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Morphological and Genetic Diversity of Temperate Phages in *Clostridium difficile*▽

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Eight temperate phages were characterized after mitomycin C induction of six *Clostridium difficile* isolates corresponding to six distinct PCR ribotypes. The hypervirulent *C. difficile* strain responsible for a multiinstitutional outbreak (NAP1/027 or QCD-32g58) was among these prophage-containing strains. Observation of the crude lysates by transmission electron microscopy (TEM) revealed the presence of three phages with isometric capsids and long contractile tails (*Myoviridae* family), as well as five phages with long noncontractile tails (*Siphoviridae* family). TEM analyses also revealed the presence of a significant number of phage tail-like particles in all the lysates. Southern hybridization experiments with restricted prophage DNA showed that *C. difficile* phages belonging to the family *Myoviridae* are highly similar and most likely related to previously described prophages ϕC2, ϕC5, and ϕCD119. On the other hand, members of the *Siphoviridae* phage family are more genetically divergent, suggesting that they originated from distantly related ancestors. Our data thus suggest that there are at least three genetically distinct groups of temperate phages in *C. difficile*; one group is composed of highly related myophages, and the other two groups are composed of more genetically heterogeneous siphophages. Finally, no gene homologous to genes encoding *C. difficile* toxins or toxin regulators could be identified in the genomes of these phages using DNA hybridization. Interestingly, each unique phage restriction profile correlated with a specific *C. difficile* PCR ribotype.

Clostridium difficile is now one of the leading bacterial pathogens responsible for nosocomial infections, and hypervirulent strains, such as NAP1/027 (North American pulsed-field type 1 and PCR ribotype 027), are emerging and are associated with increased morbidity and disease severity (33, 42, 43, 60). The development of *C. difficile*-associated diarrhea in individuals normally not at risk suggests that the epidemiology and virulence of *C. difficile* are changing (13, 42, 43). The main virulence factors are two exotoxins, TcdA and TcdB (for a review, see reference 59). In addition, about 6% of the *C. difficile* strains analyzed possess a binary toxin encoded by two genes, *cdtA* and *cdtB* (44, 52). The expression of this binary toxin may be associated with increased disease severity and morbidity (25) and was found to correlate with the presence of an 18-bp deletion in the *tcdC* gene (33). It is noteworthy that TcdC was recently shown to act as a negative regulator of toxin production (36). Various hydrolytic enzymes (48), as well as the capsule and flagella, also likely play a role in virulence (6, 14, 56). TcdA and TcdB are encoded in the pathogenicity locus (PaLoc) located on an ~19.6-kb chromosome fragment of all toxigenic strains (59). Twenty-four variations in the PaLoc have been identified thus far, and they are used as markers for toxinotyping of *C. difficile* strains (28, 45).

For many years, bacteriophages have been associated with major pathogens, such as *Vibrio cholerae*, *Escherichia coli* O157:H7, or *Clostridium botulinum*. Often, these pathogens became virulent after acquisition of one or more prophages carrying powerful toxins or other virulence factors, like superantigens and various enzymes (for reviews, see references 7 and 8). With the sequencing of a number of microbial genomes, the role of prophages in bacterial evolution and virulence has become evident (2–4, 17, 40). It is noteworthy that numerous prophages, like those found in *Streptococcus pyogenes*, share extensive DNA similarity (10), which makes phage evolution possible through the exchange of functional modules (12, 26). In addition, genetically related prophages may serve as anchor points leading to bacterial genome reorganization (8, 9). Finally, they may also evolve through interactions with the virulent phage population (29).

