *M. tuberculosis* life cycle is its ability to establish asymptomatic latent infections and reactivate to cause active disease and transmission to new hosts (reviewed in reference 2). During latency, *M. tuberculosis* exists in a state of nonreplicating persistence (NRP), a poorly understood physiological state in which the bacterium can utilize alternative carbon sources and energy-generating pathways for long-term survival within the host (3–6). While a live attenuated vaccine for TB is available (*Mycobacterium bovis* bacillus Calmette-Guérin [BCG]), its efficacy at preventing adult pulmonary TB and thus *M. tuberculosis* retransmission is highly varied (7, 8). Furthermore, *M. tuberculosis* exhibits increased phenotypic resistance to anti-TB antibiotics during NRP (2, 9), making it more difficult to kill this organism in latently infected individuals. Therefore, new strategies and/or therapeutics are urgently needed to help eradicate TB. This will require an improved understanding of the mechanisms utilized by *M. tuberculosis* to persist and/or reactivate within the host.
A hallmark of latent *M. tuberculosis* infection is the formation of host granulomatous lesions at sites of infection (10). Within the granuloma, *M. tuberculosis* is exposed to numerous environmental and/or biological “stressors” that alone, or in combination, are capable of damaging essential constituents of the bacterium. These stressors include low oxygen tension (5), limited nutrient availability (11), reactive oxygen and nitrogen species (12), and cell envelope-perturbing agents (13, 14). The recognition of and successful adaptation to these stressors in *M. tuberculosis* are mediated by the activities of regulatory proteins, including two-component signal transduction systems, extracytoplasmic function sigma factors, serine-threonine protein kinases, and other stress-responsive elements. MprAB, SigE, and PknB are three such regulatory factors that collectively mediate the resistance of *M. tuberculosis* to cell envelope stressors (reviewed in references 15 and 16). MprAB is a two-component signal transduction system that recognizes misfolded or unfolded proteins in the extracytoplasmic compartment (17). This system regulates a diverse set of >300 genes, including determinants encoding sigma factors, regulatory proteins, chaperones, proteases, and metabolic enzymes (18). SigE is an extracytoplasmic function sigma factor that controls a regulon of >70 genes in response to a variety of cell envelope-perturbing agents (19, 20). Finally, PknB is a serine-threonine protein kinase that regulates cell envelope stress resistance through posttranslational modifications (phosphorylation) of target substrates (21, 22). Recent evidence indicates that the stress-responsive pathways controlled by MprAB, SigE, and PknB are integrated at multiple levels. For example, MprAB directly regulates sigE expression (18), while SigE directly regulates the operon encoding MprA-MprB-PepD-MoaB2 (20). Similarly, PknB phosphorylates the SigE anti-sigma factor RseA, leading to the degradation of RseA by the ClpC1P2 proteases and the subsequent release of SigE for downstream gene regulation, including at the mprAB operon (21). However, in addition to this higher level integration, a subset of downstream determinants within each regulon are shared and are directly or indirectly coregulated by MprAB, SigE, and PknB. One such locus is the four-gene operon encoding Rv2745c (ClgR)-Rv2744c-Rv2743c-Rv2742c (20, 21, 23), which recently was reported to encode a minimal functional unit of an *M. tuberculosis* phage shock protein (Psp) system (24).

In Gram-negative and Gram-positive bacteria, the Psp system helps maintain homeostasis of the plasma membrane under conditions of cell envelope stress (25). PspA is a key component of the Psp system, acting to repress or activate the Psp response depending on the stress status of the cell. In the absence of stress, PspA localizes to the cytoplasmic compartment, where it binds to and negatively regulates PspF, a transcription factor controlling the psp operon (*pspABCDE*) and other PspA regulon members (26–28). In the presence of stress, PspA dissociates from PspF, relocates to the inner face of the plasma membrane, binds to other membrane-associated components of the Psp system, and homooligomerizes to form a higher-order scaffold-like structure to physically stabilize the plasma membrane and help prevent the loss of proton motive force (29). In *M. tuberculosis*, *Rv2744c* has been predicted to encode an ortholog of *PspA* (30). *Rv2744c* contains a PspA/IM30 domain, and it has been shown to interact with itself (31) and other components of the proposed *M. tuberculosis* Psp system, including transcription factor *Rv2745c* (ClgR) and *Rv2743* (a protein of unknown function) (24). However, apart from these observations, the function and/or role of *Rv2744c* have yet to be elucidated. In this report, we present evidence demonstrating that *Rv2744c* shares structural and physical features consistent with its designation as a PspA family member. However, while *Rv2744c* is upregulated in *M. tuberculosis* upon exposure to cell envelope stress, loss of this determinant does not alter resistance to cell envelope perturbants. Furthermore, *Rv2744c* localizes to the surface of lipid droplets (LDs) and regulates the characteristics of these quasio rganelles, including their number and size. In addition, dysregulation of *Rv2744c* production negatively impacts the survival of *M. tuberculosis in vitro* under conditions of NRP. Based on these observations, we propose that *Rv2744c* is a PspA ortholog that may function in a novel manner in *M. tuberculosis* relative to other archetypical PspA family members.

**MATERIALS AND METHODS**

**Bacterial strains, media, and growth conditions.** All strains, plasmids, and mycobacteriophages used in the study are described in Table 1. Escherichia coli TOP10 or DH5α was used for cloning. *E. coli* BL21(DE3)/pLysS (EMD Millipore, Billerica, MA) was used to overexpress and purify recombinant proteins following induction with isopropyl-β-D-thiogalactoside (IPTG) (Thermo Fisher Scientific, Waltham, MA). *E. coli* BTH101 was used for bacterial adenylate cyclase two-hybrid assays. *E. coli* HB101 was used as a recipient for transduction with *in vitro*-packaged lambda phages. *E. coli* strains were grown in Luria-Bertani (LB) broth, LB agar (Thermo Fisher Scientific), or MacConkey base agar medium (BD, Franklin Lakes, NJ) containing maltose as the sole carbon source. The medium was supplemented with 100 μg/ml ampicillin (Thermo Fisher Scientific), 50 μg/ml kanamycin sulfate (Thermo Fisher Scientific), 150 μg/ml hygromycin B (AG Scientific, San Diego, CA), or 25 μg/ml chloramphenicol (Thermo Fisher Scientific) when necessary. The *Mycobacterium* strains used in this study were from ATCC (Manassas, VA) and are derivatives of *Mycobacterium tuberculosis* H37Rv (ATCC 27294) or *Mycobacterium smegmatis* mc²155 (ATCC 700084). For routine culturing, *Mycobacterium* strains were grown with shaking (150 rpm) at 37°C in Middlebrook 7H9 broth (BD) or on Middlebrook 7H10 agar medium (BD) supplemented with 0.5% glycerol, 10% albumin-dextrose-catalase (ADC) or oleic acid-ADC (OADC) (BD), and 0.05% Tween 80 (Sigma, St. Louis, MO), unless otherwise noted. To prepare lipid droplets, *Mycobacterium* strains were grown with shaking at 37°C in Sauton’s medium (32). When required, the mycobacterial medium was supplemented with 25 μg/ml kanamycin sulfate (Thermo Fisher Scientific), 50 μg/ml hygromycin B (AG Scientific), and/or 10 ng/ml anhydrotetracycline (Sigma). *Mycobacteriophage* production and transduction of *M. tuberculosis* H37Rv were described previously (33). Electroporment cells of *M. tuberculosis* were prepared as described previously (34).

