

# The *dlt* operon confers resistance to cationic antimicrobial peptides in *Clostridium difficile*

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The *dlt* operon in Gram-positive bacteria encodes proteins that are necessary for the addition of D-alanine to teichoic acids of the cell wall. The addition of D-alanine to the cell wall results in a net positive charge on the bacterial cell surface and, as a consequence, can decrease the effectiveness of antimicrobials, such as cationic antimicrobial peptides (CAMPs). Although the roles of the *dlt* genes have been studied for some Gram-positive organisms, the arrangement of these genes in *Clostridium difficile* and the life cycle of the bacterium in the host are markedly different from those of other pathogens. In the current work, we determined the contribution of the putative *C. difficile* *dlt* operon to CAMP resistance. Our data indicate that the *dlt* operon is necessary for full resistance of *C. difficile* to nisin, gallidermin, polymyxin B and vancomycin. We propose that the D-alanylation of teichoic acids provides protection against antimicrobial peptides that may be essential for growth of *C. difficile* in the host.

## INTRODUCTION

*Clostridium difficile* causes a potentially fatal intestinal disease that is increasing in incidence and severity (Dubberke *et al.*, 2010). Infection is often chronic and difficult to eradicate (O'Brien *et al.*, 2007). *C. difficile* is transmitted as dormant endospores that are highly resistant to killing by heat, antibiotics, oxidizing agents, UV radiation and many other treatments that are usually effective for killing bacteria (Vonberg *et al.*, 2008; Setlow, 1995). *C. difficile* spores germinate in the intestine to produce vegetatively growing cells capable of producing toxins A and B, which have been implicated in disease progression and severity (Bartlett *et al.*, 1977; McDonald *et al.*, 2005).

Upon germination, actively growing *C. difficile* must contend with the onslaught of host defences present in the intestinal environment. The host produces a number of innate immune factors, such as cationic antimicrobial peptides (CAMPs) and bacteriolytic enzymes that protect the intestine from invading bacterial pathogens (Müller *et al.*, 2005; Gudmundsson & Agerberth, 1999). Some of these CAMPs are products of host cells and others are produced by the intestinal microflora. These host effectors are attracted to the overall negative charge of bacterial cells and, as a consequence, bacterial resistance to these effectors can be achieved by charge modification of cell wall and membrane components (Nizet, 2006; Peschel, 2002). In many Gram-positive bacteria, this charge modification occurs by D-alanyl esterification of teichoic acid in the cell

wall, which results in an increased positive charge on the cell (Neuhaus & Baddiley, 2003). The addition of D-alanine esters to teichoic acids is typically mediated by the products of the *dlt* operon, which encodes four proteins: DltA, a D-alanine:D-alanyl carrier protein ligase; DltB, a D-alanyl transfer protein; DltC, the D-alanyl carrier protein; and DltD, a D-alanine esterase. All four proteins are required for successful addition of D-alanine to the cell wall (Neuhaus & Baddiley, 2003). Incorporation of D-alanine into teichoic acid has been demonstrated to increase resistance of bacteria to CAMPs and bacteriolytic enzymes, as well as contribute to the virulence of pathogens such as *Bacillus anthracis* (Fisher *et al.*, 2006), *Listeria monocytogenes* (Abachin *et al.*, 2002), *Streptococcus pneumoniae* (Wartha *et al.*, 2007) and *Staphylococcus aureus* (Weidenmaier *et al.*, 2005).

We report that transcription of the *C. difficile* *dlt* operon is induced in response to CAMPs and is important for resistance to CAMPs during vegetative growth. In addition, we identified a putative regulatory gene as part of the *dlt* operon in *C. difficile*. This work represents what is believed to be the first report examining the role of the *dlt* operon in any *Clostridium* species.

## METHODS

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *C. difficile* strains were grown in BHIS medium (Smith *et al.*, 1981) supplemented with 0.1% L-cysteine. Media for growth of *C. difficile* were supplemented with 250 µg D-cycloserine ml<sup>-1</sup>, 50 µg kanamycin ml<sup>-1</sup> and 20 µg thiamphenicol ml<sup>-1</sup>; 5 µg erythromycin ml<sup>-1</sup>; or 5 µg erythromycin ml<sup>-1</sup> and 50 µg kanamycin ml<sup>-1</sup>, as needed. *C. difficile* strains were

Abbreviations: CAMP, cationic antimicrobial peptide; qPCR, quantitative reverse-transcription PCR.

maintained at 37 °C in an anaerobic chamber (Coy Laboratory Products) with an atmosphere of 10% H<sub>2</sub>, 5% CO<sub>2</sub> and 85% N<sub>2</sub>. *Bacillus subtilis* strains were routinely grown at 37 °C in L broth (Luria & Burrous, 1957) or BHIS medium supplemented with 1 µg erythromycin ml<sup>-1</sup> when needed. For propagation of *B. subtilis* in the anaerobic chamber, the growth medium was supplemented with 5 mM KNO<sub>3</sub>. *Escherichia coli* strains were grown at 37 °C in L or BHIS medium supplemented with 20 µg chloramphenicol ml<sup>-1</sup> or 100 µg ampicillin ml<sup>-1</sup>, as needed.

