Washington University School of Medicine
Digital Commons@Becker

Open Access Publications

2009

The Bacillus anthracis protein MprF is required for synthesis of lysylphosphatidylglycerols and for resistance to cationic antimicrobial peptides

Shalaka Samant
University of Illinois at Chicago

Fong-Fu Hsu
Washington University School of Medicine in St. Louis

Alexander A. Neyfakh
University of Illinois at Chicago

Hyunwoo Lee
University of Illinois at Chicago

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs

Recommended Citation

Samant, Shalaka; Hsu, Fong-Fu; Neyfakh, Alexander A.; and Lee, Hyunwoo, ,"The Bacillus anthracis protein MprF is required for synthesis of lysylphosphatidylglycerols and for resistance to cationic antimicrobial peptides." Journal of Bacteriology. 191,4. 1311-1319. (2009).
https://digitalcommons.wustl.edu/open_access_pubs/2454

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.

Journal of Bacteriology

The *Bacillus anthracis* Protein MprF Is Required for Synthesis of Lysylphosphatidylglycerols and for Resistance to Cationic Antimicrobial Peptides

Shalaka Samant, Fong-Fu Hsu, Alexander A. Neyfakh and Hyunwoo Lee

J. Bacteriol. **2009, 191(4):1311. DOI: 10.1128/JB.01345-08.**
Published Ahead of Print 12 December 2008.

Updated information and services can be found at:
http://jb.asm.org/content/191/4/1311

These include:

SUPPLEMENTAL MATERIAL Supplemental material

REFERENCES This article cites 40 articles, 20 of which can be accessed free at: http://jb.asm.org/content/191/4/1311#ref-list-1

CONTENT ALERTS Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: **http://journals.asm.org/site/misc/reprints.xhtml**
To subscribe to to another ASM Journal go to: **http://journals.asm.org/site/subscriptions/**

Journals.ASM.org

Downloaded from http://jb.asm.org/ on March 20, 2014 by Washington University in St. Louis

Journal of Bacteriology, Feb. 2009, p. 1311–1319 Vol. 191, No. 4
0021-9193/09/$08.00+0 doi:10.1128/JB.01345-08
Copyright © 2009, American Society for Microbiology. All Rights Reserved.

The *Bacillus anthracis* Protein MprF Is Required for Synthesis of Lysylphosphatidylglycerols and for Resistance to Cationic Antimicrobial Peptides▽†

Shalaka Samant,[1]‡ Fong-Fu Hsu,[2] Alexander A. Neyfakh,[1]§ and Hyunwoo Lee[1]*

Center for Pharmaceutical Biotechnology, University of Illinois at Chicago, M/C 870, 900 S. Ashland Ave., Chicago, Illinois 60607,[1] and Mass Spectrometry Resource, Department of Internal Medicine, Division of Endocrinology, Diabetes, and Metabolism, Washington University School of Medicine, St. Louis, Missouri 63110[2]

Received 26 September 2008/Accepted 25 November 2008

During inhalational anthrax, *Bacillus anthracis* survives and replicates in alveolar macrophages, followed by rapid invasion into the host's bloodstream, where it multiplies to cause heavy bacteremia. *B. anthracis* must therefore defend itself from host immune functions encountered during both the intracellular and the extracellular stages of anthrax infection. In both of these niches, cationic antimicrobial peptides are an essential component of the host's innate immune response that targets *B. anthracis*. However, the genetic determinants of *B. anthracis* contributing to resistance to these peptides are largely unknown. Here we generated Tn*917* transposon mutants in the ΔANR strain (pXO1⁻ pXO2⁻) of *B. anthracis* and screened them for altered protamine susceptibility. A protamine-sensitive mutant identified carried the transposon inserted in the BA1486 gene encoding a putative membrane protein homologous to MprF known in several gram-positive pathogens. A mutant strain with the BAS1375 gene (the orthologue of BA1486) deleted in the Sterne 34F2 strain (pXO1⁺ pXO2⁻) of *B. anthracis* exhibited hypersusceptibility not only to protamine but also to α-helical cathelicidin LL-37 and β-sheet defensin human neutrophil peptide 1 compared to the wild-type Sterne strain. Analysis of membrane lipids using isotopic labeling demonstrated that the BAS1375 deletion mutant is unable to synthesize lysinylated phosphatidylglycerols, and this defect is rescued by genetic complementation. Further, we determined the structures of these lysylphosphatidylglycerols by using various mass spectrometric analyses. These results demonstrate that in *B. anthracis* a functional MprF is required for the biosynthesis of lysylphosphatidylglycerols, which is critical for resistance to cationic antimicrobial peptides.

Bacillus anthracis is an endospore-forming gram-positive pathogen that causes the infectious disease anthrax in mammals, including humans. Infections can occur via intradermal inoculation, ingestion, or inhalation of spores (24). Although anthrax infections via the former two routes are usually self-contained, inhalational anthrax is often lethal (23). In a mouse model of inhalational anthrax, inhaled *B. anthracis* spores are phagocytosed by alveolar macrophages that are believed to migrate to local lymph nodes (10). During migration, the spores germinate inside the macrophage phagolysosome to give rise to vegetative bacilli. The newly formed vegetative cells lyse the phagolysosome and replicate inside the macrophage cytoplasm (6), eventually escaping from the macrophage into the bloodstream. Therefore, in order to establish a successful anthrax infection, *B. anthracis* must survive and replicate intracellularly inside the macrophage, as well as extracellularly in the host's blood.

Upon entering the bloodstream, *B. anthracis* is targeted by an array of innate immune mediators circulating in the host's blood, such as the complement proteins and cellular components such as neutrophils and platelets in humans. However, inhalational anthrax infection in animals is characterized by rapid progression into systemic bacteremia and the heavy growth of *B. anthracis* in the bloodstream (21). This observation indicates that *B. anthracis* is able not only to evade complement-mediated lysis and but also to resist the antibacterial activities of innate immune cells.

One important antibacterial activity of innate immune cells in the human blood relies on the production of cationic antimicrobial peptides. These peptides are present in the cytosolic granules of neutrophils, eosinophils, and platelets and are released upon contact with bacterial pathogens (18). Cationic antimicrobial peptides interact electrostatically with negatively charged cell surface molecules, such as teichoic acids and phosphatidylglycerols of gram-positive bacteria, subsequently inducing disintegration of membrane structures and ultimately causing bacterial cell death (41). Some gram-positive pathogens, however, possess resistance mechanisms, by which they change cell surface properties and avoid killing by cationic antimicrobial peptides. For example, gram-positive pathogens, such as *Staphylococcus aureus* (29, 30), *Listeria monocytogenes* (1, 37), and *Streptococcus pneumoniae* (16), are able to be modify teichoic acids and phospholipids with D-alanine by DltABCD and L-lysine by MprF, respectively. Since these modifications contribute to a net positive

* Corresponding author. Mailing address: Center for Pharmaceutical Biotechnology, University of Illinois at Chicago (m/c 870), 900 S. Ashland Ave., MBRB #3018, Chicago, IL 60607. Phone: (312) 996-3371. Fax: (312) 413-9303. E-mail: hlee31@uic.edu.

† Supplemental material for this article may be found at http://jb.asm.org/.

‡ Present address: Section of Microbial Pathogenesis, Boyer Center for Molecular Medicine, Yale University School of Medicine, 295 Congress Ave., New Haven, CT 06511.

§ Deceased 20 April 2006.

▽ Published ahead of print on 12 December 2008.