**DNA manipulations.** Restriction enzyme digestion, cloning, subcloning, and DNA electrophoresis were done according to standard techniques (35). Oligonucleotides and primers were synthesized by Eurofins MWG Operon (Huntsville, AL) and are listed in Table S1 in the supplemental material. PCR was performed using Platinum Pfx or Taq polymerase (Thermo Fisher Scientific, Waltham, MA). All amplified products were first cloned into pCR2.1-TOPO (Thermo Fisher Scientific) and sequenced (MCLAB, San Francisco, CA) to confirm the absence of mutations. Cloned inserts were then removed by restriction enzyme digestion (NEB, Ipswich, MA), resolved on a 1% agarose gel, recovered by gel purification, and subcloned into the appropriate vector. Ligations were performed using T4 DNA ligase (NEB). When necessary, plasmid DNA was treated with Antarctic phosphatase (NEB) to prevent religation of the vector ends. Plasmid DNA was prepared using the QIAprep spin miniprep kit (Qiagen, Venlo, the Netherlands), as recommended by the manufacturer. DNA fragments were purified using either the QIAquick gel extraction kit or QIAquick PCR purification kit (Qiagen).
TABLE 1 Strains and plasmids used in this study

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<td>pHZE1246</td>
<td>pHAE87 derivative containing pTZ1246</td>
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* Kan', kanamycin resistant; Amp', ampicillin resistant; Hygr, hygromycin resistant.

**DNA and RNA extraction and RT-PCR.** Mycobacterial genomic DNA was isolated as described previously (34). For isolation of RNA, M. tuberculosis or M. smegmatis was grown in 7H9 or Sauton’s broth to mid-log phase (optical density at 600 nm [OD600], 0.5). RNA was fixed by treating cultures with RNAlater (Thermo Fisher Scientific, Waltham, MA) at 4°C prior to processing. Bacteria were collected by centrifugation, washed twice with phosphate-buffered saline (PBS), and then mechanically disrupted in the presence of RNA-Bee (Tel-Test, Friendswood, TX) by bead beating. RNA was then extracted with chloroform, precipitated with isopropanol, and stored at −20°C until use. The resulting RNA was
suspended in diethylpyrocarbonate (DEPC)-treated water, treated with Turbo DNase (Thermo Fisher Scientific) to remove contaminating genomic DNA, and purified using an RNaseasy mini prep column (Qiagen, Venlo, the Netherlands). For reverse transcription-PCR (RT-PCR), cDNA from M. tuberculosis or M. smegmatis strains was prepared by incubating 500 ng of total RNA with random M. tuberculosis decamers (36) in a reverse transcription reaction mixture containing SuperScript III reverse transcriptase (Thermo Fisher Scientific). To test for the presence of DNA contamination, identical reaction mixtures lacking SuperScript III reverse transcriptase were also run. The reaction mixtures included primer sets that amplified 100- to 150-bp fragments from specific coding sequences. Amplification conditions consisted of 40 rounds of denaturation at 95°C for 30 s and annealing/extension at 60°C for 15 s using iQ SYBR green Supermix (Bio-Rad, Hercules, CA). The reactions were run on an iCyler iQ real-time PCR detection system (Bio-Rad). Single PCR products were confirmed by performing a postamplification melting curve analysis for each reaction. For relative quantification of transcript levels, the expression of target genes was normalized to the gene encoding the constitutively expressed SigA sigma factor. Absolute quantification of transcript levels was performed by preparing a standard curve for each primer set using 10-fold serial dilutions of chromosomal DNA as the template. The transcript number of the desired gene was then normalized to SigA, whose expression was set at 1.

**Protein extraction, SDS-PAGE, and Western blotting.** Proteins were extracted from E. coli or Mycobacterium spp. by French press or bead-beating. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein-containing fractions were incubated with 2× SDS-PAGE loading dye and denatured for 5 min at 95°C. The proteins were then separated on 12% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes using the Trans-Blot Turbo RTA transfer kit and Trans-Blot Turbo transfer system (Bio-Rad, Hercules, CA). For Western blotting, membranes were blocked in TTBS (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.5% Tween 20) containing 5% skim milk for 1 h and probed with antisera diluted in TTBS overnight at 4°C. The primary antibodies included anti-His (1:1,500) (Covance, Princeton, NJ), anti-FLAG (1:3,000) (Sigma, St. Louis, MO), anti-MmprB (1:50,000) (Covance), anti-GroES (1:3,000) (BEI Resources, National Institute of Allergy and Infectious Diseases [NIAID], NIH), and anti-green fluorescent protein (anti-GFP) (1:5,000) (Covance). Membranes were then washed in TTBS and incubated for 2 h at room temperature with goat anti-mouse IgG (1:5,000) (Thermo Fisher Scientific, Waltham, MA) or goat anti-rabbit IgG (1:5,000) secondary antibody conjugated to horseradish peroxidase. Blots were developed using the SuperSignal West Pico chemiluminescent substrate kit (Thermo Fisher Scientific) and visualized on CL-Xposure X-ray film (Thermo Fisher Scientific).

**Plasmid construction.** To generate the vector for Rv2744c protein production, Rv2744c was amplified from M. tuberculosis genomic DNA by PCR using primers Rv2744cF-WD-Nhel and Rv2744cRev4-NotI. The product was cloned into pCR2.1-TOPO, digested with NheI and NotI, and subcloned into the same sites of pET24b. The resulting plasmid (pTZ1168) was then transformed into E. coli BL21 (DE3)/pLysS to generate TCZ1820. To generate the vector for MS_2695 deletion, 1.0-kb regions upstream and downstream of MS_2695 were individually amplified from M. smegmatis mc^1^5^5^ genomic DNA using the primer pairs Ms2695kupFp and Ms2695kdnRP, and Ms2695kdnF and Ms2695kupR. The resulting PCR products were then purified and assembled into a contiguous fragment by overlap PCR using primers Ms2695kp1F and Ms2695kd1R. The product was cloned into pCR2.1-TOPO, released following digestion with XbaI, and subcloned into the XbaI site of pTZ898 (31) to generate pTZ1231. To generate the vector for Rv2744c deletion, 1.0-kb regions upstream and downstream of the Rv2744c coding sequence were PCR amplified from M. tuberculosis H37Rv genomic DNA using the primer pairs Rv2744kp1F and Rv2744kd1R and Rv2744kd2F and Rv2744kp2R, respectively. The resulting products were cloned into pCR2.1-TOPO, released following digestion with Spel-XhoI and XbaI-EcoRI, respectively, and fragments directionally subcloned into the corresponding sites flanking the *res-hyg-res* cassette present in pYUB854 (33). The resulting plasmid, pTZ1246, was then used to prepare high-titer transducing phage for allelic exchange. To generate the Rv2744c complementation plasmid, the Rv2744c coding sequence along with N-terminal 3× FLAG and C-terminal 6× His epitope tags were amplified from pTZ1171 (31) using primers pET24fwd-PstI and pET24rev-HindIII, cloned into pCR2.1-TOPO, released by digestion with PstI-HindIII, and directionally subcloned into the corresponding sites of Mycobacterium expression construct pSE100 (37), resulting in pTZ1227. The untagged wild-type allele of Rv2744c was also amplified from M. tuberculosis genomic DNA using primers Rv2744cFpSE100PstI and Rv2744cRpSE100EcoRV, cloned into pCR2.1-TOPO, released by digestion with PstI-EcoRV, and directionally subcloned into the corresponding sites of pSE100, resulting in pTZ1489. To generate the vector producing TetR, the tet(R) coding sequence was amplified from pmC1s (37) using primers TetRF-EcoRI and TetRR-EcoRV, cloned into pCR2.1-TOPO, released by digestion with PstI, and subcloned into the corresponding site of pSE100. The resulting vector, pTZ1463, was then electroporated into M. tuberculosis ΔRv2744c, resulting in TB314.

