The dltABCD Operon of Bacillus anthracis Sterne Is Required for Virulence and Resistance to Peptide, Enzymatic, and Cellular Mediators of Innate Immunity

Nathan Fisher,1 Lynne Shetron-Rama,1 Amy Herring-Palmer,1 Brian Heffernan,1 Nicholas Bergman,1,2 and Philip Hanna1*

Department of Microbiology and Immunology1 and Program in Bioinformatics,2 University of Michigan Medical School, Ann Arbor, Michigan 48104

Received 17 October 2005/Accepted 23 November 2005

In the environment, the gram-positive bacterium Bacillus anthracis persists as a metabolically dormant endospore. Upon inoculation into the host the endospores germinate and outgrow into vegetative bacilli able to cause disease. The dramatic morphogenic changes to the bacterium during germination and outgrowth are numerous and include major rearrangement of and modifications to the bacterial surface. Such modifications occur during a time in the B. anthracis infectious cycle when the bacterium must guard against a multitude of innate immune mediators. The dltABCD locus of B. anthracis encodes a cell wall D-alanine esterification system that is initiated by transcriptional activation during endospore outgrowth. The level of transcription from the dltABCD operon determined B. anthracis resistance to cationic antibacterial peptides during vegetative growth and cationic peptide, enzymatic, and cellular mediators of innate immunity during outgrowth. Mutation of dltABCD was also attenuating in a mouse model of infection. We propose that the dltABCD locus is important for protection of endosporeforming bacteria from environmental assault during outgrowth and that such protection may be critical during the establishment phase of anthrax.

The cell surface of gram-positive bacteria is composed of many structural macromolecules including peptidoglycan, teichoic acids, and cell surface proteins (22). Teichoic acids are long chains of repeating glycerophosphate residues that can be found covalently linked to either peptidoglycan (wall-associated teichoic acid) or membrane-anchored glycolipids (lipoteichoic acid [LTA]). Interestingly, in some species or under certain environmental conditions, teichoic acids are not found and in these instances, they are generally replaced with a similar polyanionic structure(s) that seems to serve the same general purposes as teichoic acids (22).

While teichoic acids or similarly functioning structures are thought to be essential for life in gram-positive bacteria (34), they also serve as powerful attractants for cationic antibacterial compounds, including those central to the innate immune system of higher organisms, by significantly contributing to the overall negative charge of the bacterial cell wall (22). As an effective means of counteracting this potential threat, bacteria can adjust the net negative charge of these polymers by covalent addition of cationic molecules, most often D-alanine (34). Interestingly, in some species or under certain environmental conditions, teichoic acids are not found and in these instances, they are generally replaced with a similar polyanionic structure(s) that seems to serve the same general purposes as teichoic acids (22).

For many gram-positive bacteria, the addition of D-alanine esters to teichoic acids is catalyzed by four protein products encoded by the dltABCD operon. The dltA gene of this operon encodes a D-alanine:D-alanyl carrier protein ligase (Dcl) that covalently attaches D-alanine to the 4′ phosphopantetheine prosthetic group present on the D-alanyl carrier protein (Dcp), encoded by dltC. The dltB and dltD gene products are also required for the synthesis of D-alanyl teichoic acids. The former is believed to be a transmembrane channel involved in transport of D-alanyl Dcp to the extracellular space, while the latter serves as a chaperone ensuring the fidelity of D-alanine ligation to Dcp among the cellular pool of possible carrier molecules (22). After transport, no additional proteins are required, and D-alanyl Dcp is thought to associate with LTA, wall-associated teichoic acid, or potentially other surface structures to form a “thioesterase-like” enzyme mimic. Loss of function in any one of the four dltABCD gene products is sufficient to terminate the entire pathway and loss of D-alanine modifications (22).

In the pathogenic bacteria Staphylococcus aureus and Listeria monocytogenes, lack of D-alanyl teichoic acids has been linked to a decrease in virulence (1, 3). Bacillus anthracis is a gram-positive bacterium that causes anthrax after the endospore morphotype enters into a host through ingestion, inhalation, or contact with a cutaneous lesion. Most mammals, including humans, are considered susceptible to anthrax. Systemic disease, characterized by massive septicemia and toxemia, is often highly fatal even if treated. Due, at least in part, to the robust resistance properties of the dormant endospore, B. anthracis remains a viable threat as an agent of biological terrorism. Endospores, not vegetative bacilli, are considered the contagion (6).