Downloaded from http://jb.asm.org/ on March 20, 2014 by Washington University in St. Louis

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Strains		
B. anthracis		
ΔANR	A host strain for Tn*917* transposon mutagenesis, pXO1$^-$ pXO2$^-$	A. M. Friedlander (2)
Sterne 34F2	Wild type, pXO1$^+$ pXO2$^-$	P. C. Hanna (36)
SH0001	A Sterne strain containing pCN55 plasmid, Spec^r	This study
SH0002	Δ*mprF*::Km^r, a Sterne 34F2 strain carrying deletion of bp −8 to 2512 of *mprF* (2,586 bp)	This study
SH0003	SH0002 containing p*mprF* plasmid, Km^r Spec^r	This study
SH0004	*mprF*::Tn*917*-Erm^r, a ΔANR strain carrying Tn*917* inserted in the BA1485 gene	This study
E. coli		
GM2163	F$^-$ *ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 rpsL136 dam13*::Tn*9 xylA5 mtl-1 thi-l mcrB1 hsdR2*; Cm^r Str^r	NEB (28)
Plasmids		
pCN55	A shuttle vector, Amp^r for *E. coli*, Spec^r for *B. anthracis*	E. Charpentier (5)
p*mprF*	pCN55 harboring the *mprF* open reading frame with its own promoter	This study

[a] Cm^r, chloramphenicol resistance; Spec^r, spectinomycin resistance; Erm^r, erythromycin resistance; Amp^r, ampicillin resistance; Str^r, streptomycin resistance; Km^r, kanamycin resistance.

charge on the cell surface, they are believed to facilitate repulsion of the cationic peptides.

Identifying the *B. anthracis* genes that contribute to cationic peptide resistance can elucidate the molecular basis of this virulence trait. A recent study has shown that the *B. anthracis* genome contains a functional *dltABCD* operon (7). A *B. anthracis* mutant strain inactivated in this operon exhibits hypersusceptibility to various cationic antimicrobial peptides, decreased survival in macrophages, and virulence attenuation in a mouse model of inhalational infection. To date, the *dltABCD* operon is the only genetic determinant of *B. anthracis* experimentally proven to contribute to cationic antimicrobial peptide resistance.

In the present study, we have identified a *B. anthracis* gene (BA1486 in the ΔANR [pXO1$^-$, pXO2$^-$] strain; BAS1375 in the Sterne 34F2 [pXO1$^+$, pXO2$^-$] strain) whose knockout leads to hypersusceptibility to protamine, as well as to human cationic antimicrobial peptides, α-helical LL-37, and β-sheet human neutrophil peptide 1 (HNP-1). We show that inactivation of this gene results in a strain that is unable to synthesize phosphatidylglycerols modified with lysine. Our results demonstrate that the *B. anthracis* genome carries a functional *mprF* gene required for cationic antimicrobial peptide resistance.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. The bacterial strains and plasmids used in the present study are listed in Table 1. *B. anthracis* ΔANR strain (pXO1$^-$, pXO2$^-$) was the host strain used for generation of Tn*917* insertional mutants. *B. anthracis* Sterne 34F2 strain (pXO1$^+$, pXO2$^-$) was the parental strain used for the construction of a clean knockout mutant strain. Plasmid pTV1-OK (11) was used for Tn*917* transposon mutagenesis in *B. anthracis* ΔANR, and pCN55 (5) was the backbone plasmid for the construction of p*mprF* used for complementation. OneShot TOP10 chemically competent *Escherichia coli* cells (Invitrogen) were used as the host for all of the cloning procedures. *E. coli* strain GM2163 (New England Biolabs) was used to obtain unmethylated plasmid DNA for transformation of *B. anthracis*. All strains were cultivated in Luria-Bertani (LB) medium (32) at 37°C. When necessary, appropriate antibiotics were added to the culture as follows: 100 μg of kanamycin/ml and 5 μg of erythromycin/ml for *B. anthracis* and 40 μg of kanamycin/ml and 200 μg of erythromycin/ml for *E. coli*.

Construction of Tn*917* transposon mutants in *B. anthracis*. The temperature-sensitive plasmid pTV1-OK was used to deliver the Tn*917* transposon to the ΔANR strain of *B. anthracis* (11, 15). pTV1-OK harbors a selectable antibiotic resistance gene, *aphA3*, which expresses kanamycin resistance in both *E. coli* and *B. anthracis* (38). It also encodes a Tn*917* transposon element, consisting of an erythromycin resistance marker and the Tn*917* transposase flanked by inverted repeats.

Electrocompetent *B. anthracis* cells prepared as described previously (34) were transformed with pTV1-OK isolated from *E. coli* GM2163, and transformants were selected on LB agar containing both kanamycin (100 μg/ml) and erythromycin (5 μg/ml) at 30°C. A single transformant was grown in LB medium overnight at 30°C, and the next day the overnight culture was diluted 1:200 into fresh LB medium supplemented with 0.04 μg of erythromycin/ml. A sublethal concentration of erythromycin is known to induce the expression of erythromycin resistance and transposase genes of Tn*917* (11). After growth at 42°C for 5 h, the culture was diluted and plated on LB agar containing 5 μg of erythromycin/ml and incubated at 42°C. After an overnight incubation, 40,000 to 50,000 colonies were generally obtained. To verify the loss of the transposon delivery plasmid, randomly chosen colonies were streaked onto LB agar plates containing either 100 μg of kanamycin/ml or 5 μg of erythromycin/ml. A total of 5,000 transposon mutants (erythromycin resistant and kanamycin sensitive) were picked and tested for protamine sensitivity.

Screening for protamine-sensitive *B. anthracis* Tn*917* transposon mutants. To identify protamine-sensitive mutants, *B. anthracis* Tn*917* transposon mutants were screened as follows. Individual mutant strains, along with several (ΔANR) wild-type controls, were grown in LB medium in 96-well plate formats. Using a 96-pin replicator, each culture was patched onto freshly made LB agar supplemented with protamine (600 and 800 μg/ml, respectively), followed by incubation overnight at 37°C. The MIC of protamine in LB agar for wild-type *B. anthracis* ΔANR and Sterne 34F2 strains is 850 to 900 μg/ml. Mutants that failed to grow on protamine-containing plates were further confirmed for protamine sensitivity. Genomic DNA isolated from protamine-sensitive mutants was used to determine the location of the Tn*917* insertion sites as described below.

Determination of Tn*917* transposon insertion sites. To determine transposon insertion sites in *B. anthracis* Tn*917* transposon mutants (erythromycin resistant and kanamycin sensitive), an arbitrary PCR method was used (3, 26). Genomic DNA was isolated from each *B. anthracis* Tn*917* insertion mutant by using a GenElute bacterial genomic DNA kit (Sigma-Aldrich) and used as a template. A transposon-specific primer (Tsp1, 5′-CCCATAGATAAGAAATACACCTG-3′) and an arbitrary primer (AP1, 5′-CCAGGCCTGCAGATGATGNNNNNNNNNNGTAT-3′) were used in the first round of PCR at the following conditions: 95°C for 2 min; followed by 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 5 min; and followed finally by 72°C for 10 min. The first PCR product (5 μl), purified by using the Wizard SV gel and PCR clean-up system (Promega), was then used as a template in a second nested PCR with a pair of nested primers

Downloaded from http://jb.asm.org/ on March 20, 2014 by Washington University in St. Louis

Tsp2 (transposon specific, 5′-AACCGTTACCTGTTTGTGCCA-3′) and AP2 (arbitrary, 5′-CCAGGCCTGCAGATGATG-3′). The conditions for the second PCR were as follows: 95°C for 2 min; followed by 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 2 min; and followed in turn by 72°C for 5 min. Products from the second PCR, purified using the Wizard SV gel and PCR clean-up system, were used as a template for sequencing with the Tsp2 primer to determine the site of Tn*917* insertion.