**Purification of Rv2744c.** M. tuberculosis Rv2744c was purified from E. coli using Ni affinity chromatography. Briefly, TCZ1820 was grown in LB with selection at 37°C until cells reached mid-exponential phase. Protein was then induced with 1.0 mM IPTG for 3 h at 37°C. Following induction, cultures were centrifuged and the resulting bacterial pellet suspended in lysis buffer (50 mM Na$_2$HPO$_4$ [pH 8.0], 300 mM NaCl, 10 mM imidazole, 3 μg DNAase/ml, 3 μg RNase/ml, and protease inhibitor cocktail (Sigma, St. Louis, MO)) before lysis with a French pressure cell. The total cell lysate was then purified by centrifugation at 25,000 × g for 30 min, rocked with nickel-nitrirotiacetic acid (NTA)-agarose (Qiagen, Venlo, the Netherlands) for 90 min at 4°C, and then processed by batch purification. Bound protein was washed with binding buffer (50 mM Na$_2$HPO$_4$ [pH 8.0], 300 mM NaCl, 10 mM imidazole) and then wash buffer (50 mM Na$_2$HPO$_4$ [pH 8.0], 300 mM NaCl, 25 mM imidazole). Finally, NTA resin was sequentially incubated with 1-ml aliquots of elution buffer (50 mM Na$_2$HPO$_4$ [pH 8.0], 300 mM NaCl, 150 mM imidazole) to remove protein from the resin. Protein purity was determined by subjecting recovered fractions to SDS-PAGE and staining with Coomassie brilliant blue (Thermo Fisher Scientific, Waltham, MA).

**Sucrose gradient ultracentrifugation.** The molecular mass of purified Rv2744c was determined by sucrose gradient ultracentrifugation. Briefly, a Gradient Master (BioComp Instruments, Fredericton, Canada) was used to generate linear 10% to 50% sucrose gradients prepared in protein extraction buffer. Approximately 0.5 mg of purified Rv2744c-His, or protein standards, including bovine serum albumin (66 kDa), β-amylase (200-kDa), thyroglobulin (669 kDa), and blue dextran (2,000 kDa) (all from Sigma, St. Louis, MO), were layered on top of individual gradients and enriched by ultracentrifugation in a SW55 Ti rotor (Beckman Coulter, Brea, CA) at 100,000 × g for 16 h at 4°C. Half-milliliter fractions were then removed sequentially from the tops of tubes and collected to quantify the amount of protein at 260 using a NanoDrop (Thermo Fisher Scientific, Waltham, MA) or to determine protein identity by SDS-PAGE, followed by Western blotting.

**Bacterial two-hybrid assays.** Specific protein-protein interactions were investigated using the bacterial two-hybrid system BACTH (Euromedex, Soufflweyersheim, France), as per the manufacturer’s
directions. BACTH vectors expressing Rv2744c have been described, including pTZ1182, pTZ1183, and pTZ1185 (31). To generate BACTH vectors expressing Rv2745c (clgR) or Rv2743c, the sequencing code for these genes was PCR amplified from M. tuberculosis H37Rv genomic DNA using the primer pairs 2745cXbaI-F2H and 2745cKpnI-R2H, and FBamH1Rv2743c and RKpn1Rv2743c, respectively. The resulting products were cloned into pCR2.1-TOPO, released using XbaI-KpnI, and directly subcloned into BACTH vectors pKT25, pUT18, or pUT18 digested with the same enzymes, resulting in pTZ1345, pTZ1346, pTZ1364, pTZ1379, and pTZ1380. Vector combinations were then cotransformed into the E. coli BTH101 reporter strain, and the isolated transformants were streaked onto MacConkey/maltose agar medium containing ampicillin and kanamycin. Plates were incubated at 30°C for 48 to 72 h, and bacteria were subsequently screened for the presence of a red colony phenotype indicative of protein-protein interactions. Two-hybrid vectors expressing DNA sequences coding for leucine zipper motifs (T25-zip and T18-zip) were included as positive controls, while negative controls were performed with the empty T18 or T25 plasmids.

Homology modeling, BLAST searches, and phylogenetic analyses. PspA orthologs were identified via PSI-BLAST search (blast.ncbi.nlm.nih.gov) using the M. tuberculosis Rv2744c, Escherichia coli PspA, or Arabidopsis thaliana Vipp1 protein sequence as a query. Searches were directed at bacteria phyla that included M. tuberculosis PspA orthologs were identified via PSI-BLAST search (blast.ncbi.nlm.nih.gov). Actinobacteria, Cyanobacteria, Proteobacteria, and Acidobacteria were included as positive controls, while negative controls were performed with the empty T18 or T25 plasmids. M. smegmatis mutant was generated by specialized allelic exchange event by PCR using primers Rv2744cF100 and Rv2744cR100, resulting in strain TB284. To complement the unmarked deletion in MS_2695 and Rv2744c, pTZ1227 was electroporated into TCCZ2054 or TB284, resulting in strain TCCZ2365 or TB303, respectively. Expression of 3xFLAG-Rv2744c-His in these strains was then confirmed by Western blotting using α-FLAG and α-His antibodies.

In vitro survival assays. The sensitivity of Mycobacterium spp. to in vitro stressors was assessed using standard survival assays or MIC growth assays. M. tuberculosis or M. smegmatis derivatives were grown to mid-log phase in 7H9 medium, diluted in fresh medium to an OD600 of 0.02, and exposed to specific stressors, including SDS (0.5% for M. tuberculosis and 0.1% for M. smegmatis), H2O2 (1.25 mM to 80 mM), thiordiazide (4 μg/ml to 256 μg/ml), vancomycin (0.5 μg/ml to 32 μg/ml), ethanol (0.3% to 5.0%), and NaCl (500 mM and 1 M). Bacterial survival was then measured by removing aliquots at specific times and plating for viable CFU on agar medium (survival assay) or by measuring culture growth at specific times postexposure (MIC assay) using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). To assess the survival of M. tuberculosis derivatives during NRP, strains were grown using the rapid anaerobic dormancy (RAD) model, with slight modifications (39). Briefly, wild-type M. tuberculosis H37Rv, TB284, or TB303 was grown in Sauton’s medium to mid-exponential phase and diluted to an OD600 of 0.05 in Sauton’s medium. A 6.7-ml aliquot of culture was then dispensed into individual 16 by 100-mm blood collection tubes (product no. 366441; BD, Sparks, MD), each containing a 3 by 10-mm micro-stir-bar plate (product no. 1451365; Thermo Fisher Scientific, Waltham, MA). The tubes were tightly sealed with rubber stoppers, resulting in a defined headspace of 0.5 within each tube. Control tubes lacking bacteria or containing 9 μg/ml methylene blue to monitor oxygen depletion in the culture medium were also included for each strain assayed. The cultures were stirred at 200 rpm on a four-position micro-stir plate (Wheaton, Millville, NJ) that was placed inside a 37°C incubator. At defined time points, individual sacrificial tubes were opened and the CFU quantified on 7H10 agar medium. For each time point, three sacrificial tubes were processed for each strain assayed. Nutrient stress was induced by growing M. tuberculosis cultures to mid-log phase in Sauton’s medium, washing in PBS, and then incubating bacteria in 1× PBS containing 0.05% tyloxapol (Thermo Fisher Scientific) at 37°C with shaking. At various times poststarvation, aliquots were removed, diluted, and plated on agar medium to quantify viable bacteria.