The switch from dormant endospore to virulent, rapidly dividing bacilli represents an important early establishment stage in the anthrax infectious cycle (6, 7, 11). The initial events, breaking of endospore dormancy and start of metabolic functions, are termed germination and outgrowth and occur...
The overall goal of this work is to gain an understanding of the bacillus's success in survival and growth within the host. Although direct evidence for the presence of teichoic acids is lacking for B. anthracis (20), this study uses temperature-dependent plasmid insertion mutagenesis (9) to show that the enzymes encoded within the dltABCD operon are expressed during endospore outgrowth and control the presence of cell wall-associated, ester-linked D-alanine. Additionally, this operon was found to be critical for resistance to peptide, enzymatic, and cellular mediators of innate immunity as well as virulence in a murine model of inhalation anthrax.

**MATERIALS AND METHODS**

**Bacterial strains and antibiotics.** The B. anthracis Sterne 34F2 strain was cultured on brain heart infusion (BHI, Difco) broth or plates containing 15 g/liter agar. Escherichia coli DH10B and INV110 were maintained on LB broth or plates. Plasmids were maintained by the addition of 50 μg/ml kanamycin sulfate or 10 μg/ml tetracycline as appropriate. Endospores were prepared by nutrient exhaustion in modified G medium as described (9, 17).

**FIG. 1. Integration of pNFd13 into the dltABCD locus for construction of the DLTe mutant strain.** A 500-base-pair fragment, including the Shine-Dalgarno sequence and a truncated 5′ region of dltA, was inserted into pNFd13 via gateway cloning. The resulting plasmid was transferred to B. anthracis where the temperature-sensitive gram-positive origin of replication allows efficient transformation and recovery of kanamycin-resistant clones at 30°C. Subsequent transfer to the nonpermissive temperature of 39°C, with continued kanamycin selection, allows isolation of clones in which pNFd13 has inserted into the chromosome via homologous recombination with the 5′ region of dltA. The resulting chromosomal structure of the DLTe mutant was verified and monitored extensively. Integration of pNFd13 into the targeted locus results in (i) P_dlt complementation studies without further genetic manipulation (9).

**Disruption of dltABCD by pNFd13.** A 0.572-kilobase fragment (from positions –22 to 552 with respect to the transcriptional start site of dltA) of the 5′ region of dltABCD, termed dltA′, was amplified from B. anthracis chromosomal DNA using the primers attB1, CGAAATTTAGGGTGGGAACGTTATGAAG and attB2-GCCCGTGTTTAAGTTGAAAGTCTTTTCAACCTCC where attB1 and attB2 are modified recombinase sequences that allow entry into the Gateway cloning system (Invitrogen). The resulting amplicon was transferred first to pDONRet (9), then to pNFd13 (9), which was integrated into the B. anthracis chromosome at the dltABCD locus as described (9) (Fig. 1). Integration into the chromosome occurs randomly by homologous recombination between the plasmid born dltA′ sequence and that on the chromosome.

Clones in which this recombination occurred are isolated by shifting the temperature of cultivation to 39°C, which completely cures cells of all replicating pNFd13, rendering only cells with integrated plasmid capable of continued growth on kanamycin selection. After integration of pNFd13 into dltABCD, the native dltABCD promoter transcribes only the truncated dltA′ product and a plasmid-encoded lacZ gene. All four open reading frames within the dltABCD operon remain intact but are removed from transcriptional control of the native promoter and are instead placed downstream of a plasmid-encoded P_promoter that is normally silent unless activated by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) to the culture (Fig. 1). This strategy allows for detailed characterization of dltABCD transcription with P_promoter (9). The resulting mutant strain is designated B. anthracis DLTe.

