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Lipoprotein biosynthesis by prolipoprotein diacylglyceryl transferase is required for efficient spore germination and full virulence of *Bacillus anthracis*

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Abstract

Bacterial lipoproteins play a crucial role in virulence in some Gram-positive bacteria. However, the role of lipoprotein biosynthesis in *Bacillus anthracis* is unknown. We created a *B. anthracis* mutant strain altered in lipoproteins by deleting the *lgt* gene encoding the enzyme prolipoprotein diacylglyceryl transferase, which attaches the lipid anchor to prolipoproteins. ^{14}C-palmitate labeling confirmed that the mutant strain lacked lipoproteins, and hydrocarbon partitioning showed it to have decreased surface hydrophobicity. The anthrax toxin proteins were secreted from the mutant strain at nearly the same levels as from the wild-type strain. The TLR2-dependent TNF-α response of macrophages to heat-killed *lgt* mutant bacteria was reduced. Spores of the *lgt* mutant germinated inefficiently *in vitro* and in mouse skin. As a result, in a murine subcutaneous infection model, *lgt* mutant spores had markedly attenuated virulence. In contrast, vegetative cells of the *lgt* mutant were as virulent as those of the wild-type strain. Thus, lipoprotein biosynthesis in *B. anthracis* is required for full virulence in a murine infection model.

Keywords

bacterial lipoprotein; prolipoprotein diacylglyceryl transferase; anthrax; *Bacillus anthracis*; spores; germination; Toll-like receptor 2; lgt

Introduction

Bacillus anthracis is a Gram-positive, rod-shaped bacterium and the causative agent of anthrax. *B. anthracis* undergoes a developmental life cycle, alternating between two distinct forms, spores and vegetative cells. Spores are metabolically dormant and can survive for long periods under harsh conditions. However, once they gain access to an animal host, the spores germinate and grow out as vegetative bacteria (Setlow, 2003). Vegetative cells secrete several virulence factors, most prominently the anthrax toxins, which are composed of the three proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF). These proteins combine to form lethal toxin (LT, the combination of PA and LF) and edema toxin (ET, the combination of PA and EF) (Leppla, 2006; Young and Collier, 2007). The toxins individually and cooperatively attenuate the host innate immune system, allowing massive bacteremia and a resulting toxemia that rapidly kills the host (for reviews see (Moayeri and Leppla, 2009; Moayeri and Leppla, 2011)).

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Microbial lipoproteins are expressed on the bacterial cell surface and have important functions in the growth and survival of bacteria. These include substrate binding for ABC transport systems, processing of exported proteins, sporulation, and germination (Kontinen and Sarvas, 1993; Khandavilli *et al.*, 2008; Igarashi *et al.*, 2004; Deka *et al.*, 2006; Dartois *et al.*, 1997; Hutchings *et al.*, 2009), as recently reviewed (Kovacs-Simon *et al.*, 2011). Microbial lipoproteins are synthesized as precursors carrying a conserved sequence termed a "lipobox" at the C-terminus of the signal peptide. A diacylglyceryl moiety is transferred to the cysteine residue within the lipobox by lipoprotein diacylglyceryl transferase (Lgt), and the signal peptide of the prolipoprotein is then cleaved by the signal peptidase (Tokunaga *et al.*, 1982; Hayashi *et al.*, 1985). In Gram-negative bacteria, lipoproteins are further modified by N-acyltransferase (Lnt), which transfers an N-acyl group to the diacyl-glyceryl cysteine, yielding mature triacylated lipoproteins, which are then often transferred to the outer membrane (Robichon *et al.*, 2005). Although an equivalent enzyme has not been found in Gram-positive bacteria, some N-acylation of lipoproteins was reported to occur in *Bacillus subtilis* and *Staphylococcus aureus* (Hayashi *et al.*, 1985; Navarre *et al.*, 1996). The lipoprotein biosynthetic pathway is essential for growth of Gram-negative bacteria, but is dispensable for growth of Gram-positive bacteria such as *B. subtilis*, *S. aureus*, and *Streptococcus pneumoniae* (Stoll *et al.*, 2005; Petit *et al.*, 2001; Leskela *et al.*, 1999).

In an infected animal host, microbial lipoproteins are recognized by Toll-like receptors (TLRs), which play a central role in the innate immune system by sensing pathogen-associated molecular patterns (Akira *et al.*, 2006). MyD88 is a crucial adaptor protein in TLR signal transduction except that occurring through TLR3. MyD88-deficient mice have increased susceptibility to *B. anthracis* (Okugawa *et al.*, 2011). TLR2, one of the TLR family proteins, is the primary receptor recognizing diacylated and triacylated lipoproteins and leading to induction of cytokine and chemokine synthesis (Takeuchi *et al.*, 2002; Takeuchi *et al.*, 2001). Inactivation of *lgt* eliminates lipoproteins and allows bacteria to escape from TLR recognition. As a result Lgt deficiency in bacteria such as *S. aureus* and *Streptococcus agalactiae* produces a hypervirulent phenotype in mouse infection models (Henneke *et al.*, 2008; Bubeck Wardenburg *et al.*, 2006). In contrast, Lgt-deficient mutants of *Listeria monocytogenes* and *S. pneumoniae* are attenuated in virulence (Petit *et al.*, 2001; Baumgartner *et al.*, 2007). Thus, microbial lipoproteins appear to have species-specific roles in virulence.

In this study, we investigated the role of lipoproteins in *B. anthracis*. We constructed a *B. anthracis lgt* mutant that was unable to carry out lipid modification of prelipoproteins and used this to investigate the role of lipoprotein biosynthesis in *B. anthracis*.

Results

Identification of candidate lipoproteins in *B. anthracis*

The programs ScanProsite and G+LPPv2 identified 145 candidate lipoproteins in the genome of the *B. anthracis* Ames 35 strain. Because the Ames strain sequence does not include the *B. anthracis* pXO1 and pXO2 plasmid sequences, these sequences were analyzed from the *B. anthracis* Ames Ancestor strain. Three proteins encoded on each of the two plasmids were identified as putative lipoproteins, making a total of 151 candidates. The program LipoP scored 138 of the 151 candidates as lipoproteins (Table S1). Forty-one proteins could confidently be assigned a name and function, with most of the rest identified only as putative lipoproteins or ABC transporters.

The construction and complementation of *lgt*-deficient *B. anthracis*

To determine the role of lipoproteins in *B. anthracis*, we constructed an *lgt*-deficient mutant. The *lgt* gene (BA5391 in the Ames strain) was disrupted without polar effects using the Cre-*loxP* system (Pomerantsev *et al.*, 2006) as shown in Fig. 1A. We confirmed by PCR and sequencing that the *lgt* gene was deleted (Fig. 1B). We complemented the *lgt* gene in two ways. The first ("*in situ*") method placed an intact gene back into the chromosome. This was accomplished with the single crossover plasmid pSC, into which the DNA region encompassing BA5389, BA5390, BA5391, and BA5392 was cloned at the multi-cloning site between the *loxP* sites, allowing the insertion of *lgt* (and neighboring genes) into the chromosome (Fig. 1C). Because the region containing BA5389 to BA5392 was reported to be operonic (Passalacqua *et al.*, 2009), we inserted the *lgt* gene along with the putative co-operonic genes to avoid any disruption of transcription of the adjacent genes. After the single-crossover, Cre recombinase treatment was performed to remove the erythromycin resistance gene along with the pSC backbone and duplicated genes. Although we anticipated that a single-crossover would spontaneously remove the second copy of BA5392, it was still duplicated after several passages as was shown by PCR and sequencing (Fig. 1C and D). To exclude the possibility that the duplicated BA5392 affected the phenotype in these *in situ* complemented strains, we also complemented the *lgt* mutation with the plasmid pSW4-*lgt* in which *lgt* transcription is driven by the *pagA* promoter of *B. anthracis*. For each phenotype examined below, the *in situ* complemented strain displayed properties like those of the original wild-type strain.

Lgt is required for lipid modification of prelipoproteins

To determine whether *lgt* performs the expected protein lipidation in *B. anthracis*, we carried out metabolic labeling experiments with [^{14}C]-palmitic acid. Autoradiography of lipoprotein extracts separated by SDS-PAGE revealed several[^{14}C]-labeled protein bands in the samples of the wild-type strain and the *lgt* mutant complemented with either the pSW4-*lgt* plasmid or by *in situ* gene replacement (Fig. 2A and B). In contrast, no labeled proteins were detected in the *lgt* mutant (Fig. 2A and B). The strains having the mutation complemented in either of the two ways showed the same [^{14}C]-labeled proteins as the wild-type strain, suggesting that the duplicated BA5392 gene in the *in situ* complemented mutant did not affect lipoprotein processing. These results demonstrated that *lgt* is required for the lipid modification of prolipoproteins in *B. anthracis*.