Lipid droplet purification, subcellular fractionation, and protein localization. Lipid droplets (LDs) were isolated from 1.0-liter cultures of M. smegmatis derivatives after growth in Sauton’s medium for 1 week at 37°C to 5.0%, and NaCl (500 mM and 1 M). Bacterial survival was then measured by removing aliquots at specific times and plating for viable CFU on agar medium (survival assay) or by measuring culture growth at specific times postexposure (MIC assay) using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). To assess the survival of M. tuberculosis derivatives during NRP, strains were grown using the rapid anaerobic dormancy (RAD) model, with slight modifications (39). Briefly, wild-type M. tuberculosis H37Rv, TB284, or TB303 was grown in Sauton’s medium to mid-exponential phase and diluted to an OD600 of 0.05 in Sauton’s medium. A 6.7-ml aliquot of culture was then dispensed into individual 16 by 100-mm blood collection tubes (product no. 366441; BD, Sparks, MD), each containing a 3 by 10-mm micro-stir-bar plate (product no. 1451365; Thermo Fisher Scientific, Waltham, MA). The tubes were tightly sealed with rubber stoppers, resulting in a defined headspace of 0.5 within each tube. Control tubes lacking bacteria or containing 9 μg/ml methylene blue to monitor oxygen depletion in the culture medium were also included for each strain assayed. The cultures were stirred at 200 rpm on a four-position micro-stir plate (Wheaton, Millville, NJ) that was placed inside a 37°C incubator. At defined time points, individual sacrificial tubes were opened and the CFU quantified on 7H10 agar medium. For each time point, three sacrificial tubes were processed for each strain assayed. Nutrient stress was induced by growing M. tuberculosis cultures to mid-log phase in Sauton’s medium, washing in PBS, and then incubating bacteria in 1× PBS containing 0.05% tyloxapol (Thermo Fisher Scientific) at 37°C with shaking. At various times poststarvation, aliquots were removed, diluted, and plated on agar medium to quantify viable bacteria. Lipid droplet purification, subcellular fractionation, and protein localization. Lipid droplets (LDs) were isolated from 1.0-liter cultures of M. smegmatis derivatives after growth in Sauton’s medium for 1 week at 37°C to 5.0%, and NaCl (500 mM and 1 M). Bacterial survival was then measured by removing aliquots at specific times and plating for viable CFU on agar medium (survival assay) or by measuring culture growth at specific times postexposure (MIC assay) using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). To assess the survival of M. tuberculosis derivatives during NRP, strains were grown using the rapid anaerobic dormancy (RAD) model, with slight modifications (39). Briefly, wild-type M. tuberculosis H37Rv, TB284, or TB303 was grown in Sauton’s medium to mid-exponential phase and diluted to an OD600 of 0.05 in Sauton’s medium. A 6.7-ml aliquot of culture was then dispensed into individual 16 by 100-mm blood collection tubes (product no. 366441; BD, Sparks, MD), each containing a 3 by 10-mm micro-stir-bar plate (product no. 1451365; Thermo Fisher Scientific, Waltham, MA). The tubes were tightly sealed with rubber stoppers, resulting in a defined headspace of 0.5 within each tube. Control tubes lacking bacteria or containing 9 μg/ml methylene blue to monitor oxygen depletion in the culture medium were also included for each strain assayed. The cultures were stirred at 200 rpm on a four-position micro-stir plate (Wheaton, Millville, NJ) that was placed inside a 37°C incubator. At defined time points, individual sacrificial tubes were opened and the CFU quantified on 7H10 agar medium. For each time point, three sacrificial tubes were processed for each strain assayed. Nutrient stress was induced by growing M. tuberculosis cultures to mid-log phase in Sauton’s medium, washing in PBS, and then incubating bacteria in 1× PBS containing 0.05% tyloxapol (Thermo Fisher Scientific) at 37°C with shaking. At various times poststarvation, aliquots were removed, diluted, and plated on agar medium to quantify viable bacteria.

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insoluble pellet remaining in the bottom of the tube following the first ultracentrifugation was washed 3 times with PBS containing 0.05% Tween 20 and then solubilized in PBS containing 1% Triton X-100 overnight at 4°C with gentle rocking. The resulting material was designated the membrane fraction.

Triglyceride quantification of LDs. To quantify the triacylglycerol concentration in LDs, total protein concentration of the isolated LDs was first determined using a Qubit protein assay kit (Thermo Fisher Scientific, Waltham, MA). Samples were then adjusted so that the final protein concentration was 25 μg/ml. Ten microliters of each sample was processed using a serum triglyceride kit (Cell Biolabs, San Diego, CA), as per manufacturer’s recommendations, and run alongside triglyceride standards that were prepared in parallel. After incubation, plates were read at an absorbance of 570 nm, values from replicate wells were averaged, and the triacylglycerol concentration was calculated.

