Journal of Virology, Dec. 2009, p. 12037–12045 Vol. 83, No. 23
0022-538X/09/$12.00 doi:10.1128/JVI.01256-09
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Bacteriophage-Mediated Toxin Gene Regulation in *Clostridium difficile*▽

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Received 17 June 2009/Accepted 14 September 2009

Clostridium difficile **has been identified as the most important single identifiable cause of nosocomial antibiotic-associated diarrhea and colitis. Virulent strains of** ***C. difficile*** **produce two large protein toxins, toxin A and toxin B, which are involved in pathogenesis. In this study, we examined the effect of lysogeny by ΦCD119 on** ***C. difficile*** **toxin production. Transcriptional analysis demonstrated a decrease in the expression of pathogenicity locus (PaLoc) genes** ***tcdA*****,** ***tcdB*****,** ***tcdR*****,** ***tcdE*****, and** ***tcdC*** **in ΦCD119 lysogens. During this study we found that** ***repR*****, a putative repressor gene of ΦCD119, was expressed in** ***C. difficile*** **lysogens and that its product, RepR, could downregulate** ***tcdA*****::*****gusA*** **and** ***tcdR*****::*****gusA*** **reporter fusions in** ***Escherichia coli*****. We cloned and purified a recombinant RepR containing a C-terminal six-His tag and documented its binding to the upstream regions of** ***tcdR*** **in** ***C. difficile*** **PaLoc and in** ***repR*** **upstream region in ΦCD119 by gel shift assays. DNA footprinting experiments revealed similarities between the RepR binding sites in** ***tcdR*** **and** ***repR*** **upstream regions. These findings suggest that presence of a CD119-like temperate phage can influence toxin gene regulation in this nosocomially important pathogen.**

Clostridium difficile, a gram-positive, anaerobic, spore-forming bacterium, has been identified as one of the major causative agents of antibiotic-associated diarrhea and pseudomembranous colitis. *C. difficile* produces toxins A and B that damage intestinal mucosa and cause fluid accumulation in the colon (1). The toxin genes *tcdA* and *tcdB*, along with accessory genes *tcdR*, *tcdC*, and *tcdE*, are part of a 19.6-kb pathogenicity locus (PaLoc). Toxin genes *tcdA* and *tcdB* are positively regulated by TcdR (previously TxeR) (27), and *tcdC* is involved in the negative regulation of toxin genes (16, 29). In pathogenic *C. difficile* strains, the PaLoc is present at identical locations in the chromosome, whereas it is completely absent in nontoxinogenic strains. This observation has led to the suggestion that the presence of the toxin gene cluster may be associated with a transposable genetic element (3). In other clostridial species, toxins are known to be encoded by mobile genetic elements such as bacteriophages and plasmids (6, 9, 10, 31).

Following publication of the genome of ΦCD119 (15), the genome of a second *C. difficile* temperate phage (ΦC2, a member of the *Myoviridae*) was published (13). More recently, eight temperate phages were characterized from six different *C. difficile* isolates, including the hypervirulent strain responsible for a multi-institutional outbreak (NAP1/027 or QCD-32g58) (11). In addition, the multidrug-resistant *C. difficile* strain CD630 was found to harbor two highly related prophages (13, 39) as part of its mosaic genome, where nearly 11% is made of mobile genetic elements. Thus, it appears that *C. difficile* strains often harbor temperate phage(s) as part of their genetic makeup. No direct evidence of lysogenic conversion of a nontoxinogenic *C. difficile* strain to toxin production was shown. However, preliminary results showed that toxin A and/or toxin B production is modified in a toxigenic *C. difficile* lysogen (12). In our lab we have successfully used a *C. difficile* phage for treating *C. difficile*-associated disease in hamster models (37). Later, we characterized and presented the first complete *C. difficile* phage genome (15). During these studies it was found that ΦCD119 could modulate toxin production in its *C. difficile* host strains. Hence, we have conducted a detailed study on the effect of lysogenization by this temperate phage on toxin production in *C. difficile* and characterized the role of phage-encoded protein RepR on transcriptional regulation of the PaLoc genes. This is the first evidence demonstrating the role of a temperate phage in virulence gene regulation in *C. difficile*.

MATERIALS AND METHODS

C. difficile **growth conditions, phage propagation, and purification.** The *C. difficile* strains were stored in chopped meat broth (Carr Scarborough Microbiologicals, Inc., Decatur, GA) at room temperature. When required, the broth cultures were subcultured onto brain heart infusion agar and incubated anaerobically at 37°C. Bacteriophage was induced as described by Mahony et al. (26), propagated, and purified according to methods described previously (15).

Generating ΦCD119 lysogens. The *C. difficile* ΦCD119 lysogen F10 and the ΦCD119 host *C. difficile* strains 602, 660, and 460 were obtained from Rosanna Dei, Universitá degli Studi di Firenze, Italy. Bacteriophage ΦCD119 was induced by mitomycin C treatment from ΦCD119 lysogen F10 and was isolated by techniques described by Mahony et al. (26). ΦCD119 (1×10^9 PFU) was spotted on a lawn of *C. difficile* strains 602, 660, or 460 on brain heart infusion agar plates and incubated overnight at 37°C, under anaerobic conditions. Bacterial colonies within the lysis zone were isolated and tested for phage production following mitomycin C (10 μg ml^{-1}) treatment. Putative lysogens were confirmed by probing their genomic DNA with α-^{32}P-labeled ΦCD119 DNA. Three ΦCD119 lysogens were isolated in each host *C. difficile* strain and were named after their parental isolate number, followed by an alphabetical character with a phage sign (e.g., 602ΦA, 602ΦB, and 602ΦC are the three individually isolated lysogens derived from strain 602).

Toxin A assay. Toxin A was measured using an enzyme-linked immunosorbent assay (ELISA) kit from Meridian Diagnostics Inc., Cincinnati, OH. Bacterial cultures were harvested at different time points from *C. difficile* strains by centrifugation and were resuspended in Tris buffer (0.05 M Tris-HCl, pH 7.5). The cytosolic contents of the harvested cells were obtained by sonication followed by brief centrifugation. Equal amounts of cytosolic proteins were taken, and their relative toxin A content was determined using an ELISA kit according to the

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▽ Published ahead of print on 23 September 2009.

TABLE 1. Sequences of primers used for reverse transcription-PCR

Gene name	Primer	
	Direction	Sequence(5′ to 3′)
tcdA	Forward	GCAGCTACTGGATGGCAAAC
	Reverse	ATCTCGAAAAGTCCACCAGC
tcdB	Forward	TCATTTGACGATGCAAGAGC
	Reverse	CCTTTCCTCAACAATTTGCG
tcdR	Forward	TCAAAGTAAGTCTGTTTTTGAGGAA
	Reverse	TGCTCTATTTTTAGCCTTATTAACAGC
tcdC	Forward	GGTTCAAAATGAAAGACGACG
	Reverse	GCACCTCATCACCATCTTCA
tcdE	Forward	TGGAGGAATCAGAAAAGTAGCA
	Reverse	CATTTCATCTGTCATTGCATCT
16SrRNA	Forward	ACACGGTCCAAACTCCTACG
	Reverse	AGGCGAGTTTCAGCCTACAA
int	Forward	ATGAATATCAAATCAGCTTTTAT
	Reverse	TGAATAAATACTCCCATGTACTT
repR	Forward	CTGTCATCATCATTGAGAGAATA
	Reverse	GGGAAAGTGATAGAAGATTTCCT
CD2693	Forward	GTGCCCAAACTAATCATCGG
	Reverse	GCTAACATTCCTGCCTCTGG
CD2694	Forward	AAAATGCTAAATTTGGTTTGT
	Reverse	CTCCAAATTAAAACTATAGCATCA

manufacturer's directions. Tris buffer (0.05 M Tris-HCl, pH 7.5) alone was used as a negative control.