Construction of an *mprF* deletion mutant in *B. anthracis* Sterne 34F2 strain and p*mprF* for genetic complementation. A mutant carrying a kanamycin resistance marker (*aphA3*) replacing *mprF* was constructed in the Sterne 34F2 strain of *B. anthracis* using the method developed by Shatalin and Neyfakh (34). *B. anthracis* Sterne 34F2 genomic DNA isolated using the GenElute bacterial genomic DNA kit was used as a template to PCR amplify the upstream and downstream regions, respectively, of *mprF*. The upstream 531-bp DNA fragment was amplified using the primer pair mprFUpNotI (5′-TTATGCGGCCGCGCCAATATTTCTTGTACTTTCAAC-3′) and mprFUpPstI (5′-ATAACTGCAGCTCTCCTTGTATAAGAAAACTGA-3′). The downstream 573-bp DNA fragment was amplified using the primer pair mprFDnHindIII (5′-TTTAAAGCTTAGAAAGAATCATTCTTTACCAATTA-3′) and mprFDn XhoI (5′-TTTGCTCGAGATGGATCATATAAAGTATCTTCTAA-3′). (The restriction enzyme sites are underlined in the primer sequences.) After restriction digestion, the PCR products were cloned, flanking the kanamycin antibiotic cassette in a temperature-sensitive plasmid pKS1 (34). The resultant plasmid was transformed into GM2163 *E. coli* to obtain unmethylated plasmid DNA and used for the transformation of *B. anthracis*, selecting for resistance to kanamycin (100 μg/ml) and erythromycin (5 μg/ml). A culture of a single transformant grown overnight at 30°C in LB medium was diluted in fresh LB medium containing kanamycin (100 μg/ml) and grown overnight at 42°C for the first round of recombination, followed by dilution in fresh LB medium and growth overnight at 30°C for the second round of recombination. A serially diluted culture was then plated onto LB agar containing kanamycin (100 μg/ml). Finally, erythromycin-sensitive and kanamycin-resistant colonies were identified. In the Δ*mprF*::Km^r mutants isolated, the loss of pKS1 and the replacement of the *mprF* gene with the kanamycin resistance cassette was verified by both antibiotic susceptibility and PCR analyses. The deleted region of *mprF* (2,520 bp) extended from bp −8 to bp 2512.

To construct a complementing plasmid for the *B. anthracis mprF* mutant strain, a shuttle vector pCN55 was used as a backbone plasmid (5). The *B. anthracis mprF* gene, along with an ~300-bp upstream region, was PCR amplified using the Sterne 34F2 genomic DNA as a template and the primer pair mprFComFwdBamHI (5′-ACTCGGATCCCGCATAACCTATTATAAT-3′) and mprFComRevEcoRI (5′-GAAAAAGAATTCATTGTACAAGTT-3′). The PCR product restriction digested with BamHI and EcoRI was cloned into pCN55 digested with the same restriction enzymes to obtain the complementing plasmid p*mprF*. Unmethylated p*mprF* isolated from *E. coli* GM2163 was used to transform the *B. anthracis mprF* strain.

Susceptibility test for cationic antimicrobial peptides. For determination of susceptibility of *B. anthracis* strains to protamine, 2 μl of an exponential culture of either wild-type Sterne, wild type/pCN55, Δ*mprF*::Km^r, *mprF*::Tn*917*-Erm^r, or Δ*mprF*::Km^r/p*mprF* strain was spotted onto LB agar supplemented with 200, 400, 600, or 800 μg of protamine/ml and incubated for 48 h at 37°C.

For antimicrobial peptide killing assay, LL-37 (82% purity) was custom-synthesized from Sigma, and HNP-1 was purchased from Bachem (Torrance, CA). Peptides were dissolved in 0.01% acetic acid supplemented with 0.2% bovine serum albumin at appropriate concentrations. Overnight culture of *B. anthracis* strains grown in LB medium was diluted 1:50 in fresh LB and grown to optical density of ~1.0 (~5×10^7 cells/ml) at 600 nm. The cells were harvested, washed twice with 0.5% tryptone, and then resuspended in 0.5% tryptone. Subsequently, 45 μl of the cell suspension was mixed with 5 μl of a respective peptide (10× concentration) in 96-well polypropylene plates. After incubation for 45 min at 37°C with shaking, the cultures were serially diluted and plated on LB agar to determine the number of viable cells. The percent survival was calculated as follows: (the number of surviving cells/the number of cells in the initial inoculum) × 100.

Analysis of radiolabeled polar membrane lipids by two-dimensional thin-layer chromatography (2D-TLC). Overnight cultures of *B. anthracis* strains (wild-type Sterne 34F2, Δ*mprF*::Km^r, or Δ*mprF*::Km^r/p*mprF*) grown in LB medium were diluted 1:200 in 2 ml of fresh LB medium. After the addition of 3 μCi of [^{14}C]lysine (50 μCi/ml; GE Healthcare Life Sciences) or 2 μCi of [^{32}P]orthophosphate (10 mCi/ml; Perkin-Elmer) to each culture, the cultures were incubated for 6 h with aeration at 37°C. The cells were harvested, washed once with phosphate-buffered saline (PBS), and used for isolation of total polar membrane lipids according to the method of Bligh and Dyer with a slight modification (4, 27, 33). The cell pellet was suspended in 2 ml of 0.12 M sodium acetate (pH 4.8), to which chloroform (2.5 ml) and methanol (5.5 ml) were added. After incubation for 2 h at room temperature, the solids were removed by centrifugation. The supernatant was mixed with chloroform (2.5 ml) and water (2.5 ml), and the lower phase was transferred to a new glass tube and dried under a stream of air. Dried total lipid material was dissolved in 50 to 100 μl of chloroform-methanol (2:1 [vol/vol]) and spotted onto silica gel 60 F_{254} HPTLC plates (M5628-5; Merck). The silica plates were run in the first dimension with chloroform-methanol-water (65:25:4 [vol/vol/vol]) and in the second dimension with chloroform-methanol-acetic acid-water (80:12:16:4 [vol/vol/vol/vol]) (37). [^{14}C]lysine- or ^{32}P-labeled lipids were visualized by using a phosphorimager (GE Healthcare Life Sciences).

Analysis of lysylphospholipids using mass spectrometry. To prepare lipid samples, wild-type *B. anthracis* Sterne 34F2 was grown to an optical density at 600 nm of ~1 in 1 liter of LB medium. The cells were harvested, washed once with PBS, and resuspended in 200 ml of 0.12 M sodium acetate (pH 4.8). The total lipid material was isolated as described above and spotted onto preparatory silica gel 60 F_{254} plates (M13895-7; Merck). After running the silica plates in two directions as described above, spots on the silica plates were visualized by staining with iodine. Individual spots were scraped off from the silica plates and suspended in chloroform and methanol (2:1 [vol/vol]). After overnight extraction, samples were filtered with Whatman filter paper, and the filtrates were concentrated for mass spectrometric analysis.

Low-energy collisionally activated dissociation (CAD) tandem mass spectrometry experiments were conducted on a Finnigan (San Jose, CA) TSQ 7000 mass spectrometer equipped with the ICIS data system or on an LTQ linear ion-trap mass spectrometer with the Xcalibur operation system. Lipid samples were continuously infused into the electrospray ionization (ESI) source with a syringe pump at a flow rate of 2 μl/min. The skimmer was at ground potential, and the electrospray needle was set at 4.5 kV. The temperature of the heated capillary was 260°C. For CAD tandem mass spectra obtained with a linear ion-trap mass spectrometer, the automatic gain control of the ion trap was set to 7×10^4, with a maximum injection time of 100 ms. Helium was used as the buffer and collision gas at a pressure of 10^{-3} mbar. The mass resolution was 0.6 Da at half peak height. The MS^n spectra ($n \geq 2$) were obtained with a relative collision energy varied from 16 to 20% and an activation time varies from 30 to 50 ms with an activation Q value at 0.25. For neutral loss spectra obtained with a triple-stage quadrupole instrument, both first quadrupole (Q1) and third quadrupole (Q3) were scanned simultaneously with a set mass difference at 300.2, which represents a phospho-1′-lysyl-glycerol residue cleaved from the $[M+H]^+$ ions of lysylphosphatidylglycerol upon CAD with Ar (2.3 mtorr) under a collision energy of 35 eV in the rf-only second quadrupole (Q2). Both Q1 and Q3 were tuned to unit mass resolution and scanned at a rate of 3 s/scan. All of the mass spectra were accumulated in the profile mode.

RESULTS

Characterization of a Tn*917* library of *B. anthracis*. To identify *B. anthracis* genetic determinants of resistance to cationic antimicrobial peptides, we generated a library of transposon insertion mutants using Tn*917* transposon mutagenesis. Transposon Tn*917* is usually delivered on a temperature-sensitive plasmid for easy curing after the event of transposon insertion. We used one such plasmid, pTV1-OK (11). During transposition, the Tn*917* transposon is known to preferentially insert into plasmids (pXO1 and pXO2) rather than the chromosome of *B. anthracis* (12, 40). To circumvent this problem, we used *B. anthracis* strain ΔANR as a host that lacks both plasmids but for which the protamine MIC is the same as for *B. anthracis* Sterne 34F2 carrying pXO1 plasmid.

