Effect of phage infection on toxin production by *Clostridium difficile*

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Infection with *Clostridium difficile* and subsequent production of toxins A and B may result in *C. difficile*-associated diarrhoea and pseudomembranous colitis in hospital patients. The effect of four temperate phages, obtained by induction of clinical *C. difficile* isolates, on toxin production by *C. difficile* was determined. None of these phages converted a lysogenized non-toxigenic *C. difficile* strain to toxin production. One of the accessory toxin genes, *tcdE*, was detected in three phages, φC2, φC6 and φC8; however, the non-repeating regions of *tcdA* and *tcdB* encoding the enzymic domains were not carried on phage DNA. Phage infection of toxigenic strains increased toxin B production in four of six lysogens, although the level of *tcdB* transcription as determined by real-time RT-PCR was not significantly altered. However, levels of toxin A transcription in two lysogens were significantly altered without any corresponding differences in toxin A production.

INTRODUCTION

*Clostridium difficile* is a spore-forming anaerobe and the causative agent of pseudomembranous colitis and many cases of antibiotic-associated diarrhoea in hospital patients (Bartlett et al., 1978). Toxins A and B are major virulence factors causing enterotoxicity and cytotoxicity, respectively, to cells (Lyerly et al., 1985). The secretory mechanism of both toxins is unknown; the toxins lack signal peptides, suggesting that an alternative to the type II pathway of the general secretory pathway is present (Mukherjee et al., 2002). A toxin-associated gene, *tcdE*, which lies in the pathogenicity locus (PaLoc) between *tcdA* and *tcdB*, was shown to possess a phage holin-like function when expressed in *Escherichia coli*. As phage holins are involved in cell membrane disruption (Wang et al., 2003), TcdE was speculated to play a role in toxin release through formation of membrane lesions (Tan et al., 2001). We wished to investigate whether the presence of prophage would enhance toxin production in *C. difficile*. To date, production of *C. difficile* toxins has not been shown to be regulated or encoded by temperate phages, although the PaLoc possesses characteristics of a mobile genetic element (Braun et al., 1996; Hammond & Johnson, 1995).

Previous studies of the potential effects of phages on *C. difficile* toxin production are limited to the description of two phages that did not convert non-toxigenic strains to toxin production (Mahony et al., 1985). In our study, four temperate phages that were induced from three clinical *C. difficile* isolates were tested for toxin conversion without success, although *tcdE* was detected in phage DNA. Phage infection of toxigenic strains increased toxin B production in four of six lysogens, although the level of *tcdB* transcription as determined by real-time RT-PCR was not significantly altered. However, levels of toxin A transcription in two lysogens were significantly altered without any corresponding differences in toxin A production.

METHODS

Bacteria and bacteriophages. Fifty-six *C. difficile* isolates were isolated from stool samples of diarrhoeal patients at Sir Charles Gairdner Hospital (SCGH), Western Australia, as previously described (Riley et al., 1994). Overnight broth cultures were induced with 3 μg mitomycin C ml⁻¹ for phage, as previously described (Nagy & Foldes, 1991). Three of the 56 isolates harboured temperate phages as determined by plaque assay: φC2 from CD242, φC5 from CD578, and φC6 and φC8 from CD371 (Table 1). Isolates that produced a high number of plaques for each phage were determined through plaque assay (see below) and used as propagating strains. All *C. difficile* isolates used in this study are shown in Table 1. Phages were purified as described by Sambrook et al. (1989) and determined to be *Myoviridae* (φC2, φC3 and φC8) and *Siphoviridae* (φC6) phages (unpublished data).

Culture media, bacterial growth and bacteriophage propagation. *C. difficile* isolates were cultured in brain heart infusion broth (BHIB; Oxoid) for overnight or 2.5–3 h (exponential phase) at 37 °C in 80 % N₂, 10 % CO₂ and 10 % H₂ (Don Whitley D3161). Cultures for toxin assays were incubated for 48 h. Anaerobe basal agar (ABA; Oxoid),
Extraction of phage DNA was carried out as previously described followed by chloroform, precipitated with 0.3 M sodium acetate (pH equal volumes of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) 40 mM EDTA and 500 µl of 10–8 p.f.u. ml–1) was mixed with an indicator culture (4 ml of 108 c.f.u. ml–1) and overlaid onto 1 % ABA for phage propagation, or 10 µl phage was spotted onto plates seeded with 600 µl indicator for phage assay.