Reverse transcriptase PCR. Total RNA was prepared from *C. difficile* cultures using an RNeasy minikit (Qiagen). After DNase treatment for 30 min at 37°C, the RNA was cleaned with RNeasy columns and was checked for DNA contamination by PCR. The cDNA was synthesized using 1 μg of DNA-free RNA and random hexamer primers and employing a first-strand cDNA synthesis kit for reverse transcription-PCR (using avian myeloblastosis virus reverse transcriptase) from Roche Biochemicals (Indiana). The synthesized cDNA and the gene-specific primers (Table 1) were used in the subsequent PCRs. Except for *tcdC*, all transcriptional analyses were performed using RNA extracted from 16-h-old *C. difficile* cultures. For *tcdC* analysis RNA was extracted from a culture of *C. difficile* grown for 8 h.

Expression of six-His tagged ΦCD119 repressor. The PCR was used to amplify *repR* from ΦCD119 DNA. DNA was isolated from purified bacteriophage with a High Pure Lambda Isolation Kit (Roche). The *repR* coding sequence was cloned into pET22b (Novagen), such that it was under the T7 promoter and contained a C-terminal six-histidine tag, employing primers containing NdeI and BamHI (Forward, 5′-GTCGCATATGACTAACACATTTGGAAAC-3′; Reverse, 5′-CCGGGGATCCATTCTCTTCTTTCTTCG-3′). The cloned *repR* was verified by DNA sequence analysis, and the recombinant vector (pETRepR) was transformed into *Escherichia coli* BL21-CodonPlus(DE3) strain (Stratagene), carrying the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 RNA polymerase. The transformants were grown to mid-log phase (optical density at 600 nm [OD_{600}] of 0.5) before inducing *repR* expression (0.5 mM IPTG). After 2 h of induction, the cells were harvested and lysed by sonication. The presence of RepR with a His_6 tag was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis employing anti-His_6 antibodies (Invitrogen). The ΦCD119 genome sequence and repressor protein sequence can be found in the GenBank under accession numbers AY855346 and AAX53417, respectively.

Reporter fusion plasmids for *E. coli*. Plasmids pTUM512, pTUM515, pTUM521, and pTUM525 were the kind gift of A. L. Sonenshein of Tufts University. Plasmids pTUM512 and pTUM515 carry the *tcdA* upstream (527 bp from translational start) and *tcdR* upstream (511 bp from the translational start) fused in frame with the *E. coli gusA* gene, respectively. Plasmids pTUM521 and pTUM525 carry the same respective promoter fusions along with the *tcdR* gene (28). Reporter constructs from pTUM512, pTUM515, pTUM521, and pTUM525 were excised with SalI-EagI digestion, and fragments were subcloned into the pACYC184 vector, resulting in the construction of pACYC512, pACYC515, pACYC521, and pACYC525, respectively (Table 2). To construct the *gdh-gusA* fusion, the *gdh* promoter region was PCR amplified from *C. difficile* chromosomal DNA using primers *gdh*F (GGATCCTAGCTGGGATATCGGC, with BamHI) and *gdh*R (TCTAGAAAAGCCCCCTTATAAA, with XbaI) and was cloned in pGEMT Easy vector (Promega) to create pGEM-*gdh*. The *gusA* gene was excised from pTUM515 using XbaI and PstI and was subsequently cloned in pGEM-*gdh* to create pGEM (*gdh-gusA*). The *gdh-gusA* fusion was then excised from pGEM (*gdh-gusA*) using BamHI and SalI and cloned in pACYC184 digested with the same enzymes to create pACYC528.

***E. coli* reporter strains for β-glucuronidase assays.** The *E. coli gusA* mutant, GM241 (33), was obtained from the *E. coli* Genetic Stock Center (Yale University, CT) and lysogenized with λDE3 to create the GM241 (DE3) strain (Table 2) to facilitate expression of *repR* cloned under the T7 promoter in a pET22-b vector (Novagen). DE3 λ phage carries an IPTG-inducible T7 RNA polymerase gene, and lysogenization with DE3 λ facilitates the expression of *repR* cloned in

TABLE 2. *E. coli* strains and plasmids used in this study

E. coli strain or plasmid	Description	Source or reference
Strains		
BL21-codon plus (DE3)	Host for pET expression system	Stratagene
GM241	*gusA* mutant	33
GM241(DE3)	*gusA* mutant lysogenized with λDE3 phage and host for *gusA* reporter plasmids	This study
Plasmids		
pET22-b	Cloning vector; T7 expression system; C-terminal His tag; Cb^r	Novagen
pETRepR	Plasmid carrying *repR* under inducible T7 promoter; Cb^r	This Study
pTUM512	*tcdA* promoter-*gusA* fusion; Cb^r	Sonenshein laboratory, Tufts University
pTUM515	*tcdR* promoter-*gusA* fusion; Cb^r	Sonenshein laboratory, Tufts University
pTUM521	*tcdA* promoter-*gusA* fusion and *tcdR* structural gene; Cb^r	28
pTUM525	*tcdR* promoter-*gusA* fusion and *tcdR* structural gene; Cb^r	28
pACYC184	Cloning vector with p15A origin of replication; coexists with plasmids that use colE1 origin; Tet^r Cm^r	New England Biolabs
pACYC512	SalI-EagI fragment from pTUM512 cloned in pACYC184; Cm^r	This study
pACYC515	SalI-EagI fragment from pTUM515 cloned in pACYC184; Cm^r	This study
pACYC521	SalI-EagI fragment from pTUM521 cloned in pACYC184; Cm^r; carries *tcdR* structural gene	This study
pACYC525	SalI-EagI fragment from pTUM525 cloned in pACYC184; Cm^r; carries *tcdR* structural gene	This study
pACYC528	*tcdA* promoter in pACYC515 was replaced with *gdh* promoter; Cm^r	This study

the pET vector under the control of the T7 promoter. To measure the effect of RepR on the expression of the *tcdR*, *tcdA*, and *gdh* promoters, plasmids (Table 2) pACYC512, pACYC515, pACYC525, pACYC521, and pACYC528 carrying *gusA* reporter fusions were introduced into *E. coli* strain GM241 (DE3) carrying either pETRepR or vector pET22-b. GM241 (DE3) transformants were grown in Luria-Bertani broth for 1 h, and then IPTG was added at a 0.5 mM final concentration for inducing the expression of RepR from the T7 promoter. Culture samples were removed at regular time intervals for up to 24 h following IPTG induction, and the amount of β-glucuronidase activity was assessed as described by Mani et al., with minor modifications (28). Cells were washed and suspended in 0.8 ml of Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$ [pH 7.0], 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM 2-mercaptoethanol), and it was permeabilized with 50 μl of 0.1% SDS plus chloroform. The enzyme reaction was started by the addition of 0.16 ml of 6 mM *p*-nitrophenyl β-D-glucuronide (Sigma) and stopped by the addition of 0.4 ml of 1.0 M $NaHCO_3$. β-Glucuronidase activity units were calculated as described before by Dupuy and Sonenshein (8).