B. anthracis ΔANR strain harboring pTV1-OK was grown at 42°C, the nonpermissive temperature for replication of pTV1-OK, in the presence of erythromycin at a low concentration (0.04 μg/ml), and plated onto LB agar selecting for transposon insertional mutants resistant to erythromycin at 5 μg/ml. To check the efficiency of plasmid curing, we tested ~600 randomly chosen clones for kanamycin sensitivity. About 92% of the clones tested were kanamycin sensitive. This curing effi-

Downloaded from http://jb.asm.org/ on March 20, 2014 by Washington University in St. Louis

ciency is about the same as that reported for pTV1-OK-mediated Tn*917* mutagenesis in another gram-positive bacterium, *Streptococcus mutans* (11), and indicates that most of the cells properly lost the delivery plasmid.

To assess the distribution of Tn*917* insertions on the chromosome, we determined the transposon insertion sites in 50 randomly picked transposon mutants by arbitrary PCR and sequencing as described in Materials and Methods. Sequencing results revealed that each mutant carried a single insertion of Tn*917* transposon, but many of the 50 transposon mutants were redundant, representing 16 independent Tn*917* insertions. Surprisingly, most of the transposon insertions were strongly biased to regions in the first half of the chromosome (see Fig. S1 in the supplemental material). This transposition tendency was very different from that observed in other gram-positive bacteria such as *Enterococcus faecalis* (8) and *S. aureus* (3), in which Tn*917* preferentially inserts into chromosomal regions near the replication terminus. In *E. faecalis*, a 29-bp consensus sequence centered on TATAA was known as a preferred Tn*917* insertion site (8). We did not detect such a consensus sequence, although 10 of 16 independent Tn*917* inserted regions contain a 5-bp sequence similar to TATAA within 10 bp of the transposon insertion sites (see Table S1 in the supplemental material). Collectively, these results suggest that Tn*917* transposition, even in the strain of *B. anthracis* lacking both plasmids (pXO1 and pXO2), is biased and occurs in a manner distinct from that known in other gram-positive bacteria.

Of 50 transposon mutants sequenced, the most common ones (18/50 [36%]) carried the transposon inserted in a gene (BA0743) encoding a major facilitator family transporter protein. Other frequently obtained mutants carried a transposon insertion in genes encoding a hypothetical protein (BA2743; 7/50 [14%]), another major facilitator family transporter (BA1858; 4/50 [8%]), the YfhP protein (BA0521; 4/50 [8%]), SpoIIE (BA0061; 3/50, [6%]), a putative DNA helicase (BA0934; 2/50 [4%]) or a putative virulence factor (BA1486; 2/50 [4%]) and in the intergenic region (2/50 [4%]) between genes encoding an arginyl-tRNA synthetase (BA2175) and a hypothetical protein (BA2176). Mutants retrieved only once harbored the transposon in the following genes (BA0410 encoding a putative heavy-metal transporting ATPase; BA1664, a hypothetical protein; BA1779, FlgG; BA3902, a putative bacteriocin ABC transporter; and BA4670, a sensor histidine kinase) and the intergenic regions (BA2075/BA2076, BA2805/BA2807, and BA5331/BA5332). Based on mapping for Tn*917* insertion sites, we speculated that ~32% (16/50) of the Tn*917* library generated for the present study contains unique transposon insertions.

Identification of protamine-sensitive *B. anthracis* transposon mutants. To identify the *B. anthracis* genes involved in cationic antimicrobial peptide resistance, we screened 5,000 Tn*917* transposon mutants for altered protamine sensitivity. Individual 5,000 transposon mutants were grown in 96-well plates and replica plated onto LB agar containing two different concentrations of protamine (600 and 800 μg/ml). Each 96-well plate contained several wells of the wild-type ΔANR strain as a control, which can grow at protamine concentrations up to 800 to 850 μg/ml. A total of 65 mutants were identified as hypersusceptible to protamine compared to the wild type. We

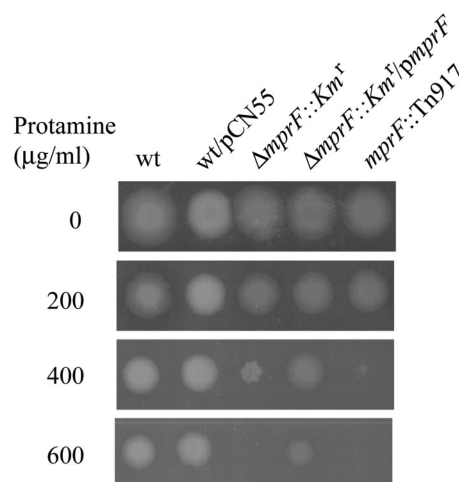


FIG. 1. The *B. anthracis mprF* mutant is hypersusceptible to a linear cationic peptide protamine. Wild-type Sterne 34F2, wild-type/pCN55 (empty vector) (SH0001), Δ*mprF*::Km^r mutant (SH0002), Δ*mprF*::Km^r/p*mprF* (SH0003), and *mprF*::Tn*917*erm^r (SH0004) strains (~5 × 10^4 cells) were spotted onto LB agar plates supplemented with protamine at the indicated concentrations. The images scanned were obtained after 48 h of incubation at 37°C.

also identified mutants that could grow at protamine concentrations of ≥1 mg/ml. These protamine-resistant mutants carried the transposon inserted in genes BA0410 and *yfhP*, respectively, but they were not further characterized in the present study. Determination of transposon insertion sites in protamine-hypersusceptible mutants, as described in Materials and Methods, revealed that all 65 mutants carry the transposon inserted at the same nucleotide position in the BA1486 gene.

The BA1486 gene coding for a putative cytoplasmic membrane protein of 861 amino acid residues exhibited high homology to known MprF proteins of gram-positive bacteria such as *S. aureus* (29) and *L. monocytogenes* EGD-e (37): 34% identity and 54% similarity to either MprF throughout the whole amino acid sequence. MprF is known to be required for resistance to cationic antimicrobial peptides. Similar to the known MprF proteins, the putative *B. anthracis* MprF is predicted to have 13 transmembrane domains at the N terminus (amino acid residues 8 to 527) by TMpred analysis (www.ch.embnet.org/software/TMPRED_form.html). This analysis and the protamine-sensitive phenotype of the identified transposon mutant suggested that the BA1486 gene in *B. anthracis* is a functional orthologue of *mprF* known in other gram-positive bacteria. BA1486 in the ΔANR strain and its orthologue, BAS1375, in the Sterne 34F2 strain of *B. anthracis* are referred to as *mprF* hereafter.

The *B. anthracis mprF* gene is required for resistance to structurally different cationic antimicrobial peptides. The transposon mutant identified as protamine sensitive harbored Tn*917* inserted at the 3′ end of the BA1486 gene (2,583 bp) between nucleotides 2551 and 2552. The transposon insertion at the very end of the gene raised the possibility that gene function was not completely inactivated. We also noted that a downstream region of the BA1486 gene was possibly deleted during transposition, which potentially removes ~20 bp from the 3′ end of the neighboring BA1485 gene.

Downloaded from http://jb.asm.org/ on March 20, 2014 by Washington University in St. Louis

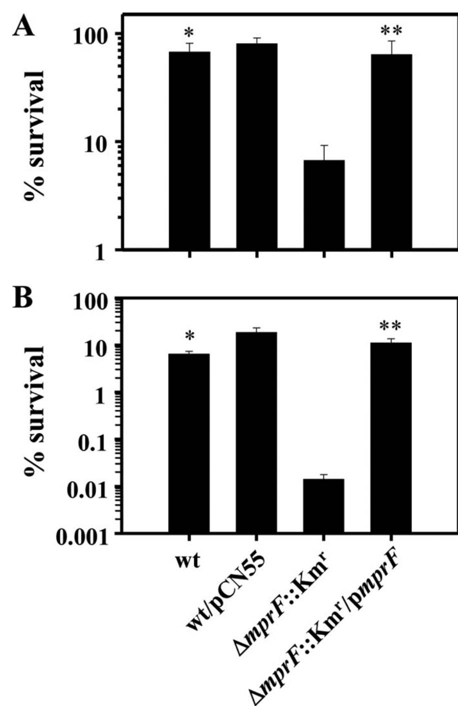


FIG. 2. The *B. anthracis mprF* mutant is hypersusceptible to α-helical LL-37 and β-sheet HNP-1. Antimicrobial peptide killing assay was performed as described in Materials and Methods. Strains (~2×10^6 cells) were incubated with HNP-1 at 2 μg/ml (A) and LL-37 (B) at 25 μg/ml. The percent survival was calculated as follows: (the number of surviving cells/the number of cells in the initial inoculum) × 100. Error bars indicate the standard deviation of three independent experiments. Asterisks indicate statistically significant differences ($P < 0.01$) in the paired Student *t* test (*, wild type versus Δ*mprF*::Km^r; **, Δ*mprF*::Km^r versus Δ*mprF*::Km^r/p*mprF*).