Deletion of *lgt* alters bacterial surface hydrophobicity

Bacterial surfaces are composed of hydrophobic and hydrophilic components, and many of them are proteins (Doyle, 2000). We considered whether the loss of surface-associated lipoproteins in the *lgt* mutant would alter bacterial surface hydrophobicity. Thus, to determine surface hydrophobicity for the wild-type, *lgt* mutant, and *in situ* complemented strains, we employed the Microbial Adhesion to Hydrocarbons (MATH) test. This test is based on the adhesion of bacteria to hexadecane in a buffer with high ionic strength that minimizes electrostatic effects (Rosenberg, 1981). As shown in Fig. 3, wild-type and *in situ* complemented *B. anthracis* exhibited higher surface hydrophobicity and readily adhered to hexadecane, evidenced by the lower optical density of the (lower) aqueous phase. In contrast, the *lgt* mutant strain was preferentially located in the aqueous phase. When the assay was performed with spores instead of vegetative bacteria, no differences in surface hydrophobicity was found (data not shown), indicating that the hydrophobicity of the spore surface was not altered. Taken together, these results show that the surface of vegetative bacteria of the *lgt* mutant is more hydrophilic than that of the wild-type or *in situ* complemented strains.

Deletion of *lgt* impairs germination

It was previously reported that inactivation of *lgt* impairs germination in *B. subtilis* (Igarashi *et al*., 2004). To test whether the same is true for *B. anthracis*, we assessed germination by measuring optical density at 600 nm and the loss of heat resistance. The *lgt* mutant showed significantly decreased germination efficiency in both assays (Fig. 4A and B), when compared to the fully germination-proficient *in situ* complemented mutant and wild-type strains. These data show that lipoproteins are needed for efficient spore germination in *B. anthracis*. Once germination occurred, both the *lgt* mutant and the complemented strain grew as well as the wild-type strain in BHI medium and modified G medium (data not shown).

Anthrax toxin secretion is not prevented in the *lgt* mutant

The anthrax toxins play a crucial role in virulence toward animal hosts (Moayeri and Leppla, 2011). Because *lgt* mutants of *B. subtilis* were reported to be impaired in protein secretion due to decreased levels of the PrsA lipoprotein chaperone/foldase (Leskela *et al.*, 1999; Kontinen and Sarvas, 1993), we considered the possibility that the lack of *lgt* in *B. anthracis* would prevent anthrax toxin secretion. We found that PA, LF, and EF were secreted from the wild-type, *lgt*$^{-}$, and *in situ* complemented strains grown in BHI medium in air (data not shown) or in NBY medium supplemented with 0.9% Na_2HCO_3 in 9% CO_2 (Fig. 5). (NBY medium was used because it appears to mimic the induction of toxin secretion by Na_2HCO_3/CO_2 that occurs in a host which is infected with *B. anthracis* (Bartkus and Leppla, 1989; Chitlaru *et al*., 2006).) Quantitation of the band intensities showed no significant differences in the amounts of PA, EF, and LF in supernatants derived from the *lgt* mutant compared to the parental and complemented strains (Fig. 5). Thus, *lgt* deficiency appears not to effect anthrax toxin secretion or stability in *B. anthracis in vitro.*

B. anthracis lacking *lgt* is a less potent activator of the macrophage inflammatory response

The lack of *lgt* in several Gram-positive pathogens was reported to attenuate host immune responses (Bubeck Wardenburg *et al.*, 2006; Henneke *et al*., 2008). To determine whether *lgt* deficiency in *B. anthracis* affects immune responses, we measured TNF-α production in macrophages exposed to heat-killed bacteria of the wild-type, *lg*t mutant, and *in situ* complemented strains. We compared the cytokine responses of macrophages from wild-type C57BL/6J, TLR2, and MyD88-deficient mice (Fig. 6A). The Lgt-deficient bacteria caused significantly less TNF-α induction in the macrophages from C57BL/6J strains than did wild-type or complemented bacteria. A significant portion of the TNF-α response induced by wild-type and *in situ* complemented bacteria was mediated by TLR2. Other signaling pathways also contributed to the TNF-α response, as the lower cytokine response induced by the *lgt* mutant was not TLR2 dependent (Fig. 6A). As expected, none of the bacterial strains induced a cytokine response in macrophages from MyD88-deficient mice (Fig. 6A).

Next, we asked whether lipoproteins of *B. anthracis* induce a TNF-α response through TLR2-mediated immune activation, as occurs with lipoproteins of other Gram-positive bacteria such as *S. aureus* and *S. pneumoniae*. We isolated lipoprotein-enriched fractions from the wild-type strain using Triton X-114 (TX-114) detergent extraction and partition procedures, as described in Experimental Procedures. Coomassie blue-stained gels showed several bands in the TX-114 fraction (Fig. 6B). The lipoprotein samples strongly activated TNF-α production from C57BL/6J macrophages, but had little effect on macrophages from TLR2-deficient mice. To address the concern that the TX-114 fraction may contain other bacterial components such as DNA and peptidoglycan that may activate macrophages, the TX-114 fraction was treated with lipoprotein lipase to inactivate the lipoproteins. Lipoprotein lipase was reported to eliminate the ability of lipoproteins samples to activate

via TLR2 (Shimizu *et al.*, 2005). This treatment significantly reduced the TNF-α response of the wild-type macrophages but did not affect the response of the TLR2-deficient macrophages (Fig. 6C). We confirmed that the decrease in cytokine production was not due to macrophage cell death by measuring viability (data not shown). These results indicate that lipoproteins of *B. anthracis* play a role in inducing inflammatory cytokines, acting at least in part through TLR2.

Lipoprotein biogenesis is required for efficient germination and virulence *in vivo*

To investigate the contribution of lipoproteins to *B. anthracis* virulence, we challenged two strains of mice. Complement-deficient A/J mice are highly susceptible to non-encapsulated toxigenic *B. anthracis* (Loving *et al.*, 2007), whereas complement-sufficient C57BL/6J mice require much higher doses of bacteria or spores for infection (Moayeri *et al.*, 2010). Mice were infected with wild-type, *lgt*$^{-}$, and *in situ* complemented strain spores or vegetative cells at 1 × LD_{100} doses for the particular mouse strain. Both A/J and C57BL/6J mice infected with the *lgt* mutant spores had significantly decreased mortality compared to mice infected with the wild-type strain (Fig. 7A and C). In contrast, no differences between strains were seen when vegetative bacteria were used (Fig. 7B and D). Complementation with the *lgt* gene restored virulence (Fig. 7A–7D). These results suggested that the reduced virulence of the *lgt* mutant strains results from their impaired germination.

To address in another animal model the question of whether the lgt mutant is impaired for germination *in vivo*, we measured edema at early times after spore injection, which we previously showed was representative of the outgrowth of vegetative bacteria (Moayeri *et al.*, 2010). A/J mice were infected with equal numbers of *lgt* mutant or *in situ* complemented spores in the foreleg in a small volume (50 μl). ET-mediated local edema was quantitated by measuring anterior to posterior and sagittal dorsal/ventral dimensions at various times post-infection. Even at the earliest (3-h) measurement, the *lgt* mutant induced significantly less edema than the complemented strain (Fig. 8). This difference persisted through the 6- and 24-h measurements (Fig. 8).

For a more direct quantification of the *lgt* mutant's germination ability, we assessed bacterial growth and survival (as colony forming units (CFU)) in mouse skin and internal organs. Skin was homogenized in a germination-prohibitive buffer 6 h after subcutaneous (SC) injection of spores. Plating of the homogenates before and after heat treatment (to kill vegetative bacteria) allowed enumeration of ungerminated spores and vegetative bacteria. In parallel, intravenous (IV) injection of spores provided an alternative *in vivo* environment for germination and rapid delivery to organ sites. Spleens and livers from mice were pooled and processed together to quantify vegetative bacteria vs. total bacteria (ungerminated spores + vegetative bacteria). Results from these studies are presented in Fig. 9A (skin) and Fig. 9B (liver + spleen). While the wild-type and complemented strain bacteria were present as vegetative bacteria and spores in both skin and the internal organs, the *lgt* mutant appeared to be present only as spores, reflecting little to no germination. Interestingly, the total number of bacteria isolated from the skin of mice injected with the *lgt* mutant was consistently lower than from those injected with the wild-type and the complemented mutant by 6 h (Fig. 9A). This could be due in part to a more efficient ingestion and killing of the *lgt*- vegetative bacteria, present in smaller numbers due to slower spore germination, and their subsequent inability to produce sufficient anthrax toxins to inactivate immune cells. This explanation is consistent with our recent demonstration that germination and the resultant production of anthrax toxin is required for effective evasion of myeloid cell-mediated killing of *B. anthracis* (Liu *et al.*, 2010).