Isolation of stable lysogens of \(\phi C2\), \(\phi C6\) and \(\phi C8\). Five \(C.\ difficile\) isolates were infected with the four phages, producing six stable lysogens of \(\phi C2\), \(\phi C6\) and \(\phi C8\) (Table 1). \(\phi C5\) did not lysogenize these isolates. Centres of turbid plaques were stabbed with a sterile wire and cultured on blood agar (BA) for 48 h. Single colonies arising from phage-resistant cells within the plaque, due either to mutation or to resistance conferred by prophage, were induced with mitomycin C and assayed with the uninfected strain to determine the presence of prophage. Stability of lysogens after –70 °C storage was tested by mitomycin C induction and colony blots using phage genome probes labelled with digoxigenin (Roche), as previously described (Karcher, 1995). Stable lysogens were named by the parental isolate number followed by the phage type, e.g. lysogen CD382C2 was host strain CD382 lysogenized by \(\phi C2\).

DNA extraction from \(C.\ difficle\) and phages. An overnight broth culture (10 ml) of \(C.\ difficle\) was concentrated in 1 ml TE buffer, treated with 2 µg lysozyme ml–1 at 37 °C for 30 min followed by 1 % (v/v) SDS, 40 mM EDTA and 500 µg proteinase K ml–1 at 55 °C for 1–2 h, and 100 µg RNase A ml–1 at 37 °C for 30 min. DNA was extracted twice with equal volumes of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) followed by chloroform, precipitated with 0.3 M sodium acetate (pH 5–2) and ethanol, and purified (Wizard Clean-up system by Promega). Extraction of phage DNA was carried out as previously described (Sambrook et al., 1989).

Detection of toxin A and B levels in uninfected hosts and lysogens. Broth cultures of \(C.\ difficle\) were standardized to obtain 1–9×10^3 c.f.u. ml–1 and filtered (0.22 µm; Acrodisc). Crude toxin in the filtrate was serially diluted twofold and toxin A and B were assayed by ELISA (Techlab) and cell culture, respectively. One toxin A unit was defined as the reciprocal of the ELISA end point dilution to logarithmic base 2. Toxin A assays were performed once only due to reagent costs. Toxin B was assayed by inculating 20 µl crude toxin onto Vero cells with a seeding concentration of 50000 cells per well (Mahony et al., 1989). Cells were observed after 48 h and the minimum cytotoxic titre (MCT), defined as the reciprocal of the dilution that resulted in 50 % of cell death, was determined by neutral red assay (Mahony et al., 1989). MCTs were compared by mean and variance of cytotoxic titre units, which was defined as the logarithmic base 2 of the MCT (Freeman & Wilcox, 2003; Ketley et al., 1984). Assays were done in triplicate and repeated twice.

Detection of toxin genes in phage DNA by PCR. Primers for amplification of \(tcdA\) (NK 2/3) and \(tcdB\) (NK 104/105) were described previously (Kato et al., 1991). A PCR reaction of 30 µl consisted of 1 µl template DNA, 0.18 µM of each primer, 200 µM dNTP, 2.5 mM MgCl2, 1× reaction buffer and 0.75 U Taq gold polymerase (Perkin Elmer). Cycling conditions were 94 °C for 9 min, 35 cycles of 95 °C for 20 s and 55 °C for 120 s, then 74 °C for 5 min. Primers for \(tcdE\) (KST 1/2) were also described previously (Tan et al., 2001). The 20 µl PCR reaction consisted of 7 µl template DNA, 0.2 µM of each primer, 200 µM dNTP, 2 mM MgCl2, 1× reaction buffer and 0.75 U Taq gold polymerase (Perkin Elmer). Cycling conditions were 94 °C for 10 min, 45 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and 72 °C for 7 min. Primer sequences are shown in Table 2. Chromosomal DNA from four toxigenic and non-toxigenic isolates served as control for amplification of toxin genes.

