DltX of *Bacillus thuringiensis* Is Essential for D-Alanylation of Teichoic Acids and Resistance to Antimicrobial Response in Insects

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The *dlt* operon of Gram-positive bacteria is required for the incorporation of D-alanine esters into cell wall-associated teichoic acids (TAs). Addition of D-alanine to TAs reduces the negative charge of the cell envelope thereby preventing cationic antimicrobial peptides (CAMPs) from reaching their target of action on the bacterial surface. In most gram-positive bacteria, this operon consists of five genes *dltXABCD* but the involvement of the first ORF (*dltX*) encoding a small protein of unknown function, has never been investigated. The aim of this study was to establish whether this protein is involved in the D-alanylation process in *Bacillus thuringiensis*. We, therefore constructed an in-frame deletion mutant of *dltX*, without affecting the expression of the other genes of the operon. The growth characteristics of the *dltX* mutant and those of the wild type strain were similar under standard *in vitro* conditions. However, disruption of *dltX* drastically impaired the resistance of *B. thuringiensis* to CAMPs and significantly attenuated its virulence in two insect species. Moreover, high-performance liquid chromatography studies showed that the *dltX* mutant was devoid of D-alanine, and electrophoretic mobility measurements indicated that the cells carried a higher negative surface charge. Scanning electron microscopy experiments showed morphological alterations of these mutant bacteria, suggesting that depletion of D-alanine from TAs affects cell wall structure. Our findings suggest that DltX is essential for the incorporation of D-aryl esters into TAs. Therefore, DltX plays a direct role in the resistance to CAMPs, thus contributing to the survival of *B. thuringiensis* in insects. To our knowledge, this work is the first report examining the involvement of *dltX* in the D-alanylation of TAs.

**Keywords:** *B. thuringiensis, dltX, antimicrobial peptides, virulence, D-alanylation, insects, innate immunity

**INTRODUCTION**

The cell wall of gram-positive bacteria contains highly charged anionic polymers called teichoic acids (TAs) that consist of alditol phosphate repeats. These polymers are either anchored to the cytoplasmic membrane via a lipid anchor [lipoteichoic acid (LTA)] (Fischer, 1988; Fischer et al., 1990) or covalently linked to N-acetylmuramic acid residues of the peptidoglycan layer...
The high prevalence of esters, or both (Fischer and Rösel, 1980; Fischer et al., 1990). Dickeya spp.), where they have been shown to confer resistance to AMPs, (Schneewind and Missiakas, 2014). The function of TAs is not fully understood; they have long been considered to be essential for Gram-positive bacteria. However, this is no longer the case since mutants of S. aureus or B. subtilis devoided of WTA have been obtained (D’Elia et al., 2006a,b). Nevertheless, they play crucial roles in cell morphology and division and are important for many cell envelope-dependent processes such as the activity of autolytic enzymes, the binding of divalent cations, and susceptibility to innate host defenses (Weidenmaier and Peschel, 2008). WTAs and LTAs can be covalently modified by substitutions with either glycosyl residues or D-alanyl esters, or both (Fischer and Rösel, 1980; Fischer et al., 1990). The high prevalence of D-alaninylation among Gram-positive species suggests that D-alaninylation of TAs is biologically relevant, especially for pathogenic bacteria in the context of host–pathogen relationship (Abachin et al., 2002; Chan et al., 2007). Indeed, they play crucial roles in cell morphology and division and are important for many cell envelope-dependent processes such as the activity of autolytic enzymes, the binding of divalent cations, and susceptibility to innate host defenses (Weidenmaier and Peschel, 2008). WTAs and LTAs can be covalently modified by substitutions with either glycosyl residues or D-alanyl esters, or both (Fischer and Rösel, 1980; Fischer et al., 1990).