**β-Galactosidase assays.** An enzymatic reporter system was used to monitor dltABCD transcriptional expression throughout logarithmic growth, stationary phase, and sporulation. Four ml of a culture grown to stationary phase in BHI broth was added to 100 ml fresh BHI or modified G medium and incubated at 39°C with vigorous aeration. Since the mutant grew poorly in this medium, 4 mM IPTG was added to the culture at the time of inoculation in order to restore growth to parental levels. This treatment had no detectable effect on sporulation efficiency. At 30-min intervals throughout growth, the optical density at 600 nm (OD600) was recorded and cells were collected by centrifugation from a 1-ml aliquot and stored at –80°C until analysis.

For expression analysis during outgrowth, endospores were heat activated by incubation at 65°C for 20 min in deionized, distilled water. After cooling to room temperature, endospores were suspended in an optical density at 600 nm of 1.0
in 1 ml of BHI broth. Samples were incubated at 37°C for either 30 min or 1 h, after which the cells were collected by centrifugation and stored at −80°C. In order to differentiate between the two β-galactosidase production during outgrowth and β-galactosidase associated with dormant endospores, 200 μg/ml chloramphenicol was added to parallel samples to prevent nascent protein synthesis during outgrowth.

For analysis of β-galactosidase activity, cell pellets were suspended in 1 ml Z buffer (60 mM Na2HPO4, 40 mM Na2HPO4, 10 mM KCl, 1 mM MgSO4 and 50 mM β-mercaptoethanol, pH 7.0) and were lysed by the addition of 20 μl tolune followed by vigorous vortexing for 15 seconds. Lysates were warmed to 37°C and the reaction was initiated by the addition of 200 μl 40 mg/ml o-nitrophenyl-β-D-galactoside in Z buffer. After development, the reaction was terminated by addition of 0.5 ml 1 N Na2CO3. Cell debris was removed by centrifugation, the absorbance at 420 nm was recorded, and Miller units were calculated as (1,000 x A420nm)/(reaction time in minutes x OD600).

Chemical analysis and quantification of nonpeptidoglycan surface D-alanine. Ester-linked nonpeptidoglycan D-alanine was isolated from bacterial cell walls essentially as described in (14); 4 ml broth cultures of B. anthracis 34F2 or DLTd were grown to early stationary phase (OD600 ~ 1.5) in BHI at 39°C and back-diluted into 400 ml fresh BHI in 1-liter baffled flasks. Cultures were incubated at 39°C, 300 rpm and growth was monitored by recording the optical densities of the cultures at 600 nm. Cells were harvested by centrifugation from 50-ml (lag phase and early logarithmic growth phase) or 15-ml (middle logarithmic to stationary phase) aliquots each hour after inoculation. Pellets were stored at −80°C prior to analysis. After thawing to room temperature, cells were washed three times with 1 ml 0.1 M morphology-ethanol-sulfonfolic acid (MES), pH 6.0. After washing, cells were boiled in 0.5 ml 0.2% sodium dodecyl sulfate, 1.0 M MES, pH 6.0, for 15 min. The cell wall material was collected by centrifugation and washed four times with 0.5 ml 0.1 M MES, pH 6.0, and then dried in a tablet oven at 10°C and weighed.

Ester-linked (nonpeptidoglycan) D-alanine was released from the cell wall material by suspending the dried pellet in 0.5 ml sodium pyrophosphate, pH 8.3 and incubation at 60°C for 3 h. The insoluble material was then removed by centrifugation and discarded and 0.1 ml of the supernatant was combined with 0.25 ml of 1% lauryl reagent (4 vol 0.1 M sodium pyrophosphate, pH 8.3, 2 vol 0.2% mg/ml flavin adenine dinucleotide in 0.1 M sodium pyrophosphate, 1 vol 50 mg/ml horseradish peroxidase (200 units/mg), 1 vol 5 mg/ml dianisidine sulfate, and 0.1 vol 5 mg/ml α-tetramino acid oxidase (15 units/mg)). The reaction was allowed to proceed for 15 min at 37°C after which it was terminated by the addition of 1 ml 0.1% sodium dodecyl sulfate. The absorbance at 460 nm for the supernatant was measured.