Alternatively, the lower total number of bacteria may be due to inefficient germination of the *lgt* mutant spores retrieved from the animals. However it is clear that almost all the

bacteria retrieved from mice injected with the mutant were spores, and analyses of skin sections from mice injected with spores also showed a higher number of ungerminated *lgt* mutant spores compared to the complemented strain spores (Fig. 9C). Altogether, these data verify that *lgt* deficiency leads to an attenuation of virulence primarily through impaired *in vivo* germination, with a possible consequence of higher efficiency of killing by the innate immune response.

Discussion

B. anthracis, like other Gram-positive bacteria, is predicted from genomic sequence analysis to have a large number of cell surface-associated lipoproteins (Hutchings *et al.*, 2009), but these have not been studied as a group. In fact, no putative *B. anthracis* lipoproteins appear to have experimentally been localized to the cell or spore membranes through a lipid anchor. Several proteins that are expected to be anchored in the cell wall by lipid modifications are implicated as playing important roles in normal physiology and/or pathogenesis. Examples include the manganese ABC transporter MntA (BA3189) (Gat *et al.*, 2005), the polyglutamic capsule anchoring protein CapD (pXO2 BA0063) (Richter *et al.*, 2009), the InhA protease (BA0672) (Chung *et al.*, 2009), and the three PrsA chaperone/foldases (BA1041, 1169, and 2336) (Kontinen and Sarvas, 1993). (See also Table S1 and comments below.)

To further understand the role of lipoproteins in *B. anthracis*, we began by screening databases using bioinformatic tools. We used ScanProsite with the improved G+Lppv2 motif, followed by LipoP. G+LPPv2 was reported to have 100% sensitivity to 90 experimentally verified Gram-positive bacterial lipoproteins whereas LipoP has a higher specificity and ability to discriminate against false positives than G+LPPv2 (Rahman *et al.*, 2010). This analysis identified 138 proteins as putative lipoproteins (Table S1), indicating that lipoproteins represent about 2.5% of the proteome. This percentage is similar to that in the proteomes of other Gram-positive bacteria (Sutcliffe and Harrington, 2002; Babu *et al.*, 2006). The largest group of the candidate lipoproteins in *B. anthracis* consists of substrate-binding proteins of ABC transporter uptake systems. The next largest group comprises proteins involved in sensing germinants and triggering germination. This analysis helped to suggest possible phenotypic changes that might be observed in bacteria deficient in lipoprotein biosynthesis.

We disrupted the *lgt* gene using the previously reported Cre-*loxP* system (Pomerantsev *et al.*, 2009). To verify that the phenotypes described here are due to the *lgt* mutation, it was essential to complement the mutation. The *in situ* complementation method used here took advantage of the residual *loxP* site generated during gene deletion (Fig. 1A). This method also allowed complementation to be done without having to maintain a plasmid under selective pressure. The strain complemented in this way retained two copies of the gene adjacent to *lgt*, BA5392 (annotated as a bifunctional histidine-containing protein kinase/phosphatase), but this had no detectable effect on any of the phenotypes of *B. anthracis* studied here. The strain complemented with a plasmid expressing *lgt* also behaved in every regard like the wild-type strain.

While *lgt* is essential for the survival of Gram-negative bacteria, it is dispensable in Gram-positive bacteria, as shown for *S. aureus* and *S. pneumoniae* (Stoll *et al.*, 2005; Petit *et al.*, 2001). In *B. anthracis*, the *lgt* mutant grows as well as the wild-type strain in either nutrient-rich medium (BHI medium) or poor-nutrient medium (e.g., modified G medium) (data not shown). This is somewhat surprising given the large number of ABC transporters that are lipoproteins. ABC transporters mediate the uptake of many nutrients, such as peptides, amino acids, metals, and vitamins (Davidson *et al.*, 2008), and the uptake of these materials

could promote growth in nutrient-poor media. However, *B. anthracis* can apparently satisfy its essential requirements through de novo synthesis, or by using other uptake mechanisms that do not involve lipoproteins.

Although *B. subtilis* lacking *lgt* shows impairment of protein secretion (Kontinen and Sarvas, 1993), there were no significant changes in anthrax toxin secretion in the *B. anthracis lgt* mutant under the conditions used here. A secretion defect might have been expected since the three similar putative *B. anthracis* PrsA secretion chaperone/foldases are predicted to be involved in protein secretion and all are lipoproteins (Table S1). In fact, these three *B. anthracis* genes supported modest increases in production of recombinant PA when expressed in *B. subtilis* (Williams *et al.*, 2003).

The largest phenotypic changes we found in the *lgt* mutant involved spore germination. Spore germination plays a crucial role in anthrax disease initiation (Carr *et al.*, 2009) and the ability to germinate efficiently may be viewed as a key virulence factor. In our infection model we found that the *lgt* mutant has reduced virulence, likely caused by its impaired germination. Genomic and genetic analyses have identified five distinct germination operons in *B. anthracis*, *GerH*, *GerK*, *GerL*, *GerS*, and *GerX* (Fisher and Hanna, 2005; Carr *et al.*, 2009). Each operon contains three genes, denoted A, B, and C (e.g., *gerHA*, *gerHB*, *gerHC*, etc.), of which the C gene in each operon encodes a lipoprotein (Table S1). The three proteins encoded by each operon associate to form a receptor that recognizes the specific small molecule germinants such as amino acids and purine nucleosides that initiate germination. In addition, two less well-characterized genes that affect germination, *gerD* (Pelczar *et al.*, 2007) and *gerM* (Rigden and Galperin, 2008), are also predicted to encode lipoproteins (Table S1). The GerD protein is believed to co-localize with the germinant receptor proteins and accelerate their signaling to downstream effectors. Mutation of the cysteine in the lipobox of GerD leads to spores lacking the protein and having a severe germination defect like that of a complete *gerD* deletion (Pelczar *et al.*, 2007; Mongkolthanaruk *et al.*, 2009). Both types of *gerD* mutations therefore accurately phenocopy the *lgt* mutation described here. Although we did not directly demonstrate that specific germinant proteins and pathways are impaired in the *lgt* mutant strain, the key roles that lipoproteins play in the germinant sensing pathways appear sufficient to account for the impairment of germination. It is interesting to note that the first identification of *lgt* in *B. subtilis* came from a search for germination mutants, and the gene was first designated *gerF* (Igarashi *et al.*, 2004), prior to its biochemical role in lipoprotein biosynthesis being recognized.

While lipoproteins were found to be essential in a spore-initiated infection, they were dispensable when vegetative bacteria were inoculated into mice. This retention of virulence was surprising given that up to 150 proteins probably depend on a lipid anchor for correct localization and function, and that *lgt* is an essential gene in Gram-negative bacteria. A possible decrease in protein secretion (affecting proteinaceous virulence factors) might have been expected to decrease virulence (Leskela *et al.*, 1999), but appears to be of little significance in the infection model used here. Furthermore, the apparent ability to dispense with a large number of ABC transporters during growth in animals suggests that this is a rich environment where nutrient acquisition is not limiting.

It should be noted, in considering explanations for the restricted impact of the *lgt* mutation, that the precise effect of Lgt deficiency on the anchoring of specific lipoproteins may vary. The prolipoproteins will be secreted onto the surface of the mutant bacteria, or into the matrix of the peptidoglycan cell wall, and will initially be anchored there by their signal peptides. In the absence of the Lgt enzyme, the prolipoproteins may remain cell surface associated and retain some function. For certain lipoproteins, this residual function may

approach that of the normal, fully-lipidated protein that would be produced in a wild-type bacterium (Hutchings *et al.*, 2009). This may reconcile our finding that the *lgt* mutant retains virulence (Fig. 7) with the report that the deletion of the manganese ABC transporter and lipoprotein MntA (BA3189) severely impairs virulence (Gat *et al.*, 2005). Some insights into this question could come from a comparison of the "surfaceomes" (Desvaux *et al.*, 2006) of the *lgt*$^{-}$ and wild-type strains, as might be achieved by mass spectrometry of protease-released peptides.