To clearly establish that the protamine hypersusceptibility of the identified transposon mutant was specifically due to inactivation of the putative *mprF* gene, we constructed a clean knockout mutant in the Sterne 34F2 strain of *B. anthracis*. In the newly constructed deletion mutant, most (bp −8 to 2152) of the *mprF* gene is removed by replacing the region with a kanamycin resistance cassette (34). Similar to phenotypes observed with the *mprF* transposon mutant, the *mprF* deletion mutant grew slightly more slowly than the wild-type strain in LB or BHI medium and exhibited protamine hypersusceptibility: it was able to grow on LB agar containing up to 400 μg of protamine/ml but failed to grow on LB agar containing 600 μg of protamine/ml compared to the wild type (Fig. 1). Moreover, genetic complementation of the *mprF* deletion mutant with a plasmid carrying a wild-type copy of the *mprF* gene restored wild-type resistance to protamine (Fig. 1). These results confirm that inactivation of the putative *mprF* gene is responsible for protamine hypersusceptibility in *B. anthracis*.

Bacterial strains hypersusceptible to protamine do not always display increased sensitivity to other cationic antimicrobial peptides with different structures (9). Thus, we further determined the susceptibility of the *mprF* deletion mutant to structurally different cationic antimicrobial peptides such as α-helical cathelicidin LL-37 and β-sheet defensin HNP-1. The wild-type and mutant strains were incubated for 45 min with respective peptides at the indicated concentrations, and cell survival was determined by viable cell counts. Approximately 70% survival of the wild-type Sterne strain was seen after treatment with HNP-1 (2 μg/ml) (Fig. 2A), whereas ~6% survival was observed in the presence of LL37 (25 μg/ml) (Fig. 2B). Compared to the wild type, the *mprF* mutant strain exhibited increased susceptibility to respective peptides: ~10-

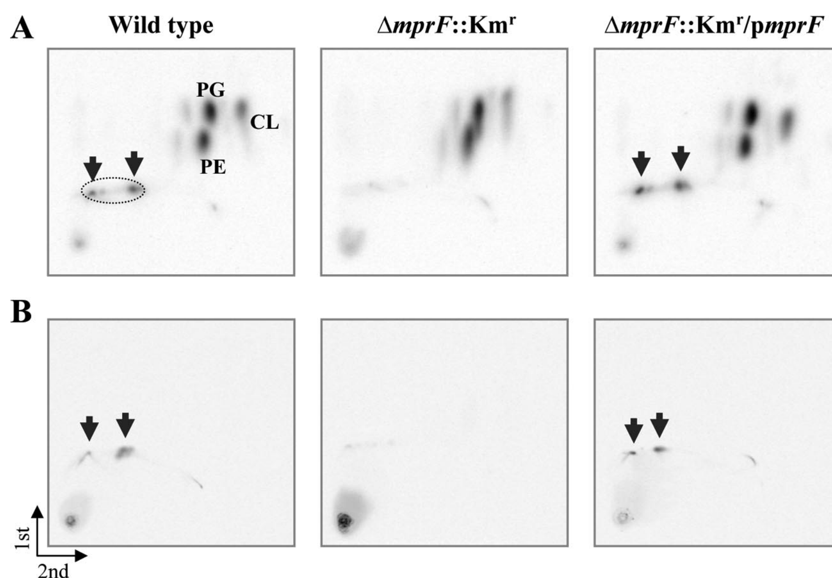
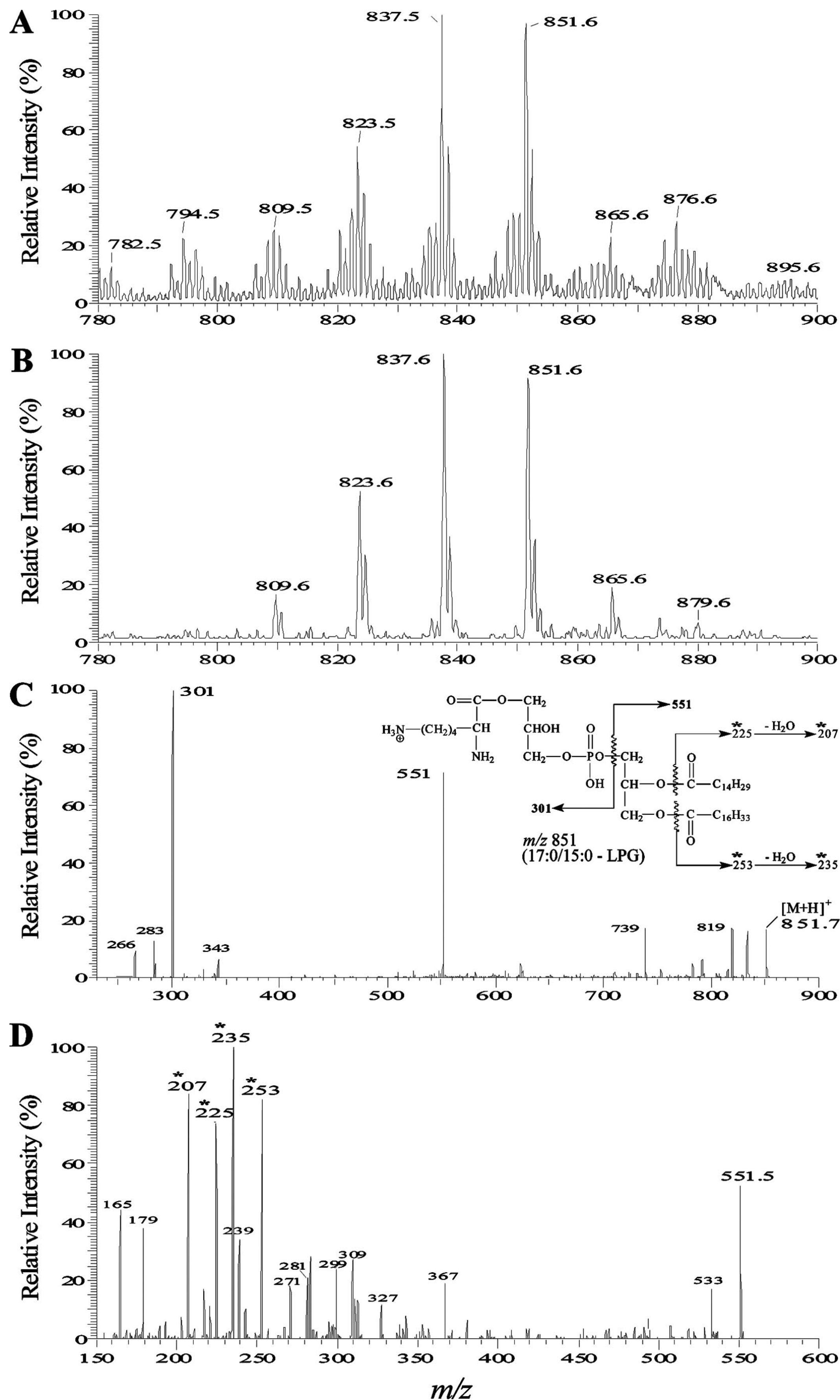