D-alanine esterification (Perego et al., 1995; Neuhaus et al., 1996). These genes have also been found in a few Gram-negative bacteria, notably in soft-rot plant pathogenic enterobacteria (Dickeya and Pectobacterium spp.), where they have been shown to confer resistance to AMPs, probably by modifying the surface lipopolysaccharides (LPSs) (Pandin et al., 2016). DltA is a D-alanine-D-alanyl carrier protein ligase that catalyzes the D-alanylation of the D-alanyl carrier protein DltC (Heaton and Neuhaus, 1992, 1994; Debabov et al., 1996). The roles of DltB and DltD are less clear. Two models have been proposed: according to Neuhaus and Baddiley, DltD is thought to facilitate D-alanine ligation to DltC and DltB is believed to be involved in the translocation of Alanylated-DltC across the membrane where it may then transfer D-alanine directly onto LTA (Debabov et al., 2000; Neuhaus and Baddiley, 2003). A second model has been proposed, by Fisher and colleagues, to account for the contributions of DltB and DltD; in this model DltB transfers D-alanine from DltC to undecaprenyl-phosphate (C55-P) to produce D-Ala-P-C55. This lipid linked intermediate is then, flipped across the membrane, whereas DltD, active at the outer side of the membrane, transfers D-Ala to LTA (Fischer, 1994). Reichmann et al. (2013) recently reexamined the function of the dlt operon and found that DltC does not pass through the membrane and, therefore, that it is unlikely that this protein is involved in the final D-alaninylation step of LTAs. They also showed that DltD is targeted to the outside of the cell as was suggested by Fisher and colleagues in their model. Nevertheless, the existence of a D-Ala-P-C55 lipid linked intermediate has never been confirmed experimentally. In Dickeyadadantii, the dltB mutant is more sensitive to antimicrobial peptides than the wild type (WT) strain, but a dltD mutant is as resistant as the WT strain (Pandin et al., 2016).

Mutants with TAs lacking D-alanine esters exhibit a variety of phenotypic changes. For example, mutants of S. aureus lacking a functional dlt operon are deficient in their ability to regulate the anionic charge of the outer envelope and show poor survival both when they are exposed to CAMPs in vitro and when they are introduced to a host in vivo (Peschel et al., 1999). Similarly, mutants of the intracellular pathogen Listeria monocytogenes lacking functional dltA are highly susceptible to CAMPs (Abachin et al., 2002). B. subtilis also uses dlt to modify TAs, and thus reduce its sensitivity to CAMPs (Perego et al., 1995). In Bacillus anthracis, the dlt genes are turned on during spore germination in the host and are necessary for resistance to innate host defenses (Fisher et al., 2006). We have also previously shown that a Bacillus cereus mutant lacking a functional dlt operon is highly sensitive to colistin and polymyxin B, two standard antimicrobial compounds that have long been used to define the mechanisms by which CAMPs kill bacteria. Virulence of this mutant in an insect model was also significantly attenuated (Abi Khattar et al., 2009).

The dlt operon has been studied in many organisms and inactivation of any single gene (dltA to dltD) is enough to completely abrogate operon function (Peschel et al., 1999; Collins et al., 2002; Vélez et al., 2007; Abi Khattar et al., 2009). In B. subtilis, each gene of the dltABCD operon is required for the D-alaninylation of LTA and these four proteins are believed to be the only proteins that are necessary and sufficient for D-Ala incorporation (Perego et al., 1995). However, in B. subtilis, as in most gram-positive bacteria, the operon contains a fifth gene encoding a small protein of less than 50 amino acids in length, DltX. Wang et al. (2004) using a mathematical algorithm have predicted that this small ORF upstream from dltA is part
of the dlt operon and several experimental analyses, in several species, have shown that, all five genes of the operon belong to the same transcriptional unit (Koprivnjak et al., 2006; Palumbo et al., 2006; Bron et al., 2012). Nevertheless, despite being encoded upstream of dltA in several gram-positive bacteria, the role of DltX in TAs d-alanylation has not yet been investigated. Therefore, the aim of this study was to determine whether dltX encodes a novel protein with a role in d-alanylation of TAs. We therefore constructed a ∆dltX mutant strain in Bacillus thuringiensis strain 407 (Bt 407), by allelic replacement, without affecting the expression of the other four genes of the operon. We then analyzed the effect of the deletion on the morphological and physicochemical properties of the bacterial surface, and operon and several experimental analyses, in several species, have shown that, all five genes of the operon belong to the same transcriptional unit (Koprivnjak et al., 2006; Palumbo et al., 2006; Bron et al., 2012). Nevertheless, despite being encoded upstream of dltA in several gram-positive bacteria, the role of DltX in TAs d-alanylation has not yet been investigated. Therefore, the aim of this study was to determine whether dltX encodes a novel protein with a role in d-alanylation of TAs. We therefore constructed a ∆dltX mutant strain in Bacillus thuringiensis strain 407 (Bt 407), by allelic replacement, without affecting the expression of the other four genes of the operon. We then analyzed the effect of the deletion on the morphological and physicochemical properties of the bacterial surface, and examined its effects on bacterial virulence and resistance to host immunity. We report here that the dltX of B. thuringiensis is essential for the incorporation of d-alanyl esters into TAs and is required for resistance to CAMPs and for full virulence of the bacterium following its injection into the model insects Galleria mellonella and Drosophila melanogaster.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

