INFECTION AND IMMUNITY, July 2006, p. 3756–3764 Vol. 74, No. 7
0019-9567/06/$08.00+0 doi:10.1128/IAI.00307-06

Bacillus anthracis Phospholipases C Facilitate Macrophage-Associated Growth and Contribute to Virulence in a Murine Model of Inhalation Anthrax

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Received 23 February 2006/Returned for modification 27 March 2006/Accepted 7 April 2006

Several models of anthrax pathogenesis suggest that early in the infectious process *Bacillus anthracis* endospores germinate and outgrow into vegetative bacilli within phagocytes before being released into the blood. Here, we define the respective contributions of three phospholipases C (PLCs) to the pathogenesis of *B. anthracis*. Genetic deletions of the PLCs were made in the Sterne 7702 background, resulting in the respective loss of their activities. The PLCs were redundant both in tissue culture and in murine models of anthrax. Deletion of all three PLC genes was required for attenuation of virulence in mice after intratracheal inoculation. This attenuation may be attributed to the inability of the PLC-null strain to grow in association with the macrophage. Complementation of these defects in both models of anthrax was achieved by expression of the PLC genes in *trans*. The functional redundancy between PLCs in the virulence of *B. anthracis* implies that their activities are important for anthrax pathogenesis.

Anthrax results from the introduction of *Bacillus anthracis* endospores into the body through abrasions in the skin, by the ingestion of contaminated foods, or by inhalation (5). During the establishment of inhalation anthrax, *B. anthracis* is believed to have a transient intracellular phase in which endospores are engulfed by alveolar phagocytes and are trafficked to regional lymph nodes (12, 28). Endospores germinate in association with the phagocytic cells resulting in the outgrowth of vegetative bacilli, which are eventually released from the phagocyte into the extracellular environment (4, 12). *B. anthracis* then enters the circulatory and lymphatic systems and grows to high titers, causing massive septicemia, toxemia, and often death.

Nascent *B. anthracis* bacilli associated with macrophages were shown to express *atxA* (AtxA), a transcriptional activator of three exotoxin genes (*pag*, *lef*, and *cya*) whose products combine to form two binary A-B exotoxins: lethal toxin (LeTx) and edema toxin (EdTx) (12). LeTx and EdTx are expressed throughout anthrax infections and are key virulence factors required for *B. anthracis* pathogenesis (5). The structural genes and the transcriptional activator required for the expression of the two exotoxins are encoded on the 185-kb virulence plasmid, pXO1 (21). *B. anthracis* contains a second virulence plasmid, pXO2, which encodes the genes required for the synthesis of the poly-D-glutamic acid capsule (18). The capsule confers serum resistance and hinders the ability of immune cells to phagocytose the bacilli. *B. anthracis* strains deficient in the production of either the exotoxins or the capsule are attenuated for virulence.

Despite the importance of the two exotoxins and capsule in the pathogenesis of *B. anthracis*, they appear to be not absolutely required during the early, intracellular stages of infection. The nonencapsulated, nontoxigenic ΔSterne strain ($pXO1^-$ $pXO2^-$) replicates in the cytoplasm of cultured macrophages in a manner similar to the isogenic Sterne strain ($pXO1^+$ $pXO2^-$) (4, 29). This suggests that chromosomally encoded genes facilitate survival and replication in association with the macrophage. We hypothesized that *B. anthracis* may utilize phospholipases during these initial events in anthrax pathogenesis. Three genes were annotated on the *B. anthracis* genome that encodes putative phospholipases C (PLCs) (26). These genes have high homology to *B. cereus* and *Listeria monocytogenes* phosphatidylcholine PLC (*plcB*; PC-PLC; BA0677), sphingomyelinase (*smcA*; SMase; BA0678), and phosphatidylinositol-specific PLC (*plcA*; PI-PLC; BA3891) (26).

PLCs hydrolyze the polar head groups from phospholipids. They exhibit a broad range of specificities dependent upon recognition of the polar head group and the hydrophobic moiety. For example, PC-PLC is able to act both on phosphatidylcholine and a variety of other phospholipids, whereas SMase and PI-PLC are restricted to sphingomyelin and phosphatidylinositol, respectively (37). Phospholipases are known to contribute to the pathogenesis of a variety of bacteria by participating in phosphate acquisition, deregulation of cellular signaling, tissue destruction, and degradation of mucus layers (30). In *L. monocytogenes*, PLCs are used in combination with the pore-forming cytolysin listeriolysin O (LLO) to disrupt phagosomal membranes and aid in the escape of *L. monocytogenes* into the cytosol (9).

PC-PLC, PI-PLC, and SMase of *B. cereus* are expressed as part of a regulon under the control of the transcriptional activator PlcR (8). *B. anthracis* encodes a PlcR homologue that has a C-terminal truncation due to a nonsense mutation but still expresses the regulon, albeit weakly (20). Transcript was detected for *plcB*, *smcA*, and *plcA* in vitro during *B. anthracis* infections of cultured macrophages (15). In addition, plasma