Purification of RepR-His$_6$ protein. To purify RepR-His$_6$, a 1-liter culture of the BL21-CodonPlus(DE3) strain carrying pETRepR was grown at 37°C for 4 h in the presence of IPTG (0.5 mM). The cells were harvested by centrifugation (13,000 × *g*), resuspended in 20 ml of buffer A (50 mM sodium-phosphate, 300 mM NaCl, 20 mM imidazole), and lysed by sonication and the cell debris was removed by centrifugation (14,000 × *g*). The supernatant was loaded on a 5-ml Ni^{++} Sepharose column (Amersham Biosciences) and washed with buffer A. RepR was eluted sequentially with buffer B (50 mM sodium phosphate, 300 mM NaCl) containing imidazole at concentrations of 50 mM, 100 mM, and 150 mM and analyzed for purity by SDS-PAGE. Fractions with RepR were pooled, dialyzed against water, and lyophilized. Protein concentration was measured by the Bradford method (2).

Gel mobility shift experiments. Sequences upstream of *tcdR* and *repR* were amplified by PCR, with the primer pair TXR2 (5′-TAATGATGCTTTATTTG AAAATTTTG-3′) and TXR3 (5′-TTATTGACTAAATTATAAAGTTTC-3′) and the pair REP1 (5′-AGTCATAGTATTCACCTTCCGTTTTT-3′) and REP2 (5′-TCAACTCCTTTTGTTTTCATTTTGCT-3′), respectively, and labeled with biotin using a 3′ biotin end-labeling kit (Pierce), following the manufacturer's recommendations. DNA binding with RepR protein was performed using a LightShift Chemiluminescent electrophoretic mobility shift assay kit (Pierce) following the manufacturer's instructions. Briefly, binding reactions with 10 picomoles of biotinylated DNA targets were performed with different concentrations of purified RepR protein in 20-μl reaction mixtures in 1× buffer (10 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol at pH 7.5) supplemented with 50 ng/μl poly(dI-dC) and 10% glycerol. After incubation for 30 min at room temperature, binding reaction mixtures were loaded onto 8% polyacrylamide gels in 0.5× Tris-borate-EDTA buffer. After electrophoresis, the reaction mixtures were transferred from the gel onto a nylon membrane using a Bio-Rad minigel wet transfer system at 380 mA for 30 min. The DNA was cross-linked with UV (at 312 nm) for 10 to 15 min with the membrane face down on a transilluminator. The biotin-labeled DNA was detected using streptavidin-horseradish peroxidase conjugate as per the manufacturer's instructions. The amount (moles) of purified RepR used in the binding reaction mixtures was calculated based on the molecular size of monomer RepR.

DNA footprinting experiments. Upstream regions of *repR* and *tcdR* were PCR amplified using specific primers. For *repR* upstream regions, the REP1 (see above) forward phosphorylated primer and the reverse REP3 (5′-CTTAATTA TAGTTAACATTTTGCTAAC-3′) primer were used. For the *tcdR* upstream region, the forward TXR2 primer (see above) and the reverse phosphorylated primer TXR4 (5′-TGTTTTTACAATACTTTATTAATATAAAG-3′) were used. The amplified products were gel extracted and end labeled with γ-^{32}P using T4 polynucleotide kinase. Approximately 20,000 cpm of the labeled probe was used in each reaction mixture, and the footprinting experiments were carried out as per the instructions of the Core Footprinting System (Promega). The RepR binding reactions were performed at room temperature in a 50-μl reaction mixture containing binding buffer (50 mM Tris-HCl [pH 7.8], 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, and 20% glycerol) for 10 min. Fifty microliters of Ca_2/Mg_2 solution (5 mM $CaCl_2$ and 10 mM $MgCl_2$) and 3 U of RQ1 DNase were then added, and the mixture was incubated for 1 min. Following digestion, the DNA was precipitated, resuspended in 4 μl of sequencing gel loading buffer (38), heated to 80°C for 5 min, and separated in a 9% polyacrylamide gel with 7 M urea. For the markers, Maxam-Gilbert G+A sequencing reactions were performed on the PCR-amplified products (30).

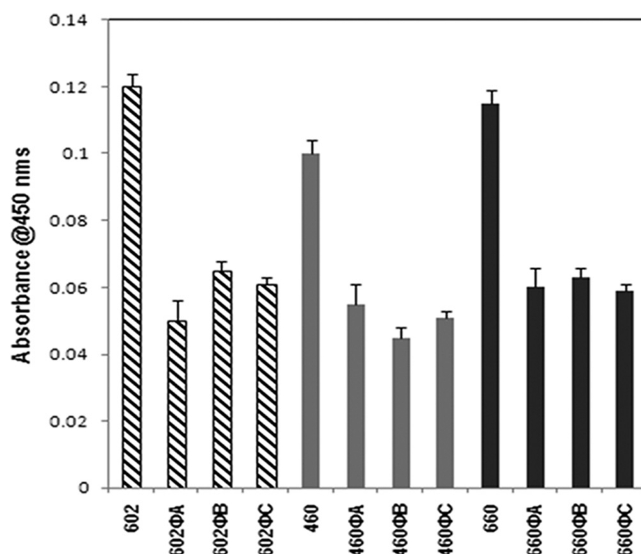

FIG. 1. Toxin A titers, expressed as absorbance units, were determined by ELISA. Toxin A production in overnight cultures of ΦCD119 lysogens was compared to parental strains. The ELISA signal was recorded as absorbance at 450 nm relative to the background signal, and the data shown are the mean plus standard error of three replicative samples. Experiments were repeated three times, and the figure represents the data from a single experiment.

RESULTS

Effect of ΦCD119 infection on toxin production in *C. difficile*. Our preliminary studies suggested that lysogenization of ΦCD119 could modulate toxin production in *C. difficile* (R. Govind and J. A. Fralick, unpublished data). To understand whether this effect is strain specific, we tested toxin production in ΦCD119 lysogens derived from three different *C. difficile* strains, 602, 660, and 460. Cytosolic proteins from the lysogens and their respective parent strains were harvested from overnight cultures, and the relative amount of toxin A was determined by ELISA. Tris buffer used for the protein preparation was used as a negative control in the ELISAs, and it recorded zero absorbance. In all three backgrounds the ΦCD119 lysogens produced approximately 50% less toxin A than their respective parent strains (Fig. 1). These results suggest that the effect of ΦCD119 lysogenization on *C. difficile* is not strain specific. For further analyses on the effect of ΦCD119 lysogenization on toxin production, we selected two lysogens derived from the strain 602, 602ΦA, and 602ΦB. We compared toxin A production of 602ΦA and 602ΦB lysogens with their parent (602) throughout their growth cycles. It was found that the rates of cell growth are similar in lysogens and the parent strain (Fig. 2). However, the relative amount of toxin A per OD_{600} in the lysogens was consistently less than that present in the parent throughout the growth cycle (Fig. 2).