Detection of toxin genes by Southern hybridization. Phage and bacterial DNA was digested by HindIII and/or XbaI (Promega), separated in 1 % agarose, transferred to a nylon membrane (Amersham Pharmacia Biotech) (Sambrook et al., 1989) and fixed by heating in a microwave for 2–5 min (Angeletti et al., 1995). Southern hybridization was carried out with the DIG High Prime DNA Labelling and Detection Starter Kit 1 (Roche), according to the manufacturer’s instructions. Purified (Wizard Clean-up system; Promega) undigested phage DNA was DIG-labelled for use as probes (Roche). Probes for \(tcdA\), \(tcdB\) and

Table 1. \(C.\ difficle\) isolates used for phage induction, propagation and infection, and in toxin assays

<table>
<thead>
<tr>
<th>(C.\ difficle) isolates*</th>
<th>Purpose in this study</th>
<th>Toxin B†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD242</td>
<td>Induction of (\phi C2)</td>
<td>Negative</td>
<td>This study</td>
</tr>
<tr>
<td>CD578</td>
<td>Induction of (\phi C5)</td>
<td>Negative</td>
<td>This study</td>
</tr>
<tr>
<td>CD371</td>
<td>Induction of (\phi C6) and (\phi C8)</td>
<td>Positive</td>
<td>This study</td>
</tr>
<tr>
<td>CD602</td>
<td>Propagation of (\phi C2) and (\phi C5)</td>
<td>Negative</td>
<td>This study</td>
</tr>
<tr>
<td>CD60</td>
<td>Propagation of (\phi C6)</td>
<td>Positive</td>
<td>This study</td>
</tr>
<tr>
<td>CD843</td>
<td>Propagation of (\phi C8)</td>
<td>Positive</td>
<td>This study</td>
</tr>
<tr>
<td>VPI 10463‡</td>
<td>Toxin A and B positive control</td>
<td>Positive</td>
<td>Lyerly et al. (1982)</td>
</tr>
<tr>
<td>CD33</td>
<td>Toxin A and B negative control</td>
<td>Negative</td>
<td>This study</td>
</tr>
<tr>
<td>CD382</td>
<td>Test for toxin conversion by phages</td>
<td>Negative</td>
<td>This study</td>
</tr>
<tr>
<td>CD594</td>
<td>Isolation of (\phi C8) lysogen</td>
<td>Positive</td>
<td>This study</td>
</tr>
<tr>
<td>CD727</td>
<td>Isolation of (\phi C6) lysogen</td>
<td>Positive</td>
<td>This study</td>
</tr>
<tr>
<td>CD839</td>
<td>Isolation of (\phi C2) lysogen</td>
<td>Positive</td>
<td>This study</td>
</tr>
<tr>
<td>CD1017</td>
<td>Isolation of (\phi C6) and (\phi C8) lysogens</td>
<td>Positive</td>
<td>This study</td>
</tr>
<tr>
<td>CD6938</td>
<td>Isolation of (\phi C6) lysogen</td>
<td>Positive</td>
<td>This study</td>
</tr>
</tbody>
</table>

*All CD prefix isolates from diarrhoeal patients at SCGH, Western Australia.
†Determined by cytotoxicity assays on Vero cells.
‡Kindly provided by Dr Maja Rupnik, University of Ljubljana, Slovenia.
Table 2. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Sequence (5′–3′)</th>
<th>Accession no.</th>
<th>Reference</th>
</tr>
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<tr>
<td>NK 2/3</td>
<td>tcdA</td>
<td>CCCAATAGAAGATTCATAATTGAAGCTT</td>
<td>M30307</td>
<td>Kato et al. (1991)</td>
</tr>
<tr>
<td>ToxA-283/360</td>
<td>tcdA</td>
<td>GGAAGAAAAAGAATCTGCTGCTAGG</td>
<td>M30307</td>
<td>This study</td>
</tr>
<tr>
<td>TOXA-315</td>
<td>tcdA</td>
<td>AAACCTTAGTGCAAGAATTAGGA</td>
<td>M30307</td>
<td>This study</td>
</tr>
<tr>
<td>NK 104/105</td>
<td>tcdB</td>
<td>CCCCCTTACGCTTTAATTCTTTTTT</td>
<td>X53138</td>
<td>Kato et al. (1998)</td>
</tr>
<tr>
<td>ToxB-23/101</td>
<td>tcdB</td>
<td>TTTCTGATGCATAGTGACTAAA</td>
<td>X53138</td>
<td></td>
</tr>
<tr>
<td>TOXB-48</td>
<td>tcdB</td>
<td>CTCGTATATGTCCCAAGTATACAA</td>
<td>X53138</td>
<td></td>
</tr>
<tr>
<td>KST 1/2</td>
<td>tcdE</td>
<td>CCGGGGATCCAGTGGACTAGTCCACTTT</td>
<td>M30308</td>
<td>Tan et al. (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCCCAAGCTCCTCAACTGACCAGCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Reporter dye FAM (6-carboxyfluorescein).
†Minor groove binder/non-fluorescent quencher.