The acrystalliferous strain B. thuringiensis 407 Cry- belonging to serotype 1 (Lereclus et al., 1989) was used throughout this study and designated as Bt 407. Escherichia coli K-12 strain TG1 was used for the construction of plasmids and cloning experiments, and E. coli ET 12567 Dam- Dcm- (Stratagene, La Jolla, CA, United States) was used for the construction of plasmids and cloning experiments, and E. coli ET 12567 Dam- Dcm- (Stratagene, La Jolla, CA, United States) was used for the construction of plasmids and cloning experiments in accordance with the manufacturer’s recommendations. Oligonucleotide primers used in this study are listed in Table 1 and were synthesized by Sigma-Proligo (Paris, France). PCR was performed in a thermocycler, Applied Biosystems 2720 Thermal cycler (Applied Biosystems, United States). Amplified fragments were purified with the QIAquick PCR purification Kit (QIAgen). Digested DNA fragments were separated on 1% agarose gels and were extracted from gels with the QIAquick gel extraction Kit (QIAgen). Nucleotide sequences were determined by Beckman Coulter Genomics (Takeley, United Kingdom). Electroporation to transform Bt 407 was carried out as previously described (Lereclus et al., 1989).

**Manipulation of DNA**

Chromosomal DNA was extracted from *Bacillus* cells harvested in the mid-exponential growth phase, with the GENTRA Puregene DNA Purification bacteria Kit (QIAGen, France). Plasmid DNA was extracted from *E. coli* by a standard alkaline lysis procedure using QIAprep spin columns (QIAGen, France). Restriction enzymes and T4 DNA ligase (New England Biolabs, United States) were used in accordance with the manufacturer’s recommendations. Oligonucleotide primers used in this study are listed in Table 1 and were synthesized by Sigma-Proligo (Paris, France). PCR was performed in a thermocycler, Applied Biosystems 2720 Thermal cycler (Applied Biosystems, United States). Amplified fragments were purified with the QIAquick PCR purification Kit (QIAgen). Digested DNA fragments were separated on 1% agarose gels and were extracted from gels with the QIAquick gel extraction Kit (QIAgen). Nucleotide sequences were determined by Beckman Coulter Genomics (Takeley, United Kingdom). Electroporation to transform Bt 407 was carried out as previously described (Lereclus et al., 1989).

**Construction of a dltX Deletion Mutant**

A B. thuringiensis strain containing a dltX deletion was generated by precise, in frame allelic exchange and deletion replacement without antibiotic resistance cassettes. The thermosensitive plasmid MAD (pMAD) was used in these experiments. The 846 bp sequence immediately upstream from dltX was amplified with the primers dltX-a, dltX-b, and a 1064 bp sequence immediately downstream from dltX was amplified with the primers dltX-c and dltX-d. The primers dltX-b and dltX-c introduce overlapping PCR products. The two amplicons were then subjected to another PCR cycle with the primers dltX-a and dltX-d, such that a modified dlt operon, from which 159 bp had been deleted, was amplified. This amplicon was digested with NcoI and BglII and was introduced between the corresponding cloning sites of pMAD. Bacterial Strains and Growth Conditions in the mid-exponential growth phase, with the GENTRA Puregene DNA Purification bacteria Kit (QIAGen, France). Plasmid DNA was extracted from *E. coli* by a standard alkaline lysis procedure using QIAprep spin columns (QIAGen, France). Restriction enzymes and T4 DNA ligase (New England Biolabs, United States) were used in accordance with the manufacturer’s recommendations. Oligonucleotide primers used in this study are listed in Table 1 and were synthesized by Sigma-Proligo (Paris, France). PCR was performed in a thermocycler, Applied Biosystems 2720 Thermal cycler (Applied Biosystems, United States). Amplified fragments were purified with the QIAquick PCR purification Kit (QIAgen). Digested DNA fragments were separated on 1% agarose gels and were extracted from gels with the QIAquick gel extraction Kit (QIAgen). Nucleotide sequences were determined by Beckman Coulter Genomics (Takeley, United Kingdom). Electroporation to transform Bt 407 was carried out as previously described (Lereclus et al., 1989).