Transcriptional analyses in ΦCD119 lysogens. Using reverse transcriptase PCR, transcriptional analyses of different genes were performed with strain 602 and the 602Φ lysogens. To determine the effect of ΦCD119 lysogenization on the regulation of the pathogenicity loci of *C. difficile*, we compared the transcription of all five genes of the PaLoc. Several studies (8, 18, 23, 35) have shown increased toxin production at the

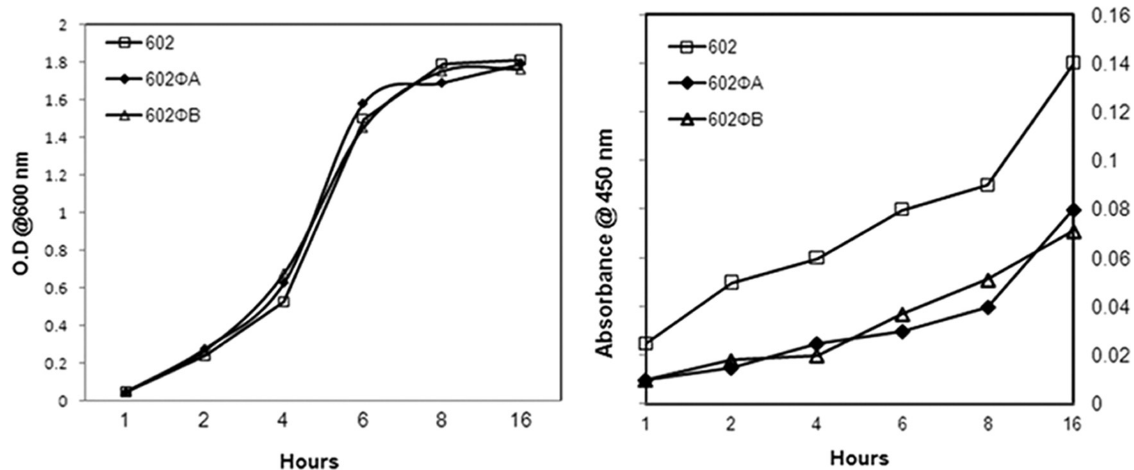

FIG. 2. Toxin A production in the *C. difficile* 602 parent strain and the lysogens 602ΦA and 602ΦB at different time points in the growth cycle. (Left) Growth curve. (Right) Toxin A ELISA.

end of exponential growth phase, and hence we prepared RNA from the overnight cultures (16 h old) for transcription analysis. RNA from an 8-h culture was used to analyze *tcdC* transcription, a stage at which it was found to be transcribed (18). The transcripts of *tcdA*, *tcdB*, *tcdR*, *tcdE*, and *tcdC* were found to be downregulated in the lysogens compared with the parent strain (Fig. 3a). Among the toxin genes, *tcdA* was transcribed at a higher rate than *tcdB* in the parent strain, supporting an earlier observation that the difference in *tcdA* and *tcdB* mRNA levels was approximately twofold (8). Equal levels of 16S rRNA were amplified from both the lysogens and the parent and served as internal controls. In our previous study we defined the integration site of ΦCD119 to be present between *C. difficile* genes CD2693 and CD2694 (15). The integration site of ΦCD119 in between genes CD2693 and CD2694 was confirmed through PCR in lysogens 602ΦA and 602ΦB (data not shown). We then examined the effect of the integration of ΦCD119 on the expression of host genes CD2693 and CD2694 that are present on either side of the prophage ΦCD119 in lysogens. The transcripts of both of these genes were found to be unaffected by lysogenization with ΦCD119 (Fig. 3b), suggesting that the effect of ΦCD119 lysogenization on *C. difficile* PaLoc expression is specific for the PaLoc.

We cloned, sequenced, and annotated the genome of the ΦCD119 (15) and identified several open reading frames ([ORFs] ORF44, ORF45, and ORF46) which code for putative transcriptional regulators. Of these ORFs, only ORF44 is transcribed in ΦCD119 lysogens along with the gene that encodes integrase, *int* (Fig. 3c) (15). ORF 44 (*repR*) contains an N-terminal helix-turn-helix domain (IPR001387), which belongs to the XRE family of repressors (25). Similar to many other prophages, *repR* and *int* may be involved in the maintenance of lysogeny of ΦCD119 in *C. difficile*.

RepR represses *tcdA* and *tcdR* expression in *E. coli*. Since we found that *repR* is expressed in ΦCD119 lysogens and that the PaLoc genes are downregulated in ΦCD119 lysogens, we examined the effect of RepR on toxin gene regulation. To accomplish this, we measured the effect of RepR on the expression of the *tcdA-gusA* and *tcdR-gusA* promoter fusions in *E. coli*. Initially, we transformed the *gusA* mutant *E. coli* strain

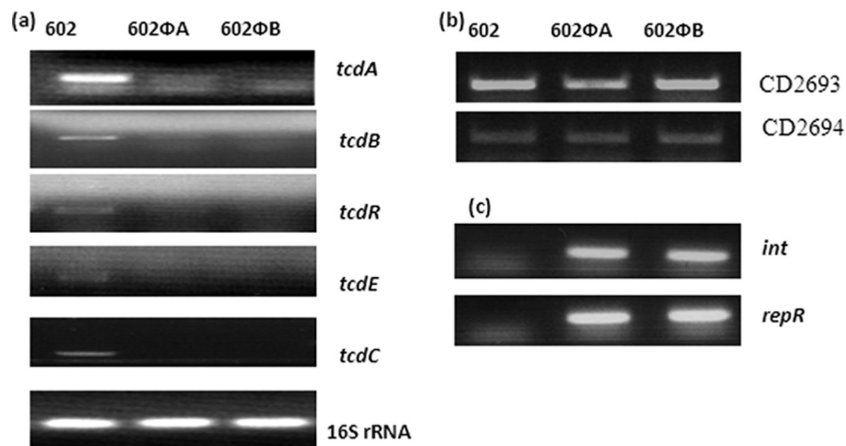

FIG. 3. Reverse transcriptase PCR analyses of (a) PaLoc genes (a), *C. difficile* genes near the integration site of the phage (b), and ΦCD119 genes (c) in strains 602 (parent), 602ΦA, and 602ΦB. 16S rRNA was used as a control.