Epifluorescence, immunofluorescence, and electron microscopy. Epifluorescence microscopy was used to visualize Rv2744c-GFP in M. tuberculosis Δrv2744c. Briefly, TB314 was grown in Sauton’s medium for 1 week. Bacteria were then pelleted and suspended in 4% paraformaldehyde (PFA) for 1 h at 4°C to fix the bacteria. Fixed bacteria were washed 3 times with PBS, and aliquots were plated on agar medium to confirm culture sterilization. Once confirmed, samples were removed from the biosafety level 3 (BSL3) laboratory and incubated with 1 mg/ml Nile red for 1 h in the dark at room temperature to stain the LDs. Bacteria were then washed 3 times in PBS and mounted on slides using a glass coverslip and ProLong Gold antifade reagent (Thermo Fisher Scientific, Waltham, MA). To visualize LDs by immunofluorescence, LDs were isolated from E. coli and ProLong Gold antifade reagent (Thermo Fisher Scientific) were washed 3 times in PBS–10% BSA, and then incubated with a 1:2,000 dilution of anti-mouse IgG–Alexa Fluor 488 (Thermo Fisher Scientific) for 1 h at room temperature, washed 3 times in PBS, permeibilized with 0.1% Triton X-100 for 1 h at room temperature, washed 3 times in PBS, and then blocked with PBS containing 10% bovine serum albumin (BSA) for 1 h at room temperature. LDs were incubated with a 1:1,000 dilution of anti-FLAG antibody (Sigma, St. Louis, MO) for 1 h at room temperature, washed 3 times in PBS–10% BSA, and then incubated with a 1:2,000 dilution of anti-mouse IgG–Alexa Fluor 488 (Thermo Fisher Scientific) for 1 h at room temperature. Finally, LDs were washed 3 times in PBS–10% BSA and then 3 times in PBS. Coverslips were mounted onto glass slides using the ProLong Gold antifade reagent. Bacteria and LDs were observed with a Nikon CFI Plan Apochromat VC 100×/1.40-numerical-aperture oil immersion lens mounted on a Nikon Eclipse 80i upright microscope outfitted with a multifluorescence filter set and Nikon DS-C2MBW monochromatic digital camera (Nikon, Inc., Melville, NY). Image processing and analysis were conducted using the NIS-Elements software (Nikon, Inc.). For electron microscopy (EM) of LDs within bacteria, M. smegmatis, M. tuberculosis, and E. coli were grown in Sauton’s medium for 1 week, pelleted, washed 2 times in 100 mM sodium cacodylate buffer, and fixed in a 4% glutaraldehyde–2% PFA solution overnight. Samples were then immunoized into epoxy resin and sections viewed on a Hitachi H600 transmission electron microscope (TEM) equipped with an AMT digital camera. For EM of purified Rv2744c-His, an aliquot of protein from fraction 17 of the sucrose gradient was diluted to 1 mg/ml. Protein was adsorbed onto ionized Formvar/copper 400 mesh grids, stained with 2% uranyl acetate, and imaged on the Hitachi H600 TEM at an accelerating voltage of 75 kV with a magnification from ×100,000 to ×300,000.

Statistical analysis. Statistical differences were determined using Student’s t test. A P value of <0.05 was considered significant.

RESULTS
Rv2744c is a member of a novel clade of PspA-like proteins. PSI-BLAST analysis indicates that M. tuberculosis Rv2744c contains a PspA/IM30 domain similar to that of other established PspA family members. Furthermore, Rv2744c lies in an operon along with other genes thought to encode a minimal functional unit of a Mycobacterium Psp system (24). To begin investigating whether Rv2744c possesses characteristics consistent with its designation as a PspA family member, Rv2744c was analyzed by bioinformatics. First, the amino acid sequence of Rv2744c was aligned to PspA family members from a representative group of organisms in which PspA had been characterized and/or identified (27, 42–46). While M. tuberculosis Rv2744c exhibited 86% and 76% sequence identity to the PspA ortholog present in Mycobacterium smegmatis and Rhodococcus jostii sp. strain RHA1, respectively (see Fig. S1 in the supplemental material), the sequence identities between Rv2744c and PspA orthologs in Escherichia coli, Yersinia enterocolitica, Streptomyces lividus, and Arabidopsis thaliana were <20% (see Fig. S1). Next, M. tuberculosis Rv2744c, E. coli PspA, and A. thaliana Vipp1 (vesicle inducing protein in plastids, a PspA ortholog in plants and photosynthetic bacteria), were used in individual BLAST searches to identify high-scoring PspA-like proteins from the general protein database. Amino acid sequences from protein hits were then compared using Clustal W alignment (DNAStar, Madison, WI) to determine the extent of conservation between PspA family members. PspA family members segregated into three distinct clades, with M. tuberculosis Rv2744c comprising an atypical PspA clade more closely related to A. thaliana Vipp1 than to E. coli PspA (Fig. 1A). Finally, it has been reported that PspA family members possess a conserved secondary structure containing 4 to 6 α-helical domains (28, 47).

To investigate whether M. tuberculosis Rv2744c shared this characteristic, Rv2744c was modeled using PsiPred and other secondary structure prediction programs alongside E. coli PspA (48). Similar to PspA, the secondary structure of Rv2744c was predominantly α-helical, consisting of 5 distinct α-helical domains (Fig. 1B). Interestingly, Rv2744c contained an extra C-terminal region that was not present in E. coli PspA (Fig. 1B; see also Fig. S1 in the supplemental material). M. tuberculosis Rv2744c and E. coli PspA were also modeled using Rosetta ab initio application (49) due to the absence of structural data on PspA family members. This analysis produced models in which secondary and tertiary structures were conserved between Rv2744c and E. coli PspA, including a central helix–helix region (Fig. 1C and D). Thus, M. tuberculosis Rv2744c shares conservation in its predicted secondary and tertiary structures with E. coli PspA even though the primary amino acid sequence of these proteins is poorly conserved.

Rv2744c forms homo-oligomers of high molecular weight and distinct structure. A common characteristic of PspA family members is their ability to homo-oligomerize and form structured complexes of high molecular weight (42, 50–52). E. coli PspA forms a 36-mer spheroid-like homo-oligomeric complex consisting of 4 stacked rings of PspA 9-mers (42). Similarly, PspA from Burkholderia pseudomallei homo-oligomerizes to form ring-shaped complexes, rod-like species of various lengths, and mesh-like structures (51). As M. tuberculosis Rv2744c interacts with itself in vivo (31), we next determined whether Rv2744c also formed homo-oligomeric complexes in vitro. To facilitate the purification and detection of Rv2744c, a histidine-tagged variant (Rv2744c-His) was generated and used for these assays. Rv2744c-His was produced at high levels in E. coli and could be purified from the soluble fraction to near homogeneity (Fig. 2A). Next, Rv2744c-His was resolved by size exclusion chromatography to determine its mass; however, Rv2744c-His was unable to migrate in and/or around the S300 Sephacryl column matrix (data not shown), indicating that Rv2744c either forms a large aggregate and/or it
FIG 1 Phylogenetic and modeling analysis of distantly related PspA family members. (A) Phylogenetic tree generated from representative protein BLAST search results with Rv2744c (M. tuberculosis), PspA (E. coli), and Vip1 (A. thaliana) against bacterial and plant families, including Cyanobacteria, Actinobacteria, Spirochaetes, Fusobacteria, Acidobacteria, and Proteobacteria. Specific species analyzed included Escherichia coli, Shigella flexneri, Enterococcus gallinarum, Salmonella enterica, Pectobacterium carotovorum, Yersinia enterocolitica, Photobacterium luminiscens, Photobacterium profundum, Vibrio cholerae, Idiomarina loihiensis, Shewanella oneidensis, Zymomonas mobilis, Mastigocoleus testarum, Desulfovibrio vulgaris, Leptotrichia hofstadii, Paenibacillus sp., Sideroxydans paludicola, Rv2744c Regulates Mycobacterium Lipid Droplets June 2016 Volume 198 Number 11 jb.asm.org 1651 Journal of Bacteriology on November 12, 2017 by guest http://jb.asm.org/ Downloaded from
FIG 2 Rv2744c forms higher-order homo-oligomeric structures in vitro. (A) Rv2744c-His was purified via Ni affinity chromatography. Protein fractions were resolved by SDS-PAGE to confirm purity (M, molecular mass marker; WCL, whole-cell lysate; FT, flowthrough; W1 to W3, washes 1 to 3; E1 to E4, elutions 1 to 4). (B) Purified Rv2744c-His was resolved on a continuous 10 to 50% sucrose gradient along- side protein standards of known mass. Following ultracentrifugation, Rv2744c-His localized predominantly in fractions 17 to 19 (Fig. 2B and C). Rv2744c-His was also detected in fractions 21 to 23 (Fig. 2B and C). This banding pattern places the mass of Rv2744c-His between the protein standards thyroglobulin (669 kDa) and blue dextran (>2,000 kDa). To determine whether Rv2744c-His present in fractions 17 to 19 formed a structured complex, an aliquot of fraction 17 from the sucrose gradient was visualized by negative-stain electron microscopy. The protein present in this fraction formed higher-ordered homo-oligomeric complexes consisting of individual spheroids and organized tube-like structures (Fig. 2D and E). These structures strongly resembled those seen with other characterized PspA family members. Collectively, these data, along with the bioinformatic studies, suggest that Rv2744c possesses structural and physical characteristics consistent with its designation as a PspA family member.