**Strain and plasmid construction.** Oligonucleotides used in this study are listed in Table 2. *C. difficile* strain 630 DNA, GenBank accession number AM180355 (Sebahia *et al.*, 2006), was used as a template for PCR amplification unless otherwise noted. Sequencing of cloned DNA fragments was performed by the Tufts University Core Facility using an ABI 3130XL DNA sequencer. A null mutation in *dltD* was created in several steps. First, plasmid pMC111 was created by retargeting of the group II intron from pCE240 (kindly supplied by C. Ellermeier, University of Iowa) using primers oMC74, oMC75, oMC76 and the EBS Universal primer as outlined in the TargeTron users' manual (Sigma-Aldrich), followed by initial cloning of the retargeted fragment in pCE240 digested with *BsrGI/HindIII*. Plasmid pCE240 is a derivative of pJIR750ai (Sigma-Aldrich) and is similar to pMTL007 (Heap *et al.*, 2007). pMC111 was then introduced by transformation into *E. coli* strain HB101(pRK24), resulting in strain MC116. pRK24 is a derivative of the broad-host-range plasmid RP4, which mobilizes IncP *oriT* plasmids. MC116 was then mated with *C. difficile* strain JIR8094, resulting in transfer of pMC111 by conjugation, as previously described (Dineen *et al.*, 2007), except that transconjugants were selected on BHIS plates supplemented with D-cycloserine, kanamycin and thiamphenicol. Inactivation of *dltD* was selected for by screening transconjugants for erythromycin resistance and thiamphenicol sensitivity, resulting in strain MC120. Insertional disruption of *dltD* was confirmed using primers oMC21 and oMC22, which are located outside the region of insertion in the *dltD* locus.

To complement the *dltD* disruption, a 4812 bp fragment containing the *dltDABC* operon and its upstream region was amplified using oMC98 and oMC100 as primers and cloned between the *Bam*HI and *Sph*I sites of pSMB47, generating pMC118. MC128 was created by integration of pMC118 into the chromosomal Tn916 locus of *B. subtilis* strain BS49. MC129 is a transconjugant from the mating of *B. subtilis* strain MC128 and *C. difficile* strain MC120, resulting in the integration of the plasmid::Tn916 fusion of the donor strains into the *C. difficile* chromosome, as previously described (Haraldsen & Sonenshein, 2003).

**MIC determination.** Susceptibility tests were performed anaerobically in BHIS broth as follows. *C. difficile* strains were grown to OD<sub>600</sub> 0.5 and then diluted 1:100 in 1 ml fresh medium to give an initial concentration of approximately 5 × 10<sup>5</sup> c.f.u. ml<sup>-1</sup>. The medium was supplemented with a range of concentrations of nisin (MP Biomedicals), polymyxin B (Sigma-Aldrich, ≥6000 USP units mg<sup>-1</sup>), gallidermin (Alexis Biochemicals), LL-37 (cathelicidin) (AnaSpec), Magainin II (Phoenix Pharmaceuticals) or vancomycin (Sigma-Aldrich) and cultures were incubated for 18 h at 37 °C. The MIC was defined as the lowest concentration of antimicrobial peptide that prevented visible turbidity after 18 h. MICs were determined a minimum of three times for each isolate and represent the highest concentration of antimicrobial at which growth was consistently observed.

**CAMP growth assays.** To assess the effects of CAMPs on growth, *C. difficile* strains were grown to late-exponential phase in BHIS and diluted 1:50 into fresh BHIS medium, BHIS with 10 µg nisin ml<sup>-1</sup> or BHIS with 75 µg polymyxin B ml<sup>-1</sup> to OD<sub>600</sub> ~0.025. Samples were taken at hourly intervals to assess growth by optical density.

**Quantitative reverse-transcription PCR (qPCR) analysis.** Exponential phase cultures of *C. difficile* grown in BHIS medium or BHIS supplemented with nisin, gallidermin or polymyxin B were

**Table 1.** Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or features	Source or reference
<b><i>E. coli</i> strains</b>		
HB101	F <sup>-</sup> <i>mcrB mrr hsdS20</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20</i>	B. Dupuy*
MC116	HB101 containing pRK24 and pMC111	This study
<b><i>C. difficile</i> strains</b>		
630	Clinical isolate	Wüst & Hardegger (1983)
JIR8094	Erm <sup>S</sup> derivative of strain 630	O'Connor <i>et al.</i> (2006)
MC120	JIR8094 <i>dltD</i> :: <i>ermB</i>	This study
MC129	MC120::Tn916 ( <i>dltDABC</i> ); Erm <sup>R</sup>	This study
<b><i>B. subtilis</i> strains</b>		
BS49	CU2189::Tn916	P. Mullany†
MC128	BS49 Tn916::pMC118	This study
<b>Plasmids</b>		
pRK24	Tra <sup>+</sup> Mob <sup>+</sup> ; <i>bla tet</i>	Thomas & Smith (1987)
pCE240	<i>C. difficile</i> TargeTron construct based on pJIR750ai (group II intron, <i>ermB</i> ::RAM, <i>ltrA</i> ); <i>catP</i>	C. Ellermeier‡
pSMB47	Tn916 integrational vector; Cm <sup>R</sup> Erm <sup>R</sup>	Manganelli <i>et al.</i> (1998)
pMC111	pCE240 retargeted to <i>dltD</i>	This study
pMC118	4812 bp DNA sequence corresponding to <i>dltDABC</i> plus 500 bp upstream sequence cloned as <i>Bam</i> HI/ <i>Sph</i> I fragment in pSMB47	This study

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**Table 2.** Oligonucleotides used in this study

Underlined bases denote retargeted sequences.