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**Complementation of the dltX Mutant**

For the complemented strain, the entire dltX open reading frame of Bt 407 strain with its promotor region was amplified from genomic DNA of Bt 407 by PCR with Taq DNA polymerase (Extensor High-Fidelity; Thermo Scientific). The forward primer Comp F included a restriction site for XbaI and the reverse primer Comp R included a restriction site for

### Table 1 | Primer sequences used in this study.

| Primer name | Nucleotide sequence (5’-3’)
|-------------|---------------------------
| dltX-a      | CATGCCATGGTCGAAATACCTTCTTGGC |
| dltX-b      | CAATTTGTCATTAACTGTTGTCATTCTTCTCTTAACTG |
| dltX-c      | CATTTAAAGAGAGAAAAGACTGAAATTTAGAAGAAATAATG |
| dltX-d      | GAAGATCTACAGGCAGCCACACAAATAC |
| Comp-F      | GCTCTAGAATGGCTTTCTCCATTCAGC |
| Comp-R      | CCCCCGGGGTCTTGGGAGCATAGTTGG |
| XbaI-dlt-gfp | CGTCCTAGAAGAGCTTGAGTTGAGT |
| EcoRI-dlt-gfp | GGGATCCATCAGCCATTCAGAATCATTGCT |
| BGF1372.Q3  | AATTGAAAGTTGGGCTGGAGAA |
| BCR1372.Q3  | CGGATCTCGGCGCAACAAAA |

* Restriction sites are underlined.
XmaI. The resulting 611 kb fragment was digested with XbaI and XmaI, gel-purified, and ligated to the pHT304-18 shuttle vector previously digested with the same enzymes. An aliquot of ligation mixture (~50 ng DNA) was used to transform E. coli TG1 by electroporation. The resulting construct, pHT304-18ΔdltX, was verified by restriction mapping and transferred into E. coli ET 12567 by electroporation. Unmethylated plasmid pHT304-18ΔdltX from E. coli ET 12567 was then introduced into strain Bt 407ΔdltX by electroporation.

Construction of PdltΩgfp Fusion Reporter Plasmid
The upstream promoter region of the dlt operon (Pdlt) was amplified by PCR with Taq DNA polymerase (New England Biolabs) and with the primer pair XbaI-dlt-gfp and EcoRI-dlt-gfp (Table 1). The 239 bp PCR product was digested with XbaI and EcoRI, and ligated into the same sites upstream of the promoterless gfp gene carried on pHT315Ωgfp (Dau et al., 2009) resulting in the creation of the plasmid pHT315Ωdlt-gfp. The plasmid was verified by restriction analysis, PCR, and sequencing and was transferred into E. coli ET 12567 by electroporation. Unmethylated plasmids from E. coli ET 12567 were then introduced into Bt 407 and Bt 407ΔdltX mutant strains by electroporation.

Quantitative RT-qPCR
The amounts of dltA transcripts in Bt407 and ΔdltX strains were measured by real time reverse transcription. RNA extraction and cdDNA synthesis were performed as described previously (Réjasse et al., 2012). Primers BCF1372.Q3 and BCR1372.Q3 located inside the dltA gene were designed with Primer Express software from Applied Biosystems. Real time PCR was carried out with Sybr green PCR master mix (Applied Biosystems) as recommended by the supplier. Mean values were calculated from two separate experiments in which qPCR reactions were performed in triplicate. The cycle threshold was used to determine the relative dltA gene expression levels in two genetic backgrounds. Data were analyzed by the comparative threshold cycle (ΔΔCT) method with the Relative Expression Software Tool (REST 2009 V2.0.13, QIAGen). Expression ratios were normalized to two Bt 407 endogenous reference housekeeping genes, pur and tpi.

Scanning Electron Microscopy (SEM) Analysis
Scanning electron microscopy (SEM) was performed at the Microscopy and Imaging Platform MIMA2 (Micalis, B2HM, Massy, France) of the INRA research center of Jouy-en-Josas (France). Samples were critical point dried × progressively dehydrated with increasing concentrations of a solution of 0.1 M sodium cacodylate. Samples were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 at room temperature (France). Samples were reared on beeswax and pollen (La Ruche Roannaise, Roanne, France). For the infection experiments, the larvae are starved 24 h.
prior to infection and 10^4 bacterial vegetative cells suspended in 10 µl PBS buffer were injected into the hemocoel of last instar G. mellonella larvae (weighing about 200 mg) with a 0.5-by-25 mm needle and a microinjector (KdScientific syringe pump). The larvae in the control group were injected with PBS buffer. Following inoculation, the larvae were kept by groups of five in small Petri-dishes without food (Bouillaut et al., 2005) and mortality was recorded after 48 h of incubation at 30°C. For each strain, 20 larvae were used for toxicity assay and the results shown are the means of at least three independent experiments.