Rv2744c-MS_2695 is upregulated by, but does not mediate resistance to, cell envelope stressor SDS. To begin investigating the importance of PspA in Mycobacterium physiology, unmarked deletion mutations were generated in the coding sequences for Rv2744c or its homolog in M. smegmatis, MS_2695 (Fig. 3A). PCR analyses confirmed the loss of these genes in both mutant strains (Fig. 3B). To verify that loss of Rv2744c or MS_2695 did not impact Mycobacterium growth, wild-type and mutant strains of M. tuberculosis or M. smegmatis were cultured in standard laboratory medium, and replication kinetics were monitored. The deletion of Rv2744c or MS_2695 did not alter growth characteristics in either M. tuberculosis (Fig. 3C) or M. smegmatis (Fig. 3D). To determine whether Rv2744c or MS_2695 was regulated in response to cell envelope stress, gene expression levels were measured in wild-type M. tuberculosis or M. smegmatis by reverse transcription-quantitative PCR (qRT-PCR) following exposure to 0.05% SDS for 90 min (18). Rv2744c or MS_2695 expression levels were first normalized to the housekeeping gene sigA, whose expression is constitutive, and then compared to untreated controls, whose expression was set to 1.0. Exposure of M. tuberculosis to SDS induced Rv2744c expression by ~3.0-fold (Fig. 3E), while the expression of MS_2695 was upregulated by ~40-fold in M. smegmatis following SDS exposure (Fig. 3F). Finally, the importance of Rv2744c and MS_2695 for cell envelope stress resistance was assessed by exposing wild-type and mutant strains to bactericidal concentrations of SDS. Bacterial survival was monitored over time by plating for CFU. While exposure to SDS reduced the viability of M. tuberculosis (Fig. 3G) and M. smegmatis (Fig. 3H) over time, the loss of Rv2744c or MS_2695 did not increase sensitivity of either species.
FIG 3 Rv2744c and MS_2695 are upregulated by, but do not mediate protection against, extracellular stress. (A) Organization of loci containing Rv2744c or MS_2695 in M. tuberculosis or M. smegmatis, respectively. The numbers above the genes refer to gene length (in base pairs), and the numbers below the genes refer to the distance (in base pairs) between genes. Unmarked deletion mutations were generated via homologous recombination in Rv2744c or MS_2695. (B) Loss of Rv2744c or MS_2695 was confirmed by PCR using primers flanking the gene (small arrows in panel A). M, 1-kb plus DNA ladder. (C and D) Growth of the wild type (○) or ΔRv2744c or MS_2695 mutants (Δ) of M. tuberculosis (C) or M. smegmatis (D) was assessed in 7H9 medium supplemented with OADC (M. tuberculosis) or ADC (M. smegmatis) and containing 0.05% Tween 20. (E and F) The expression of Rv2744c (E) or MS_2695 (F) was measured in the absence of or following exposure to 0.05% SDS for 90 min. The expression of Rv2744c or MS_2695 was measured via qRT-PCR and expressed as the ΔΔC\textsubscript{t} relative to the control without SDS. (G and H) The survival of the wild type (○) or ΔRv2744c or MS_2695 mutants (Δ) was assessed following exposure of M. tuberculosis to 0.5% SDS (G) or exposure of M. smegmatis to 0.1% SDS (H). Bacterial survival was quantified by plating serial 10-fold serial dilutions to determine the CFU per milliliter. All error bars represent the standard error of the results from three replicates (*, P < 0.05).
possible to isolate LDs directly from M. tuberculosis due to biosafety reasons. Here, GFP was fused to the C terminus of Rv2744c, cloned into pSE100, and transformed into M. tuberculosis H9004 Rv2744c. The resulting recombinant strain was then grown under conditions promoting LD production, fixed, incubated with Nile red to stain neutral fatty acids within LDs, and visualized by epifluorescence microscopy (56, 57). Rv2744c can be seen associating with clusters of LDs produced in M. tuberculosis (Fig. 4F).

Thus, Rv2744c localizes to and associates with LDs produced by M. smegmatis and M. tuberculosis.

Rv2744c/MS_2695 regulates the size and number of LDs produced by Mycobacterium species. Given that Rv2744c formed large homo-oligomeric complexes in vitro and localized to LDs in vivo, we next assessed whether Rv2744c regulated aspects of LD homeostasis. To investigate this possibility, wild-type M. smegmatis mc2155, the M. smegmatis ΔMS_2695 mutant, or the M. smeg-
mutant overproducing the epitope-tagged form of Rv2744c (pTZ1227) was grown under conditions promoting LD production. Strains were fixed, stained, and visualized using transmission electron microscopy. Electron-lucent LDs were observed in all three strains (Fig. 5A), demonstrating that Rv2744c/MS_2695 is not necessary for LD formation. Next, the impact of Rv2744c/MS_2695 on LD size was examined. For these studies, the diameters of >300 LDs from >200 bacteria present in >20 different microscopic fields were quantified for each strain, using a counter blinded to the strain genotype. The M. smegmatis ΔMS_2695 mutant exhibited an increased number of LDs possessing a smaller diameter (<50 nm) compared to that of the wild-type control (Fig. 5B). In contrast, overproduction of the tagged Rv2744c variant in the M. smegmatis ΔMS_2695 mutant (TCZ2365) led to an increased number of LDs possessing a larger diameter (≈100 nm) compared to that of the wild-type parent (Fig. 5B). The observed increase in LD diameter was not an artifact of the epitope tags present on Rv2744c, as a similar phenotype was...
observed with an *M. smegmatis ΔMS_2695* mutant expressing the wild-type Rv2744c allele from the same vector (TCZ2654) (see Fig. S2A in the supplemental material).

To further confirm that Rv2744c regulated LD size, TCZ2365 was transformed with an integrating plasmid constitutively producing the TetR repressor. The operator sequence for tetR is positioned between the promoter and Rv2744c coding sequence present on pTZ1227, allowing Rv2744c expression in this new strain (TCZ2455) to be regulated by anhydrotetracycline (ATc), which can be included in the growth medium. TCZ2455 was grown under conditions inducing LD production either in the absence or presence of 10 nM ATc, fixed, and processed for electron microscopy. The size of LDs was then quantified and compared to the size distribution of LDs observed in wild-type *M. smegmatis mc²155*, the *M. smegmatis ΔMS_2695* mutant (TCZ2054), and TCZ2365 (Fig. 5B). Rv2744c and MS_2695 expression levels in these strains were also quantified by qRT-PCR relative to a standard curve, so that Rv2744c and MS_2695 expression levels could be directly correlated with LD size. In the absence of ATc, TCZ2455 expressed a small amount of Rv2744c, likely due to the inability of TetR to completely repress the expression of this determinant from the pTZ1227 multicopy plasmid (see Fig. S2B in the supplemental material). Interestingly, this level of Rv2744c expression was similar to that observed in wild-type *M. smegmatis mc²155* (see Fig. S2B) and resulted in LDs that had a similar size distribution to that observed in the wild-type parent (compare Fig. S2C with Fig. 5B). Taken together, these results suggest that LD size distribution is directly correlated to the amount of Rv2744c produced within the bacteria.