Primer	Sequence	Use and location, or source
oMC21	5'-GCGAATTCGGTGAATTTATTTGGTGCTCC-3'	<i>dltD</i> sequencing
oMC22	5'-GCGGATCCCATATAAGAATGCTCCTTTAAAATCC-3'	<i>dltD</i> sequencing
oMC38	5'-AAAGACGGAGTCACAAGTCACC-3'	<i>dltD</i> qPCR
oMC39	5'-CTGCTTTATACTCGTCACTTCCC-3'	<i>dltD</i> qPCR
oMC44	5'-CTAGCTGCTCCTATGTCTCACATC-3'	<i>rpoC</i> qPCR
oMC45	5'-CCAGTCTCTCCTGGATCAACTA-3'	<i>rpoC</i> qPCR
oMC48	5'-CTTCAGGAAGTACAGGAAAGCC-3'	<i>dltA</i> qPCR
oMC49	5'-TCCATGGATAAGACCAGGGTA-3'	<i>dltA</i> qPCR
oMC74	5'-AAAAGCTTTTGCAACCCACGTCGATCGTGAAA <u>AAAGTTGTCTTGGT</u> GCGCCCAGATAGGGTG-3'	<i>dltD</i> intron retargeting
oMC75	5'-CAGATTGTACAAATGTGGTGATAACAGATAAGT <u>CGTCTTGTTTAACTTACCTTTCTTTGT</u> -3'	<i>dltD</i> intron retargeting
oMC76	5'-CGCAAGTTTCTAATTTCGGTT <u>ACTTTTCGATAGAGGAAAGTGTCT</u> -3'	<i>dltD</i> intron retargeting
EBS	5'-CGAAATTAGAACTTGCCTCAGTAAAC-3'	Sigma-Aldrich
Universal		
oMC96	5'-CGTTCAGGTCAATTTCTCTAGGC-3'	<i>cprA</i> qPCR
oMC97	5'-GGTCAAGACCATTTGTAGGCTC-3'	<i>cprA</i> qPCR
oMC98	5'-GCGGATCCAGAGATGGAAGAGAAGA-3'	<i>PdltDABC</i> cloning
oMC100	5'-GCGCATGCGTCCATCAAATATTAGTATCAGATGG-3'	<i>PdltDABC</i> cloning
oMC101	5'-GCCATCTTGTCCAGAACTC-3'	<i>dltB</i> sequencing
oMC102	5'-GAGTTCTGGACAAGATGGC-3'	<i>dltB</i> sequencing
oMC103	5'-GCCATTAGACCTAAAATTACTG-3'	<i>dltB</i> sequencing
oMC104	5'-CCTCGGTTGGTCCATATC-3'	<i>dltA</i> sequencing
oMC105	5'-GCTCCTATCATAATGCCAT-3'	<i>dltA</i> sequencing
oMC106	5'-CATCTTTGTCTCTTCCTTTGT-3'	<i>dltD</i> sequencing
oMC107	5'-GCTGTAACCCCTTTATCTTT-3'	<i>dltD</i> sequencing
oMC197	5'-CTTCCCTCTTCCCTGTATTTC-3'	CD2855 qPCR
oMC198	5'-CTTCAAACCTGGAGTGGATGGAG-3'	CD2855 qPCR
oMC199	5'-GATATTGGACATGCTGTCTTTGG-3'	CD2850 qPCR
oMC200	5'-CGAGAATCAAGGTTTGTGGATGG-3'	CD2850 qPCR

harvested at OD<sub>600</sub> 0.2 (active growth), and RNA was prepared as previously described (McBride & Sonenshein, 2011; Dineen *et al.*, 2007). RNA quantity and quality were measured by absorbance ( $A_{260}$  and  $A_{260}/A_{280}$  ratio, respectively) on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Primers for qPCR were designed using the online PrimerQuest tool from Integrated DNA Technologies (<http://www.idtdna.com/Scitools/Applications/Primerquest>), and amplification efficiencies for each primer set were determined prior to use. To control for chromosomal DNA contamination, mock cDNA synthesis reactions containing no reverse transcriptase were used as negative controls in subsequent amplifications. cDNA samples were diluted fourfold and used as templates for qPCR of *rpoC* (primers oMC44/oMC45), *dltD* (oMC38/oMC39), *dltA* (oMC48/oMC49), CD2855 (oMC197/oMC198) and CD2850 (oMC199/oMC200), using Roche SYBR Green I PCR mix and a Roche LightCycler 480 II thermocycler. Reactions were performed in a final volume of 20  $\mu$ l using 4  $\mu$ l diluted cDNA and 1  $\mu$ M of each primer. Reactions were performed in triplicate using cDNA extracted from each of a minimum of three biological replicates, and results are presented as the means and SDs of the data obtained. Amplification included 40 cycles of the following steps: 10 s at 95 °C, 10 s at 53 °C, 15 s at 72 °C. Results were calculated using the comparative cycle threshold method (Schmittgen & Livak, 2008), in which the amount

of target mRNA is normalized to that of an internal control transcript (*rpoC*). The two-tailed Student's *t* test was used to analyse the data.