Virulence in Drosophila melanogaster
The WT Oregon R strain and the relish^B20 mutant strain (impaired in IMD signaling and production of antimicrobial peptides) were used in this analysis (Hedengren et al., 1999). Overnight bacterial cultures were washed in PBS and diluted to OD 600 = 2. Twenty adult female flies aged between 2 and 5 days were pricked with a thin tungsten needle previously dipped into the bacterial preparation as previously described (Ligoxygakis et al., 2002). Surviving flies were counted every 2 h.

Minimal Inhibitory Concentrations
Polymyxin B has long been used to define the mechanisms by which AMPs kill bacteria [46]. Susceptibility to polymyxin B was evaluated by determining the half inhibitory concentration (IC_{50}) from dose-response curves obtained with various concentrations of polymyxin B. The tests were performed in 96-well microplates containing 7–9 concentrations (from 200 to 800 µg/ml) of polymyxin B (Sigma) for WT 407 and 407ΔdltX complemented strains, and from 3 to 25 µg/ml for 407ΔdltX. Bacterial growth was scored after inoculation of strains at an initial OD_{600} of 0.1 and incubation at 30°C for 6 h. IC_{50} corresponds to the concentration of polymyxin B required to inhibit inoculum viability by half and was determined as the concentration required to bring the curve down to point half way between its top and bottom plateau. The results shown are the means of at least three independent experiments performed in duplicate.

Statistical Analysis
Results concerning D-alanine quantification of TAs, electrophoretic mobility and in vivo pathogenicity assays were analyzed by the two-tailed Student’s t-test. A p-value of 0.05 was considered to be significant.

RESULTS

In Silico Analysis of DltX
In silico BLAST searches indicate that the dltX gene is present in 809 species of which 805 are Firmicutes, including Bacillus, Staphylococcus, Listeria, Lactobacillus, Streptococcus, and Enterococcus. In most cases, dltX is located immediately upstream from dltA. In B. thuringiensis this operon consists of five genes dltXABCD. In strain Bt 407, transcripts of dltX have been detected by mapping RNA-seq datasets, obtained in vitro 2 and 5 h after the end of exponential phase and in vivo in infected G. mellonella cadavers, 36 h post mortem, with the Bt 407 reference genome (Sébastien Gélis-Jeanvoine, personal communication). These data prompted us to carry out a detailed analysis of the role of this protein in the process of D-alanylation. DltX belongs to the DUF3687 superfamily of proteins. To date, 811 sequences with this domain are listed in Pfam. Proteins in this family are approximately 50 amino acids in length and their protein sequences are highly conserved among the firmicutes. There are two completely conserved residues (L and Y, at positions 23 and 45 of the protein, respectively) that may be functionally important. A number of entries are annotated as D-Ala-teichoic acid biosynthesis protein; however, there is no direct evidence to support this annotation. In Bt 407, DltX contains 11 positively charged amino groups clustered near the N-terminus followed by a hydrophobic region of 22 amino acids and by a putative non-cytoplasmic domain of 15 amino acids in the C-terminus. The 22 amino acids hydrophobic region is predicted to form an α-helix, which suggests that DltX is a transmembrane protein. The topology of DltX present in the Gram negative species is the same, although there is no sequence similarity. In addition, DltX does not have a predicted signal peptide, which suggests that it is probably not exported (Figure 1).

Deletion of dltX Does Not Affect the Expression of the dlt Operon
We sought to study the influence of dltX on teichoic acid D-alanylation; therefore, we constructed a mutant harboring a precise in frame allelic deletion of the B. thuringiensis dltX gene, to avoid impact on the transcription of the downstream located dlt genes. We used the overlap extension (OE-PCR)
protrusions on their surface (cells had an irregular and wrinkled shape, and showed rib-like expression (Figure 3A). WT cells had a typical rod shape with a regular and smooth surface (Figure 3B). Deletion of dltX leads to significant changes in cell morphology, as the dltX mutant cells have an irregular and wrinkled shape, in contrast to the regular rod shape of WT cells. Complemented mutant cells (Figure 3C) showed an appearance similar to that of WT cells.

The dltX Mutant Is Sensitive to Polymyxin B and Nisin In Vitro

We compared the growth of WT Bt 407, Bt 407ΔdltX mutant and complemented mutant strain Bt 407ΔdltX (pHT304-18Δdlt) exposed to polymyxin B or Nisin, to determine if dltX has a direct role in resistance to CAMPs. Addition of 150 µg polymyxin B ml−1 or 75 µg Nisin ml−1 to a growing culture caused immediate growth interruption of the mutant whereas WT and complemented strains were not affected (data not shown). Therefore, the dltX mutant is highly susceptible to CAMPs and its complementation with dltX completely restored the resistant parental phenotype.