Results

Identification and mutation of dltBCD. The dltBCD operon of B. anthracis was identified by comparison of the sequenced genome (27) with the known homologues from Bacillus subtilis (24). The open reading frames within the B. anthracis operon spanning BA1389 to 1386 were found to encode proteins 66.7, 70.4, 69.6, and 63.1% identical to, and 52.9, 54.3, 51.9, and 46.7% identical to, DltA, DltB, DltC, and DltD from B. subtilis, respectively.

In order to initially characterize this locus, the B. anthracis operon was targeted for temperature-dependent, plasmid-insertion mutagenesis as described in (9). This approach results in (i) Pdlt being separated from the coding region of the operon creating a protein-null strain, (ii) a lacZ reporter gene, encoding β-galactosidase, placed downstream of Pdlt, for determination of promoter expression levels, and (iii) the intact coding regions of dltBCD placed under the control of the inducible Pspac promoter, allowing complementation studies without further genetic manipulation (Fig. 1). The mutant chromosomal structure was verified by extensive PCR analysis as described (9), which was shown to be the most accurate method for detection of any rare plasmid excision events.

In addition to the phenotypes detailed below, the resulting mutant strain exhibited morphological defects that included a high frequency of abnormally long cells when grown on BHI agar plates (not shown). In BHI broth, germination, outgrowth and vegetative growth of the DLtD mutant and the parental strain were indistinguishable and elongated cells could not be found, however the mutant was incapable of growth in modified G medium without induction of dltBCD by the addition of IPTG. Enumeration of vegetative cells and endospores on modified G medium agar plates resulted in a 5 or 4 log de-
crease, respectively, in the viable CFU count compared to BHI or modified G medium plates supplemented with IPTG. The nature of this defect is unknown. However, the observation that growth could not be restored by addition of glucose suggests that the defect is not merely the result of an inability to grow in a nutrient poor medium. It has been reported that \textit{dltABCD} plays a role in cation homeostasis (22). Therefore, it is likely that \textit{dltABCD} is required for \textit{B. anthracis} survival in high concentrations of cations, including magnesium, manganese, zinc and calcium found in modified G medium (17).

**Expression analysis of \textit{P}_{dlt}** Integration of \textit{pNFd13} into the chromosome results in the replacement of the \textit{dltABCD} open reading frames with the \textit{lacZ} gene, encoding \(\beta\)-galactosidase (Fig. 1) under the control of the wild-type \textit{dltABCD} promoter region. In order to monitor native promoter activity, cell lysates were collected throughout the growth cycle and the level of \(\beta\)-galactosidase was scored by a standard enzymatic assay. Expression levels and patterns from \textit{P}_{dlt} did not vary significantly between growth in either BHI (irrespective of the addition of IPTG, not shown) or modified G medium supplemented with 4 mM IPTG (Fig. 2A). However, growth in BHI resulted in an extended stationary phase and asynchronous sporulation (not shown) whereas growth in IPTG-supplemented modified G medium allowed the culture to rapidly and synchronously progress to sporulation, and is shown for this reason. This analysis shows that the \textit{dltABCD} promoter is highly active during early logarithmic growth but diminishes during stationary phase and sporulation. This pattern of expression is similar to that observed for \textit{P}_{dlt} in \textit{B. subtilis} except that optimal expression of \textit{dltABCD} was detected slightly later, during mid-logarithmic growth in that organism (24).

Since high-level expression of \textit{dltABCD} was detected from \textit{B. anthracis} cultures at very early timepoints during exponential growth, expression was monitored during outgrowth. Endospores were efficiently (>97% germination in 10 min, data not shown) germinated by inoculation into BHI medium. Significant levels of \(\beta\)-galactosidase activity were detectable as early as 30 min following germination, well before the first round of vegetative replication occurs at around 90 min postgermination (Fig. 2B). Comparison with cultures treated with chloramphenicol to block protein translation ensured that the majority of the detected signal originated from de novo synthesis during outgrowth and was not the result of protein captured in the dormant endospore during the preceding sporulation. As expected, detectable levels of \(\beta\)-galactosidase activity were not associated with the parental strain under any condition tested (not shown).