GM241 (DE3) carrying either *tcdA-gusA* (pACY512) or *tcdR-gusA* (pACY515) with pET22b and tested for β-glucuronidase activity and found that the activity was below detectable levels (data not shown). These results suggested that the *tcdA* and *tcdR* promoters in pACYC512 and pACYC515 were not active in *E. coli* strain GM241 (λDE3) due to the absence of TcdR, the positive regulator. In 1997, Moncrief et al. provided the first evidence for a positive role of TcdR in toxin gene regulation using a reporter fusion in *E. coli* (32). These results were confirmed by similar experiments using *Clostridium perfringens* (27) and later in *C. difficile* (28). Our results are in agreement with these previous observations and justify the use of *E. coli* strain GM241 (λDE3) for studying *tcdA* and *tcdR* promoter activity. After these initial studies, we then introduced the plasmid expressing RepR (pETRepR) or the control pET22b into *E. coli* strains carrying either pACYC521 or pACYC525. The plasmids pACYC521 and pACYC525 carry the *tcdR-gusA* and *tcdA-gusA* fusions, respectively, along with the *tcdR* structural gene under its own promoter. Since the *repR* gene is under the control of IPTG-inducible T7 promoter, we measured β-glucuronidase activity with respect to growth with and without IPTG RepR induction. Expression of *tcdA-gusA* and *tcdR-gusA* was recorded in *E. coli* strains carrying pACYC521 or pACYC525 along with control pET22b. Production of RepR reduced the level of the *gusA* product when it was under the control of either the *tcdA* or *tcdR* promoter, and the reduction was observed throughout the growth cycle (Fig. 4a to d). The effect of RepR on the *gdh* promoter was measured in cells carrying the pETRepR plasmid along with pACYC528, and it was found that RepR has no effect on the *gdh* promoter (Fig. 4e and f). These results suggest that RepR specifically represses the *tcdA* and *tcdR* promoters when TcdR is coexpressed. This might be due to either the direct interaction of RepR with these promoters or the regulation of *tcdR* expression. To examine these possibilities, we purified RepR and conducted DNA binding studies.

Expression and purification of ΦCD119 RepR. To purify RepR, we transformed the pETRepR plasmid into the BL21-CodonPlus(DE3) strain, and the expression of the RepR protein was followed by SDS-PAGE and Western blot analyses employing His_6 antibodies (Invitrogen). The immunoblot of protein extracts of *E. coli* strain carrying the pETRepR (Fig. 5a) showed a band around 16 kDa, corresponding to the predicted size of the recombinant RepR-His_6 protein. No such protein was detected in the absence of the RepR-encoding plasmid. Western blotting using anti-histidine antibodies also confirmed the expression of RepR protein. A band around 32 kDa was also detected in the Western blot, suggesting that RepR may form a dimer, like many other DNA binding proteins (19) (Fig. 5a). The expressed protein was purified (Fig. 5b) using Ni^{++} affinity columns and used in gel mobility shift and DNA footprinting experiments.

Gel shift experiments. Initially, partially purified RepR protein (Fig. 5b, lane 4) was used in DNA binding experiments with *tcdA* and *tcdR* upstream DNAs, and it was found that RepR could bind only with *tcdR* and not with *tcdA* upstream DNA (data not shown). We then purified RepR and used different dilutions to perform DNA binding experiments with *tcdR* upstream DNA as well as with *repR*'s own upstream DNA. The phage repressor proteins have been shown previously to bind to upstream operator regions to control promoters that decide lytic versus lysogenic cycles of the phage (42). Our results demonstrated that RepR can bind to its own upstream region, and competition by the addition of unlabeled excess probe abolished the binding, indicating the specificity of the interactions (Fig. 6b). Similarly, when RepR was added to a *tcdR* upstream DNA fragment, the mobility of the DNA was decreased, depending on the amount of RepR added, and the interaction was found to be specific through competition experiments (Fig. 6a). These results suggest that RepR modulates toxin gene expression indirectly by controlling the expression of *tcdR*, the toxin gene regulator.

Identification of RepR binding sequences in *tcdR* and *repR* upstream regions. To identify the specific binding sites of RepR, a DNase I footprinting assay was performed with the *tcdR* and *repR* upstream DNA regions. RepR protected a region extending from positions −68 to −118 relative to the *tcdR* ATG start codon (Fig. 7a), and it lies downstream of the *tcdR* predicted promoters (Fig. 7c) (28). RepR binds to the repeat sequences (Fig. 7b and d) upstream of the *repR* start codon. These repeat sequences may represent the *repR* operators. However, the implication of RepR's binding to these sequences in controlling the lytic versus lysogenic cycles of ΦCD119 is yet to be determined. When we aligned the RepR binding sequences in both *repR* and *tcdR* promoter regions with the LALIGN program (http://www.ch.embnet.org/software/LALIGNform.html), we identified multiple sequence similarities between the two RepR binding regions (Fig. 7e). This could explain RepR's ability to bind to *tcdR* upstream, which resulted in reduced toxin gene expression. BLAST analysis against the *C. difficile* genome using the putative operator sequence (Fig. 7d) revealed many possible RepR binding sequences, and, interestingly, one was found within the coding region of the *tcdC* gene (data not shown).

DISCUSSION

In the genus *Clostridium*, several toxin-encoding genes are located on mobile genetic elements such as nonintegrative lysogenic phages, lysogenic phages, and plasmids (6, 9, 17, 31). In *C. difficile*, it has been shown that PaLoc genes share homology with phage genes, *tcdE* with phage holins (41), *tcdA* with a *Clostridium tetani* phage CT2 gene (4), and *tcdC* with ORF22 of *Lactobacillus casei* phage A2 (14). In this study, by demonstrating the interaction of a phage regulator with that of a PaLoc gene, we are presenting another piece of evidence for the possible relationship of the PaLoc with a temperate bacteriophage. We examined the effect of a phage repressor, RepR, on the expression of PaLoc genes of *C. difficile*. We found that the expression of the ΦCD119 RepR protein in *C. difficile* ΦCD119 lysogens decreased toxin production. Transcriptional analysis revealed a decreased level of RNA from all five PaLoc genes (*tcdR*, *tcdB*, *tcdE*, *tcdA*, and *tcdC*) (Fig. 3a), and reporter gene fusion experiments in *E. coli* carrying *tcdR* indicated that the presence of RepR causes the downregulation of *tcdA* and *tcdR* promoters (Fig. 4). Furthermore, DNA binding studies indicated that RepR binds specifically to DNA sequences upstream of *tcdR* as well as to its own gene (Fig. 6). TcdR is an autoregulator that acts as an activator for *tcdB* and *tcdA* (8, 27, 28). Hence, our results suggest that RepR is acting through *tcdR* to downregulate *tcdB* and *tcdA*. RepR also down-