Deletion of dltX Leads to Attenuated Virulence in Galleria mellonella

The finding that dltX is essential for the resistance of Bt407 to CAMPs led us to investigate the effect of its deletion on bacterial virulence in two insect models. We injected 104 vegetative cells of the WT, mutant, or complemented strains into fifth instar G. mellonella larvae and compared their virulence by monitoring the mortality level of Galleria infected with the different strains (Figure 5). The WT and complemented strains were significantly more virulent in insects (p < 0.0001) than the dltX mutant strain (Figure 5). In fact, virulence in G. mellonella was almost completely abolished in the dltX mutant (13% mortality) whereas...
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FIGURE 3 | Effects of dltX deletion on Bt 407 cells. Scanning electron micrographs show exponential growth phase cells of WT (A), ΔdltX (B), and ΔdltX complemented cells (C) in Y buffer (pH 5.6). Magnifications are x20k and x30k. The dotted lines on pictures in the top panel represent a scale of 1.5 µm (x20K magnification) and those in the bottom panel represent a scale of 1 µm (x30K magnification).

WT and complemented strains were highly virulent and gave almost 100% mortality 48 h post-infection.

The Low Virulence of the dltX Mutant Is Directly Correlated with Its Sensitivity to the Humoral CAMP Response In Vivo

The immune system in insects comprises cellular and humoral responses that have been largely investigated in the model organism Drosophila melanogaster (Lemaitre and Hoffmann, 2007). The humoral response involves the infection-induced activation of NF-κB transcription factors, Dif and Relish, which in turn activate AMP encoding genes. Dif is downstream from the Toll pathway and Relish is downstream from the IMD pathway (Hedengren et al., 1999; Rutschmann et al., 2000). These signaling cascades are specifically elicited by microbial associated molecular patterns (MAMPs) that are detected by cognate pattern recognition receptors (PRRs), which are members of the Peptidoglycan Recognition Receptor and Glucan Binding Receptor families (Ferrandon et al., 2007). Notably, the Toll pathway is triggered upon sensing of Lysine (Lys)-type peptidoglycan, which is a common component of most Gram-positive bacteria, whereas the IMD pathway is activated by the mesodiaminopilimelic acid (DAP)-type peptidoglycan common to Gram-negative bacteria (Leulier et al., 2003; Ferrandon et al., 2007). The wall of all B. cereus group species also consists of a DAP-type peptidoglycan (Vollmer et al., 2008). We therefore, compared the virulence of WT, ΔdltX, and the complemented strain in WT oregon R and relish mutant flies to confirm that the loss of virulence phenotype of the ΔdltX mutant is associated with its susceptibility to CAMPs in vivo. Bt407 was resistant to the fly humoral response because WT and relish immunodeficient insects exhibited similar survival curves with high lethality of the infected flies at 15 h post-infection (Figure 6). Similar to findings obtained with Galleria larvae, survival curves of WT flies infected with the ΔdltX mutant showed that loss of dltX is sufficient to significantly impair virulence. Indeed, 75% of the flies infected with the ΔdltX mutant survived 24 h post-infection. Interestingly however, the virulence of the ΔdltX mutant was fully restored in relish mutant flies which do not produce CAMPs in response to the infection. This phenotype is associated with the loss of DltX because the complemented strain was as virulent as the WT strain in both WT oregon R and relish mutant flies. These findings clearly demonstrate that DltX is required for the resistance of Bt 407 to the humoral antimicrobial response of Drosophila infected by injection into the hemocoel.

DltX Is Involved in the D-Alanylation of TAs

We sought to determine whether the sensitivity of the dltX mutant to CAMPs was due to impairment in the incorporation of D-alanyl esters into TAs of the cell wall. D-Alanine was released by mild alkaline hydrolysis from whole heat-inactivated bacterial cells and quantified by HPLC analysis. Almost no D-alanine was released from the cell walls of the ΔdltX mutant, contrary to what is observed for the WT Bt 407, indicating that D-alanylation of TA was significantly impaired in the mutant. D-alanine amount released from the complemented strain was significantly higher than the one released from WT strain. This result can be explained by the copy number of the plasmid used for complementation (Figure 7).

Deletion of dltX Significantly Alters Ionic Surface Charge

The loss of D-alanylation in the dltX mutant may result in a net change in electrical charge of the bacterial surface, which may explain the higher sensitivity of the dltX mutant to CAMPs both in vitro and in vivo. We measured the electrophoretic mobility (EM) of each strain to see if the deletion of dltX was associated
with a change of the global charge at the bacterial surface. EM was significantly higher in the ΔdltX mutant than in the WT strain (p = 0.0007) (Figure 8), showing that deletion of dltX alters electrical surface charge. Thus, the ΔdltX strain has a high negative charge at its surface.