Recent epidemics caused by hypervirulent strains, such as *C. difficile* NAP1/027, and the renewed interest in phage therapy (51, 54, 57) have prompted an increasing number of research groups to study phages of *C. difficile*. However, data on this group of phages are still scarce (15, 34, 35, 39, 49), and the possible impacts of these phages on the biology of their bacterial hosts are unknown. The first report of molecular characterization of temperate phages in *C. difficile* was published by Goh et al. in 2005 (22). In this study, 3 of 56 clinical isolates of *C. difficile* yielded four double-stranded DNA phages (ϕC2, ϕC5, ϕC6, and ϕC8) upon mitomycin C induction (22). Phages ϕC2, ϕC5, and ϕC8 were morphologically similar (family *Myoviridae*), and ϕC2 and ϕC5 were closely related, as revealed by DNA-DNA hybridization experiments (22). In contrast, phage ϕC6 belonged to the *Siphoviridae* family and shared only a little DNA similarity with the three other phages. In 2006, the

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first complete genomic sequence and annotation of a *C. difficile* temperate phage (ϕCD119, a member of the *Myoviridae*; 53,325 bp) were made available (23). The genome of a second temperate phage (ϕC2, a member of the *Myoviridae*; 56,538 bp) was also published recently (21). In addition, the multidrug-resistant *C. difficile* strain CD630 was found to harbor in its genome two highly related prophages whose sequences were identical in more than 60% of their entire genomes (47). These prophages were recently shown to be inducible by mitomycin C and are members of the *Myoviridae* family (21).

Previous studies have revealed some homology between genes in the *C. difficile* PaLoc and phage genes (20, 21, 23, 55). For example, the *tcdA* gene encoding toxin A was found to be related to a gene with an unknown function in phage ϕCT2 of *Clostridium tetani* (10). Furthermore, there is 55% identity between ORF22 of *Lactobacillus casei* phage A2 and the last 103 amino acids of TcdC found in *C. difficile* VPI 10463 (20). A low level of sequence identity was also noted between ORF41 of *C. difficile* phage ϕC2 and TcdB (21). Interestingly, ORF46 of ϕC2 and ORF41 of ϕCD119 shared similarity with a putative penicillinase repressor (Cdu1) encoded by a gene bordering the *C. difficile* PaLoc. Finally, the gene product of *tcdE* was shown to be structurally and functionally related to a phage holin, as revealed by bioinformatics analyses and in vivo expression in *Escherichia coli* (55). Accordingly, ORF34 of ϕCD119 was shown to be similar to TcdE (23), and DNA hybridization experiments revealed the presence of *tcdE* homologs in the genomes of *C. difficile* phages ϕC2, ϕC5, and ϕC8 (20, 21). Altogether, these results suggest that some of the genes in the PaLoc are genetically and, in the case of *tcdE*, functionally related to phage genes, thus supporting the assumption that the origin of PaLoc (or fragments of it) might have been an ancient prophage.

Complete genome sequencing and analysis of phages ϕCD119 and ϕC2, as well as the two prophages in strain CD630, failed to reveal any direct evidence of virulence factors (21, 23, 47). Moreover, none of the phages in *C. difficile* described so far could convert a nontoxigenic strain into a toxigenic strain. However, Goh et al. suggested that lysogenization of toxigenic strains of *C. difficile* with certain phages could lead to increased toxin A and/or toxin B production (20). Although *tcdA* transcription was shown to be significantly increased in a strain lysogenized with phage ϕC8, these authors could not find a correlation between the level of transcription and the level of toxin production in their lysogens, nor could they find a correlation with a specific phage. Nevertheless, this study suggests that some phages may play a role in the regulation of the expression of toxin genes or other genes in *C. difficile*. For example, the five putative transcriptional regulators identified in the genome of ϕCD119 may be involved in host gene regulation (23).

In this study, we performed a survey of the diversity of inducible prophages residing in the genomes of *C. difficile* clinical isolates. Morphological and genetic analyses were performed, and a preliminary classification of the phages was established. Screening for genes encoding toxins and toxin regulators in the PaLoc failed to identify homologous genes in the genomes of these phages.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. difficile* isolates were kindly provided by Jacques Pépin of the Université de Sherbrooke (Québec, Canada). Strain CD630 was a kind gift from Julian Parkhill of the Wellcome Trust Sanger Institute (Cambridge, United Kingdom). *C. difficile* strains CD44 and CD52 were isolated from human fecal samples provided by Mirabelle Kelly of the Jean-Talon Hospital (Montréal, Québec, Canada). Bacterial strains were routinely cultured at 37°C under anaerobic conditions in brain heart infusion (BHI) broth (Oxoid, Nepean, Ontario, Canada) which had been prereduced under an anaerobic atmosphere for 24 h at 37°C prior to use.