Apart from altering LD diameter, the deletion of *Rv2744c* did not impact the amount of TAG detected within pTZ1227 (Fig. 5C) strains were examined using a modified version of the rapid anaerobic dormancy (RAD) model (A) or a PBS starvation assay (B). The data represent the mean survival of bacteria from triplicate cultures ± the standard error of the mean (SEM) (*, *P < 0.05*).

**Fig. 6** Rv2744c regulates *M. tuberculosis* survival under conditions of NRP. Growth and survival of *M. tuberculosis* H37Rv (●), ΔRv2744c (○), and ΔRv2744c/pTZ1227 (□) strains were examined using a modified version of the rapid anaerobic dormancy (RAD) model (A) or a PBS starvation assay (B). The data represent the mean survival of bacteria from triplicate cultures ± the standard error of the mean (SEM) (*, *P < 0.05*).
PspA is a key component of the phage shock protein (Psp) system present in many bacteria. This system is thought to mediate resistance to cell envelope stress by stabilizing the plasma membrane and by preventing the loss of proton motive force. PspA family members possess a number of evolutionarily conserved characteristics, including (i) a largely α-helical secondary structure, (ii) the ability to homo-oligomerize and form structured complexes of high molecular weight, and (iii) association with lipids. In this study, the structural and functional attributes of Rv2744c were characterized to determine whether this protein represented an ortholog of PspA in *M. tuberculosis*. While Rv2744c possessed characteristics consistent with its designation as a PspA family member, Rv2744c did not function to regulate resistance to cell envelope stress. Rather, Rv2744c localized to the surface of LDs, where it regulated LD number, size, and survival of *M. tuberculosis* during NRP. These findings indicate that Rv2744c represents a novel PspA family member whose function includes the regulation of LD homeostasis and *M. tuberculosis* survival under conditions of NRP.

Rv2744c shares limited primary amino acid sequence homology with *E. coli* PspA and PspA-like proteins from other organisms. Rv2744c aligns to a unique clade of atypical PspA-like proteins that are found predominantly in members of the *Actinobacteria*. This clade is evolutionarily distant from the atypical clade containing *E. coli* PspA, as well as the clade containing *A. thaliana* Vipp1, a PspA family member whose function includes membrane maintenance and thylakoid biogenesis in *Cyanobacteria* and plants. In contrast to the minimal primary amino acid sequence homology, bioinformatics predicts that Rv2744c possesses secondary and tertiary structures that are highly conserved with other PspA family members. Rv2744c is largely α-helical, and our Rosetta models predict that it may form five distinct helical domains organized in a fashion similar to those in *E. coli* PspA. Interestingly, Rv2744c contains a 48-amino-acid C-terminal extension that is absent in proteins from the PspA clade but that is present in PspA members from the Vipp1 clade. While the exact role of this extension in Rv2744c function remains unknown, it has been proposed that the C-terminal extension present in Vipp1 confers specificity and allows this protein to carry out specialized functions related to its role in oxygenic photosynthesis (47). Rv2744c also possesses physical characteristics that are conserved with *E. coli* PspA and other PspA family members. Rv2744c self-associates and forms homo-oligomeric complexes of high molecular weight. While the molecular weight of Rv2744c could not be resolved using size exclusion chromatography, sucrose gradient ultracentrifugation studies indicate that Rv2744c forms homo-oligomeric complexes between 650 kDa and 2,000 kDa. When imaged by negative-stain electron microscopy, Rv2744c assumed a spheroid-like structure and elongated filamentous-like superstructures that likely consist of stacked spheroidal subunits. These structural characteristics are consistent with those observed with other PspA proteins (42, 47, 51) and indicate that Rv2744c is a bona fide ortholog of PspA.

Rv2744c is located within an operon (Rv2745c-Rv2744c-Rv2743c-Rv2742c) whose organization and transcriptional arrangement are highly conserved in members of the *M. tuberculosis* complex (24). However, nontuberculous *Mycobacterium* species, including *M. smegmatis*, carry a smaller version of this locus that includes homologs of Rv2745c-Rv2744c-Rv2743c but that lacks Rv2742c (24). While the significance of this difference is unclear, the presence of Rv2742c in human TB-causing species indicates that inclusion of this gene may confer additional capabilities to this system that are associated with virulence and/or disease elicitation. Rv2745c encodes the ClgR transcription factor, which is an ortholog of the Clp protease gene regulator (23, 62). ClgR regulates the expression of its own operon and various other determinants (23), including the ClpC1 and ClpP2 proteases responsible for degradation of the SigE anti-sigma factor, RseA (21). Interestingly, clgR expression is induced by a variety of different stress conditions, including redox stress, cell envelope stress, stress induced following heat shock, and acid stress (62–67). Furthermore, clgR is upregulated under conditions of low oxygen tension and following reaeration from hypoxia-induced dormancy (62). However, in contrast to the well-characterized nature of ClgR, virtually nothing is known about the function of the other two determinants present in this operon, Rv2743c and Rv2742c. Both are conserved alanine-rich proteins, and Rv2743c is predicted to possess a transmembrane region, which suggests that this protein interacts with the plasma membrane.

Recently, it has been proposed that genes contained within the Rv2745c-Rv2744c-Rv2743c-Rv2742c operon may encode a minimal functional unit of the Psp system, helping to regulate plasma membrane homeostasis in *M. tuberculosis* under conditions of cell envelope stress (24). In this scenario, ClgR would function in a role analogous to PspF, Rv2744c to PspA, Rv2743c to PspB, and Rv2742c to PspC. However, our studies characterizing Rv2744c and its ortholog in *M. smegmatis*, MS_2695, suggest that this protein may function in a role that is independent of or in addition to its proposed role in plasma membrane homeostasis. For example, although the expression of both MS_2695 and Rv2744c is upregulated in response to the cell envelope perturbant SDS, neither *M. smegmatis* nor *M. tuberculosis* derivatives deleted in these determinants exhibited increased sensitivity to SDS or other stressors compared to their wild-type counterparts. This contrasts the SDS-sensitive phenotype seen with clgR and Rv2743c mutants of *M. tuberculosis* (24). In addition, although Rv2744c has been shown to immunoprecipitate or pull down ClgR (a cytoplasmic protein) and Rv2743c (a predicted membrane protein) when coproduced in a surrogate *E. coli* host (24), we failed to observe Rv2744c interaction with either ClgR or Rv2743c in *E. coli* in vivo when assayed using a bacterial adenylate two-hybrid assay (data not shown). While the reason(s) underlying this discrepancy is currently unclear, it is possible that the association of Rv2744c with ClgR and Rv2743c occurs indirectly through another protein. Alternatively, it is possible that the interaction between Rv2744c and ClgR or between Rv2744c and Rv2743c is not stable enough to promote an association between the adenylate cyclase subunits used in the BACTH assay.