**DISCUSSION**

The dlt operon encodes proteins that are responsible for the incorporation of D-alanine into TAs, which is an important process for bacterial resistance to CAMP’s. However, the functions of several dlt genes remain unclear and are still under investigation. Moreover, few studies have indicated that dltX may be part of the dlt operon despite the fact that the gene is present in the genomes of 807 bacteria belonging to the Firmicutes phylum, as revealed by our in silico analysis. This is probably due to the fact, that, in the original genomes annotations, the small ORF preceding the dltABCD genes has been frequently missed due to its small size. Therefore, the involvement of the gene product of dltX in the function of this operon has never been investigated until now despite the fact that its sequence is highly conserved among Gram positive Firmicute family bacteria, which suggests that the product of this gene could be important and involved in the D-alanylation process.

In the present study, we used the human opportunistic and entomopathogenic bacterium *B. thuringiensis* crystal minus strain 407 and investigated the role of dltX in the resistance of this pathogen to cationic antimicrobial host components. In strain *Bt* 407 dltX was transcribed along with the other genes of the operon such as already found in several other species such as *S. aureus* or *Lactobacillus plantarum* (Koprivnjak et al., 2006; Palumbo et al.,...
DltX Is Involved in the D-Alanylation Process in *B. thuringiensis*

2006; Bron et al., 2012). We first constructed a *dltX* mutant by allelic exchange, in which the *dltABCD* genes were intact and expressed under the control of their endogenous promoter. The resulting *dltX* mutant had a pleiotropic phenotype, including changes in bacterial cell morphology, high susceptibility to bacterial and insect cationic AMPs, and attenuated virulence in two insect infection models. These observations demonstrate that bacterial and insect cationic AMPs, and attenuated virulence in changes in bacterial cell morphology, high susceptibility to *dltX* resulting expressed under the control of their endogenous promoter. The findings suggest that DltX does not act as a *cis* or *trans* element that regulates transcription of the operon, but plays a direct biosynthetic, transport or addresser role in D-alanylation. We have also performed a complementation of the *ΔdltX* mutant with a mutated form of *dltX* in which the start codon (ATG) was replaced by a stop codon (TGA) thus impeding the translation of the putative DltX protein (data not shown). Unlike the native *dltX* sequence, this mutated form of *dltX* could not restore the parental phenotype of resistance to CAMPs and virulence toward *G. mellonella*. However, the exact function of DltX remains to be elucidated.

We also observed that disruption of *dltX* strongly affected the cell morphology of *B. thuringiensis* since the *ΔdltX* mutant strain presented irregular shapes which were not observed in the parental strain (Figure 3). Such dramatic changes in cell morphology have not been reported for other *Δdlt* mutants of gram positive bacteria, except for *L. plantarum*. Indeed, scanning and transmission electron microscopy (SEM and TEM) of a *L. plantarum* *Δdlt* mutant showed perforations of the cell envelope (Palumbo et al., 2006). The authors suggested that this effect may result from a high rate of autolysis. We observed no differences between the growth curve of the WT parental strain and that of the *ΔdltX* mutant (data not shown). This indicates that the surface modifications are unlikely to influence the rate of autolysis, at least *in vitro* and under the conditions tested. Moreover, we showed that the cell surface of the *ΔdltX* mutant was significantly more negatively charged than that of the WT strain. This is not surprising because the cell wall is predicted to be largely more anionic in the absence of positively charged D-alanyl esters. However, in contrast with our results, Giàouris et al. (2008) found that the level of D-alanylation in the cell wall of *Lactococcus lactis* did not significantly modify the global surface charge. They suggested that most D-alanyl substituents of TAs are located inside the cell wall and are not exposed at the cell surface, which may explain why electrophoretic mobility was not affected in this mutant strain. Thus, the inactivation of the *dlt* operon has a wide range of physiological consequences in different bacteria, but the absence of D-alanyl esters in the TAs does not result in a clear morphological phenotype that is common to all the bacterial species harboring a *dlt* operon except their higher sensitivity to CAMPs (Peschel et al., 2000; Fabretti et al., 2006).