Bacterial DNA extraction. Genomic DNA was extracted using the following procedure. All centrifugations were performed at room temperature. Ten milliliters of an overnight culture was centrifuged in 15-ml conical tubes for 15 min in a clinical centrifuge. The cell pellets were suspended in 1 ml saline (0.85% NaCl), transferred to an Eppendorf tube, and centrifuged for 2 min at full speed in a microcentrifuge. Cells were then suspended in 1 ml of a 20% sucrose solution containing 120 mg/ml lysozyme and incubated at 37°C for 30 min. After centrifugation for 2 min, the pellet was suspended in 600 μl of 2% sodium dodecyl sulfate. The preparation was incubated at room temperature with frequent agitation until complete lysis, after which 300 μl of ice-cold potassium acetate (3 M, pH 7.0) was added. After mixing, the lysate was incubated on ice for 5 min and then centrifuged at full speed for 5 min. The cleared lysate was transferred to a new tube, and 650 μl of isopropanol was added. After mixing by inversion, the DNA was precipitated by centrifugation for 10 min. The resulting pellet was rapidly dissolved in 320 μl of distilled water, after which 200 μl of 7.5 M potassium acetate and 350 μl of a phenol-chloroform solution (1:1, vol/vol) were added. After vigorous mixing (no vortex), the phases were separated by centrifugation for 5 min at full speed. The upper aqueous phase was transferred to a new tube, and a second phenol-chloroform extraction was performed. The DNA was precipitated by addition of 1 ml of anhydrous ethanol and centrifugation for 15 min at full speed. The DNA pellet was dried for a few minutes and dissolved in 40 μl of 10 mM Tris-HCl–1 mM EDTA (pH 8.0).

PCR ribotyping. The PCR ribotyping method was used as an initial typing tool for classification of our *C. difficile* isolates (5). The 16S rRNA primer 5′-GTGC GGCTGGATCACCTCCT-3′ and the 23S rRNA primer 5′-CCCTGCACCCTT AATAACTTGACC-3′ described by Bidet et al. (5) were used. The PCR was performed with a PTC-200 thermal cycler (MJ Research, Waltham, MA) using a 50-μl (final volume) mixture containing 1× reaction buffer (Invitrogen, Burlington, Ontario, Canada), 125 μM of each deoxynucleoside triphosphate, 2 mM $MgCl_2$, 100 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (Invitrogen). Two microliters of a 1/10 dilution of *C. difficile* DNA extract (see above) was added as a template. A similar amount of sterile water was used for the negative control. The cycling conditions were 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s, and elongation at 72°C for 1 min. A final elongation step of 5 min at 72°C was included. The final reaction mixture was combined with 8 μl of 6× gel loading buffer (46), and 6 μl was separated using a 1.5-mm-thick 5% nondenaturing polyacrylamide gel in 1× Tris-acetate-EDTA buffer using a Mini Protean II apparatus (Bio-Rad Laboratories, Mississauga, Ontario, Canada). The gel was run for ~45 min at 150 V and then stained with ethidium bromide before it was exposed to UV light. Recorded images were analyzed using the Quantity One software (Bio-Rad Laboratories).