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TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or phage	Relevant genotype[a]	Source or reference
Strains		
B. anthracis		
Sterne 7702	pXO1$^+$ pXO2$^-$	24
ΔSterne	pXO1$^-$ pXO2$^-$	This study, Sterne cured of pXO1
BJH035	7702, Δ*lef*::Km^r	This study
BJH108	7702, Δ*plcB*::Sp^r	This study
BJH109	7702, Δ*plcR*::Sp^r	This study
BJH121	7702, Δ*plcBsmcA*::Sp^r	This study
BJH217	7702, Δ*plcA*::Em^r	This study
BJH236	7702, Δ*smcA*::Sp^r	This study
BJH238	7702, Δ*smcA*::Sp^r Δ*plcA*::Em^r	This study
BJH249	7702, Δ*plcB*::Sp^r Δ*plcA*::Em^r	This study
BJH250	7702, Δ*plcBsmcA*::Sp^r Δ*plcA*::Em^r	This study
BJH251	7702, Δ*lef*::Km^r Δ*plcBsmcA*::Sp^r Δ*plcA*::Em^r	This study
B. subtilis 168	*trpC2*	1
E. coli		
XL1-Blue MRF′	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^r)]	Stratagene
DH5α	F$^-$ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ$^-$	Invitrogen
One Shot TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80 *lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*araleu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
One Shot INV110	F′ [*tra*Δ*36 proAB lacI*^{q} *lacZ*ΔM15] *rpsL* (Str^r) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) Δ(*mcrC-mrr*) *102*::Tn*10* (Tet^r)	Invitrogen
CGSC 6478	GM272 (*dam-3 dcm-6*)	22
Plasmids		
pUC19	pBR322 derivative *lacZ*α; Ap^r	43
pDG641	pJRD184::Em^r	11
pDG783	pSB118::Km^r	11
pDG1726	pSB119::Sp^r	11
pCR-XL-TOPO	P_{lac} *lacZ*α *ccdB* Km^r pUC_{ori}	Invitrogen
pHP13	$pUC19_{ori}$ $pTA1060_{ori}$; Cm^r Em^r	14
pKSV7	$pUC19_{ori}$ $pE194_{ori(ts)}$; Ap^r Cm^r	33
pBH010	pKSV7(Δ*lef*::Km^r)	This study
pBH024	pKSV7(Δ*smcA*::Sp^r)	This study
pBH027	pKSV7(Δ*plcBsmcA*::Sp^r)	This study
pBH032	pKSV7(Δ*plcB*::Sp^r)	This study
pBH040	pKSV7(Δ*plcR*::Sp^r)	This study
pBH093	pKSV7(Δ*plcA*::Em^r)	This study
pBH095	pHP13::*plcB smcA plcA*	This study
Phage CP-51	Generalized transducing phage	35

[a] Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance; Ap^r, ampicillin resistance; Sp^r, spectinomycin resistance; Str^r, streptomycin resistance; Em^r, erythromycin resistance; Km^r, kanamycin resistance.

from guinea pigs dying of anthrax displayed higher levels of PLC activity versus culture filtrates of *B. anthracis*, implying that they may play a role in the disease (45). Recently, it was shown by ectopic expression of each *B. anthracis* PLC gene in *Escherichia coli* and *L. monocytogenes* that the genes encode functional proteins with activities similar to their corresponding *B. cereus* orthologues (25, 40). In the present study we used a genetic approach to determine the contributions of PLCs to *B. anthracis* pathogenesis. The genes encoding each PLC were disrupted, both individually and in combination, in order to investigate their functions in vitro using a cultured macrophage model of infection and in vivo with mice challenged via an intratracheal route.

MATERIALS AND METHODS

Growth conditions and strain construction. Bacterial strains, plasmids, and phages relevant to the present study are listed in Table 1. *E. coli* and *B. anthracis* strains were cultured in Luria-Bertani (LB) medium and brain heart infusion (BHI; Difco) medium, respectively. The medium was supplemented with antibiotics to maintain selection at the following concentrations: ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), erythromycin (1 μg/ml), kanamycin (50 μg/ml), and spectinomycin (100 μg/ml). CCY medium was used for *B. anthracis* sporulation (34). Endospores were prepared as previous described and stored in 1-ml aliquots of sterile water after a 30-min incubation at 65°C (4). Endospore germination was analyzed at 37°C by measuring the decrease in optical density as described previously (6). Briefly, endospores were suspended in double-distilled H_2O to an optical density at 600 nm of about 0.5, and 2× BHI was added at a 1:1 ratio to initiate the germination reaction.

Plasmid constructs were made in *E. coli* XL1-Blue or DH5α backgrounds except for the complementation vector, which was constructed in *B. subtilis* strain 168 due to the apparent selection against a functional *plcB* gene in *E. coli*. Oligonucleotide primers were designed using the genome sequence of the *B. anthracis* Ames strain. Primer sequences used in the present study are listed in Table 2. The Expand High Fidelity System (Roche) was used for PCR amplification. Deletion constructs were made as previously described (3). Briefly, the gene of interest was cloned into either pUC19 or pCR-XL-TOPO vectors, and the internal portion was deleted through inverse PCR and replaced with an