FIG. 3. The *B. anthracis mprF* mutant is unable to synthesize lysinylated phospholipids. 2D-TLC profiles of ^{32}P-labeled phospholipids (A) and L-[^{14}C]lysine-labeled lipids (B) from wild-type Sterne 34F2, Δ*mprF*::Km^r, or Δ*mprF*::Km^r/p*mprF* strains. The total radiolabeled lipid samples were prepared from each strain and separated by 2D-TLC and visualized using a phosphorimager as described in Materials and Methods. Representative results of two independent experiments are shown. Wild-type lipid samples subjected to preparatory 2D-TLC and iodine-stained displayed profiles similar to that of the wild-type sample in panel A (data not shown). The circled area (indicated approximately with a dotted line) on the preparatory 2D-TLC with wild-type lipid samples was extracted as a mixture for mass spectrometric analyses (see the text for details). CL, cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

Downloaded from http://jb.asm.org/ on March 20, 2014 by Washington University in St. Louis



Downloaded from http://jb.asm.org/ on March 20, 2014 by Washington University in St. Louis

fold more susceptible to HNP-1 (Fig. 2A) and ~360-fold more susceptible to LL37 (Fig. 2B). The resistance of the *mprF* mutant to these peptides was restored to the wild-type level upon genetic complementation with p*mprF* carrying a wild-type copy of *mprF* with its native promoter (Fig. 2). Collectively, these results demonstrate that the *B. anthracis* MprF is required for resistance not only to a linear peptide protamine but also to α-helical and β-sheet cationic antimicrobial peptides.

The *B. anthracis mprF* mutant is unable to synthesize phospholipids modified with lysine. In *S. aureus* and *L. monocytogenes*, MprF was shown to be required for the synthesis of lysylphosphatidylglycerols (27, 29, 37). To investigate the function of the *B. anthracis* MprF, we first compared the phospholipid profiles of the wild-type Sterne, Δ*mprF*::Km^r, and Δ*mprF*::Km^r/p*mprF* strains. Total polar membrane lipids were prepared from each strain grown in the presence of [^{32}P]orthophosphate, separated by using 2D-TLC, and visualized by using a phosphorimager as described in Materials and Methods. The phospholipids from the wild-type and genetically complemented *mprF* strains consisted of at least five major phospholipid species (Fig. 3A). In contrast, the phospholipids of the *mprF* deletion mutant strain was composed of only three major species (Fig. 3A), indicating that MprF is required for synthesis of certain phospholipid species. To further determine whether the phospholipid species missing in the *mprF* deletion mutant strain are lysinylated, we compared the total polar membrane lipids isolated from respective strains grown in the presence of L-[^{14}C]lysine (Fig. 3B). Indeed, the two major phospholipid species that appeared only in the wild-type and genetically complemented strains were labeled with L-[^{14}C]lysine. These apparently lysinylated phospholipids were missing in the *mprF* deletant (Fig. 3B). Taken together, these results suggest that the *B. anthracis* MprF mediates the synthesis of certain lysylphospholipids.

Structural determination of the *B. anthracis* lysylphospholipids by using mass spectrometry. To elucidate the structure of putative lysylphospholipids observed by radiolabeling experiments (Fig. 3), the wild-type lipid samples were separated by using 2D-TLC. Respective TLC spots stained with iodine were extracted with organic solvent and subjected to various mass spectrometric analyses (see Materials and Methods). TLC spots encompassing at least two putative lysylphospholipid species as shown by radiolabeling experiments were extracted and analyzed as a mixture because of poor separation on preparative TLC (Fig. 3A). TLC spots corresponding to three major phospholipid species shown to be present in the wild-type, Δ*mpr*::Km^r, and Δ*mpr*::Km^r/p*mprF* strains (Fig. 3A) were determined to be cardiolipin, phosphatidylglycerol, and phosphatidylethanolamine, respectively (data not shown).

Upon ESI in a linear ion-trap (LIT) or tandem quadrupole instrument in the positive-ion mode, the putative lysylphospholipid mixture isolated was shown to contain $[M+H]^+$ ions at

TABLE 2. Composition of lysylphosphatidylglycerols

$[M+H]^+$	Structure[a]	Relative abundance
809	ND	15
823	15:0/15:0-LPG	50
837	17:0/14:0-LPG	100
851	17:0/15:0-LPG	90
865	18:0/15:0-LPG	20

[a] Deduced from product-ion analysis with an LIT mass spectrometer. LPG, lysylphosphatidylglycerol. ND, structure not determined.

m/z 809, 823, 837, 851, 865, and 879 (Fig. 4A), corresponding to various lysylphosphatidylglycerols (Table 2). The $[M-H]^-$ ions that are 2 Da lighter were also observed in the negative-ion mode (data not shown) confirming the results obtained by the positive-ion mode. The profile of the tandem mass spectrum from neutral loss scan of 300 (Fig. 4B) is nearly identical to the ESI mass spectrum (Fig. 4A), further supporting the presence of the various molecular species of lysylphosphatidylglycerols.

The structural assignments (Table 2) were conducted on both the $[M+H]^+$ and $[M-H]^-$ ions by using multiple-stage LIT mass spectrometry. The product-ion spectrum from MS^2 on the $[M+H]^+$ ion at *m/z* 851 (Fig. 4C) contained a prominent ion at *m/z* 551, likely arising from cleavage of 1′-lysylphosphoglycerol residue of the molecule (see the scheme depicted in Fig. 4C, inset). This was consistent with the observation of the ion at *m/z* 301, corresponding to a protonated ion of a 1′-lysyl-phosphoglycerol. This fragmentation pathway leading to neutral loss of the 1′-lysyl-phosphoglycerol residue (300 Da) was also observed for all of the other lysylphosphatidylglycerols shown in Table 2 (data not shown). This finding was in agreement with the notion that the profiles of the tandem mass spectrum from neutral scan of 300 (Fig. 4B) and of the ESI mass spectrum (Fig. 4A) are nearly identical as described earlier. Further dissociation of the ion at *m/z* 551 (851→551, Fig. 4D) gave rise to ions at *m/z* 253 and 225, corresponding to the 17:0- and 15:0-acyl (RCO^+) cations, respectively, a finding consistent with the presence of ions at *m/z* 235 (253-H_2O) and 207 (225-H_2O) arising from the further loss of a water residue (14). The ions at *m/z* 327 and 309 arose from losses of 15:0-fatty acid substituent as a ketene and as an acid, respectively, while the ions at *m/z* 299 and 281 arose from the corresponding losses of the 17:0-fatty acid residue. The results were in accordance with the presence of 17:0- and 15:0-fatty acids in the molecule.

By contrast, the product-ion spectrum from MS^2 on the $[M-H]^-$ ion at *m/z* 849 observed in the negative-ion mode was dominated by the ion at *m/z* 721, which was equivalent to a deprotonated ion of a phosphatidylglycerol arising from loss of a lysine (128 Da) (see the scheme in Fig. S2A in the supplemental material) (13). This was further supported by the LIT MS^3 spectrum of the ion at *m/z* 721 (849→721, see Fig. S2A in

FIG. 4. Mass spectrometric analyses of lysylphospholipids of *B. anthracis*. (A) ESI/MS spectrum obtained in the positive-ion mode; (B) LIT/MS^2 spectrum of neutral loss scan of 300; (C) LIT/MS^2 spectrum of the $[M+H]^+$ ion at *m/z* 851; (D) LIT/MS^3 spectrum of the same ion at *m/z* 551 (851→551). Lysylphospholipid samples were prepared and analyzed by using mass spectrometry as described in Materials and Methods.

Downloaded from http://jb.asm.org/ on March 20, 2014 by Washington University in St. Louis

the supplemental material), which contains the ion at *m/z* 647, arising from loss of the glycerol head group as a [glycerol-H_2O] residue (74 Da), along with the ion at *m/z* 405, arising from *m/z* 479 by loss of [glycerol-H_2O] or from *m/z* 497 by loss of a glycerol residue (92 Da) (13). The spectrum also contained the ions at *m/z* 269 and 241 corresponding to 17:0- and 15:0-carboxylate anions, respectively, and the ions at *m/z* 479 and 497 arising from losses of the 15:0-fatty acid substituent as an acid and as a ketene, respectively, along with the ions at *m/z* 451 and 469 corresponding to the analogous losses of 17:0-fatty acid residue. The ion pairs at *m/z* 479/497 were, respectively, more abundant than those at *m/z* 451/469, clearly demonstrating that the 17:0- and 15:0-fatty acid moiety were located at *sn*-1 and *sn*-2 positions, respectively (13). These results suggest that the compound corresponding to the $[M+H]^+$ ion at *m/z* 851 (and the $[M-H]^-$ ion at *m/z* 849) is 17:0/15:0-lysyl-phosphatidylglycerol. Identification of this and other lysylphosphatidylglycerols (Table 1) suggest that their synthesis requires the functional MprF in *B. anthracis*.