*DltA, dltB, dltC, or dltD* mutants of several bacteria show high susceptibility to CAMPs and are killed by peptides of host defense mechanisms (Poyart et al., 2003; Kovács et al., 2006; Abi Khattar et al., 2009; McBride and Sonenshein, 2011). We now show that DltX is also involved in the response to antimicrobial peptides. Indeed, inactivation of *dltX* alone (without affecting the expression of *dltABCD*) also substantially impaired the resistance of *B. thuringiensis* to CAMPs (Figure 4) and significantly attenuated *B. thuringiensis* virulence in insect larvae (Figures 5, 6).

We also took advantage of the *Drosophila melanogaster* infection model to demonstrate the prominent role of the D-alanylation of TAs during bacterial resistance to the antimicrobial response *in vivo*. Indeed, both *Oregon R* WT, and *relish* mutant flies (that do not produce AMPs in response to...
infection), were highly susceptible to infection by WT Bt 407. By contrast, virulence of Bt 407ΔdltX was significantly attenuated, as revealed by the survival of a large proportion of oregon R WT adult flies infected with the ΔdltX mutant, but retained its pathogenic effect on relish mutants (Figure 6). Hence, the process of D-alanylation may be essential for the persistence, development, and multiplication of the bacteria in an antimicrobial hostile environment. These findings also indicate that in the absence of antimicrobial peptides, the ΔdltX mutant possesses efficient resistance strategies to deal with the other mechanisms of host defense, such as cellular or melanization responses (Nielsen-LeRoux et al., 2012). It will be interesting to investigate these strategies in the B. cereus–Drosophila interaction model.

Moreover, based on our results, we propose that the two existing models that describe the functions of the proteins encoded by dltABCD should be amended to include dltX, to obtain a complete picture of the mechanism of D-alanine incorporation into the cell wall polymers of Gram-positive bacteria (Figure 9). This model takes into account the recent findings of Reichmann et al. (2013) that showed that DltC does not cross the membrane and that DltD is anchored to the outside of the cell. Therefore, it is unlikely that the carrier protein DltC is involved in the final step of D-alanylation on the outside of the cell, and that DltD, which is active at the outer side of the membrane, facilitates D-alanine ligation to DltC, as proposed by Neuhaus and Baddiley. DltB is predicted to be an integral membrane protein with 12 transmembrane spanning alpha helixes. In this model, DltX, due to its alpha-helical structure prediction and the presence of both extracellular and cytosolic domains, can theoretically interact with any of the four other proteins of the operon (DltA, DltB, DltC, or DltD), or even with several of them. We can consider first, that it is the intracytoplasmic domain of DltX which is involved, and which interact with one of the intracellular components (DltA or DltC). Could DltX play the role of the intermediate undecaprenol-phosphate (which has not yet been confirmed experimentally) or could it help in the transfer of D-alanine from DltC to this lipid linked intermediate? Another possibility is that DltX plays the role of a flippase which flips the D-alanlated
undecaprenol-phosphate across the membrane. An alternative, is that the transmembrane segment of DltX interacts with one of the transmembrane domains of DltB and contributes to the transfer of molecules (such as D-alanine) through the membrane channel formed by DltB. A fourth possibility is that it is the extracellular domain which is involved in the activity of DltX. This domain could interact with the D-alanylated undecaprenol-phosphate, once it is exposed on the outside of the membrane, and be necessary, together with DltD, for the transfer and/or ligation of D-alanine to TAs. It is interesting to note that only two amino acids are completely conserved in all DltX proteins described to date, one is located in the transmembrane alpha helix and the other in the extracytoplasmic domain. It is important now to confirm the interaction of DltX with one of these proteins. Moreover, our results show that the wall structure is strongly affected in the dltX mutant; this might indicate that DltX could interacts with other proteins or enzymes independent of the dlt operon, involved in the synthesis of some elements of the cell wall.

CONCLUSION

Our data clearly demonstrate that the synthesis of D-alanyl-TAs cannot be accomplished only by the concerted action of the four proteins encoded by the dltA, dltB, dltC, and dltD genes, as it frequently suggested in many studies. We have shown that the gene product of dltX is also essential in this process. Future studies addressing the mechanism of D-alanine incorporation into TAs should take into account that DltX is also necessary and essential for the the D-alanylation of TAs. However, additional work is now needed to elucidate the function of DltX and with which other(s) protein(s) of the operon it interacts.

AUTHOR CONTRIBUTIONS

RK: designed experiments, performed the experiments, analyzed and wrote the manuscript, AR: performed the experiments, JJ: performed the experiments, ZA: performed the experiments, PC and M-PC-C: conceived experiments and analyzed data, CN-L and DL: conceived experiments and analyzed data, LC: designed experiments and analyzed the data, MK and VS-B: conceived and designed the study, analyzed the data and wrote the manuscript.

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