TABLE 2. Primer sequences

Primer	Sequence (5′–3′)[a]	Application	Restriction site
plcB5	AACTGCAGAACCAATGCATTGGTTAGTGTGGTCACGTTGACG	Amplify *plcB* from 5′ (deletion construct)	PstI
plcB5	TCCCCCCGGGGGGAATCAGCACGCTCACTTTGTC	Amplify *plcB* from 3′ (deletion construct)	XmaI
plcB5	TCCCCGCGGGGACAATCGCACGGTTTACAATCC	Inverse primer *plcB* (deletion construct)	SacII
plcB4	GAAGATCTTCGGAGATGTAAACCAACCGATG	Inverse primer *plcB* (deletion construct)	BglII
smcA6	AACTGCAGAACCAATGCATTGGGAAGATTGGATCCATGGAGC	Amplify *smcA* from 5′ (deletion construct)	PstI
smcA6	GGAATTCCTGAGCCCTTGCTTTGTTAGC	Amplify *smcA* from 3′ (deletion construct)	EcoRI
smcA5	GACTAGTCTGCGTCCCAATTACATGAACG	Inverse primer *smcA* (deletion construct)	SpeI
smcA5	GAAGATCTTCGCATCTGTACGTACAAACCAG	Inverse primer *smcA* (deletion construct)	BglII
plcA11	CGGGGTACCCCGAGAGCAAAGTCGAAGTGCTG	Amplify *plcA* from 5′ (deletion construct)	KpnI
plcA11	CGGGATCCCGCTTTCCGTAATTCTCCTCCAC	Amplify *plcA* from 3′ (deletion construct)	BamHI
plcA11	TCCCCGCGGGGAGATATGAAAGGTGCAGAAGGTTC	Inverse primer *plcA* (deletion construct)	SacII
plcA11	GAAGATCTTCGAACGTCCCACTATCATGTG	Inverse primer *plcA* (deletion construct)	BglII
lef1	GGGGTACCCCGTAACAGCAATTACTTTGAGTGGTC	Amplify *lef* from 5′ (deletion construct)	KpnI
lef1	CGGGATCCCGTTATGCACGTTGAATGTAATAAGC	Amplify *lef* from 3′ (deletion construct)	BamHI
lef2	TCCCCGCGGGGAGTCTGTGGGATGTTCCTTAAGC	Inverse primer *lef* (deletion construct)	SacII
lef2	GACTAGTCCAATCCATTGGAAGTACCTTG	Inverse primer *lef* (deletion construct)	SpeI
plcR1	ACTAGCCCTTTAGAGGAAGC	Amplify *plcR* from 3′ (deletion construct)	
plcR2	GCTACTTGCGAAAGAGGGAA	Amplify *plcR* from 5′ (deletion construct)	
plcR3	TTGGCGCGCCAAGCTCAATCAACAATTGGCAGG	Inverse primer *plcR* (deletion construct)	AscI
plcR3	TCCCCGCGGGGACACTTCCTAATTTTTCTGCGTGC	Inverse primer *plcR* (deletion construct)	SacII
kan2	GACTAGTCGAGGATGAAGAGGATGAGGA	Amplify *kan* from 5′ (kanamycin cassette)	SpeI
kan1	TCCCCGCGGGGAAAATTCCTCGTAGGCGCTCG	Amplify *kan* from 3′ (kanamycin cassette)	SacII
spc3	TTGGCGCGCCAACGATTTTCGTTCGTGAATACATG	Amplify *spc* from 5′ (spectinomycin cassette)	AscI
spc1	GAAGATCTTCCGATTTTCGTTCGTGAATACATG	Amplify *spc* from 5′ (spectinomycin cassette)	BglII
spc1	GACTAGTCCATATGCAAGGGTTTATTGTTTTCTAAA	Amplify *spc* from 3′ (spectinomycin cassette)	SpeI
spc2	TCCCCGCGGGGACATATGCAAGGGTTTATTGTTTTCTAAA	Amplify *spc* from 3′ (spectinomycin cassette)	SacII
erm4	TCCCCGCGGGGAAGTCGTTAAACCGTGTGCTC	Amplify *erm* from 5′ (erythromycin cassette)	SacII
erm4	GAAGATCTTCCTTTTTTCGCACCAGCGAAAAC	Amplify *erm* from 3′ (erythromycin cassette)	BglII
plcB9	TCCCCCCGGGGGGACTTGTTTACGAGCGTGGAAAG	Amplify *plcB* from 5′ (complementation)	XmaI
smcA9	AACTGCAGCCAATGCATTGGAACCTTTGCATAACCGGAACAG	Amplify *smcA* from 3′ (complementation)	PstI
plcA6	GAAGATCTTCCACCGATGAAAGGGACACTA	Amplify *plcA* from 5′ (complementation)	BglII
plcA7	GAAGATCTTCCTTTCCGTAATTCTCCTCCAC	Amplify *plcA* from 3′ (complementation)	BglII

[a] Restriction sites are underlined.

antibiotic cassette. The gene fragment containing the inserted cassette was cloned into the temperature-sensitive shuttle vector pKSV7. The plasmid construct was passaged through a methylation deficient *E. coli* strain before being transformed into Sterne 7702 as described previously (16, 39). After a series of passages at both nonpermissive and permissive temperatures for pKSV7 replication, a deletion of the target gene was obtained via allelic replacement. Deletions were confirmed by PCR and Southern blot hybridizations. All deletion strains were constructed with the allelic replacement technique except the Δ*lef* Δ*plcBsmcAplcA* strain. CP-51 transducing phage was utilized in order to introduce Δ*lef* into the Δ*plcBsmcAplcA* background. Transductions with CP-51 were carried out as described elsewhere (42). A ΔSterne strain was obtained after passage of the Δ*lef*::Km^r mutant at elevated temperatures (41°C) in Casamino Acids medium (36) and screening for the loss of the kanamycin marker. This strain was also confirmed by PCR. The complementation vector was created by cloning *plcB*, *smcA*, and *plcA*, along with at least 500 bp upstream of each start codon into the low-copy vector, pHP13.

PLC assay plates. To detect PLC activity, 5 μl of an overnight *B. anthracis* culture grown at 37°C in BHI was spotted onto the appropriate assay plate and incubated for 36 h at 37 or 40°C. PC-PLC activity was detected on McClung Toabe agar (75 g/liter; Difco) supplemented with 50% egg yolk enrichment (Difco) to achieve a final concentration of 10% egg yolk. After incubation, colonies were removed by washing the samples to reveal the opaque zones underneath. TSA II 5% sheep blood agar (Difco) was supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ to detect sphingomyelinase activity. Beta-hemolytic zones were enhanced upon incubation at elevated temperatures (40°C). PI-PLC activity was evident on BHI agar (15 g/liter) supplemented with 500 μg of 5-bromo-4-chloro-3-indoxyl-myoinositol-1-phosphate (X-PI; Glycosynth)/ml. Strains possessing PI-PLC activity were indicated by blue colonies.

Mouse infections. An intratracheal model of inhalation anthrax was utilized for virulence studies. Six- to eight-week-old female DBA/2J mice (Jackson Laboratories) were anesthetized by the intraperitoneal injection of ketamine (120 mg/kg) and xylazine (5 mg/kg) and restrained on a surgical board. A small incision was made through the skin above the trachea. A 30-gauge needle was inserted into the trachea, and a 30-μl suspension of *B. anthracis* endospores was delivered directly into the lungs. After injection, the incision was closed with a cyanoacrylate adhesive. The inoculum doses were 10^2, 10^3, 10^4, and 10^5 endospores. For infections with the Δ*lef* and Δ*lef plcBsmcAplcA* strains the following inoculum doses were used: 10^5, 10^6, 10^7, 10^8, and 10^9 endospores. A total of eight mice per dose were used, and the experiments were repeated twice. Mice were monitored for a period of 2 weeks, with a majority of the mortalities occurring over the first 3 days. *B. anthracis* strains were recovered from the spleen, blood and/or lungs following mortality and confirmed. The 50% lethal dose (LD_{50}) was estimated by the method of Reed and Muench (27). The mean time to death (MTD) was calculated by averaging the time of death for all individuals that died after receiving 10^5 endospores. Mice were housed and maintained in a specific-pathogen-free environment in a humane fashion.