PCR amplification of *C. difficile* CD630 prophage sequences. PCR primers were designed to amplify specific regions of each of the two complete prophages predicted from the genomic sequence of *C. difficile* CD630 (accession number AM180355.1) (47). Primers CD630.1F (5′-CTGTATGACCTAACCTTTCTGAAACAAC-3′; nucleotide positions 1088267 to 1088294) and CD630.1R (5′-TTGCGACTAGGAGA AGCTACTGGA-3′; nucleotide positions 1088690 to 1088667) were specific for one of the prophages, while primers CD630.2F (5′-TTGTTTGCAGTCTTCGTTCCA CCC-3′; nucleotide positions 3426729 to 3426706) and CD630.2R (5′-CCATCAC CTCGTTGCTTATTTGGG-3′; nucleotide positions 3426338 to 3426361) were specific for the other prophage.

Prophage induction. Bacterial cells were inoculated into 5 ml of BHI medium and grown at 37°C under anaerobic conditions until stationary phase (16 to 24 h). A 3% inoculum from an overnight culture was transferred into 10 ml of fresh BHI medium, and growth was monitored until the optical density at 600 nm (OD_{600}) reached 0.1. Then, depending on the strain, mitomycin C was added to a final concentration ranging from 0.5 to 5 μg/ml. Mitomycin-containing cultures were further incubated at 37°C under anaerobic conditions, and the OD_{600} was monitored regularly. A significant decrease in the cell density after 3 to 5 h suggested that prophages were released. Crude lysates were then filtered (0.45 μm) and stored at 4°C until further analysis.

TABLE 1. Primers used to generate probes for toxin genes and toxin regulators

Primer	Sequence (5′–3′)[a]	Target gene	Probe size (bp)
LCF-139	TTAATTAATTTTCTCTACAGCTATCC	*tcdC*	722
LCF-140	TCTAATAAAAGGGAGATTGTATTATG		
LCF-149	TGAACCTGGAAAAGGTGATG	*cdtA*	376
LCF-150	AGGATTATTTACTGGACCATTTG		
LCF-160	TGGTGATATGGAGGCATCACCACT	*tcdB*	501
LCF-161	TGAGCTTTAGCTCTTGCATCGTCA		
LCF-162	ACATTATGAAGAGGGAGAAACAGA	*tcdR*	385
LCF-163	AGCCTTATTAACAGCTTGTCT		
LCF-164	CCTAGGAGGCGTTATGAATATGACA	*tcdE*	366
LCF-165	GGTACTGGTAATCCACATAAGCAC		
LCF-166	AGCTCTATTGGACTAGACCGTTGGGA	*tcdA*	514
LCF-167	ACGCATAAGCTCCTGGACCACTTA		

[a] Most sequences were based on the genomic sequence of *C. difficile* strain CD630 (accession number NC_009089); the exceptions were primers LCF-149 and LCF-150, which were based on the genomic sequence of *C. difficile* strain QCD-32g58 (accession number AAML00000000).

TEM. Prior to observation, 1.5 ml of crude lysate was centrifuged for 1 h at 4°C and 24,000 × *g*. A fraction of the supernatant (approximately 1.4 ml) was gently discarded, and 1 ml of ammonium acetate (0.1 M, pH 7.5) was added to the remaining lysate, which was then centrifuged as described above. This step was performed twice. The washed phage particles were used to prepare transmission electron microscopy (TEM) grids as follows. Ten microliters of 2% uranyl acetate was deposited onto a 200-mesh Formvar/carbon-coated copper grid (Pelco International, Redding, CA). After 30 s, 10 μl of the phage preparation was mixed with the stain by pipetting the solution up and down a few times. After further incubation for 30 s, the liquid was removed from the grid by blotting with a piece of Whatman paper. To achieve good contrast, care was taken to leave a thin film of the uranyl acetate stain on the grid and to avoid complete blotting. The grids were dried for a few minutes and then observed at 80 kV with a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a DualVision digital camera (Gatan, Pleasanton, CA). Micrographs were recorded using the Gatan Digital Micrograph software (version 1.3), after the exposure time was set to 10 s.