**\(\delta\)-Alanine incorporation into the surface.** In order to determine if mutation of \textit{dltABCD} affected the incorporation of \(\delta\)-alanine into the cell surface, ester-linked \(\delta\)-alanine was isolated and quantified from partially purified cell walls from \textit{B. anthracis} 34F2 and DLTd. The alkaline hydrolysis method used releases only ester-linked \(\delta\)-alanine, not the \(\delta\)-alanine present in peptidoglycan cross-linkages (14). \(\delta\)-Alanine was found to be associated with purified cell walls from \textit{B. anthracis} 34F2 (Fig. 3), however cell walls from the DLTd mutant were devoid of ester-linked \(\delta\)-alanine. As a control, expression of \textit{dltABCD} in the mutant, induced by addition of IPTG, rescued ester-linked \(\delta\)-alanine incorporation into the cell wall extracts to levels surpassing even those of the parental strain. Collectively, these data indicate a strict relationship between the expression of the \textit{dltABCD} operon of \textit{B. anthracis} and the presence of ester-linked \(\delta\)-alanine in the cell wall.

**Resistance to antibacterial compounds.** In order to assess the role of the \textit{dltABCD} operon in the resistance of \textit{B. anthracis} to antimicrobial peptides and cell wall antibiotics, the minimum growth inhibitory concentration was calculated for the parental and DLTd strains (Table 1). When challenged by the cationic antimicrobial peptides polymyxin B, colistin, magainin II, or nisin, the DLTd mutant showed between a 50 and 80% decrease in resistance compared to the parental strain. This defect was strongly tied to the \textit{dltABCD} locus since it was restored by IPTG-induced expression of \textit{dltABCD} in the mutant strain. Furthermore, the level of cell wall associated \(\delta\)-alanine appears to be the limiting factor for \textit{B. anthracis} resistance to cationic peptides since titration of IPTG in the growth medium resulted in a dose-dependent restoration of the parental resistance phenotype (Fig. 4).

In agreement with previous reports (19, 22, 25), the increased susceptibilities associated with the loss of \textit{dltABCD} was specific to cationic peptides since the resistance levels to the neutral peptides gramicidin D and bacitracin were unchanged from those of the parental strain (Table 1). Disruption of \textit{dltABCD} also resulted in an increased sensitivity to the cell wall directed antibiotics carbencillin and vancomycin.

**Contribution of the \textit{dltABCD} operon to innate immune resistance.** Since \textit{dltABCD} expression is part of the outgrowth program of \textit{B. anthracis}, we investigated its requirement for resistance to innate immune mediators during outgrowth (Fig. 5). Secretory phospholipase A and lysozyme are two major enzymatic components of the mammalian innate immune system, while defensins are cationic peptides produced by professional phagocytes (HNP-1 and HNP-2) or epithelial cells (\(\beta\)-defensin-1 and \(\beta\)-defensin-2) (12). When outgrowing endospores of the parental \textit{B. anthracis} strain were exposed to lysozyme and \(\beta\)-defensin-1, no reduction in CFU was detected. This strain was found to be slightly susceptible to sPLA2 and HNP-1 (25 and 18% killing, respectively) and moderately susceptible to HNP-2 and \(\beta\)-defensin-2 (42 and 40% killing, respectively). In contrast, the DLTd mutant exhibits increased sensitivity to each of the mediators tested with the exception of \(\beta\)-defensin-1. This exception is consistent with previous studies which have also found \(\beta\)-defensin-1 to be, in general, the least microbicidal of those tested in vitro (8, 23).

The mechanism of \textit{B. anthracis} resistance to \(\beta\)-defensin-1 is unknown, however, it was active against \textit{B. subtilis} cells under the assay conditions used (not shown). Lysozyme exposure results in 60% killing of the DLTd mutant while sPLA2, HNP-1, HNP-2, or \(\beta\)-defensin-2 treatment resulted in greater than 90% killing of this strain. In this assay, including IPTG in the outgrowth medium resulted in only modest, statistically insignificant restoration of the parental phenotype (not shown). It is likely that the 30 min allowance for germination is insufficient for transport of IPTG into the cell and activation of the \textit{P}_{\text{spac}} regulatory system. Collectively, these data indicate that the actions of the protein products of the \textit{dltABCD} operon participate in de novo synthesis of the vegetative cell wall during outgrowth and are essential to resist stresses that may be encountered by the bacterial cell during host infection.
FIG. 2. Expression analysis of the dltABCD operon of B. anthracis.