tcdE were prepared from PCR-amplified products of C. difficile VPI 10463. Optimal hybridization temperatures were determined from dot blots using appropriate DNA controls. Southern hybridizations were repeated three times.

Colony hybridization. Lysogens grown on BA in a matrix were transferred onto a nylon membrane and lysed as previously described (Karcher, 1995). DNA was fixed and hybridized to phage probes as described above.

Extraction of bacterial RNA and real-time RT-PCR. RNA extraction from a 10 ml overnight culture of C. difficile was carried out using reagents supplied in Totally RNA (Ambion) according to the manufacturer’s instructions. Relative quantitative real-time RT-PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) on RNA extracted from uninfected (parental) and infected (lysogenic) C. difficile isolates. ToxA-283/360 and ToxB-23/101 primers were designed to flank their TaqMan probes TOXA-315 and TOXB-48 (Table 2), respectively. TaqMan probes were labelled 5′ with the reporter dye 6-carboxyfluorescein, and 3′ with minor groove binder groups for increased sequence specificity (Kutyavin et al., 2000). The reaction volume was 20 μl, which consisted of 1 × Superscript reaction mix (Invitrogen), 2-8 mM MgCl₂, 10 mM DTT, 0-3 U Superscript III RT/Platinum Taq Mix (Invitrogen), 10 U RNaseOUT (Invitrogen), 0-3 μM of each primer, 0-2 μM TaqMan probe and 40 ng template RNA. RT-PCR reactions were carried out at 50 °C for 30 min, 94 °C for 5 min, and for 45 cycles of 94 °C for 10 s, 55 °C for 10 s, and 68 °C for 30 s. A sample was used as calibrator RNA, where serial dilutions resulting in a common host to all four phages. CD382 was infected with phages for isolation of stable lysogens, which were tested for toxin B production through cell culture. The presence of tcdA and tcdB in DNA was also determined through PCR. CD382C2, CD382C5, CD382C6 and CD382C8 did not have a cytotoxic effect on Vero cells. DNA of three single colonies of each lysogen was extracted for PCR amplification with NK 2/3 (tcdA) and NK 104/105 (tcdB) primers. All samples were negative for tcdA and tcdB.

Detection of toxin genes in phage DNA
tcdA and tcdB were not detected in phage DNA by PCR or Southern hybridization (not shown). Probes were chosen for the non-repeating region of tcdA and tcdB in the interest of detecting them separately. The non-repeating nucleotide repeats in tcdA and tcdB encode the enzymic domain (Barroso et al., 1994; Faust et al., 1998). Although the phages did not harbour these sections of the toxin genes, it is possible that sequences related to the repeating units were present. Nucleotide repeating sequences at the 3′-end of both genes (Barroso et al., 1994; Dove et al., 1990) encode repeating units responsible for carbohydrate binding in TcdA and receptor binding and translocation in TcdB (Pfeifer et al., 2003).

Despite a lack of association between the toxin genes and phage DNA in the above experiments, there are two intriguing reports of homology between tcdA and genomes of phages belonging to other species. The toxin A gene was reported to be homologous to an unknown gene in phage φCT2 of Clostridium tetani (Canchaya et al., 2002). Lactobacillus casei phage A2 ORF22 was hypothesized to have a srf function (Proux et al., 2002). Homology between ORF22 and toxin A has been reported although no details of its extent were provided (Proux et al., 2002). Alignment of ORF22 (GenBank accession no. NC_004112) to TcdA of VPI 10463 (GenBank accession no. X9298) and variant strains (GenBank accession no. AJ011301, AF279457, AF217291, AJ132669 and Y10689) did not show significant similarity.