Infections of BMM. Bone marrow-derived macrophages (BMM) obtained from 6- to 8-week-old female BALB/c mice (Jackson Laboratories) were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS; Gibco) at 37°C in a 5% CO_2 chamber with saturating humidity. BMM were seeded at a concentration of 10^5 cells per well in 24-well tissue culture plates (Corning) and were challenged at a multiplicity of infection (MOI) of 10 endospores per macrophage. A brief 10-min spin at 100 × *g* was performed to initiate contact between the endospores and the BMM, followed by a 20-min incubation to allow for the uptake of endospores into the cell. Infections were washed three times with DMEM, and the medium was replaced with a gentamicin-germinant solution to allow for germination and the reduction of extracellular bacteria. The gentamicin-germinant solution consisted of DMEM–10% FBS supplemented with 5 μg of gentamicin/ml and a germinant cocktail of 1 mM alanine, 1 mM serine, and 1 mM inosine. The germinant cocktail facilitated synchronous germination of the extracellular endospores and allowed for killing of the extracellular bacilli with the gentamicin pulse. Greater than 99.9% germination and gentamicin killing was observed in this medium (data not shown). After a 30-min treatment, the monolayers were washed an additional three times

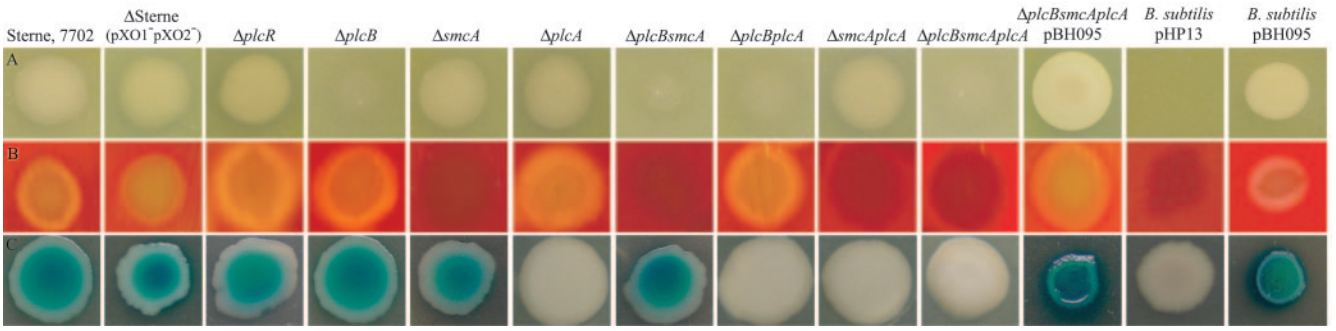

FIG. 1. PLC activity of *B. anthracis*. PC-PLC activity was assayed on McClung Toabe agar supplemented with 10% egg yolk enrichment (A), SMase activity was assayed on TSA II 5% sheep blood agar supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (B), and PI-PLC activity was assayed on BHI supplemented with 500 μg of X-PI/ml (C). Plates were inoculated with *B. anthracis* or *B. subtilis* strains, incubated at 37 or 40°C for 36 h, and monitored for opacity (PC-PLC), hemolysis (SMase), or blue-white screen (PI-PLC).

with DMEM, and the medium was replaced with DMEM–10% FBS. At the specified time points, the supernatant was aspirated, and the BMM were scraped in the presence of a 0.2% solution of saponin. Recovered samples were serially diluted, and cell-associated viable counts were enumerated by plating CFU. Changes in cell-associated growth were normalized to viable counts at the 0-h time point. For the evaluation of cytotoxicity, infections were done identically as described above except that, at 8 h after gentamicin treatment, supernatants were collected, and the macrophage cytotoxicity was measured by determining the release of lactate dehydrogenase (LDH) with the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega). The percent cytotoxicity was calculated as 100 × [(experimental LDH release − spontaneous LDH release)/(maximum LDH release − spontaneous LDH release)]. The identical scheme was also carried out for microscopic analysis, except infections were performed in Lab Tek II chamber slides (Nalge Nunc International). Slides were stained by using a HEMA 3 kit (Fisher), and photomicrographs were taken at ×1,000 magnification.