To monitor expression during vegetative growth and sporulation (A), DLTd was grown in modified G medium with 4 mM IPTG. At 30-min intervals, the optical density of the culture was measured (line) and cells were harvested and analyzed for β-galactosidase activity (columns). Results are an average from the analysis of two independent endospore preparations, each assayed in triplicate, with error bars representing one standard deviation from the mean.

For analysis during germination and outgrowth (B), dormant DLTd endospores were suspended to an OD600 of 0.25 in BHI (gray) or BHI plus 200 μg/ml chloramphenicol (black). The suspension was incubated at 37°C for either 30 or 60 min prior to analysis of β-galactosidase activity (columns). For analysis during sporulation and outgrowth (B), dormant DLTd endospores were suspended in 0.002% Z in BHI (red) or Z plus 200 μg/ml chloramphenicol (gray). The suspension was incubated at 37°C for 30 or 60 min, and cells were harvested and analyzed for β-galactosidase activity (columns) at 30-min intervals. The optical density of the culture was measured (line) and each assay run was averaged for β-galactosidase activity (columns). Results are an average from the analysis of two independent endospore preparations, each assayed in triplicate, with error bars representing one standard deviation from the mean.
Contribution of dltABCD operon to B. anthracis macrophage growth and survival. Upon inhalation, B. anthracis endospores are taken up by resident phagocytic cells (28, 11). In order to analyze the role of D-alanine in resistance to the microbicidal effects of the phagocyte, the DLTd mutant was compared to its isogenic parent in an in vitro infection of the RAW 264.7 murine macrophage-like cell line. Cell monolayers were infected with endospores, washed, harvested and plated for CFU at specified time points. Under microscopic observation, endospores for both strains appeared to be intimately associated with the RAW267.4 cells to virtually identical degrees (not shown).

Bacterial survival in macrophages was scored over time as described in materials and Methods. Macrophage bactericidal activity resulted in a decrease in viable CFU of 16.1% in 2 h and 48.6% in 5 h for parental B. anthracis 34F2 (Fig. 6).

Cell-mediated killing appears exacerbated by inactivation of the dltABCD locus since detectable CFU decreased more rapidly during infection with the DLTd strain (40.8% killing after 2 h and 83.4% after 5 h). IPTG-induced activation of dltABCD transcription in the mutant results in partial restoration of the parental phenotype (25.8% killing in 2 h and 68.8% killing in 5 h). Although enumerated CFU typically depends on both macrophage-mediated killing and bacterial growth, bacterial replication is seldom seen prior to 3 h postinoculation under the conditions tested (5, 6), presumably due to the temporal requirements for completion of germination and outgrowth prior to the resumption of the vegetative life cycle. Thus, at least for the initial time point, the observed decrease in CFU represents macrophage-mediated killing of B. anthracis cells undergoing germination and outgrowth and loss of dltABCD results in hyper-sensitivity to this killing.

![Graph showing analysis of cell wall-associated D-alanine](image)

**TABLE 1.** Minimal growth-inhibitory concentration of various antibacterial compounds against B. anthracis 34F2 and DLTd

<table>
<thead>
<tr>
<th>Compound type</th>
<th>Compound</th>
<th>MIC&lt;sup&gt;a&lt;/sup&gt; (µg/ml)</th>
<th>(% decrease&lt;sup&gt;b&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>34F2, no IPTG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>DLTd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No IPTG</td>
<td>16 mM IPTG</td>
</tr>
<tr>
<td>Cationic peptides</td>
<td>Polymyxin B</td>
<td>65</td>
<td>16 (75)</td>
</tr>
<tr>
<td></td>
<td>Colistin</td>
<td>&gt;50</td>
<td>25 (&gt;50)</td>
</tr>
<tr>
<td></td>
<td>Magainin II</td>
<td>12.5</td>
<td>3.1 (75)</td>
</tr>
<tr>
<td></td>
<td>Nisin</td>
<td>12.5</td>
<td>2.5 (80)</td>
</tr>
<tr>
<td>Neutral peptides</td>
<td>Gramcidin D</td>
<td>25</td>
<td>25 (0)</td>
</tr>
<tr>
<td></td>
<td>Bacitracin</td>
<td>50</td>
<td>50 (0)</td>
</tr>
<tr>
<td>Cell wall antibiotics</td>
<td>Carbenicillin</td>
<td>3</td>
<td>0.8 (73)</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>2.5</td>
<td>1.25 (50)</td>
</tr>
</tbody>
</table>

<sup>a</sup> As determined by broth dilution method (three replicates).