Regardless of the potential protein-interacting partners of Rv2744c, the loss or overproduction of MS_2695/Rv2744c alters the number and size distribution of LDs produced in *M. smegmatis* and negatively impacts the survival of *M. tuberculosis* following the establishment of NRP. Interestingly, overproduction of Rv2744c exacerbates the phenotypes observed in both *M. smegmatis* and *M. tuberculosis*; overproduction of Rv2744c increases the size of LDs produced over that seen in the wild-type parent and also results in a greater survival defect during NRP than that seen in the uncomplemented ΔRv2744c mutant in *M. tuberculosis*. This
FIG 7 Model of Rv2744c/MS_2695 function in LD homeostasis. Within the granuloma, M. tuberculosis persists within alveolar macrophages, where the bacterium is exposed to external stimuli and stressors that activate components of the cell envelope stress response network, including the two-component system MprAB, extracytoplasmic function sigma factor SigE, and serine-threonine protein kinase PknB. These signaling pathways are integrated at the locus that contains Rv2744c, encoding an ortholog of the phage shock protein, PspA. Following production, Rv2744c (PspA) localizes to the surface of LDs that are also produced by M. tuberculosis under various conditions of stress. We hypothesize that PspA functions to help stabilize the phospholipid monolayer of LDs so that the TAG contained within these quasiganelles can be used to maintain energy production under conditions of nonreplicating persistence (NRP) and/or during the reactivation from NRP. Loss of PspA leads to unstable LDs and a corresponding loss in TAG availability for mobilization under conditions of stress. In contrast, overproduction of PspA on the LD surface leads to occluded LDs in which TAG is unable to be accessed and mobilized for use in energy-generating pathways. PG, peptidoglycan. RseA is the anti-sigma factor for SigE, which is phosphorylated by PknB following activation and dephosphorylation, leading to the release of SigE from the plasma membrane; red circles encompassing LDs represent the phospholipid monolayer; proteins comprising the LD proteome are designated with colored rectangles and circles encompassing LDs represent the phospholipid monolayer; proteins. In conclusion, our results suggest that Rv2744c may not be involved in plasma membrane homeostasis but rather may carry out a unique function among PspA family members in Mycobacterium that includes regulation of the homeostasis of LDs.

The production of LDs is a common characteristic of most eukaryotes (reviewed in reference 68). Of note, “foamy” macrophages, a predominant cell type comprising the M. tuberculosis granuloma, contain a high abundance of LDs containing TAGs, and M. tuberculosis is often seen within or in close proximity to these cells (69–71). TAG-containing LDs are also produced by a small number of prokaryotes, primarily those from the Actinobacteria family, to which Mycobacterium belongs (reviewed in reference 41). In vitro, Mycobacterium spp. induce LD production when grown under a variety of stressful conditions, including those thought to be present within the granuloma (i.e., low oxygen tension, low nutrient availability, nitric oxide exposure, etc.) (41, 58, 72). Furthermore, pathogenic Mycobacterium species usurp and hydrolyze TAG from host LDs within foamy macrophages, incorporating the resulting fatty acid products into its own LDs for use as an energy source during NRP (58, 69, 71). Consistent with this observation, Mycobacterium bovis BCG strains that are inhibited in their ability to mediate TAG hydrolysis are attenuated for survival in vitro during and following resuscitation from NRP (60, 61). Similarly, an M. tuberculosis mutant deleted in triacylglycerol-1 synthase (Δgls1), an enzyme used to make TAGs, is compromised in its ability to accumulate intracellular TAGs and is unable to enter into NRP in an in vitro human TB granuloma model (73). Finally, an M. tuberculosis mutant deleted in lipase lipY, which is required for TAG hydrolysis, is compromised in its ability to mobilize stored TAGs and is unable to reactivate from NRP in the granuloma model system (73). Thus, LDs represent an important energy reservoir utilized by M. tuberculosis for survival during and resuscitation from NRP.

Based on the data presented here, we propose the following working model whereby Rv2744c/MS_2695 represents a unique ortholog of PspA that associates with and regulates LD homeostasis in Mycobacterium species under conditions of stress (Fig. 7). Within the granuloma, M. tuberculosis is exposed to a variety of stressors that are capable of (i) activating regulatory components of the cell envelope stress response network, including MprAB, SigE, and PknB, and (ii) leading to the production of LDs containing TAG for use as an energy source during NRP and/or reactivation from NRP. Activation of the MprAB, SigE, and PknB signaling networks following exposure to cell envelope stressors leads to upregulation of the locus that encodes Rv2744c/MS_2695, herein called pspA. Once produced, PspA localizes to LDs, where the protein homo-oligomerizes on the LD surface to help stabilize the phenotypes due to the production of Rv2744c at levels much higher than those normally seen in the wild-type parent, as introduction of the TetR repressor into the Rv2744c overexpression strain reduces Rv2744c production to wild-type levels and correspondingly restores the LD distribution pattern to one resembling that of the wild-type parent. While the exact role of Rv2744c remains unknown, we speculate that Rv2744c may function to stabilize the phospholipid monolayer surrounding the TAG core. As such, dysregulation of Rv2744c levels would be expected to not only impact the size and number of LDs produced but also regulate accessibility to TAG contained within LDs needed for use as an energy source during NRP. Previously, we reported localization of Rv2744c to the plasma membrane/cell wall compartment (31). However, the cell lysate utilized for those studies was generated by bead-beating, which would mechanically disrupt the integrity of LDs. In the current study, bacteria were lysed using a French press and the resulting cell lysates fractionated in the presence of a low concentration of sucrose, which allows for efficient separation of intact LDs from cytoplasmic and membrane proteins. In conclusion, our results suggest that Rv2744c may not be involved in plasma membrane homeostasis but rather may carry out a unique function among PspA family members in Mycobacterium that includes regulation of the homeostasis of LDs.
phospholipid monolayer and regulate the mobilization of TAG for use as an energy source during periods of NRP.

In conclusion, Rv2744c encodes a unique ortholog of PspA in *M. tuberculosis*. While Rv2744c possesses structural and functional characteristics consistent with its designation as a PspA family member, the function of this protein is unique and differs from the archetypical role this protein plays in mediating resistance to cell envelope stressors. As Rv2744c dysregulation leads to defects in *M. tuberculosis* survival during NRP, this protein may represent a novel determinant that can be therapeutically targeted to abrogate the survival of the tubercle bacillus within the host during latent infections.

ACKNOWLEDGMENTS

We are grateful to Sabine Ehrt for providing pSE100 and pMCI1s and to Eric Rubin for providing plasmids expressing GFP variants. We also acknowledge Clive Wells and the MCW Facility for Electron Microscopy for imaging of *Mycobacterium* strains and purified Rv2744c.

This work was supported by a grant from the Potts Memorial Foundation to T.C.Z.

FUNDING INFORMATION

This work, including the efforts of Thomas C. Zahrt, was funded by Potts Memorial Foundation.

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June 2016 Volume 198 Number 11 Journal of Bacteriology jb.asm.org 1659

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