#### Quantification of D-alanine ester content in teichoic acids.

Analysis of ester-linked D-alanine incorporation into cell walls was performed as described elsewhere, with minor modifications (Hyrylainen *et al.*, 2000; Fisher *et al.*, 2006). Cultures (50 ml) of wild-type (JIR8094), *dltD* mutant (MC120) and the complemented strain (MC129) were grown to OD<sub>600</sub> 0.2–0.25 in BHIS anaerobically at 37 °C and harvested by centrifugation, and the cell pellets were stored at –80 °C prior to analysis. Cell pellets were thawed at room temperature and washed three times with 1 ml 0.1 M MES, pH 6.0 (Sigma-Aldrich). To partially purify the cell walls, cell pellets were boiled for 15 min in 0.5 ml 0.2% SDS, 0.1 M MES, pH 6.0. Walls were then pelleted by centrifugation and washed five times with 1 ml 0.1 M MES, pH 6.0, and subsequently dried under vacuum in a heated tabletop vacuum centrifuge. Dried pellets were weighed to determine the amount of cell wall content for each sample. The cell wall material was then resuspended in 0.5 ml sodium pyrophosphate, pH 8.3 (EMD Chemicals), and incubated for 3 h at 60 °C to release ester-linked D-alanine residues. After incubation, the samples were centrifuged and the supernatants removed to a fresh tube. The assay for D-alanine ester content was performed as follows. A 0.1 ml

volume of sample supernatant was mixed with 0.25 ml assay reagent [four volumes 0.1 M sodium pyrophosphate, pH 8.3, two volumes FAD (Sigma-Aldrich) at 0.2 mg ml<sup>-1</sup> in 0.1 M sodium pyrophosphate, pH 8.3, one volume horseradish peroxidase (1000 units mg<sup>-1</sup>, Sigma-Aldrich) at 10 mg ml<sup>-1</sup>, one volume *o*-dianisidine dihydrochloride (Sigma-Aldrich) at 5 mg ml<sup>-1</sup>, and 0.1 volume D-amino acid oxidase (5.7 units mg<sup>-1</sup>, Sigma-Aldrich) at 13.15 µg ml<sup>-1</sup>]. The assay reactions were incubated at 37 °C for 15 min, at which time 1 ml 0.1% SDS was added to stop the reaction. Samples were then centrifuged to remove any trace of precipitate and for each the A<sub>460</sub> of the supernatant fluid was determined. The absorbance values were compared with a standard curve performed with D-alanine (Sigma-Aldrich) to determine the total amount of D-alanine esters present in each sample. The amount of D-alanine esters was divided by the weight of cell wall for each sample to determine the relative amount of D-alanine present. Results were calculated as the means and SDs of three biological replicates, each tested in duplicate. The two-tailed Student's *t* test was used to analyse the data.

## RESULTS

### Identification and expression of the *C. difficile* *dltDABC* operon

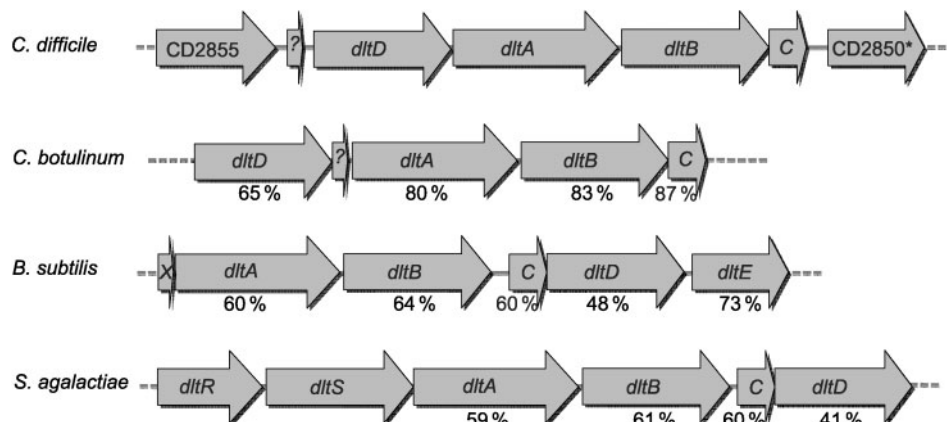
The *dlt* genes were identified as CD2851–CD2854 on the chromosome of the sequenced strain 630 (Sebahia *et al.*, 2006), based on sequence similarity to the *dlt* genes of *B. subtilis*. *C. difficile* has homologues of all four required *dlt* genes arranged in the order *dltDABC* (Fig. 1). The *dltDABC* genes are flanked upstream by a putative oxidoreductase, CD2855. The *B. subtilis* *dlt* operon also has a fifth gene, *dltE*, which encodes a putative oxidoreductase that does not have a known role in D-alanine incorporation into teichoic acid. In addition, a putative regulatory gene, CD2850, which encodes a protein similar to the DeoR family of transcriptional regulators, is located downstream of the *dltC* gene in *C. difficile*. We also searched for homologues of *dltX*, a small (144 bp) gene of

unknown function that accompanies the *dlt* genes of many Gram-positive bacteria (Abi Khattar *et al.*, 2009; Palumbo *et al.*, 2006; Allen, 2008), but were unable to find any sequences with significant similarity. However, a 123 bp ORF was present between CD2855 and *dltD*. Based on the location and size of this ORF, this sequence may have a similar, albeit unknown, function in *C. difficile* (Fig. 1).