The creation of the *lgt* mutant strain also allowed us to examine the role of lipoproteins in host innate immune responses. It was reported that heat-killed *B. anthracis* are recognized by TLR2 (Hughes *et al.*, 2005), but it was not determined which bacterial components induce TLR2 activation. Here we showed that lipoproteins of *B. anthracis* induce TNF-α production in macrophages primarily through the TLR2 pathway. We considered that TLR2 may play a protective role against *B. anthracis* infection because of its recognition of *B. anthracis*. However we found no difference in susceptibility of TLR2-deficient and wild type mice to subcutaneous *B. anthracis* infection (data not shown). This finding was similar previous findings in an inhalational anthrax mouse model (Hughes *et al.*, 2005). Thus, while TLR2 contributes to cytokine responses induced by *B. anthracis*, a protective or detrimental role for the TLR2-dependent innate immune response is not observed in mice.

In conclusion, lipoproteins synthesized and anchored to membranes through the action of the Lgt enzyme are essential for full virulence of *B. anthracis* in spore-induced infections, apparently due to the role of lipoproteins in sensing germinants present in host tissues. Lipoproteins in *B. anthracis* are recognized by TLR2; however, TLR2 activation does not play a crucial role in a mouse infection model. These results expand our understanding the pathogenesis of *B. anthracis* and could aid in developing new therapeutic approaches.

Experimental procedures

Bioinformatic analyses

We used two tools, ScanProsite with G+Lppv2 pattern and LipoP (Rahman 2008). First, protein sequence databases (UniprotKB; Taxonomy identifier 1392 *B. anthracis*) were screened using ScanProsite and the G+LPPv2 pattern ([MV]-X(0,13)-[RK]-[DERK](6,20)-[LIVMFESTAGPC]-[LVIAMFTG]-[IVMSTAGCP]-[AGS]-C). Second, proteins identified by G+LPPv2 were validated by the Hidden-Markov-Model-based tool LipoP that screens for type II signal peptidase sequences. LipoP can eliminate potential false-positives. Sequences selected by LipoP were considered as putative lipoproteins. Finally, the genes of the Ames strain which we used in this study were selected, because both Ames and Sterne strain data exist in the protein sequence database. For the screening of the pXO1 and pXO2 plasmids, *B. anthracis* Ames Ancestor strain protein sequence databases were used, because *B. anthracis* Ames strain databases does not include plasmid sequences.

Bacterial strains, growth condition and spore preparation

The bacterial strains and plasmids used in this study are listed in Table S2. *E. coli* was grown at 37°C with 225 rpm shaking in Luria Burtani broth. *B. anthracis* was grown at 37°C with shaking at 225 rpm in Brain Heart Infusion (BHI) broth (Difco). SOC medium (Quality Biologicals) was used for outgrowth of transformation mixtures prior to plating on selective medium to isolate transformants. When required, media were supplemented with antibiotics as follows: 100 μg ml^{-1} ampicillin, 20 μg ml^{-1} erythromycin, 20 μg ml^{-1} kanamycin, and 150 μg ml^{-1} spectinomycin.

Spores for germination assay and murine challenges were prepared by a previously reported method with some modifications (Hu *et al.*, 2006). Briefly, bacteria were grown at 30°C for

1 day and at room temperature for 2 days on Schaeffer's sporulation agar, and spores were collected with ice-cold distilled water. Residual vegetative cells were killed by heat at 65°C for 30 min. To remove vegetative cell debris, the spores were washed multiple times with ice-cold distilled water. Spore purity was assessed under a phase contrast microscope and was greater than 99%. Spore quantification was performed using a Petroff-Hausser counting chamber. Vegetative cells for murine challenge were grown at 37°C overnight in BHI broth, diluted 100-fold in fresh broth, and incubated with shaking at 37°C until A_{600} of 0.4–0.6 (early log phase). Cells were washed and diluted with phosphate buffered saline (PBS). Cell quantification was performed using a Petroff-Hausser counting chamber and confirmed by colony forming count on BHI agar.

DNA isolation and manipulation

Preparation of plasmid DNA from *E. coli*, transformation of *E.coli*, and recombinant DNA techniques were carried out by standard procedures (Sambrook and Russell, 2001). Recombinant plasmid construction was carried out in *E. coli* TOP10 (Invitrogen). Chromosomal DNA from *B. anthracis* was isolated with the Wizard genomic purification kit (Promega) in accordance with the protocol for isolation of genomic DNA from Gram-positive bacteria. *B. anthracis* was electroporated with unmethylated plasmid DNA isolated from *E.coli* SCS110 (*dam*$^{-}$ d*cm*$^{-}$, Stratagene). Electroporation-competent *B. anthracis* cells were prepared and transformed as previously described (Park and Leppla, 2000).

Deletion of the *lgt* gene

The primers used in this study are listed in Table S2. The Cre-*lox*P-generated deletion method was previously described (Pomerantsev *et al.*, 2009). Briefly, to delete the *lgt* gene, we amplified an upstream fragment of the *lgt* gene using the forward primer LLF containing an EcoRI site and the reverse primer LLR containing a SpeI site. The downstream fragment was amplified with the forward primer LRF containing an EcoRI site and the reverse primer LRR containing a SpeI site. These fragments were separately cloned into the temperature-sensitive pSC plasmid between *loxP* sites. pSC containing the upstream fragment was transformed into *B. anthracis* Ames 35, which was then grown at the restrictive temperature. Cre-mediated recombination was achieved by the transforming the strain with pCrePAS. The same process was repeated for the downstream fragment. After Cre-*loxP*-generated deletion, 774 bp of the 813-bp *lgt* gene were removed. Deletion of *lgt* was confirmed by PCR using forward primer BA5391genoF and reverse primer BA5391genoR, and also by sequence analysis.

Complementation of the *lgt* gene

For plasmid-based complementation of the *lgt* mutant, the *lgt* gene was cloned into plasmid pSW4 behind the *pag*A promoter. The *lgt* gene was amplified from *B. anthracis* Ames 35 strain genomic DNA by PCR using the forward primers BA5391F containing an NdeI site and the reverse primer BA5391R containing a BamHI site. Plasmid pSW4-*lgt* was identified by restriction analysis and sequencing and was transformed into the *lgt* mutant by electroporation, with selection for kanamycin resistance. For *in situ* complementation of the *lgt* gene in the genome of the *lgt*-deficient mutant, a 3413-bp fragment including BA5389 to BA5392 was amplified by PCR using the forward primer BA5389–5392F containing a PstI site and the reverse primer BA5389–5392R containing a BamHI site. The PCR product was cut with PstI and BamHI (New England Biolabs) and the fragment ligated into pSC with T4 DNA ligase (Invitrogen). Erythromycin-resistant colonies obtained by transformation with this pSC plasmid and grown at 37°C were presumed to have undergone a single crossover. The erythromycin resistance gene along with the pSC backbone and duplicated genes between the *loxP* sites were removed by Cre-mediated recombination after transforming the strain with pCrePAS at 30°C. Both pCrePAS and pSC were eliminated by growth at 37°C.

Complementation of genes was confirmed by PCR using the forward primer BA5392-BA5392F and the reverse primer BA5392-BA5392R for the junction between BA5392 and BA5392, the forward primer BA5393-BA5392F and the reverse primer BA5392-BA5392R for the junction between BA5393 and BA5392, and the forward primer BA5392-BA5391F and the reverse primer BA5392-BA5391R for the junction between BA5392 and BA5391.

Metabolic labeling of lipoproteins with [^{14}C] palmitic acid

[^{14}C] palmitic acid (MP Biomedicals) was dried under a nitrogen stream and redissolved in 0.2% Tween 80. To an exponential phase culture in BHI medium, 4 μCi [^{14}C] palmitic acid ml^{-1} was added. The bacteria were cultivated with shaking for 4 h at 37°C. The cells were disrupted by 0.1 mm diameter glass beads (Next Advance, Averill Park, NY). Lysed suspensions were centrifuged and wereseparated by sodium dodecyl sulfate-polyacrylamide gel electrophoresison a 4 to 20% Tris-glycine gel (Invitrogen) under denaturing conditions. Gels were dried and autoradiographed for 2 weeks.

Measurement of cell surface hydrophobicity

Bacteria were grown in BHI broth at 37°C and 225 rpm to late logarithmic growth phase (A_{600} nm of 4.0). Bacteria were centrifuged and washed twice in PUM buffer (17 g l^{-1} K_2HPO_4, 7.3 g l^{-1} KH_2PO_4, 1.8 g l^{-1} urea, 0.2 g l^{-1} $MgSO_4{\times}7H_2O$, pH 7.1) and resuspended in PUM to 2× the original volume. Bacteria (2 ml) were transferred to glass tubes and overlaid with 1 ml of hexadecane (Sigma-Aldrich), incubated for 10 min at 30°C, and vortexed rigorously for 2 min. The aqueous and hydrocarbon phases were allowed to separate at room temperature for 15 min before the aqueous (lower) phase was carefully removed, and its optical density determined at 400 nm.