<sup>b</sup> Percent decrease in MIC for uninduced DLTd compared to the parental 34F2 strain.

<sup>c</sup> The addition of IPTG had no effect on 34F2 MICs.
Contribution of the dltABCD operon to virulence in a murine inhalation anthrax LD₉₀ model. As a first estimate of the importance of dltABCD to establishment and maintenance of disease by B. anthracis, the susceptibility of DBA/2J mice to parental Sterne 34F2 or DLTd endospores was monitored. Although missing capsule and other pXO2-encoded genes, the Sterne strain has been used to determine genes/factors contributing to virulence (5, 11). When challenged by intratracheal delivery of 1.5 x 10⁴ B. anthracis 34F2 endospores (90% lethal dose [LD₉₀], as determined in preliminary studies, not shown), seven of eight DBA/2J mice succumbed to infection by 10 days postinoculation with 4 of the mice dying after only 48 h. However, when an equivalent dose of B. anthracis DLTd endospores was delivered, morbidity was significantly decreased (Fig. 7). Only two of eight mice succumbed to infection, with death occurring during the first 48 h in both cases.

Since the genetic system used in this study is merodiploid for a 500-base-pair region of the chromosome and is temperature dependent (9), spontaneous, low-frequency reversion to the parental genotype occurring within the host was a potential concern. The possibility of reversion makes this genetic system suitable only for initial studies of animal virulence and the potential for reversions should be considered, and monitored for, in every case. To help eliminate reversion as a possibility influencing the outcome of these infections, B. anthracis CFU were recovered from the two mice that did not survive challenge with the mutant. In both cases, all bacteria (>100 CFU per mouse) retained kanamycin resistance when plated at the nonpermissive temperature, indicating that spontaneous reversion in the animal host is either absent altogether or occurred at a very low frequency in this case. The mere presence of pNFd13 on the chromosome did not adversely affect B. anthra-

FIG. 4. Resistance to the cationic peptide nisin is determined by the level of dltABCD expression. Late-exponential-phase cells of B. anthracis 34F2 (black square) or DLTd (gray circle) were back-diluted 1:100 into fresh BHI containing serial dilutions of nisin and the indicated concentration of IPTG. Cultures were incubated 16 h at 39°C at which time the MIC of nisin was determined by measuring the OD₆₀₀. The assay was repeated four times with identical results.

FIG. 6. Intracellular survival of B. anthracis Sterne 34F2 and DLTd mutant in a RAW 264.7 murine macrophage-like cell line. Cells were seeded in a monolayer overnight and infected with parental (black squares) or DLTd (gray circles) endospores as described in the text at a multiplicity of infection of 10:1. For complementation of the mutant, 100 mM IPTG was added at the time of infection (open circles). Viable CFU were counted at the indicated time points and results are presented as the percentage of initial cell-associated CFU detected in each case. Results are the averages of three independent experiments with error bars representing one standard deviation.

FIG. 5. Resistance to innate immune mediators requires dltABCD. We germinated 10⁶ endospores of B. anthracis 34F2 (black) or DLTd (gray) in BHI broth for 30 min at 37°C, washed them twice in buffer, and suspended them in buffer containing the indicated enzyme or peptide. After a 30-min incubation at 37°C, survival was calculated by enumerating the number of viable CFU after 24 h of growth on BHI agar plates. Percent survival was calculated against a buffer only control and results are triplicate averages of two different experiments with error bars representing one standard deviation.

FIG. 7. Virulence in a mouse model of inhalational anthrax requires dltABCD. DBA/2J mice were intratracheally inoculated with 1.5 x 10⁶ endospores of B. anthracis 34F2 (black) or DLTd (gray). Survival was monitored over 10 days and results are presented as the number of mice alive at the indicated time points postinfection.
cis as several mutant strains not described in this communication resulted in infections that parallel the parental strain with regards to mouse survival (not shown).