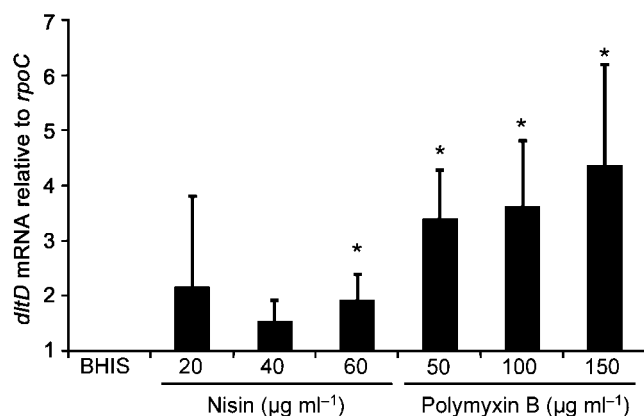
The *dlt* operons characterized to date add D-alanine esters to teichoic acid and, by changing the charge on the cell wall, provide resistance to CAMPs (Neuhaus & Baddiley, 2003). In order to determine whether the annotated *dlt* genes of *C. difficile* play a similar role, we analysed expression of these genes during growth in CAMPs. We chose to use the bacteria-derived model CAMPs nisin and polymyxin B because these CAMPs have been used extensively to study antimicrobial peptide resistance in other Gram-positive and Gram-negative bacteria, and because CAMPs are made by many bacterial species in the gut (*Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, etc.). We used qPCR to examine the expression of the operon, specifically the *dltD* transcript, during early exponential growth in standard BHIS medium and medium supplemented with a range of concentrations of the CAMPs nisin and polymyxin B. As shown in Fig. 2, transcription of *dltD* was induced when wild-type *C. difficile* was grown in the presence of either CAMP. Expression of *dltD* increased with the concentration of CAMPs in the medium and was higher in cells grown in polymyxin B than in nisin. These results are consistent with the expression profiles of *dlt* genes noted for other Gram-positive bacteria grown in media containing CAMPs (Li *et al.*, 2007a, b).

### Effects of the disruption of *dltD* on CAMP resistance

To test whether the *dltDABC* locus has a direct role in CAMP resistance, we introduced an insertion mutation



**Fig. 1.** Comparison of the *dlt* regions of *C. difficile* and of other Gram-positive bacteria. Shown is the organization of the *dlt* operon and accessory genes of *C. difficile* (GenBank accession no. AM180355), *Clostridium botulinum* (CP001078), *B. subtilis* (AL009126) and *Streptococcus agalactiae* (CP000114). Numbers below the arrows indicate percentage similarity of proteins to the corresponding *C. difficile* sequences.



**Fig. 2.** qPCR analysis of *dltD* expression in *C. difficile* during growth in polymyxin B and nisin. *C. difficile* wild-type (JIR8094) was grown to OD<sub>600</sub> 0.2 in BHIS alone or BHIS supplemented with 20, 40 or 60 µg nisin ml<sup>-1</sup>, or 50, 100 or 150 µg polymyxin B ml<sup>-1</sup>, as indicated. RNA was harvested, cDNA synthesized and qPCR performed using gene-specific primers for *dltD*. Results were normalized to an internal control gene (*rpoC*). The means and SDs of biological replicates are shown. Asterisks,  $P \leq 0.05$  by Student's *t* test.

into the first gene of the operon, *dltD*. To do so, we used a TargeTron-based group II intron (Karberg *et al.*, 2001) that was retargeted for integration into *dltD* at nucleotide 353 of the coding sequence (see Methods). The resulting strain, MC120, was then tested for the MICs of nisin, polymyxin B, gallidermin and vancomycin. The MC120 mutant had decreased MICs for all four of these compounds (Table 3). We also tested the resistance of the wild-type and MC120 strains to two animal-derived CAMPs, LL-37 and Magainin II. We observed no difference in the LL-37 MIC values of the wild-type, *dlt* mutant and complemented strains (see below). In the case of Magainin II, all of the strains studied were resistant to the highest concentration of the compound tested (>100 µg ml<sup>-1</sup>). As a result, these CAMPs were not used in further analyses.

The *dltD* mutant also demonstrated significant growth delays when exposed to sublethal concentrations of either nisin (10 µg ml<sup>-1</sup>) (Fig. 3a) or polymyxin B (75 µg ml<sup>-1</sup>) (Fig. 3b). Though the growth rate of the MC120 mutant grown in CAMPs eventually caught up with that of the parental strain, dilution of the growing mutant culture into fresh medium containing CAMPs again produced a delay in active growth (data not shown), indicating that the mutant strain neither adapted to growth in CAMPs nor acquired a resistance mutation.

The *dlt* genes in other bacteria are known to form a transcriptional unit and, as a result, disruption of the first gene in the operon results in a polar effect on transcription of the downstream genes. Because the *dlt* genes of *C. difficile* are arranged in a different orientation from that in most other species [*dltDABC* vs *dlt(X)ABCD*], it was not clear whether

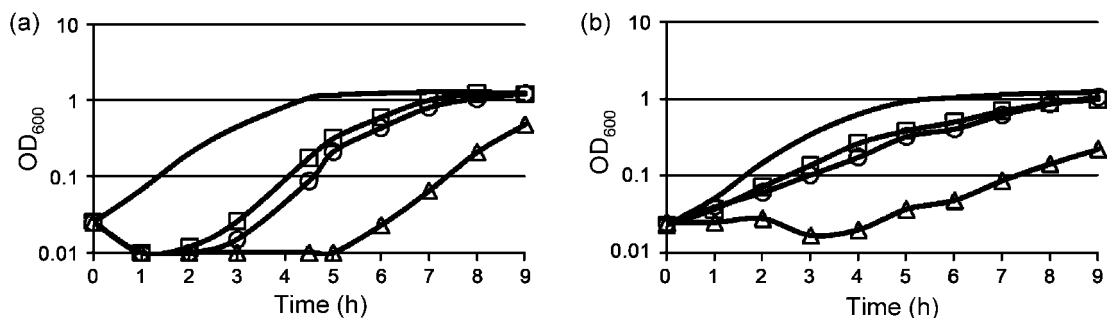
**Table 3.** MICs (µg ml<sup>-1</sup>) for *C. difficile* strains with different CAMPs