Toxin production

B. anthracis was cultured in NBY medium with or without 0.9% Na_2HCO_3 in either air or air supplemented with 9% CO_2 (Chitlaru *et al.*, 2007; Sastalla *et al.*, 2009). Cultures were grown at 37°Cwith shaking at 225 rpm until A_{600} of 2.0 (mid-log phase) and then centrifuged for 10 min at 15000 *g* and filtered using 0.22 μm polyvinylidene fluoride (PVDF) filter units (Millipore). The supernatants were denatured by the addition of 1/5 volume of 6 × sodium dodecyl sulfate (SDS) sample buffer (10% SDS, 500 mM Tris, 0.01% bromophenol blue, 0.6 M dithiothreitol, 30% glycerol, pH 7.5). The samples were boiled for 5 min and equal volumes were resolved using SDS-polyacrylamide gel electrophoresis (4 to 20% Tris-glycine gel, Invitrogen) and blotted to nitrocellulose membranes (Invitrogen). The membranes were probed for the presence of protective antigen (PA), edema factor (EF), and lethal factor (LF) using anti-PA serum (#5308 at a dilution of 1:5000), anti-EF serum (#5900 at a dilution of 1:1000), and anti-LF goat polyclonal antibodies (List Biological Laboratories, Campbell, CA, at 0.5 μg ml^{-1}), respectively. Primary antibodies were detected using IR-dye-conjugated secondary antibodies (Licor Biosciences, 1:5000) and signals were imaged and quantified with the Odyssey Infrared Imaging System (Licor Biosciences, Lincoln, NE).

In vitro germination assay

Spores were activated by incubation at 70°C for 20 min in distilled water. Subsequently, spores were washed with distilled water and resuspended in BHI medium to an A_{600} of ≈0.3 (≈2.0 × 10^7 spores ml^{-1}) and the germination processwas monitored by A_{600}. The A_{600} of the samples were measured every5 min for 120 min. Spores were routinely observed for germination by phase contrast microscopy. To evaluate the loss of heat resistance, spores incubated in BHI medium for 30 min were heated at 65°C for 30 min to kill germinated

cells. Samples taken before and after heat treatment were plated on BHI agar. One hundred percent sensitive to a heat treatment was considered 100% germinated.

Preparation of heat-killed bacteria for TNF-α assays

To generate heat-killed bacteria, vegetative cells were grown at 37°C overnight in BHI broth, diluted 100-fold in fresh broth, and incubated with shaking at 37°C until A_{600} was 0.65 (early-log phase). Thebacterial suspension was washed three times with PBS, resuspended in PBS, and A_{600} for different strains normalized to each other prior to heat inactivation (70°C for 70 min). After a final spin and removal of PBS, the bacterial cell pellet was lyophilized. Lyophilized heat-killed bacteria were weighed and resuspended in PBS prior to addition to bone-marrow-derived macrophages. To confirm that bacteria were killed, heat-killed bacteria were plated on BHI agar and incubated overnight at 37°C.

Extraction of lipoproteins

To extract a fraction containing lipoproteins, exponential phase *B. anthracis* cells at A_{600} of 1.0 were subjected to Triton X-114 (TX-114) phase partitioning (Thakran *et al.*, 2008). Briefly, the cell pellets were resuspended in PBS supplemented with 350 mM NaCl, 2% v/v TX-114 (Sigma-Aldrich), and protease inhibitor cocktail (Complete mini, Roche Diagnostics) and incubated at 4°C for 1 h. Samples were centrifuged at 10000 *g* at 4°C for 30 min and the supernatant was incubated at 37°C for 10 min to induce detergent phase separation. The upper aqueous phase was discarded and replaced with a similar volume of PBS supplemented with 350 mM NaCl. The procedure of phase separation was repeated twice and the final detergent phase was retained.. To remove the detergent, the crude lipoprotein fraction was precipitated at −20°C overnight by addition of 2.5 volumes of methanol. After centrifugation at 10000 *g* and 4°C for 30 min, the pellet was washed with methanol, air-dried, and resuspended in PBS. Protein concentration of the suspension was measured with BCA protein assay (Thermo Scientific Pierce). To digest lipoproteins, 10 μg ml^{-1} lipoprotein lipase from *Pseudomonas* sp. (Sigma-Aldrich) was added to crude lipoproteins and then incubated for 18 h at 37°C. Lipoprotein lipase was inactivated by heating at 72°C for 20 min.

TNF-α assays

Bone marrow-derived macrophages were isolated from C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), TLR2-deficient mice (knockouts on C57BL/6J background, kindly provided by Alan Sher, National Institutes of Health, Bethesda, MD) or MyD88-deficient mice (gift from Shizuko Akira, Osaka University, Osaka, Japan; received via Tod J. Merkel, Food and Drug Administration, Bethesda, MD). Cells were cultured in differentiation medium consisting of 30% L929 cell-conditioned medium in Dulbecco-modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 10 mM HEPES, 50 μg ml^{-1} gentamicin (all obtained from Invitrogen) as previously described (Newman *et al.*, 2009). On day 7, cells were plated in 96-well plates (1×10^5 cells $well^{-1}$). On day 8 cells were treated with 100 μg ml^{-1} heat-killed bacteria for 20 min and expression levels of TNF-α were measured with a TNF-α ELISA kit (R&D Systems, Minneapolis, MN).

Mouse infection studies

A/J or C57BL/6J mice (8- to 12-weeks, female) were purchased from Jackson Laboratories. In survival studies, mice were injected subcutaneously (SC, 200 μl) with spores or vegetative cells (see figure legends for doses), and observed for up to 10 days. For indirect assessment of germination by measurement of edema toxin activity, 5×10^7 spores in 50 μl PBS were injected SC into the foreleg of A/J mice and edema quantified as previously

described (Moayeri *et al*., 2010). Direct measurements of *in vivo* germination were made by completely removing skin and underlying foreleg tissue 6 h after injection of A/J mice (5×10^7 spores, SC). Tissues were immediately frozen in liquid nitrogen and homogenized in 7.5 ml of a germination inhibitory buffer (PBS + 150 μM D-alanine, 200 μM 6-thioguanosine, both from Sigma, St. Louis, MO). Dilution plating of homogenates to assess differential vegetative and spore-based colony forming units (CFUs) was performed both before and after heat treatment (70°C, 70 min). In separate experiments, C57BL/6J mice were injected with spores (1×10^6)by the intravenous (IV) route and spleen and liver from individual mice were harvested, pooled and processed for vegetative and spore-based CFU quantification as described above. For histology, A/J mouse forelegs were injected (1×10^8 spores, 50 μl, SC) and skin and underlying tissue sections were harvested 90 min and 6 h after infection, fixed overnight in 10% formalin followed by repeated 70% ethanol washes. Section preparation, hematoxylin and eosin (H&E) staining, Brown and Hopps tissue Gram stain (for vegetative bacteria), and a modified carboxyl fuchsin stain/malachite green counterstain (for spores) were performed on adjacent serial sections by Histoserv, Inc. (Gaithersburg, MD). For the spore stain, the Accustain Acid Fast Kit (Sigma) was used according to manufacturer's protocol, with a 1-h staining period in heated (steaming) carbol fuchsin solution (0.85% pararosaniline dye, 2.5% phenol, 5% glycerol, 5% DMSO, in deionized water) prior to counterstaining with malachite green (1.5% malachite green oxalate, 10% acetic acid, 17% glycerol in deionized water) for 2 min at room temperature. All protocols using mice were approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

Statistical analysis

All analyses were performed using Graph Pad Prism 5 (Graph Pad System, Inc., San Diego, CA). Statistical tests used for each experiment are listed in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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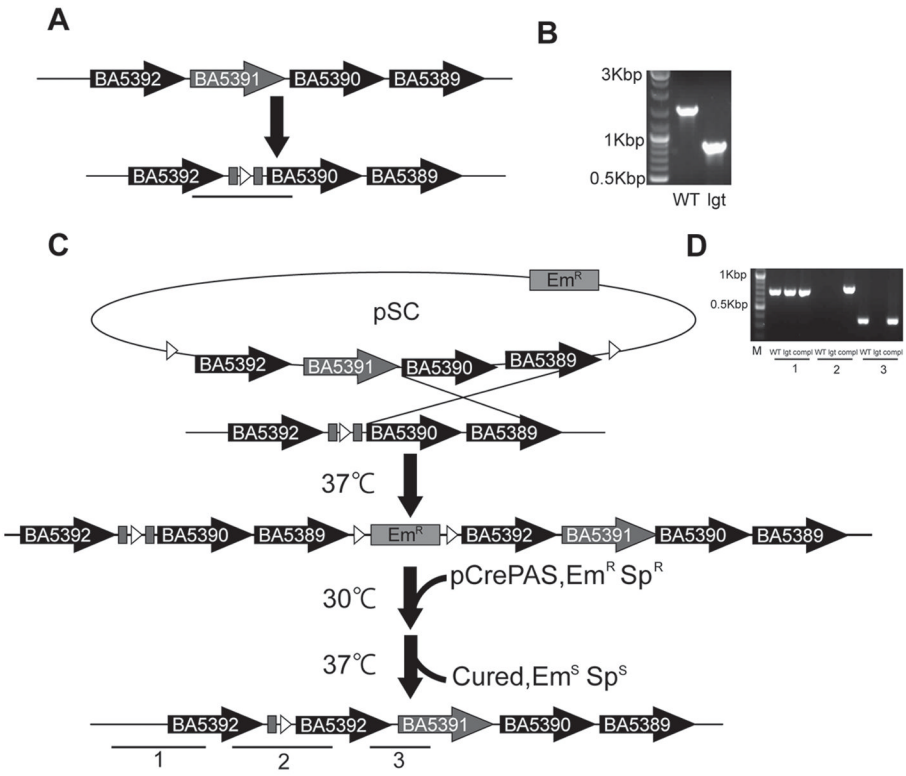

Figure 1.