**RESULTS AND DISCUSSION**

Phage did not convert non-toxigenic strain to toxin production

C. difficile strain CD382 was toxin B negative by cell culture and a common host to all four phages. CD382 was infected with phages for isolation of stable lysogens, which were tested...
However, alignment of ORF22 to TcdC of VPI 10463 showed 55% identity over the last 103 aa of TcdC with an e-value of 2e−22, but no significant similarity to the truncated TcdC of the variant strain 8864 (GenBank accession no. Y10689). Such homology between TcdA and TcdC to phage proteins suggests phage origins of toxin genes. This is interesting to note that ORF22 was absent in the variant strain with a truncated TcdC. Homology of phage genes to tcdB has not been described; however, part of the hybridizations of tcdB by PCR (not shown). To verify these results, Southern blotting using a putative ORF in sequence 584 and TcdB to suggest any significant similarities or alignments were identified between the enzymic domain or the non-repeating section of the gene, but outside of the region amplified by the PCR primers. No significant homology was detected in VPI 10463, which is dissimilar to the family may possess a different holin that is related, and both were moderately related to φC6, as determined by phage immunity assays and Southern hybridization (Goh et al., 2005). This level of relatedness was reflected in the patterns of hybridization of tcdE to phage DNA. The presence of tcdE in φC2, φC5 and φC8 is interesting because it supports the idea that phage may have some role in toxin secretion through a holin-like pathway, as suggested in two studies (Mukherjee et al., 2002; Tan et al., 2001). Holins are small membrane proteins that disrupt the bacterial membrane together with endolysins (Wang et al., 2000). To date, little is known about C. difficile toxin secretion, and although the type II pathway of the general secretory pathway is present in clostridia it is unlikely to be involved (Bruggemann et al., 2003; Mukherjee et al., 2002). Phage-infected toxigenic strains of C. difficile may be able to release toxins through a fully functional holin pathway resulting in cell lysis. Alternatively, the existing tcdE-associated holin pathway may be complemented in lysogens by phage holin genes and function at a higher efficiency to cause membrane disruption compared to uninfected toxigenic strains that rely only on the tcdE-associated holin pathway. It is also possible that the presence of a holin-like tcdE in the PaLoc is a coincidence. Putative holin sequences have been found in bacterial chromosomes, probably as remnants of cryptic phages (Wang et al., 2000).

**Toxin A and B assays of lysogens and parents**

Cytotoxicity assays were carried out on five toxigenic parental isolates and six lysogens (Table 1). Since an absorbance standard for quantification of toxin A was not provided with the ELISA kit (TechLab), the minimum end point titre was used as a measure of toxin A levels. A twofold increase in toxin A was measured in CD1017C6, while the remaining lysogens were unaltered, suggesting phage had little effect on toxin A production (Fig. 2a). Significant increases in toxin B were observed in all lysogens except for

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**Fig. 1.** Detection of tcdE in phage DNA. (a) Separation of HindIII-digested C. difficile and phage DNA in 1% agarose. Lanes: 1, λ/HindIII marker; 2, VPI 10463; 3, CD33; 4, φC2; 5, φC5; 6, φC6; 7, φC8. (b) Southern hybridization of (a) to DIG labelled tcdE probe carried out at 36°C. There was strong hybridization of the tcdE probe to VPI 10463, φC2 and φC5, and weak signals from φC8.
CD594C8 and CD727C6, which had unaltered levels (Fig. 2b). This apparent lack of coordination between toxin A and toxin B production may be due to methodology and should be confirmed by repeating the toxin A assays, and using an ELISA method for toxin B. Phage had a positive effect on toxin B production in CD839C2, CD1017C6, CD1017C8 and CD6938C6 (Fig. 2b) despite the apparent absence of tcdB in phage DNA. Hence increased toxin production was not through acquisition and transcription of an additional toxin gene. However, as discussed earlier, toxin B levels may have been increased through a phage-mediated process of toxin secretion. Alternatively, toxin expression may have been up-regulated in these lysogens.