CAMP	Strain		
	630	MC120	MC129
Nisin	90	50	110
Polymyxin B	300	150	400
Gallidermin	0.5	0.3	0.4
Vancomycin	1	0.75	1
LL-37	4	4	4

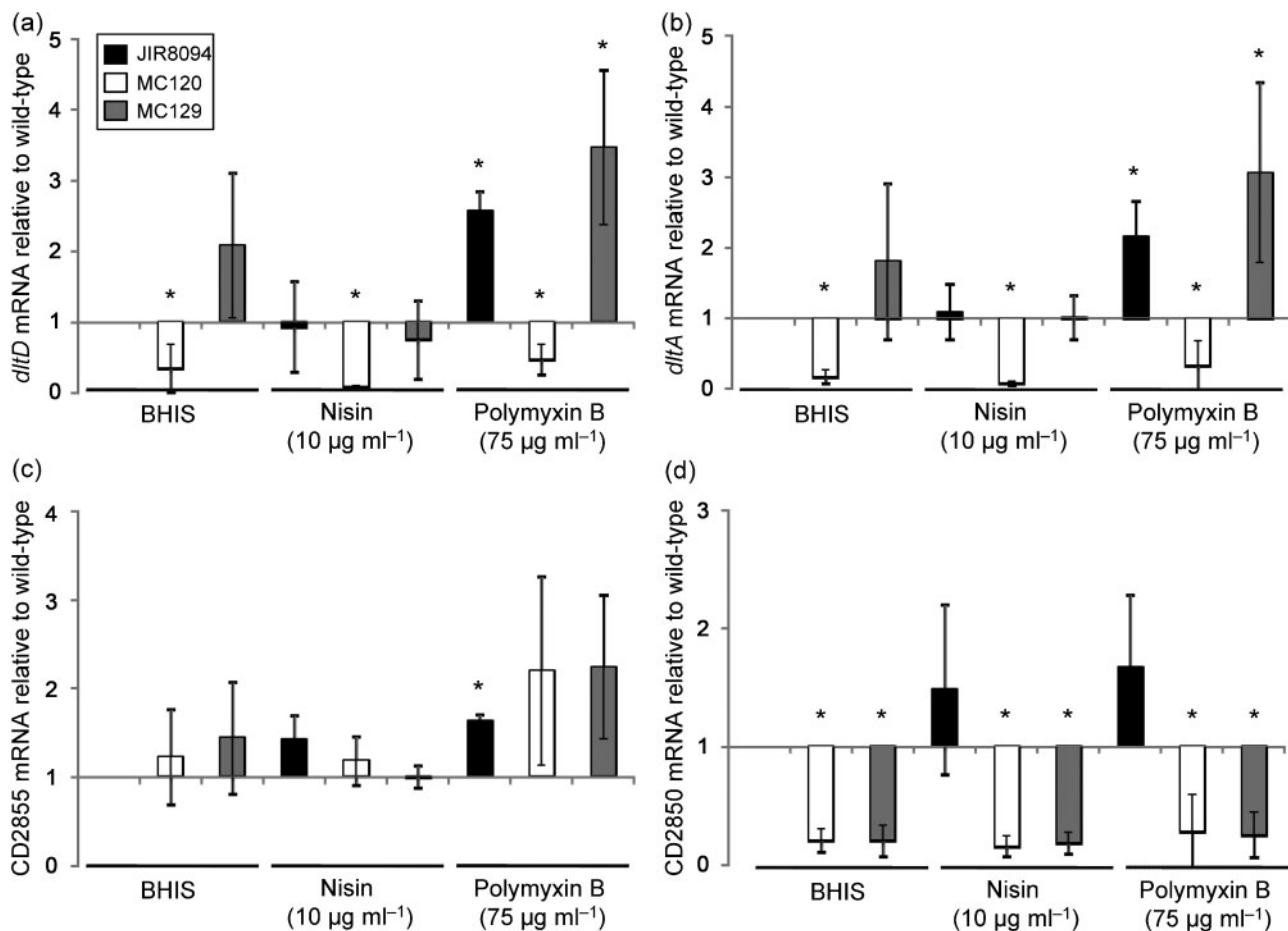
the *dltDABC* genes and the upstream oxidoreductase (CD2855) constitute a single transcriptional unit. For this reason, we performed qPCR analysis of the *dltD*, *dltA* and CD2855 transcripts during growth in the presence and absence of the CAMPs polymyxin B and nisin to determine the effects on transcription of the insertional disruption in *dltD*. As Fig. 4 illustrates, expression of *dltD* and the downstream *dltA* transcript was severely impaired in a *dltD* null mutant, as compared with the wild-type parent strain. These results confirm that targeted insertion in the *dltD* gene not only disrupts transcription of that gene but also results in markedly decreased expression of the downstream gene, providing evidence that these genes are part of a transcriptional unit. In contrast, expression of the CD2855 oxidoreductase was not induced in either the wild-type or the *dltDABC* null mutant under any condition tested (Fig. 4c), indicating that it is not part of the same transcriptional unit as *dltDABC*. Because CD2855 is not co-regulated with the *dlt* genes, it is unlikely to play a direct role in D-alanylation.

To confirm that the defect in CAMP resistance of MC120 was due to disruption of the *dlt* operon, we complemented MC120 with the native version of the *dltDABC* region, including a 500 bp upstream region predicted to contain the potential *dltX* ORF and the promoter for these genes. Complementation of MC120 with the *dltDABC* genes restored the MICs and growth properties in CAMPs to those of the wild-type (Table 3, Fig. 3). Similar to the wild-type, expression of *dltDABC* in MC129 was also induced during growth in nisin and polymyxin B (Fig. 4a, b).