Deletion of the *lgt* gene and in situ complementation. (A) *lgt* was removed by Cre-loxP system. Underlined region was amplified by PCR to confirm deletion as shown in (B). (B) Ethidium bromide stained agarose gel showing PCR analysis of lgt depletion. Primers were designed to amplify the internal portion between BA5392 and BA5390. W: wild-type *B. anthracis*, lgt: *lgt* mutant. (C) For in site complementation, the pSC plasmid containing BA5392-BA5389 was introduced into the *lgt* knockout mutant, which was then grown at the restrictive temperature. The single-crossover insertion event was selected by the EmR phenotype. Removal of the EmR along with the pSC backbone from the chromosome was achieved by Cre-mediated recombination following transformation with pCrePAS. Growth at 37°C eliminated the pSC and pCrePAS plasmids, resulting in the (re)insertion of BA5391 (*lgt* gene). Underlined regions were amplified by PCR to analyze the resulting strain as shown in (D). (D) Ethidium bromide stained agarose gel showing PCR analysis of the complementation of *lgt* gene (BA5391) and the duplication of BA5392. W: wild-type *B. anthracis*, lgt: *lgt* mutant, compl: in situ complemented mutant.

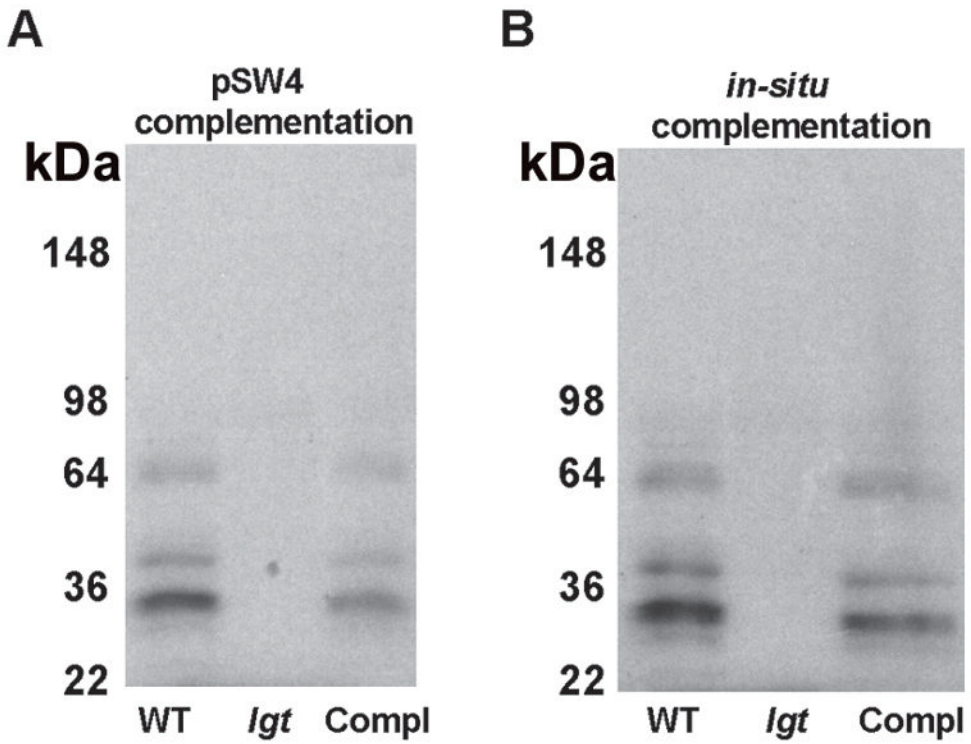

Figure 2.
Lack of lipidation in *lgt* mutant. Cells were cultivated in BHI medium with shaking for 4 h at 37°C. Lipoproteins were labeled with [^{14}C]-palmitic acid and detected by autoradiography. (A) Wild-type strain with empty vector pSW4, *lgt* mutant with empty vector pSW4, and the complemented mutant with pSW4-*lgt*. (B) Wild-type strain, *lgt* mutant, and the *in situ* complemented mutant.

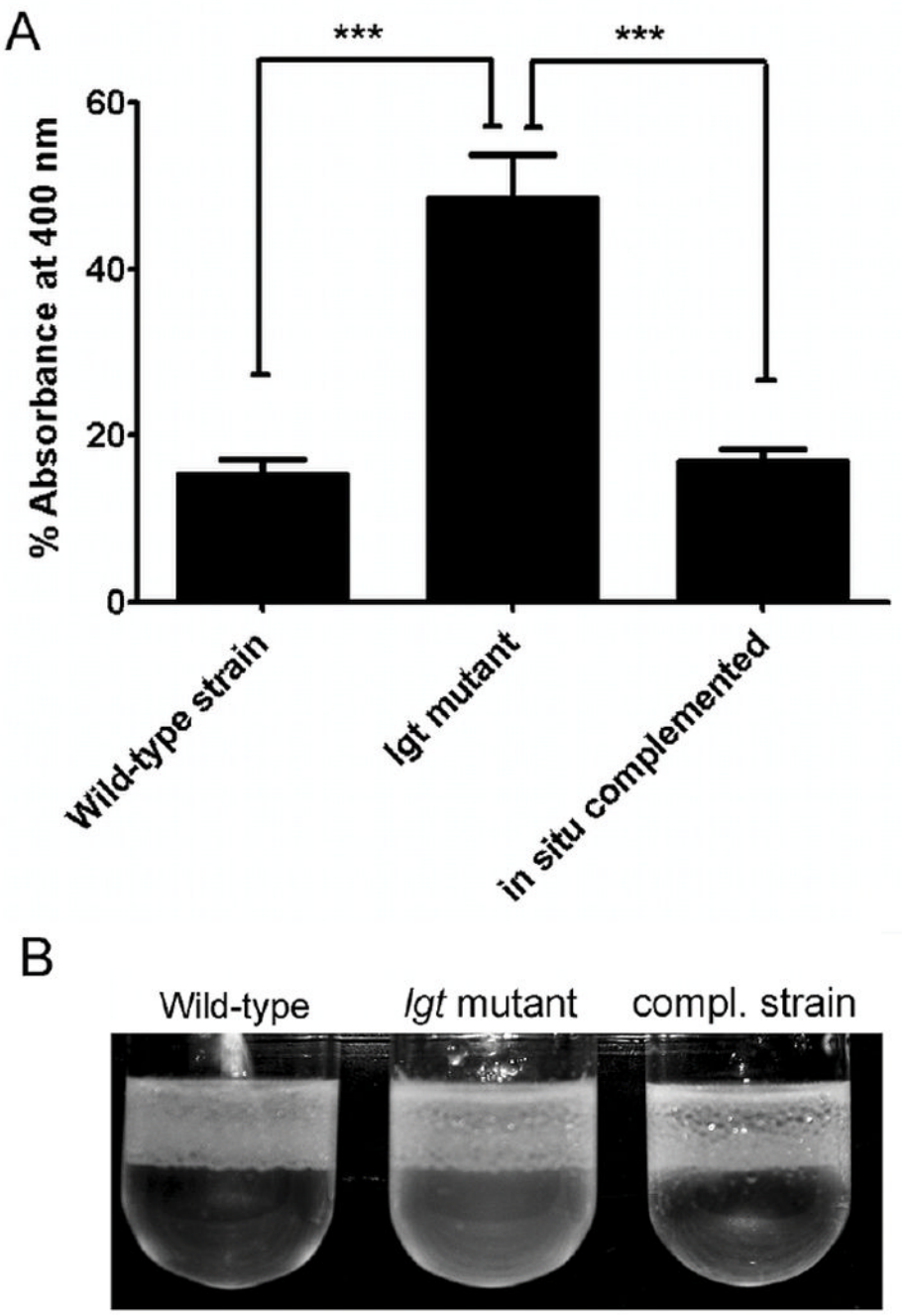

Figure 3.