RESULTS

***B. anthracis* expresses three PLCs independently of PlcR and pXO1.** The absence of hemolytic zones on blood agar has been used as a diagnostic to distinguish *B. anthracis* from other members of the *B. cereus* group. The gamma-hemolysis is thought to be a consequence of the truncated transcriptional activator PlcR, resulting in the silencing of the PlcR regulon (20). However, low levels of hemolysis have been reported for various *B. anthracis* strains, in addition to lecithinase activity (13, 19). Earlier reports imply that PLC expression may be induced under strictly anaerobic growth (15). It was postulated that the truncated PlcR may be functional under these conditions, allowing expression of the PLCs. Here, we were able to detect activity for each PLC on assay plates after growth in an aerobic environment and assign a function to the PLC genes through specific genetic deletions (Fig. 1). In contrast to the findings of Klichko et al. (15), no increase in PLC activity was observed on assay plates grown anaerobically versus those grown aerobically (data not shown). Beta-hemolytic zones were detected on sheep blood agar after 36 h when plates were supplemented with $CaCl_2$ and $MgCl_2$, which have been shown to increase the binding and activation of *B. cereus* SMase on erythrocytes (38). Hemolysis was further enhanced when blood plates were incubated at 40°C. The hemolysis seen on sheep erythrocytes was attributed to SMase since the lack of hemolytic activity coincided with the deletion of *smcA*, whereas the disruption of *plcB* and *plcA* had no effect on hemolysis (Fig. 1B). The susceptibility of sheep erythrocytes to SMase may be a result of their high sphingomyelin (50%) content and relatively negligible amounts of phosphatidylcholine and phosphatidylinositol. PC-PLC and PI-PLC activities were detected, respectively, on egg yolk agar or BHI agar supplemented with a chromogenic derivative of myoinositol phosphate (i.e., X-PI). Deletion of the *plcB* gene resulted in the loss of opaque zones beneath colonies on egg yolk agar, signifying the absence of PC-PLC activity (Fig. 1A). Strains containing a deleted *plcA* gene were white on X-PI plates due to the inability to release the blue chromogen from the myoinositol phosphate derivative as a result of the loss of PI-PLC activity (Fig. 1C). Function was restored in each null strain by complementation of the disrupted gene in *trans*, and PLC activity was conferred to *B. subtilis* via ectopic expression of the *B. anthracis* PLC genes (Fig. 1). In each case, PLC activity was detected 12 to 24 h earlier on the assay plates, suggesting that the PLC genes were overexpressed. To resolve whether the truncated PlcR is still able to mediate expression of the PLC genes, we constructed a *plcR*-null strain. The Δ*plcR* strain had PLC activities similar to that of the parental strain under all of the conditions tested, supporting earlier indications that the *B. anthracis* PlcR is indeed nonfunctional (Fig. 1) (20). In addition, ΔSterne showed no difference in expression of the PLCs, suggesting that the expression of the PLC genes can occur in the absence of both virulence plasmids (Fig. 1).

***B. anthracis* PLCs are redundant for virulence in a murine model of inhalation anthrax.** Although Sterne strains of *B. anthracis* are attenuated for virulence in most animal models of anthrax, certain strains of inbred mice remain susceptible (41). In addition, Sterne infections of inbred mice maintain similar characteristics as infections with the fully virulent *B. anthracis* strains in other species. In the present study, DBA/2J inbred mice were challenged via an intratracheal route with Sterne and isogenic *plcB*-, *smcA*-, and *plcA*-null strains, as well as strains lacking combinations thereof, in order to discern whether PLCs contribute to *B. anthracis* pathogenesis. Intratracheal infections were utilized as a model for inhalation anthrax since endospores can be reproducibly delivered directly into the lungs via this route (17). The MTD and the lethal dose of endospores required to kill 50% of the mice (LD_{50}) for Sterne infections were consistent with previous

TABLE 3. LD_{50} and MTD values for mice challenged intratracheally with *B. anthracis* strains

Strain	LD_{50}	MTD (days)[a]
Sterne 7702	8.26×10^3	2
Δ*plcB*	1.33×10^4	2
Δ*smcA*	8.40×10^3	2
Δ*plcA*	9.99×10^3	2
Δ*plcBsmcA*	2.09×10^4	2
Δ*plcBplcA*	9.55×10^3	2
Δ*smcAplcA*	1.23×10^4	2
Δ*plcBsmcAplcA*	7.71×10^4	5[b]
Δ*plcBsmcAplcA* (pBH095)	3.20×10^3	3
Δ*lef*	5.80×10^7	ND
Δ*lef* Δ*plcBsmcAplcA*	6.23×10^8	ND
Δ*plcR*	1.68×10^4	2

[a] MTD determined for a dose of 10^5 endospores. ND, no deaths were observed.
[b] $P < 0.02$ compared to Sterne 7702 as determined by the log-rank test.

TABLE 4. Germination efficiencies and doubling times of *B. anthracis* strains[a]

Strain	Mean % decrease in $OD_{600} \pm SD$[b]	Doubling time (min) ± SD[c]
Sterne 7702	58.1 ± 0.4	34 ± 2
Δ*plcB*	54.8 ± 0.5	32 ± 3
Δ*smcA*	60.2 ± 0.6	33 ± 1
Δ*plcA*	59.7 ± 0.2	32 ± 1
Δ*plcBsmcA*	58.9 ± 0.9	34 ± 2
Δ*plcBplcA*	58.5 ± 0.1	31 ± 3
Δ*smcAplcA*	60.9 ± 0.4	35 ± 3
Δ*plcBsmcAplcA*	61.8 ± 1.2	30 ± 2

[a] Results for experiments, performed in triplicate, are depicted with the standard deviation.
[b] Endospore germination in BHI at 30 min was performed as described in Materials and Methods. A decrease of ~60% in the optical density at 600 nm (OD_{600}) correlates to ~99% germination as scored by the loss of heat resistance (6).
[c] That is, doubling times during exponential growth of *B. anthracis* strains grown in BHI at 37°C.

findings in similar murine models (41) (Table 3). The disruption of the *plcR* gene had a minimal effect on the LD_{50} and no change in the MTD compared to Sterne, further supporting the notion that the truncated PlcR is, in fact, inactive (Table 3). No increase in the LD_{50} or MTD was observed when mice were infected with strains defective in individual PLC activity. Mice challenged with strains containing disruptions in any two of the three PLC genes showed virtually no distinction from the parental MTD or LD_{50} values (Table 3). The deletion of all three PLC genes was required for a moderate attenuation of virulence in mice. The LD_{50} for the Δ*plcBsmcAplcA* strain was approximately a log higher than Sterne, and the MTD of the triple PLC-null strain was increased by 3 days (Table 3). This defect in virulence can be attributed to the absence of PLC activities since each null strain showed similar germination efficiencies as well as growth rates compared to the isogenic parental (Table 4). Furthermore, we were able to complement this attenuation in virulence by expression of the PLC genes in *trans* (Table 3). A log increase in LD_{50} was observed whether the PLCs were disrupted in the Sterne strain or in a Δ*lef* background (Table 3). This suggests that the PLCs and LeTx may exert their functions separately during *B. anthracis* infections since the disruption of both factors was additive. Collectively, these results indicate that each of the PLCs play a redundant role during mouse infections, since the expression of any one of the three PLCs is sufficient to retain virulence equivalent to the parental Sterne strain.