DISCUSSION

The gram-positive pathogen *B. anthracis* is infamous for its uncontrolled, rapid growth in human blood, a host environment rich in antibacterial molecules such as cationic antimicrobial peptides produced by circulating neutrophils and platelets. In the present study, we created Tn*917* insertional mutants and identified a *B. anthracis* gene (BA1485 in the ΔANR strain; BAS1375 in the Stern strain) that is critically required for resistance to cationic antimicrobial peptides.

Tn*917* transposon mutagenesis has been successfully used in various gram-positive bacteria, facilitating the identification of genes involved in diverse functions. In *B. anthracis*, however, Tn*917* is known to insert preferentially into virulence plasmids pXO1 and pXO2 in comparison to the chromosome (12, 40). To avoid this problem, we used the ΔANR strain of *B. anthracis* that lacks both of the virulence plasmids as a host for Tn*917* mutagenesis. However, determination of Tn*917* insertion sites in a subset of transposon mutants revealed that Tn*917* mutagenesis still occurs in a severely biased manner with transposons preferentially inserting into the first half of the chromosome (see Fig. S1 in the supplemental material). This regional bias seen in *B. anthracis* is very different from that observed in other gram-positive bacteria such as *S. aureus* and *E. faecalis*, in which Tn*917* transposition is known to occur in a hot spot that coincides with the replication terminus (3, 8). Although we do not have a plausible explanation for this distinctive Tn*917* transposition in *B. anthracis*, it is clear that to achieve exhaustive genetic screens in *B. anthracis*, a better genetic technique for creating random (transposon) mutants needs to be developed.

Despite the biased representation of the Tn*917* library, we have identified a *B. anthracis* gene (BA1486 in the ΔANR strain and BAS1375 in the Sterne strain) whose inactivation causes hypersusceptibility to cationic antimicrobial peptides such as protamine, HNP-1, and LL-37 (Fig. 1 and 2). This gene encodes a putative membrane protein homologous to MprF known in a few gram-positive bacteria. In *S. aureus* and *L. monocytogenes*, MprF is shown to be required for the synthesis of lysylphosphatidylglycerols (29, 37), and the results from our study demonstrate that the *B. anthracis* orthologue is a functional MprF. The *B. anthracis* MprF exhibits the same level of homology as that of either *S. aureus* or *L. monocytogenes*. Our study shows that *B. anthracis* MprF mediates the synthesis of lysylphosphatidylglycerol species perfectly matching with those of *S. aureus* (Table 1) (29) and partly matching with those of *L. monocytogenes* (37), indicating (dis)similarity in the distribution of fatty acids among gram-positive bacterial species. Because the MprF protein in *B. anthracis* and *S. aureus* has exactly the same function, we expected that the phenotypes of *mprF* mutants in both bacterial species would be similar. The *S. aureus mprF* mutant displayed increased susceptibility to some conventional cationic (nisin and gentamicin) antibiotics. However, the *B. anthracis mprF* mutant exhibits very weak or no change in resistance to them (data not shown). For example, the MICs of nisin (0.5 to 4 μg/ml) (29) and gentamicin (0.125 to 1 μg/ml) (25), respectively, for the *S. aureus mprF* mutant strain is eightfold lower than that for the wild type. The *B. anthracis mprF* mutant, however, displayed a ≤2-fold decrease in the MIC of nisin and wild-type resistance to gentamicin (data not shown). This phenotypic difference may suggest that the basal expression level of the *B. anthracis mprF* is low in the conditions used in our study and/or that genetic determinants other than *mprF* significantly contribute to intrinsic resistance to these antibiotics in *B. anthracis*.

In gram-positive bacteria, in addition to proteolytic degradation and efflux, modifications neutralizing negative charges of cell surface, mediated by MprF and DltABCD, respectively, are known to be major resistance mechanisms for cationic antimicrobial peptides (17). The DltABCD system that incorporates D-alanine into teichoic acids has recently been shown to be functional in *B. anthracis* (7), and our study demonstrates the presence of functional MprF in *B. anthracis*. Recently, it was shown that the expression of both the *dlt* operon and *mprF* are activated by a common transcriptional regulatory system, the VirRS two-component system in *L. monocytogenes* (22), and the Aps three-component system in *S. aureus* (19) and *S. epidermidis* (20). It is therefore likely that both modification systems in *B. anthracis* are also controlled by a common regulatory pathway. We are currently investigating this possibility.

MprF homologues are also suggested to be encoded in the genomes of gram-negative bacteria such as *Pseudomonas aeruginosa* (29). Indeed, it was recently shown that the genomes of the gram-negative *Rhizobium tropici* and *Sinorhizobium medicae* carry a functional MprF that is required not only for resistance to the cationic antimicrobial peptide polymyxin B (35) but also for cell survival in lethal acidic conditions (31, 39). The presence of functional MprF in both gram-positive and gram-negative bacteria strongly supports the idea that MprF may serve as a broad-spectrum antibacterial target.

ACKNOWLEDGMENTS

This study was supported by grants U19 AI56575 and P41-RR-00954 from the U.S. National Institutes of Health.

We thank Alexander Mankin for helpful suggestions and Emmanuelle Charpentier for kindly providing the plasmid pCN55 used in this study.

This paper is dedicated to the memory of Alexander Neyfakh.

Downloaded from http://jb.asm.org/ on March 20, 2014 by Washington University in St. Louis