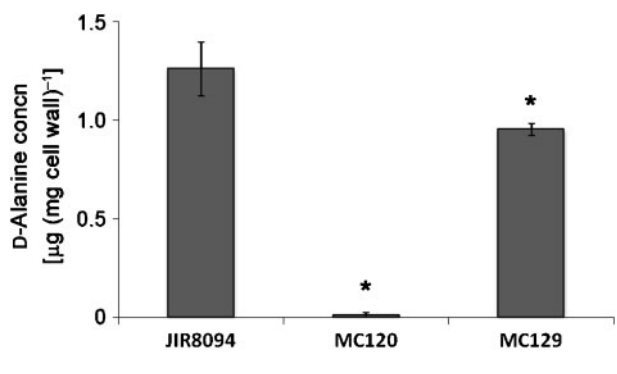
To determine whether the decreased CAMP resistance of the *dltD* mutant was due to a decrease in the incorporation of D-alanine esters into teichoic acids of the cell wall, we isolated and partially purified cell walls from the wild-type, *dltD* mutant (MC120) and *dltDABC* complemented mutant (MC129) to assay for ester-linked D-alanine. As shown in Fig. 5, the D-alanine content of the *dltD* mutant was approximately 100-fold less than that of wild-type *C. difficile*, while the D-alanine content of the *dltDABC* complemented strain was restored to near wild-type levels. Taken together, the MIC values, transcriptional analyses and D-alanine content of the *dltD* mutant indicate that the *dltDABC* gene products are responsible for incorporation of D-alanine esters into the teichoic acids of *C. difficile* and contribute to resistance to CAMPs.



**Fig. 3.** Growth of *C. difficile* wild-type and mutant isolates in nisin and polymyxin B. *C. difficile* wild-type (JIR8094,  $\square$ ), MC120 (*dltD*::intron::*ermB*,  $\triangle$ ) and MC129 (*dltD*::intron::*ermB*, *dltDABC*<sup>+</sup>,  $\circ$ ) strains were grown in BHIS medium or BHIS medium supplemented with 10  $\mu\text{g}$  nisin B  $\text{ml}^{-1}$  (a) or 75  $\mu\text{g}$  polymyxin B  $\text{ml}^{-1}$  (b). The growth rates of strains in BHIS alone were indistinguishable and are thus shown as a single solid line for clarity.



**Fig. 4.** Expression of the *dlt* genes of wild-type and mutant strains in the presence of the CAMPs nisin and polymyxin B. *C. difficile* wild-type (JIR8094), MC120 (*dltD*::intron::*ermB*) and MC129 (*dltD*::intron::*ermB*, *dltDABC*<sup>+</sup>) strains were grown to  $\text{OD}_{600}$  0.2 in BHIS or BHIS supplemented with 10  $\mu\text{g}$  nisin  $\text{ml}^{-1}$  or 75  $\mu\text{g}$  polymyxin B  $\text{ml}^{-1}$ . RNA was harvested, cDNA synthesized and qPCR performed using gene-specific primers for (a) *dltD*, (b) *dltA*, (c) CD2855 or (d) CD2850. Results were normalized to an internal control gene (*rpoC*) and are presented as the ratio of each transcript level to that of wild-type cells grown in BHIS. The means and sds of biological replicates are shown. Asterisks,  $P \leq 0.05$  by Student's *t* test.



**Fig. 5.** Analysis of ester-linked D-alanine content in *C. difficile* cell walls. Cell walls partially purified from *C. difficile* wild-type (JIR8094), MC120 (*dltD*::intron::*ermB*) and MC129 (*dltD*::intron::*ermB*, *dltDABC*<sup>+</sup>) strains harvested after growth to OD<sub>600</sub> 0.2 in BHIS were assayed for incorporation of D-alanine into teichoic acids of the cell wall. Results represent the means and SDs of three biological replicates, each replicate tested in duplicate samples. Asterisks,  $P \leq 0.05$  by Student's *t* test.

### Identification of a regulatory gene within the *dlt* operon

The regulation of the *dlt* operon and D-alanylation of teichoic acid are not known to have been studied in any *Clostridium* species. However, in the spore-forming bacterium *B. anthracis*, the *dlt* genes are turned on during spore germination in the host and are necessary for resistance to innate host defences (Fisher *et al.*, 2006). Because a potential regulatory protein is located immediately adjacent to the *dlt* operon, we examined the regulation of its expression. The gene for this regulatory protein, CD2850, is situated downstream of the operon and separated from *dltC* by 217 bp. CD2850 has homology to the DeoR family of transcriptional regulators, which often function as repressors of gene transcription and are expressed in an inverse manner relative to the genes that they regulate (i.e. regulator transcription decreases when target transcription increases; Hammer-Jespersen & Munch-Petersen, 1975), although in some circumstances they may behave as positive regulators (Haghjoo & Galán, 2007). Because of the close proximity of CD2850 to the *dlt* genes, we investigated whether expression of CD2850 coincides with expression of the *dlt* operon. Using qPCR, we found that the CD2850 regulator was expressed in response to either nisin or polymyxin B in wild-type cells (Fig. 4d). However, expression of CD2850 was drastically reduced in both the *dltD* insertional mutant (MC120) and the *dltDABC*-complemented strain (MC129). These data indicate that CD2850 expression is dependent on transcription of *dltDABC*, and provide evidence that *dltDABC* and CD2850 constitute a transcriptional unit. Considering that an increase in CD2850 expression accompanies the increase in *dltDABC* expression, it is unlikely that the CD2850 regulatory protein is a negative regulator of the *dlt* operon. However, because the *dltDABC*-complemented

strain (MC129) does not express CD2850, we can also infer that this putative regulator is not required for expression of the *dlt* operon under the conditions tested.