Cooperation between the PLCs is necessary for cell-associated growth of *B. anthracis* in BMM. During the initial stages of inhalation anthrax, *B. anthracis* is able to survive the harsh intracellular environment of the alveolar macrophage. We hypothesized that the attenuation in virulence of the Δ*plcBsmcAplcA* strain in mice may be attributed to decreased abilities to survive the innate killing mechanisms of the macrophage. Cultured BMM were infected with *B. anthracis* strains defective for PLC activities to determine whether PLCs contribute to *B. anthracis* survival and replication in association with the macrophage. Tissue culture models of anthrax are complicated by the persistence of extracellular endospores that are difficult to remove even with excessive washes. Endospore germination in this model is not uniform, and therefore treatment with antibiotics, such as gentamicin, is insufficient to completely eliminate extracellular bacteria since endospores are resistant to such insults. In an attempt to control for these parameters, the tissue culture medium was supplemented with a germinant solution consisting of alanine, inosine, and serine after phagocytosis of the endospores. This facilitated the synchronous germination of the extracellular endospores and allowed for gentamicin killing of extracellular bacteria. Greater than 99.9% germination and gentamicin killing of extracellular bacteria was observed in this medium (data not shown).

A decrease in macrophage-associated CFU was observed over the first 2 h of Sterne infections, presumably as a consequence of macrophage-mediated killing of endospores and/or recently germinated bacilli (Fig. 2A). Surviving bacilli began to replicate at around 5 to 6 h after gentamicin treatment, and by 8 h the macrophages were overcome with bacilli (average of 19 bacilli/BMM) (Fig. 2 to 4 and Table 5). The growth kinetics of Sterne in association with BMM was in close agreement with previous reports that utilized time-lapse microscopy to study the replication of *B. anthracis* during macrophage infections (29). Individual PLC-null strains showed similar growth kinetics in association with macrophages compared to the parental Sterne strain (Fig. 2A). The Δ*smcA* and Δ*plcA* strains caused comparable amounts of macrophage cell death as the Sterne strain, while the deletion of the broad-range phospholipase, PC-PLC, resulted in nearly a 30% decrease in cytotoxicity (Fig. 3). Strains containing disruptions in any two of the three PLCs showed a diminished capacity for cell-associated growth and were less cytotoxic (Fig. 2B and 3). The absence of all three PLC genes resulted in a strain that was the most deficient for macrophage-associated growth and caused limited amounts of cell death (Fig. 2B and 3). At the 8-h time point, ca. 20% of the macrophage-associated LDH was released when challenged with the Δ*plcBsmcAplcA* strain, a level roughly four times less than the amount for the parental strain. On average, macrophages infected with the triple PLC-null strain contained far fewer bacilli (5 bacilli/BMM) than those infected with the parental Sterne strain (19 bacilli/BMM) (Table 5). Limited amounts of bacterial growth were observed in association with macrophages infected with the Δ*plcBsmcAplcA* strain even at

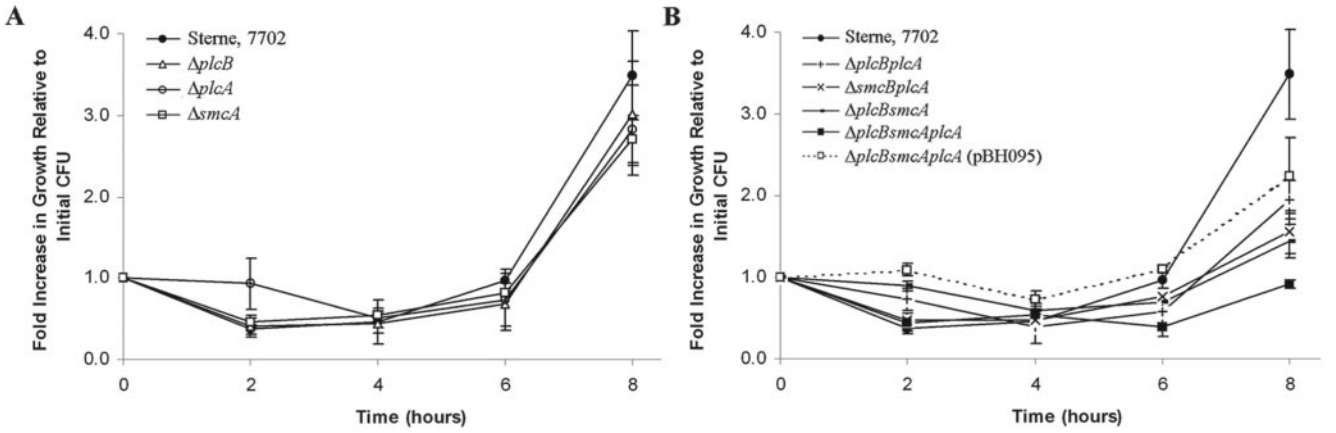

FIG. 2. Functional cooperation between *B. anthracis* PLCs is important for growth and/or survival in BMM. BMM obtained from BALB/c mice were challenged with *B. anthracis* endospores at an input MOI of 10:1 as described in Materials and Methods. Cell-associated growth was determined every 2 h for 8 h after gentamicin treatment. Changes in cell-associated growth were normalized to viable counts at the 0-h time point. Experiments were done in triplicate three separate times, and the average of three representative experiments are depicted here with the standard deviation. The differences in the fold increase in growth at 8 h after gentamicin treatment between the Δ*plcBsmcA*, Δ*plcBplcA*, and Δ*smcAplcA* strains compared to the Sterne 7702 strain were significant ($P < 0.02$ as determined by an unpaired two-tailed *t* test). The differences between the Δ*plcBsmcAplcA* strain compared to the Sterne 7702, Δ*plcB*, Δ*smcA*, Δ*plcA*, Δ*plcBsmcA*, Δ*plcBplcA*, and Δ*smcAplcA* strains were significant ($P < 0.02$ as determined by an unpaired two-tailed *t* test at 8 h).

the later time point (Fig. 2B and 4). The ability to grow and cause cytolysis was restored after complementation of the PLC genes in *trans* (Fig. 2B and 3). Since each strain germinated and was phagocytosed to a similar extent (Table 4 and data not shown), the differences seen in terms of macrophage survival can be attributed to the PLC activities. Altogether, these results indicate that the PLCs work in cooperation to resist the bactericidal effects of the phagocyte and facilitate growth of *B. anthracis* in association with BMM.