**DISCUSSION**

Upon inoculation into a host animal, pathogenic bacteria must overcome the physical, biochemical, and cellular mediators of innate immunity in order to establish infection and cause disease. Little is known regarding how *B. anthracis* resists or evades the initial biochemical and cellular mediators of innate immunity outside of the roles of its well-characterized capsule and toxins. The role of the *dltABCD* operon in this process was evaluated by mutagenesis of the entire operon and the resulting mutant exhibited the predicted deficiency in cell wall-associated \( \alpha \)-alanine. Our data suggest that modifications of the bacterial surface occurring shortly after inoculation into a host may play a significant role in *B. anthracis* resistance to host immunity.

During germination and outgrowth of bacterial endospores, the germ cell wall is deconstructed by dedicated autolysins (often referred to as spore lytic enzymes) and replaced by the *de novo* synthesis of the vegetative cell wall (26). In the case of *B. anthracis* endospore germination, this complex alteration takes place in the hostile environment of a host armed with a variety of antibacterial capabilities (13). This report is the first to demonstrate that the *dltABCD* locus of an endospore forming species is expressed immediately after germination, during outgrowth and before the first round of cellular division. In addition to showing that *dltABCD* expression is essential for resistance to cationic antimicrobial peptides and antibiotics during vegetative growth, as has been seen for a few other gram positive pathogens (1, 3, 19, 25), this study demonstrates that outgrowth-specific expression of *dltABCD* is required for resistance to peptide, enzymatic, and cellular mediators of innate immunity, presumably those seen by the bacteria early during anthrax infection.

The peptide (defensins) and enzymatic (lysozyme and secretory phospholipase) components tested each exhibit a predominately cationic charge that is crucial to efficient antimicrobial activity (18, 22). Thus, it is likely that \( \alpha \)-alanine esterification of an anionic cell wall component is crucial to proper modulation of the overall charge of the *B. anthracis* cell wall during the morphogenetic process of outgrowth. This modulation may be a general component of the outgrowth program common to all endospore forming bacteria. However, optimal expression of *dltABCD* occurs later during the growth cycle within *B. subtilis* (24) leaving the intriguing possibility that early expression of *dltABCD* may be a specific adaptation of *B. anthracis* to life in association with animal hosts.

In order to assess the importance of cell wall-associated \( \alpha \)-alanine to *B. anthracis* disease establishment and progression, DBA/2J mice were challenged by a high dose (lethal dose 90 for the parental strain) of 34F2 or DLTd endospores. Mice given the mutant strain were more resistant to infection than those that received the parental strain. The observed decrease in mortality caused by the DLTd strain is most likely related to the role of the *dltABCD* operon during both outgrowth-specific cell wall remodeling and vegetative growth. The infection system used does not allow for measure of the relative contribution of each. However, our in vitro findings strongly suggest that attenuation is at least partially related to the role of *dltABCD* in protection against host immune mediators during outgrowth.

To our knowledge, this is the first study to correlate a mutation affecting *B. anthracis* endospore outgrowth with decreased virulence in an animal model of infection. Attenuating mutants of *B. anthracis* are rarely reported, aside from those affecting toxin and capsule production, and this also is generally true for the nonencapsulated Sterne model used in this study. Thus, additional attention to the *dltABCD* operon of *B. anthracis* is warranted. Important topics to be addressed include (i) identification of the \( \alpha \)-alanine esterification target within the cell wall, since there are contradictory reports regarding the presence of teichoic acids in the very closely related *B. cereus* group of bacteria, to which *B. anthracis* belongs (15, 16, 20, 21, 29, 30, 32), (ii) discernment between the roles of *dltABCD* during outgrowth and vegetative growth in the context of infection, and (iii) analysis of mutation of *dltABCD* in a fully virulent, encapsulated strain in order to determine if, in the absence of ester-linked cell wall \( \alpha \)-alanine, capsule can mediate resistance to peptide and enzymatic mediators of innate immunity.

**ACKNOWLEDGMENTS**

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

**REFERENCES**