## DISCUSSION

The *dlt* operon of Gram-positive bacteria is responsible for the addition of D-alanine to teichoic acids, which results in a decreased negative charge on the bacterial cell surface. This change in surface charge directly diminishes the interaction of host innate immune effectors, such as CAMPs, with the bacterial surface. As a consequence, the addition of D-alanine can lead to decreased effectiveness of these molecules in protecting against Gram-positive infection. Although these genes have been studied for some organisms, the arrangement of otherwise similar genes in *C. difficile* and the life cycle of the bacterium in the host are markedly different from those of other pathogens. In the current work, we found that the expression of the *dltDABC* genes increased when wild-type *C. difficile* was grown in the presence of CAMPs, and that disruption of the *dlt* operon by insertional mutation resulted in a decrease in resistance to multiple CAMPs, including polymyxin B, nisin, gallidermin and vancomycin. These results imply strongly that the *C. difficile* *dlt* operon plays a role in protecting the bacteria against host defences, especially against bacterial CAMPs that may be produced by the indigenous microbiota.

Many regulatory schemes have been found to control transcription of the *dlt* genes of Gram-positive bacteria (Neuhaus & Baddiley, 2003). Some bacteria use a two-component system [e.g. *Streptococcus agalactiae* (Poyart *et al.*, 2001)], some use a single regulator [e.g. *L. monocytogenes* (Mandin *et al.*, 2005)] and others use multiple regulators [e.g. *S. aureus* (Li *et al.*, 2007a, b; Dunman *et al.*, 2001; Saïd-Salim *et al.*, 2003; Kraus *et al.*, 2008; Koprivnjak *et al.*, 2006)] to control transcription of the *dlt* genes. Many of the *dlt* transcriptional regulators also control the expression of virulence genes (Mandin *et al.*, 2005; Dunman *et al.*, 2001; Saïd-Salim *et al.*, 2003; Kraus *et al.*, 2008; Li *et al.*, 2007a, b). Our investigation of *dlt* transcription revealed a co-expressed regulatory protein encoded downstream of the *dltDABC* ORFs, but we could find no evidence that the putative regulator plays either a positive or a negative role in *dlt* expression. In *Streptococcus agalactiae*, the two-component regulatory system that is transcribed with the *dlt* genes is also unnecessary for *dltABCD* transcription under the conditions tested, leading to the hypothesis that these regulators are involved in regulating the level of D-alanine esters under unknown conditions (Poyart *et al.*, 2001). This may be the case for the CD2850 gene in *C. difficile* as well.

In *B. subtilis*, the *dlt* operon is regulated by an assortment of sigma factors and sporulation regulatory proteins, depending on the growth stage of the bacterium (Perego *et al.*, 1995; Cao & Helmann, 2004). The principal regulator in *B. subtilis* is SigX, a sigma factor that regulates cell

envelope metabolism and resistance to antimicrobial peptides (Cao & Helmann, 2004). Secondary transcription factors for *dlt* include SigD and Spo0A (Perego *et al.*, 1995; Estacio *et al.*, 1998). *C. difficile* does not have a SigX homologue, but does have homologues of SigD and Spo0A (Sonenshein *et al.*, 2005). However, no obvious SigD-binding sites are present in the *dlt* region of *C. difficile*. Little is known about the expression characteristics of any of these regulatory factors during growth of *C. difficile* in the host. Given that *C. difficile* in the intestinal tract encounters a very different environment from that of *B. subtilis* in the soil and has a more limited repertoire of sigma factors (Sonenshein *et al.*, 2005), it is likely that regulation of D-alanine incorporation into the cell wall of *C. difficile* will be distinct from that in the other sporulating bacteria characterized to date.

In conclusion, we have demonstrated that the *dlt* operon of *C. difficile* is expressed as a consequence of exposure to CAMPs, and that this operon is directly involved in the addition of D-alanine residues to teichoic acids as well as resistance to antimicrobial peptides. The production of CAMPs in the host represents a critical component of innate immunity. Thus, the decreased viability in the presence of any of these compounds may predicate decreased fitness in the host. Though we did not observe a decreased MIC for the *dlt* mutant with the human CAMP LL-37, the decreased MIC of the mutant for a variety of other CAMPs suggests that this strain has a general defect in growth in the presence of cationic peptides that may affect its survival *in vivo*. In *B. anthracis*, transcription of the *dlt* operon is initiated during germination and outgrowth, and mutants that do not express these genes are attenuated during outgrowth *in vitro* and for virulence in an animal model (Fisher *et al.*, 2006). Because *C. difficile* undergoes an analogous developmental evolution from the dormant spore form to a vegetative cell *in vivo*, it too must perform this task in the presence of host innate effectors, and therefore could be similarly susceptible during the outgrowth phase and periods of rapid growth. We are currently examining the role of *dltDABC* during outgrowth and vegetative growth in *in vivo* models of disease. Based on the roles of the *C. difficile dltDABC* genes in survival in the presence of CAMPs, we hypothesize that analogous genes could also affect the viability of related pathogens, such as *Clostridium botulinum*.

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