DISCUSSION

Anthrax is a disease that proceeds through discrete stages. While *B. anthracis* is considered an extracellular pathogen at later stages of anthrax, its brief intracellular phase during the establishment stage of an infection is believed to be critical. The results presented in this study identified three PLCs as virulence factors that work together to contribute to the pathogenesis of *B. anthracis* and, specifically, implicate their activities as important during the early-stage interactions with the macrophage.

TABLE 5. Distribution of bacilli per macrophage during *B. anthracis* infections

Strain	Avg no. of bacilli/ macrophage ± SEM[a]	No. of infected macrophages containing[b]:				
		1 to 5 bacilli	6 to 15 bacilli	16 to 30 bacilli	31 to 50 bacilli	>50 bacilli
Sterne 7702	19 ± 2.8	15	14	12	4	5
Δ*smcA*	15 ± 2.4	16	15	14	3	2
Δ*plcA*	14 ± 1.7	16	16	13	4	1
Δ*plcB*	14 ± 2.3	17	19	8	4	2
Δ*smcAplcA*	12 ± 1.2*	18	19	10	3	0
Δ*plcBsmcA*	11 ± 1.4*	20	17	10	3	0
Δ*plcBplcA*	11 ± 1.0*	18	17	14	1	0
Δ*plcBsmcAplcA*	5 ± 0.6†	33	17	0	0	0

[a] *, $P < 0.05$ compared to Sterne 7702 as determined by an unpaired two-tailed *t* test; †, $P < 0.02$ compared to Sterne 7702, Δ*smcA*, Δ*plcA*, Δ*plcB*, Δ*smcAplcA*, Δ*plcBsmcA*, and Δ*plcBplcA* as determined by the unpaired two-tailed *t* test.

[b] A total of 50 macrophages containing bacilli were counted for each strain at 8 h after gentamicin treatment.

PC-PLC, SMase, and PI-PLC activity was detected on assay plates after 36 h and was limited to the area directly beneath the colonies, indicating that PLC expression was weak under the conditions tested (Fig. 1). In *B. anthracis*, the *plcR* gene has a point mutation that results in a 73-amino-acid truncation in PlcR, which may explain the muted expression of the PLC genes. Indeed, increased PLC activity is observed following ectopic expression of a full-length *plcR* gene from *B. thuringiensis* (20). Further evidence that the truncated PlcR is nonfunctional is presented in this study. The deletion of the *plcR* gene did not affect PLC expression or the virulence of *B. anthracis* in a murine model of anthrax (Fig. 1 and Table 3). Expression of the PLCs was also not dependent on the presence of the pXO1 virulence plasmid and therefore, presumably, on the regulation by AtxA (Fig. 1). Consistent with these findings was that survival and growth in association with the macrophage was reported as an AtxA-independent process (4).

Functional redundancy and synergy between PLCs is not unique to *B. anthracis* pathogenesis. Lysis of human erythrocytes requires the combined efforts of *B. cereus* PC-PLC and SMase (7). During murine challenges with *L. monocytogenes* PLC-null strains, the redundant nature of the PLCs is apparent. Deletions of either *L. monocytogenes plcA* or *plcB* alone resulted in a 2- to 10-fold increase in LD_{50}, while disruption of both rendered the bacterium 500-fold less virulent (2, 32). In *B. anthracis*, we found that one functional PLC was sufficient to retain virulence in a murine model of anthrax (Table 3). Only after all three PLC genes were disrupted was there a moderate decrease in virulence (Table 3).

Anthrax LeTx, present in all PLC-null strains tested, is a major virulence determinant of *B. anthracis* that could poten-

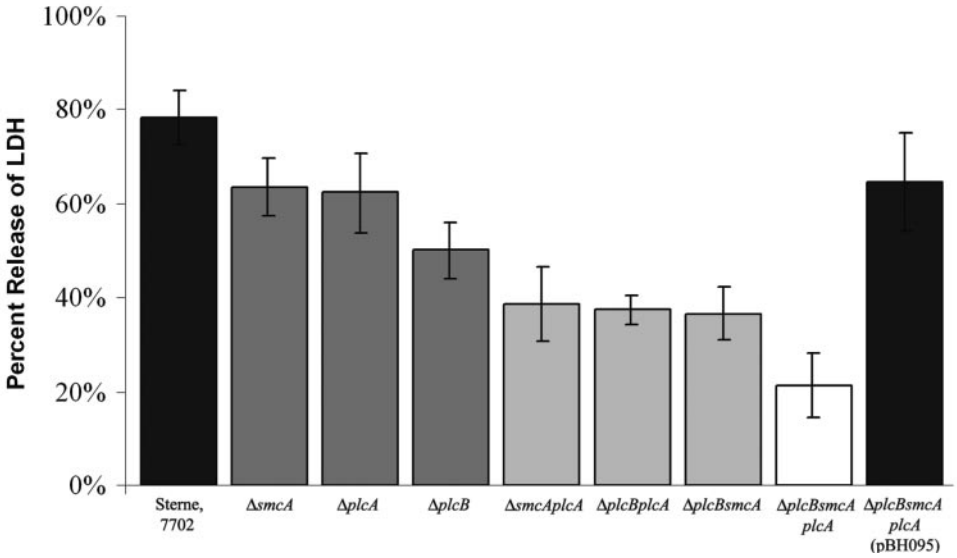

FIG. 3. *B. anthracis* PLC-null strains are less cytotoxic to macrophages. BMM were challenged with *B. anthracis* endospores at an input MOI of 10:1 as described in Materials and Methods. Supernatants were collected at 8 h after gentamicin treatment and assayed for cytotoxicity by scoring for the release of the cytoplasmic LDH. Experiments were done in triplicate, and the average of three representative experiments are depicted here with the standard deviation. The differences between Δ*plcBsmcA*, Δ*plcBplcA*, and Δ*smcAplcA* strains compared to the Sterne 7702 strain were significant ($P < 0.02$ as determined by an unpaired two-tailed *t* test). The differences between the Δ*plcBsmcAplcA* strain compared to the Sterne 7702, Δ*plcB*, Δ*smcA*, Δ*plcA*, Δ*plcBsmcA*, Δ*plcBplcA*, and Δ*smcAplcA* strains were significant ($P < 0.05$ as determined by an unpaired two-tailed *t* test).