REFERENCES

1. **Abachin, E., C. Poyart, E. Pellegrini, E. Milohanic, F. Fiedler, P. Berche, and P. Trieu-Cuot.** 2002. Formation of D-alanyl-lipoteichoic acid is required for adhesion and virulence of *Listeria monocytogenes*. Mol. Microbiol. **43:**1–14.
2. **Barnard, J. P., and A. M. Friedlander.** 1999. Vaccination against anthrax with attenuated recombinant strains of *Bacillus anthracis* that produce protective antigen. Infect. Immun. **67:**562–567.
3. **Begun, J., C. D. Sifri, S. Goldman, S. B. Calderwood, and F. M. Ausubel.** 2005. *Staphylococcus aureus* virulence factors identified by using a high-throughput *Caenorhabditis elegans*-killing model. Infect. Immun. **73:**872–877.
4. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
5. **Charpentier, E., A. I. Anton, P. Barry, B. Alfonso, Y. Fang, and R. P. Novick.** 2004. Novel cassette-based shuttle vector system for gram-positive bacteria. Appl. Environ. Microbiol. **70:**6076–6085.
6. **Dixon, T. C., A. A. Fadl, T. M. Koehler, J. A. Swanson, and P. C. Hanna.** 2000. Early *Bacillus anthracis*-macrophage interactions: intracellular survival and escape. Cell. Microbiol. **2:**453–463.
7. **Fisher, N., L. Shetron-Rama, A. Herring-Palmer, B. Heffernan, N. Bergman, and P. Hanna.** 2006. The *dltABCD* operon of *Bacillus anthracis* Sterne is required for virulence and resistance to peptide, enzymatic, and cellular mediators of innate immunity. J. Bacteriol. **188:**1301–1309.
8. **Garsin, D. A., J. Urbach, J. C. Huguet-Tapia, J. E. Peters, and F. M. Ausubel.** 2004. Construction of an *Enterococcus faecalis* Tn*917*-mediated-gene-disruption library offers insight into Tn*917* insertion patterns. J. Bacteriol. **186:**7280–7289.
9. **Groisman, E. A., C. Parra-Lopez, M. Salcedo, C. J. Lipps, and F. Heffron.** 1992. Resistance to host antimicrobial peptides is necessary for *Salmonella* virulence. Proc. Natl. Acad. Sci. USA **89:**11939–11943.
10. **Guidi-Rontani, C., M. Weber-Levy, E. Labruyère, and M. Mock.** 1999. Germination of *Bacillus anthracis* spores within alveolar macrophages. Mol. Microbiol. **31:**9–17.
11. **Gutierrez, J. A., P. J. Crowley, D. P. Brown, J. D. Hillman, P. Youngman, and A. S. Bleiweis.** 1996. Insertional mutagenesis and recovery of interrupted genes of *Streptococcus mutans* by using transposon Tn*917*: preliminary characterization of mutants displaying acid sensitivity and nutritional requirements. J. Bacteriol. **178:**4166–4175.
12. **Hoffmaster, A. R., and T. M. Koehler.** 1997. The anthrax toxin activator gene *atxA* is associated with CO_2-enhanced non-toxin gene expression in *Bacillus anthracis*. Infect. Immun. **65:**3091–3099.
13. **Hsu, F.-F., and J. Turk.** 2001. Studies on phosphatidylglycerol with triple quadrupole tandem mass spectrometry with electrospray ionization: fragmentation processes and structural characterization. J. Am. Soc. Mass Spectrom. **12:**1036–1043.
14. **Hsu, F.-F., and J. Turk.** 2005. Studies on phosphatidylserine by tandem quadrupole and multiple stage quadrupole ion-trap mass spectrometry with electrospray ionization: structural characterization and the fragmentation processes. J. Am. Soc. Mass Spectrom. **16:**1510–1522.
15. **Kok, J., J. M. van der Vossen, and G. Venema.** 1984. Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. Appl. Environ. Microbiol. **48:**726–731.
16. **Kovacs, M., A. Halfmann, I. Fedtke, M. Heintz, A. Peschel, W. Vollmer, R. Hakenbeck, and R. Bruckner.** 2006. A functional *dlt* operon, encoding proteins required for incorporation of D-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in *Streptococcus pneumoniae*. J. Bacteriol. **188:**5797–5805.
17. **Kraus, D., and A. Peschel.** 2006. Molecular mechanisms of bacterial resistance to antimicrobial peptides. Curr. Top. Microbiol. Immunol. **306:**231–250.
18. **Levy, O.** 2000. Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. Blood **96:**2664–2672.
19. **Li, M., D. J. Cha, Y. Lai, A. E. Villaruz, D. E. Sturdevant, and M. Otto.** 2007. The antimicrobial peptide-sensing system *aps* of *Staphylococcus aureus*. Mol. Microbiol. **66:**1136–1147.
20. **Li, M., Y. Lai, A. E. Villaruz, D. J. Cha, D. E. Sturdevant, and M. Otto.** 2007. gram-positive three-component antimicrobial peptide-sensing system. Proc. Natl. Acad. Sci. USA **104:**9469–9474.
21. **Lyons, C. R., J. Lovchik, J. Hutt, M. F. Lipscomb, E. Wang, S. Heninger, L. Berliba, and K. Garrison.** 2004. Murine model of pulmonary anthrax: kinetics of dissemination, histopathology, and mouse strain susceptibility. Infect. Immun. **72:**4801–4809.
22. **Mandin, P., H. Fsihi, O. Dussurget, M. Vergassola, E. Milohanic, A. Toledo-Arana, I. Lasa, J. Johansson, and P. Cossart.** 2005. VirR, a response regulator critical for *Listeria monocytogenes* virulence. Mol. Microbiol. **57:**1367–1380.
23. **Mayer-Scholl, A., R. Hurwitz, V. Brinkmann, M. Schmid, P. Jungblut, Y. Weinrauch, and A. Zychlinsky.** 2005. Human neutrophils kill *Bacillus anthracis*. PLoS Pathog. **1:**e23.
24. **Mock, M., and A. Fouet.** 2001. Anthrax. Annu. Rev. Microbiol. **55:**647–671.
25. **Nishi, H., H. Komatsuzawa, T. Fujiwara, N. McCallum, and M. Sugai.** 2004. Reduced content of lysyl-phosphatidylglycerol in the cytoplasmic membrane affects susceptibility to moenomycin, as well as vancomycin, gentamicin, and antimicrobial peptides, in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **48:**4800–4807.
26. **O'Toole, G. A., L. A. Pratt, P. I. Watnick, D. K. Newman, V. B. Weaver, and R. Kolter.** 1999. Genetic approaches to study of biofilms. Methods Enzymol. **310:**91–109.
27. **Oku, Y., K. Kurokawa, N. Ichihashi, and K. Sekimizu.** 2004. Characterization of the *Staphylococcus aureus mprF* gene, involved in lysinylation of phosphatidylglycerol. Microbiology **150:**45–51.
28. **Palmer, B. R., and M. G. Marinus.** 1994. The *dam* and *dcm* strains of *Escherichia coli*. Gene **143:**1–12.
29. **Peschel, A., R. W. Jack, M. Otto, L. V. Collins, P. Staubitz, G. Nicholson, H. Kalbacher, W. F. Nieuwenhuizen, G. Jung, A. Tarkowski, K. P. van Kessel, and J. A. van Strijp.** 2001. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. J. Exp. Med. **193:**1067–1076.
30. **Peschel, A., M. Otto, R. W. Jack, H. Kalbacher, G. Jung, and F. Gotz.** 1999. Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J. Biol. Chem. **274:**8405–8410.
31. **Reeve, W. G., L. Brau, J. Castelli, G. Garau, C. Sohlenkamp, O. Geiger, M. J. Dilworth, A. R. Glenn, J. G. Howieson, and R. P. Tiwari.** 2006. The *Sinorhizobium medicae* WSM419 *lpiA* gene is transcriptionally activated by FsrR and required to enhance survival in lethal acid conditions. Microbiology **152:**3049–3059.
32. **Sambrook, J., and D. W. Russel.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
33. **Sekimizu, K., and A. Kornberg.** 1988. Cardiolipin activation of *dnaA* protein, the initiation protein of replication in *Escherichia coli*. J. Biol. Chem. **263:**7131–7135.
34. **Shatalin, K. Y., and A. A. Neyfakh.** 2005. Efficient gene inactivation in *Bacillus anthracis*. FEMS Microbiol. Lett. **245:**315–319.
35. **Sohlenkamp, C., K. A. Galindo-Lagunas, Z. Guan, P. Vinuesa, S. Robinson, J. Thomas-Oates, C. R. H. Raetz, and O. Geiger.** 2007. The Lipid lysyl-phosphatidylglycerol is present in membranes of *Rhizobium tropici* CIAT899 and confers increased resistance to polymyxin B under acidic growth conditions. Mol. Plant-Microbe Interact. **20:**1421–1430.
36. **Sterne, M.** 1937. Avirulent anthrax vaccine. Onderstepoort J. Vet. Sci. Anim. Ind. **21:**41–43.
37. **Thedieck, K., T. Hain, W. Mohamed, B. J. Tindall, M. Nimtz, T. Chakraborty, J. Wehland, and L. Jansch.** 2006. The MprF protein is required for lysinylation of phospholipids in listerial membranes and confers resistance to cationic antimicrobial peptides (CAMPs) on *Listeria monocytogenes*. Mol. Microbiol. **62:**1325–1339.
38. **Trieu-Cuot, P., and P. Courvalin.** 1983. Nucleotide sequence of the *Streptococcus faecalis* plasmid gene encoding the 3′5″-aminoglycoside phosphotransferase type III. Gene **23:**331–341.
39. **Vinuesa, P., F. Neumann-Silkow, C. Pacios-Bras, H. P. Spaink, E. Martinez-Romero, and D. Werner.** 2003. Genetic analysis of a pH-regulated operon from *Rhizobium tropici* CIAT899 involved in acid tolerance and nodulation competitiveness. Mol. Plant-Microbe Interact. **16:**159–168.
40. **Welkos, S. L.** 1991. Plasmid-associated virulence factors of non-toxigenic ($pXO1^-$) *Bacillus anthracis*. Microb. Pathog. **10:**183–198.
41. **Yeaman, M. R., and N. Y. Yount.** 2003. Mechanisms of antimicrobial peptide action and resistance. Pharmacol. Rev. **55:**27–55.

Downloaded from http://jb.asm.org/ on March 20, 2014 by Washington University in St. Louis