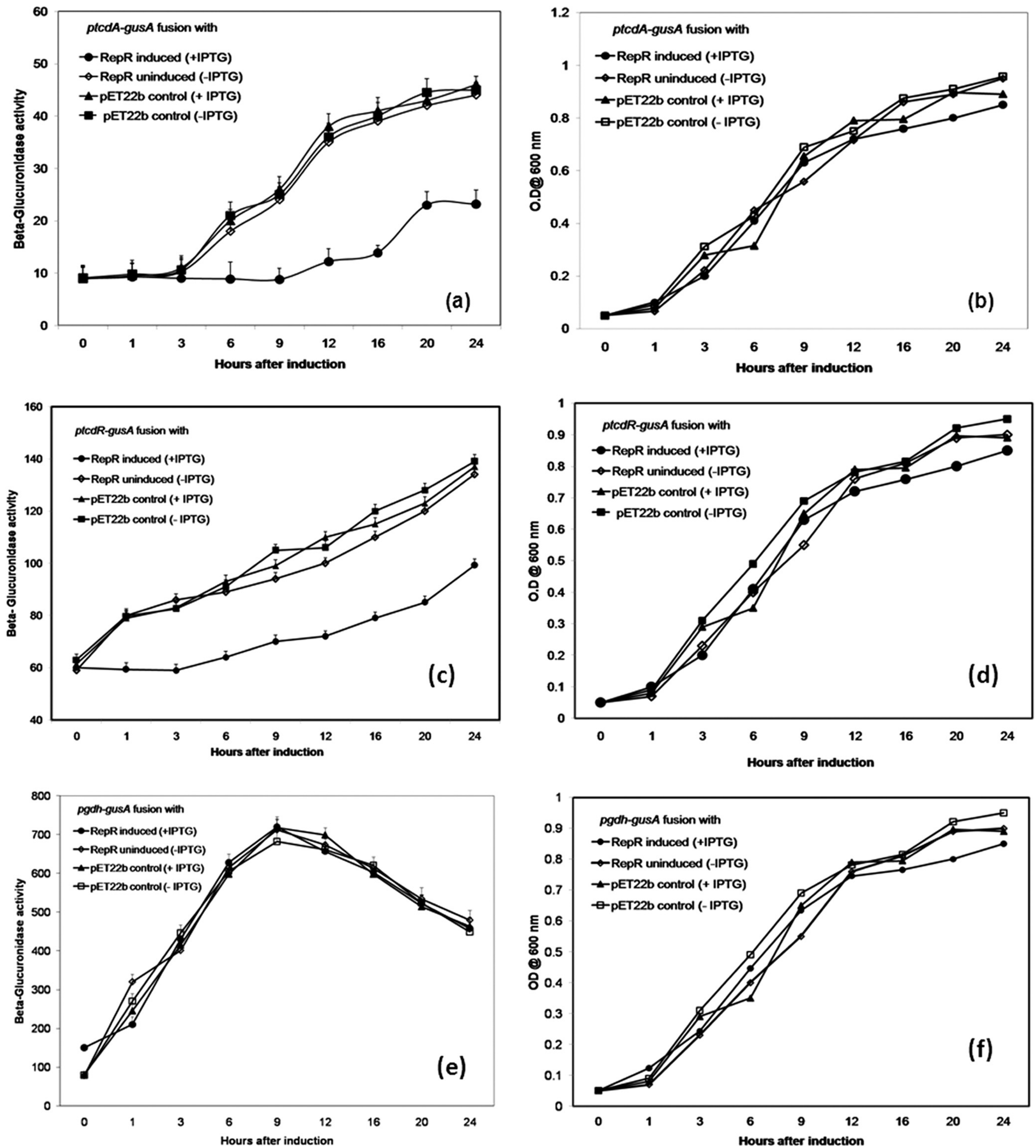

FIG. 4. β-Glucuronidase activity of *tcdA* promoter-*gusA* and *tcdR* promoter-*gusA* fusions (p*tcdA-gusA* and p*tcdR-gusA*, respectively). *E. coli* strains carrying promoter fusion plasmids along with a RepR-expressing plasmid (pETRepR) or vector (pET22-b) were grown and assayed for β-glucuronidase activity. The values represent the means of three independent experiments (plus standard error). The graphs are as follows: growth curve (b) and β-glucuronidase activity (a) of *E. coli* strains carrying reporter plasmid pACYC521 with *tcdA-gusA*; growth curve (d) and β-glucuronidase activity (c) of *E. coli* strains carrying reporter plasmid pACYC525 with *tcdR-gusA*; growth curve (f) and β-glucuronidase activity (e) of *E. coli* strains carrying reporter plasmid pACYC528 with *gdh-gusA*. Strains were grown with (+) or without (−) IPTG.

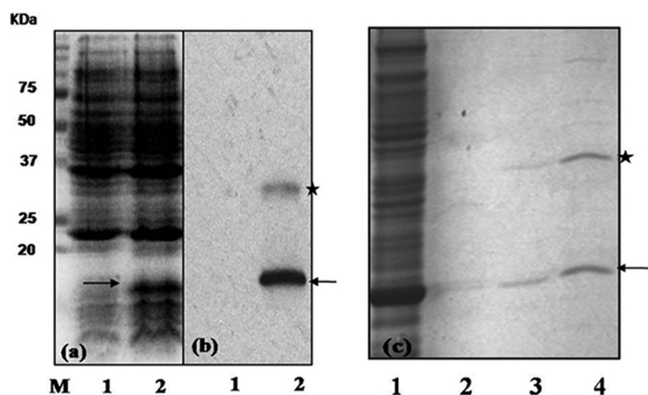

FIG. 5. Expression and purification of RepR-His_6 in *E. coli*. SDS-PAGE analysis of protein extracts from *E. coli* BL21(DE3) carrying either the pET22b vector or pET22b expressing RepR-His_6. The OD for cultures of both *E. coli* strains was adjusted to 0.1 at 550 nm, and these cultures were then sonicated and boiled with SDS-PAGE sample buffer before being loaded on the gel. Lane 1, crude cell extract from *E. coli* carrying the vector pET22b; lane 2, crude cell extract from *E. coli* carrying vector expressing RepR-His_6. (a) Proteins were stained by Coomassie brilliant blue. M, Precision plus protein marker (Bio-Rad). (b) Immunoblot using anti-His_6 antibody. (c) Purification of RepR. Lane 1, crude cell extract of *E. coli* expressing RepR-His_6; lanes 2, 3, and 4, RepR containing fractions collected from Ni^{++} affinity column for purification. Samples in lanes 3 and 4 represent the purified and partially purified RepR, respectively. The arrow indicates the monomer, and the star indicates dimeric forms of RepR.

regulates *tcdE*, a phage holin-like gene, whose product has been speculated to play a role in toxin release through the formation of membrane lesions and eventual lysis (41). This gene lies between *tcdB* and *tcdA*, and although *tcdE* does not appear to have a TcdR-dependent promoter, it has been suggested that a significant amount of transcriptional read-through occurs, resulting in bicistronic transcripts, including *tcdB-E* (18). RepR may be regulating *tcdE* through TcdR regulation of *tcdB*. Thus, reduced TcdE-mediated host cell lysis would help the prophage to successfully maintain its lysogeny. How RepR downregulates *tcdC*, a negative regulator of *tcdA* and *tcdB*, is unknown and is currently under investigation. The presence of a putative RepR binding sequence within the *tcdC* coding region could partly explain the reduced *tcdC* transcription observed in ΦCD119 lysogens. If PaLoc was once part of a prophage whose gene expression was regulated by RepR, or a RepR-like repressor, then the silencing of all five PaLoc genes might be expected. Interestingly, CodY, a global regulator in *C. difficile*, was also found to downregulate both *tcdR* and *tcdC* (7). Similar to the function of ΦCD119 RepR, a prophage-encoded repressor regulating a host bacterial gene was reported in λ phage in an earlier study (5). In *E. coli*, λ infection resulted in the complete suppression of the host *pckA* gene, which encodes phosphoenolpyruvate carboxykinase, a gene critical for gluconeogenesis (5). It was found that the lambda phage repressor *c*I, which shuts down lytic phage gene expression, also bound to the operator of the *pckA* gene and resulted in its downregulation.