Lipoproteins of *B. anthracis* influence bacterial surface hydrophobicity. Bacteria were cultured in BHI medium to an A_{600} of 4.0 and the washed bacterial suspensions were tested for adherence to hexadecane, which results in reduced optical density in the lower aqueous phase. Significant differences ($p < 0.0001$) between the *lgt* mutant, wild-type strain, and *in situ* complemented strain were determined by ANOVA with a Tukey's multiple comparison post-test and are indicated by asterisks.

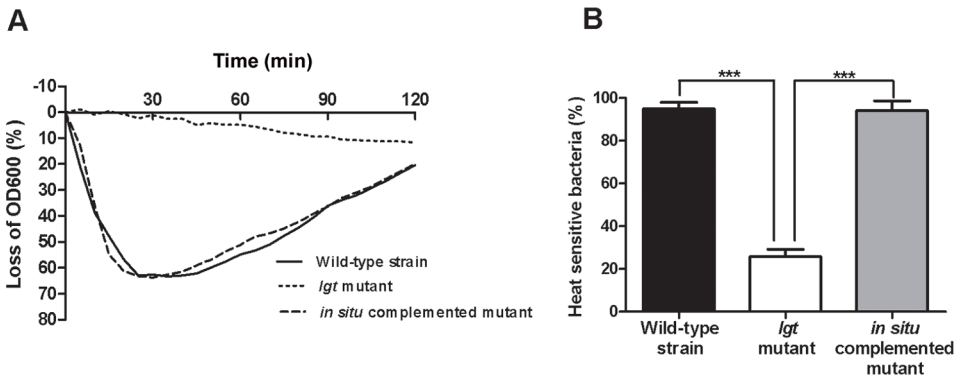

Figure 4.

Lgt deficiency attenuates germination. (A) Germination of wild-type strain, *lgt* mutant, and *in situ* complemented mutant as monitored by the decrease in A_{600} of spore suspensions during incubation in BHI medium. Results are mean values of three independent experiments. Standard deviations are < 10% of the mean. The A_{600} curve of *lgt* mutant spores differed with statistical significance from the curves of the wild-type and *in situ* complemented strain spores. (B) Spores of wild-type strain, *lgt* mutant, and complemented mutant were incubated at in (A) at 37°C for 30 min. At 30 min, samples were heat-treated at 65°C for 30 min. Before and after heat treatment, samples were plated on BHI agar. Plates were incubated overnight and colonies were counted. The fraction of total CFU that were heat sensitive (i.e., germinated) was calculated and are plotted as the mean values ± standard deviation of three independent experiments. Significant differences using one-way ANOVA with Tukey's post-test ($p < 0.001$) are indicated by three asterisks (***).

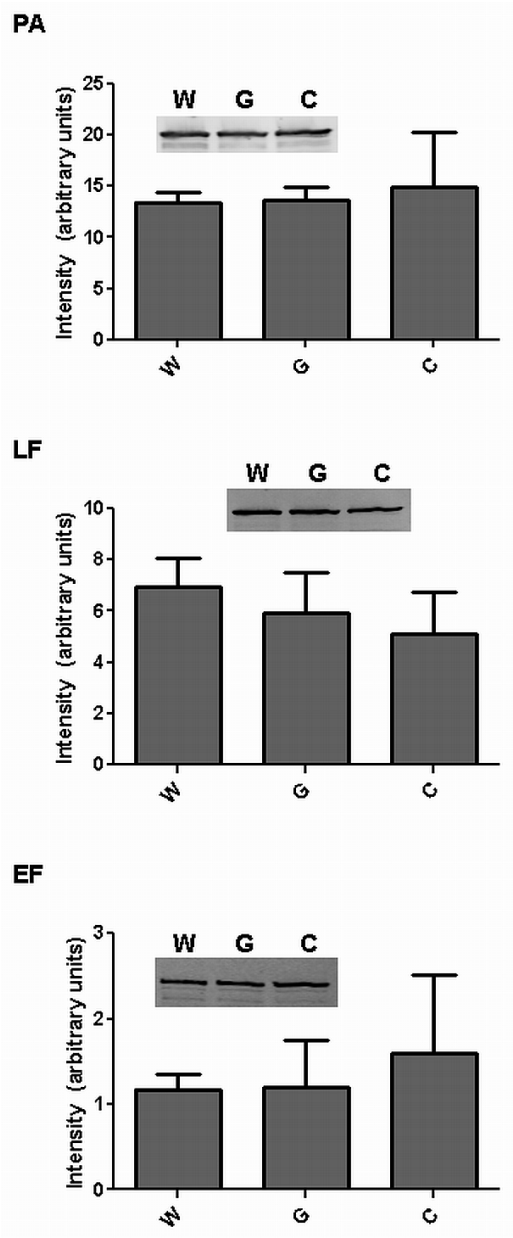

Figure 5.

Anthrax toxin protein secretion from wild-type strain, *lgt* mutant, and *in situ* complemented mutant. Wild-type strain (W), *lgt* mutant (G), and *in situ* complemented mutants (C) were cultivated in NBY medium in air, or NBY supplemented with 0.9% sodium bicarbonate and 9% CO_2 until A_{600} reached 2.0. Supernatants were separated by SDS-PAGE on 4–20% polyacrylamide gels, transferred to nitrocellulose, and blotted with antibody to protective antigen (PA), lethal factor (LF), or edema factor (EF). Intensities of bands were measured with the Odyssey software. Results shown in graph represent the mean ± SD of three independent experiments, and statistical analyses using one-way ANOVA with a Tukey's post-test for pairwise comparisons revealed no significant differences.

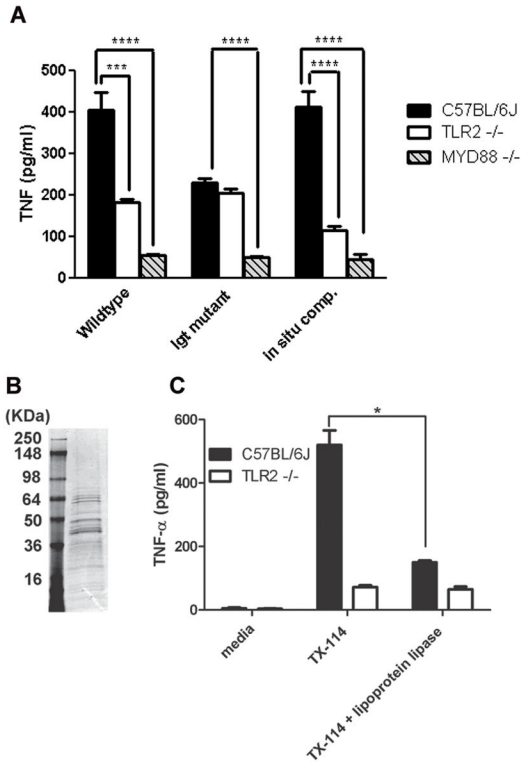

Figure 6.

Induction of TNF-α secretion from macrophages by heat-killed *B. anthracis* and lipoprotein extracts. (A) Bone marrow-derived macrophages from the three types of mice indicated were exposed to heat-killed wild-type strain, *lgt* mutant, or complemented mutant at 100 μg ml^{-1}. Macrophages were incubated for 18 h and supernatants were analyzed for TNF-α by enzyme-linked immunosorbent assay. Data are plotted as the mean values ± standard deviation of three independent experiments. Significant differences where $p < 0.001$ are indicated by three asterisks (***) and where $p < 0.0001$, indicated by four asterisks (****). (B) Coomassie blue staining of proteins in TX-114 fraction of *B. anthracis* (second lane). First lane contains molecular weight markers. (C) C57BL/6J bone marrow-derived macrophages from the two types of mice indicated were incubated with medium, TX-114 fraction, or lipoprotein lipase digested TX114 fraction for 18 h and supernatants were analyzed for TNF-α by enzyme-linked immunosorbent assay. Data are plotted as the mean values ± standard deviation of three independent experiments. Significant difference ($p < 0.05$) between lipoprotein lipase treatment and no treatment is indicated by an asterisk (*). All statistical tests used the unpaired two-tailed t-test.