**Toxin A and B expression in lysogens and parents (real-time RT-PCR)**

A relative quantitative method of real-time RT-PCR was employed to compare toxin transcripts in parental isolates and lysogens. Coordinated transcription of tcdA and tcdB after phage infection was observed in all lysogens except CD594C8 and CD6938C6, in which significant changes in the amounts of tcdA transcripts were observed (Fig. 3). This is unusual as tcdA and tcdB transcription is co-regulated (Hammond et al., 1997; Hundsberger et al., 1997) through tcdD (Mani & Dupuy, 2001), which is responsive to growth phase, constituents of medium, and temperature (Karlsson et al., 2003; Mani et al., 2002). Interestingly, phage infection increased tcdA transcription in CD594C8 but not TcdA production, while phage infection did not affect tcdB transcription but increased TcdB production in four lysogens (Table 3). This suggests that phage infection has a greater effect on toxin production or release than transcription of tcdA and tcdB, perhaps through the action of holins. In lysogens, functional holins may result in channels that are only large enough for TcdB (266 kDa) to pass through but not TcdA (308 kDa), hence extracellular TcdA levels are unaltered. However, a recent study on the size of membrane lesions formed by the λ holin showed that a protein of 480 kDa passed through the lesions. The authors suggested that holins may not form channels of a constant diameter, but rather cause general disruption of the cell membrane (Wang et al., 2003).

![Fig. 2. Toxin A and B of lysogens compared to parental strains. (a) Toxin A titres, expressed as absorbance units, were determined by ELISA. The end point dilution was the reciprocal of the minimum dilution that resulted in an A₄₅₀ < 0.09. (b) Minimum cytotoxic titre (MCT) of toxin B was determined by neutral red assay. Data are expressed as mean ± SEM. *, P values < 0.01; na, the strain was not a host of the phage, or a stable lysogen was not isolated.](image-url)
summary, toxin production was not always directly correlated with toxin transcription in lysogens. Differences in toxin levels appeared to be dependent on the parental strains rather than the infecting phage, since these changes were not associated with a specific phage, and occurred in some, but not all, lysogens of 

\[ \phi C2, \phi C5 \text{ and } \phi C8 \]. Variable toxin levels may be related to the toxinotype of parental strains, given the putative relationship of PaLoc and phage genes. Certain toxinotypes might also be more readily lysogenized and/or exhibit greater stability of lysogenic phages. Identification and sequencing of the holin genes in the phages, as well as site-directed mutagenesis of \( tcdE \), will be necessary for determining the role of phages in toxin production or secretion in \( C. difficile \).

**Table 3. Summary of toxin levels and toxin gene transcription in lysogens of \( \phi C2, \phi C5 \text{ and } \phi C8 \)**

<table>
<thead>
<tr>
<th>Parent–lysogen set</th>
<th>Transcription</th>
<th>Production</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Toxin A</td>
<td>Toxin B</td>
</tr>
<tr>
<td>CD594–CD594C8*</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>CD727–CD727C6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD389–CD389C2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD1017–CD1017C6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD1017–CD1017C8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD6938–CD6938C6</td>
<td>–</td>
<td>0</td>
</tr>
</tbody>
</table>

*Host strain CD594 lysogenized by \( \phi C8 \).

**Fig. 3.** Level of \( tcdA \) and \( tcdB \) transcripts in lysogens compared to parental strains by real-time RT-PCR. Coordinated transcription of \( tcdA \) and \( tcdB \) was observed in all lysogens except \( CD594C8 \) and \( CD6938C6 \). (a) Toxin A transcription was significantly increased in \( CD594C8 \) but significantly decreased in \( CD6938C8 \) compared to the corresponding parental strain. (b) The overall trend of toxin B transcription in lysogens is similar to that in (a). *, \( P \) values < 0.01. Data are expressed as mean ± SEM. Input RNA was determined using a calibrator sample from which a standard curve was constructed.
REFERENCES


Bruggemann, H., Baumer, S., Fricke, W. F. & 8 other authors (2003). The genome sequence of Clostridium tetani, the causative agent of tetanus disease. Proc Natl Acad Sci USA 100, 1316–1321.