Lysogens gain specific advantages from their relationship with phage that improve their overall fitness. In certain bacteria, infection by phage and subsequent conversion result in altered metabolic capabilities (24). In a recent review on marine prophage, the possible role of prophage-encoded repressors in shutting down unwanted metabolic pathways for the survival of the host bacteria under an unfavorable environment has been discussed (36). In *C. difficile*, toxin gene expression is clearly sensitive to several environmental factors such as the presence of carbon sources, temperature, biotin, and various

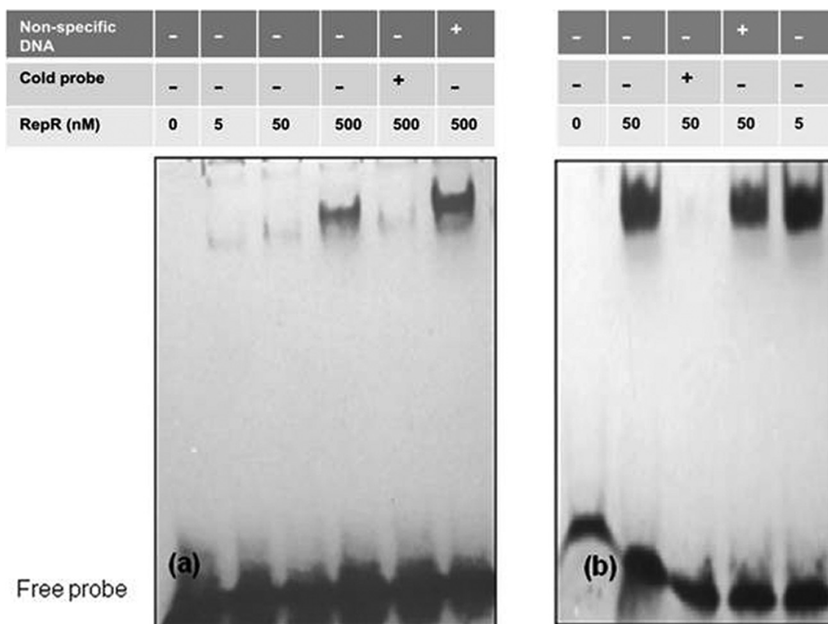

FIG. 6. Gel mobility shift assays with RepR protein. DNA fragments labeled with biotin were incubated with purified RepR protein. Gel mobility shift assays with *tcdR* upstream DNA (345 bp) (a) and with *repR* upstream DNA (415 bp) (b). Calf thymus DNA (100 ng) was used for the nonspecific competition, and a 50-fold excess cold probe was used for the specific competition.

FIG. 7. DNase I footprinting of the *tcdR* and *repR* upstream regions bound by RepR. End-labeled DNA was incubated with RepR-His$_6$. Following DNase I treatment, samples were run on a 9% acrylamide sequencing gel and visualized on a phosphorimager. (a) *tcdR* upstream DNA footprinted with RepR protein. (b) *repR* upstream DNA footprinted with RepR protein. The plus sign indicates lanes in which protein samples were incubated with the DNA, whereas the minus sign indicates lanes with naked DNA alone. The lanes in which the DNA has been chemically cleaved by Maxam-Gilbert G+A reactions are included in each panel as a reference. (c) Sequence analysis of *tcdR* upstream region. RepR binding sites in *tcdR* upstream regions are highlighted in black, and the relative positions of the predicted *tcdR* promoters P1*tcdR* and P2*tcdR* are marked. The TcdR start codon is italicized. (d) Sequence analysis of *repR* upstream region. The *repR* start codon is italicized, RepR binding sites are highlighted in black, and the direct repeats of the probable operator sequences (OP) are indicated by arrows. (e) The consensus RepR binding sequences in *tcdR* and *repR* upstream regions.

amino acids (20–22). Certain unfavorable conditions such as the absence of glucose and high temperature are known to induce toxin gene transcription through *tcdR* (8, 28). Recently, Dineen et al. have demonstrated the role of *codY* and the level of intracellular GTP in toxin gene regulation in *C. difficile* (7). The effect of some of these environmental factors on *repR* and subsequent changes in the toxin gene expression in ΦCD119 lysogens is currently under investigation. A recent study has identified the presence of a cyclic di-GMP riboswitch within the lysis module of the ΦCD119 genome (40). Riboswitches are mRNA domains that control gene expression in response to changing concentrations of their target ligands (34). The presence of such a system in ΦCD119 clearly indicates that it has evolved to monitor the physiological changes in its host through regulatory networks, and, hence, it is reasonable to assume that their effect on PaLoc genes may be part of such a network. Hence, from our study, it appears that a phage regulator(s) is another member of the complex regulatory pathways involved in *C. difficile* toxin regulation.

ACKNOWLEDGMENTS

This work was made possible through the support of NIH grant 5R03DK054816-01 and by A. R. P. Norman Hackerman Advanced Research Program grant 010674-0021-2007.

We thank A. L. Sonenshein of Tufts University for the kind gift of pTUM plasmids and for his helpful suggestions throughout the study.

REFERENCES

1. **Borriello, S. P.** 1990. 12th C. L. Oakley lecture. Pathogenesis of *Clostridium difficile* infection of the gut. J. Med. Microbiol. **33:**207–215.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Braun, V., T. Hundsberger, P. Leukel, M. Sauerborn, and C. Von Eichel-Streiber.** 1996. Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. Gene **27:**29–38.
4. **Canchaya, C., F. Desiere, W. M. McShan, J. J. Ferretti, J. Parkhill, and H. Brussow.** 2002. Genome analysis of an inducible prophage and prophage remnants integrated in the *Streptococcus pyogenes* strain SF370. Virology **302:**245–258.
5. **Chen, Y., I. Golding, S. Sawai, L. Guo, and E. C. Cox.** 2005. Population fitness and the regulation of *Escherichia coli* genes by bacterial viruses. PLoS Biol. **3:**e229.
6. **Cornillot, E., B. Saint-Joanis, G. Daube, S. Katayama, P. E. Granum, B. Canard, and S. T. Cole.** 1995. The enterotoxin gene (cpe) of *Clostridium perfringens* can be chromosomal or plasmid-borne. Mol. Microbiol. **15:**639–647.
7. **Dineen, S. S., A. C. Villapakkam, J. T. Nordman, and A. L. Sonenshein.** 2007. Repression of *Clostridium difficile* toxin gene expression by CodY. Mol. Microbiol. **66:**206–219.
8. **Dupuy, B., and A. L. Sonenshein.** 1998. Regulated transcription of *Clostridium difficile* toxin genes. Mol. Microbiol. **27:**107–120.
9. **Eklund, M. W., F. T. Poysky, S. M. Reed, and C. A. Smith.** 1971. Bacteriophage and the toxicity of *Clostridium botulinum* type C. Science **172:**480–482.
10. **Finn, C. W., Jr., R. P. Silver, W. H. Habig, M. C. Hardegree, G. Zon, and C. F. Garon.** 1984. The structural gene for tetanus neurotoxin is on a plasmid. Science **224:**881–884.
11. **Fortier, L. C., and S. Moineau.** 2007. Morphological and genetic diversity of temperate phages in *Clostridium difficile*. Appl. Environ. Microbiol. **73:**7358–7366.