tially be working in conjunction with the PLCs during anthrax infections. To address this possibility, all three PLC genes were disrupted in a lethal factor-null strain and scored for virulence. A 3-log increase in LD_{50} was observed in mice infected with the Δ*lef* strain relative to the isogenic Sterne strain (Table 3). This is comparable to previous reports (23) and highlights the importance of this toxin during Sterne infections of mice. A log increase in LD_{50} was observed in both the Sterne and the isogenic Δ*lef* background after disruption of *plcB*, *smcA*, and *plcA* (Table 3). The additive decrease in virulence following the disruption of these factors suggests that *B. anthracis* might utilize the PLCs and the LeTx separately during infections.

Cooperation between the PLCs was also evident in the in vitro macrophage model of infection. Single PLC-null strains had growth kinetics similar to those of the parental strain and displayed only a slight reduction in cytotoxicity, while strains lacking any two of the three PLCs showed intermediate phenotypes in terms of growth and cytolysis (Fig. 2 and 3). Disruption of all three PLC genes, however, resulted in pronounced differences in both bacterial growth in association with the macrophages and the ability to cause host cell death (Fig. 2B and 3). The growth of the Δ*plcBsmcAplcA* strain at the later time points, although limited, suggests that *B. anthracis* maintains still other means to survive and replicate in associ-

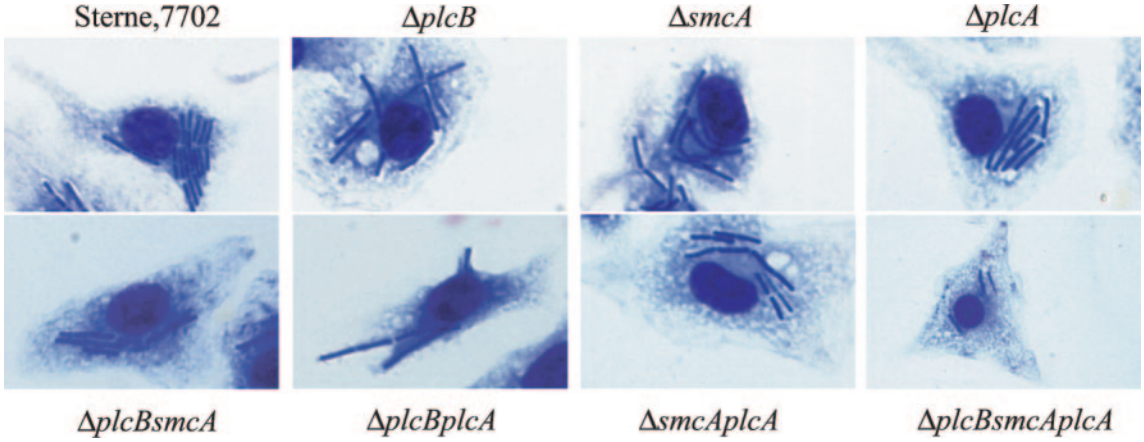

FIG. 4. PLC activity aids the growth of *B. anthracis* in association with the macrophage. BMM were challenged with *B. anthracis* endospores as described in Materials and Methods, except that infections were performed in Lab Tek II chamber slides. Slides were stained at 8 h after gentamicin treatment with Wright-Giemsa-like technique and visualized by light microscopy at ×1,000 magnification. Photomicrographs of representative fields are shown.

ation with the macrophage even in the absence of the PLCs. Recently, an orthologue of the *L. monocytogenes* pore-forming cytolysin LLO was characterized in *B. anthracis* termed anthrolysin O (31), which, in addition to other membrane active toxins encoded by the *B. anthracis* genome, may contribute to macrophage-associated survival and replication.

The growth defect observed for the triple PLC-null strain during macrophage challenges may be attributed to a defect in escape from phagocytic vacuoles after *B. anthracis* phagocytosis by the macrophage. PC-PLC, PI-PLC, and SMase activities have been shown to aid in the escape of *L. monocytogenes* and *L. ivanovii* from phagocytic vacuoles during tissue culture infections (10, 32). However, the PLCs may also be important at other points in the infection. Recently, PI-PLC was shown to decrease the ability of dendritic cells (DC) to respond to TLR ligand stimulation, thus downmodulating the immune system (44). PLC ability to interact with host cell immunity could be an important contribution to the disease at all stages of infection, but this awaits further investigation.

To summarize, *B. anthracis* produces three PLCs that are redundant in their ability to aid in macrophage-associated cell growth and cause lethality in mice. Deletion of *plcB*, *smcA*, and *plcA* was required for attenuation of *B. anthracis* in an inhalation anthrax model. Infections with cultured BMM indicate that the attenuation in mice may be attributed to a growth deficiency of the Δ*plcBsmcAplcA* strain in association with macrophages. We were able to complement this defect in both macrophage and murine challenges. The functional redundancy between PLCs implies that survival in association with the macrophage is critical for *B. anthracis* pathogenesis. Further understanding of the interactions between *B. anthracis* and its host could lead to better ways to treat the disease.

ACKNOWLEDGMENTS

This study was supported in part by HHS contract N266200400059C/N01-AI-40059, by NIH grant AI08649, and by the Great Lakes and the Southeast Regional Centers of Excellence for Biodefense and Emerging Infections.

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Editor: D. L. Burns