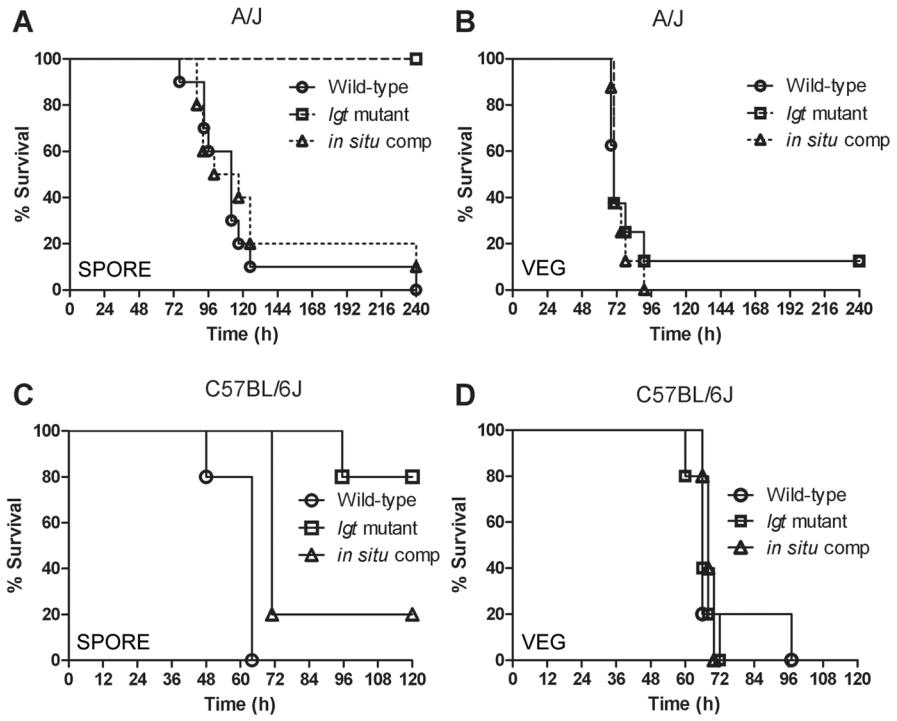

Figure 7.

Survival curves of mice challenged subcutaneously (SC) with spores or vegetative bacteria. A/J mice (A, B) or C57BL/6J (C, D) mice were injected SC with 2×10^3 spores (A, n=10), 20 vegetative cells (B, n=8), 2×10^7 spores (C, n=5) or 1×10^5 vegetative cells (D, n=5). The survival curves following *lgt* mutant spore challenge differed with statistical significance (by the logrank test) from curves of the wild-type and complemented strains for both A/J challenges ($p < 0.0001$), as well as C57BL/6J challenges ($p < 0.05$), while no significant differences were found in vegetative challenges.

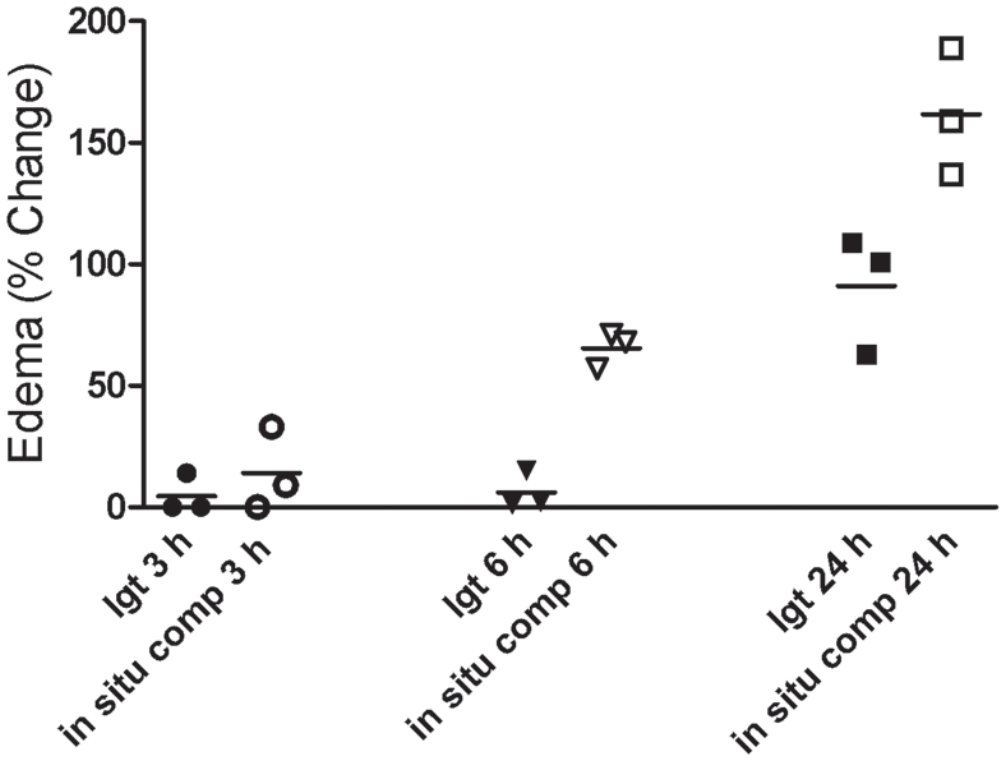

Figure 8.

Edema in mouse forelegs following infection with *lgt* mutant or *in situ* complemented strain. Groups of three A/J mice were injected with *lgt* mutant or complemented mutant (5×10^7 spores, 50 μl, SC). Percent increases in foot edema dorsal/ventral measurements relative to pre-infection measurements are shown at 3, 6, and 24 h following infection. Each symbol represents measurements of an individual mouse. P-values comparing *lgt* mutant to *in situ* complemented strain at 6 and 24 h are < 0.02 by unpaired two-tailed t-test.

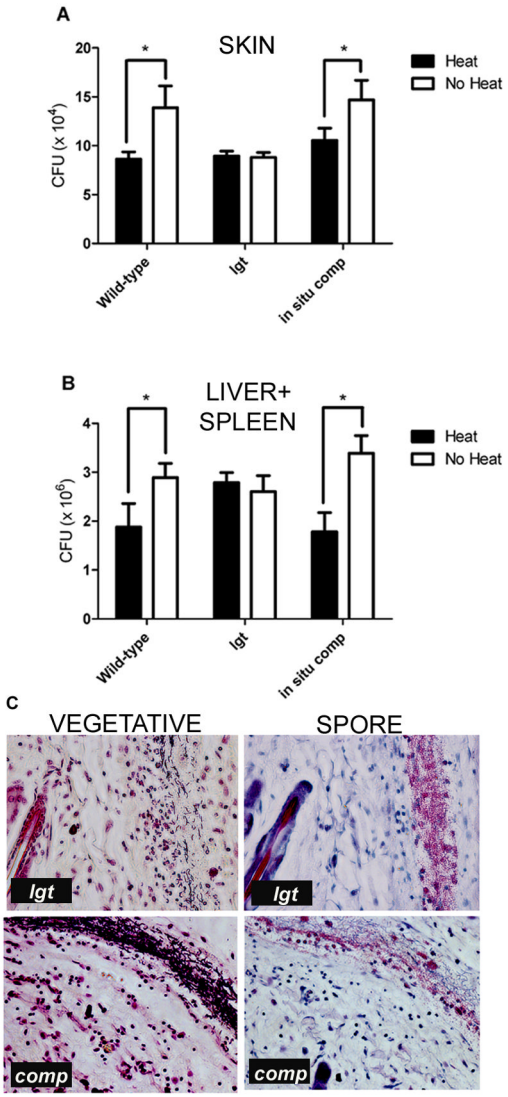

Figure 9.

Spore germination following subcutaneous (SC) and intravenous (IV) infection. (A) A/J mice (n=3/group) were injected SC in the foreleg with 5×10^7 spores in 50 μl, and (B) C57BL/6J mice (n=2/group) were injected IV with 1×10^6 spores in 200 μl. Skin and underlying tissues were obtained at 6 h from the A/J mice and spleen and liver were obtained at 90 min from the C57BL/6J mice. Tissues were processed as described in Experimental Procedures and total CFUs determined before and after heat treatment. Values plotted are the number of spores (solid bars, "Heat") and the sum of spores + germinated bacteria (open bars, "No Heat"). Asterisks indicate significant differences ($p < 0.05$) by 2-way ANOVA test. (C) Representative histology images showing staining of vegetative bacteria (left panels) and spores (right panels) in foreleg skin sections harvested 6 h post-infection from A/J mice injected SC with 1×10^8 spores. Upper panels show *lgt* mutant and lower panels show complemented strain. The upper and lower pairs of images were prepared from proximal but independent sections.