12. **Goh, S., B. J. Chang, and T. V. Riley.** 2005. Effect of phage infection on toxin production by *Clostridium difficile*. J. Med. Microbiol. **54:**129–135.
13. **Goh, S., P. F. Ong, K. P. Song, T. V. Riley, and B. J. Chang.** 2007. The complete genome sequence of *Clostridium difficile* phage phiC2 and comparisons to phiCD119 and inducible prophages of CD630. Microbiology **153:**676–685.
14. **Goh, S., T. V. Riley, and B. J. Chang.** 2005. Isolation and characterization of temperate bacteriophages of *Clostridium difficile*. Appl. Environ. Microbiol. **71:**1079–1083.
15. **Govind, R., J. A. Fralick, and R. D. Rolfe.** 2006. Genomic organization and molecular characterization of *Clostridium difficile* bacteriophage ΦCD119. J. Bacteriol. **188:**2568–2577.
16. **Hammond, G. A., and J. L. Johnson.** 1995. The toxigenic element of *Clostridium difficile* strain VPI 10463. Microb. Pathog. **19:**203–213.
17. **Hauser, D., M. Gibert, P. Boquet, and M. R. Popoff.** 1992. Plasmid localization of a type E botulinal neurotoxin gene homologue in toxigenic *Clostridium butyricum* strains, and absence of this gene in non-toxigenic *C. butyricum* strains. FEMS Microbiol. Lett. **78:**251–255.
18. **Hundsberger, T., V. Braun, M. Weidmann, P. Leukel, M. Sauerborn, and C. von Eichel-Streiber.** 1997. Transcription analysis of the genes *tcdA-E* of the pathogenicity locus of *Clostridium difficile*. Eur. J. Biochem. **244:**735–742.
19. **Jia, H., W. J. Satumba, G. L. Bidwell, 3rd, and M. C. Mossing.** 2005. Slow assembly and disassembly of lambda Cro repressor dimers. J. Mol. Biol. **350:**919–929.
20. **Karlsson, S., L. G. Burman, and T. Akerlund.** 2008. Induction of toxins in *Clostridium difficile* is associated with dramatic changes of its metabolism. Microbiology **154:**3430–3436.
21. **Karlsson, S., L. G. Burman, and T. Akerlund.** 1999. Suppression of toxin production in *Clostridium difficile* VPI 10463 by amino acids. Microbiology **145:**1683–1693.
22. **Karlsson, S., B. Dupuy, K. Mukherjee, E. Norin, L. G. Burman, and T. Akerlund.** 2003. Expression of *Clostridium difficile* toxins A and B and their sigma factor TcdD is controlled by temperature. Infect. Immun. **71:**1784–1793.
23. **Ketley, J. M., S. C. Haslam, T. J. Mitchell, J. Stephen, D. C. Candy, and D. W. Burdon.** 1984. Production and release of toxins A and B by *Clostridium difficile*. J. Med. Microbiol. **18:**385–391.
24. **Levin, B. R., and R. E. Lenski.** 1983. Coevolution in bacteria and their viruses and plasmids. Sinauer, Sunderland, MA.
25. **Li, N., Y. Chen, and J. Feng.** 1995. Cloning and analysis of prophage PBSX repressor gene from *Bacillus subtilis*. Yi Chuan Xue Bao **22:**478–486.
26. **Mahony, D. E., P. D. Bell, and K. B. Easterbrook.** 1985. Two bacteriophages of *Clostridium difficile*. J. Clin. Microbiol. **21:**251–254.
27. **Mani, N., and B. Dupuy.** 2001. Regulation of toxin synthesis in *Clostridium difficile* by an alternative RNA polymerase sigma factor. Proc. Natl. Acad. Sci. USA **98:**5844–5849.
28. **Mani, N., D. Lyras, L. Barroso, P. Howarth, T. Wilkins, J. I. Rood, A. L. Sonenshein, and B. Dupuy.** 2002. Environmental response and autoregulation of *Clostridium difficile* TxeR, a sigma factor for toxin gene expression. J. Bacteriol. **184:**5971–5978.
29. **Matamouros, S., P. England, and B. Dupuy.** 2007. *Clostridium difficile* toxin expression is inhibited by the novel regulator TcdC. Mol. Microbiol. **64:**1274–1288.
30. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74:**560–564.
31. **Miyamoto, K., J. Li, S. Sayeed, S. Akimoto, and B. A. McClane.** 2008. Sequencing and diversity analyses reveal extensive similarities between some epsilon-toxin-encoding plasmids and the pCPF5603 *Clostridium perfringens* enterotoxin plasmid. J. Bacteriol. **190:**7178–7188.
32. **Moncrief, J. S., L. A. Barroso, and T. D. Wilkins.** 1997. Positive regulation of *Clostridium difficile* toxins. Infect. Immun. **65:**1105–1108.
33. **Novel, M., and G. Novel.** 1976. Regulation of beta-glucuronidase synthesis in *Escherichia coli* K-12: pleiotropic constitutive mutations affecting *uxu* and *uidA* expression. J. Bacteriol. **127:**418–432.
34. **Nudler, E., and A. S. Mironov.** 2004. The riboswitch control of bacterial metabolism. Trends Biochem. Sci. **29:**11–17.
35. **Osgood, D. P., N. P. Wood, and J. F. Sperry.** 1993. Nutritional aspects of cytotoxin production by *Clostridium difficile*. Appl. Environ. Microbiol. **59:**3985–3988.
36. **Paul, J. H.** 2008. Prophages in marine bacteria: dangerous molecular time bombs or the key to survival in the seas? ISME J. **2:**579–589.
37. **Ramesh, V., J. A. Fralick, and R. D. Rolfe.** 1999. Prevention of *Clostridium difficile*-induced ileocecitis with bacteriophage. Anaerobe **5:**69–78.
38. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
39. **Sebaihia, M., B. W. Wren, P. Mullany, N. F. Fairweather, N. Minton, R. Stabler, N. R. Thomson, A. P. Roberts, A. M. Cerdeno-Tarraga, H. Wang, M. T. Holden, A. Wright, C. Churcher, M. A. Quail, S. Baker, N. Bason, K. Brooks, T. Chillingworth, A. Cronin, P. Davis, L. Dowd, A. Fraser, T. Feltwell, Z. Hance, S. Holroyd, K. Jagels, S. Moule, K. Mungall, C. Price, E. Rabbinowitsch, S. Sharp, M. Simmonds, K. Stevens, L. Unwin, S. Whithead, B. Dupuy, G. Dougan, B. Barrell, and J. Parkhill.** 2006. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. Nat. Genet. **38:**779–786.
40. **Sudarsan, N., E. R. Lee, Z. Weinberg, R. H. Moy, J. N. Kim, K. H. Link, and R. R. Breaker.** 2008. Riboswitches in eubacteria sense the second messenger cyclic di-GMP. Science **321:**411–413.
41. **Tan, K. S., B. Y. Wee, and K. P. Song.** 2001. Evidence for holin function of *tcdE* gene in the pathogenicity of *Clostridium difficile*. J. Med. Microbiol. **50:**613–619.
42. **Waldor, M. K., and D. I. Friedman.** 2005. Phage regulatory circuits and virulence gene expression. Curr. Opin. Microbiol. **8:**459–465.