Phage DNA analysis. Phage DNA was isolated from crude lysates obtained after mitomycin C induction using a rapid miniprep protocol described elsewhere (38). Phage DNA was digested with restriction enzymes (Roche Diagnostics, Laval, Québec, Canada) used according to the manufacturer's recommendations. The digestion mixture was heated at 70°C for 10 min to disrupt any possible cohesive end joining and immediately run through a 0.8% agarose gel. Gels were stained with ethidium bromide, exposed to UV, and photographed using a gel documentation system (Bio-Rad Laboratories).

Southern hybridization. Phage DNA was digested with restriction enzymes, and the resulting fragments were separated on a 0.8% agarose gel before they were transferred onto positively charged nylon membranes (GE HealthCare, Baie D'Urfé, Québec, Canada) using standard protocols (46). Phage DNA (~1 μg) was labeled using a digoxigenin (DIG) High-Prime kit from Roche Diagnostics according to the manufacturer's recommendations. The labeling reaction was performed for 16 h at 37°C. Probes corresponding to genes from the PaLoc (*tcdA*, *tcdB*, *tcdC*, *tcdE*, and *tcdR*) and *ctdA* encoding the binary toxin were also amplified by PCR with DIG-11-dUTP incorporation. The primer sequences used for generating the probes are shown in Table 1. Prior to hybridization, the labeled DNA probes were quantified, denatured by boiling for 10 min, diluted in DIG Easy-Hyb buffer (Roche Diagnostics) to obtain a final concentration of ~25 ng/ml, and filtered (0.45 μm) to reduce the background. Hybridization was carried out at 42°C, and the membranes were washed and detected as recommended by Roche Diagnostics. The hybridized membranes were then exposed to Kodak Biomax Light film.

RESULTS

PCR ribotyping. First, 45 isolates of *C. difficile* were obtained from various sources; these strains included toxigenic strain CD630 obtained from the Sanger Institute. All the strains except CD630 were isolated from human stools during the *C. difficile* outbreak in 2003 and 2004 in the province of Québec (Canada). In this outbreak, one strain, strain NAP1/027, was found to be predominant, (25, 33). This strain is also referred to as QCD-32g58, and its whole genome is currently being sequenced (accession number AAML00000000).

The 45 isolates were then classified by PCR ribotyping in order to subsequently analyze the prophage contents of different strains. For the 45 isolates analyzed, 11 distinct ribotypes were obtained, 6 of which were later shown to contain inducible prophages (see below). The latter six ribotypes are shown in Fig. 1A. Note that isolates CD5, CD6, and CD44 are most likely clones of the same strain as they have identical PCR ribotype profiles (Fig. 1A). In fact, 31 isolates (69%) had the same ribotype as CD5, CD6, and CD44 (data not shown). These 31 isolates also belonged to toxinotype III, were binary toxin positive, and harbored an 18-bp deletion in the *tcdC* gene (J. Pépin and É. Frost, personal communications). Based on

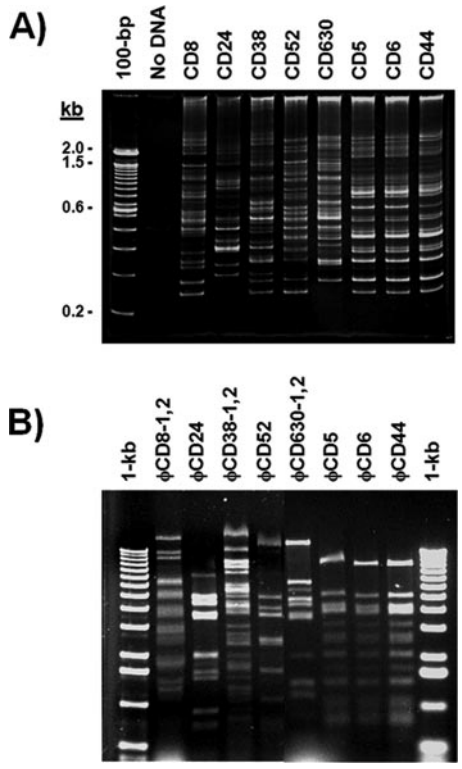


FIG. 1. (A) PCR ribotyping of *C. difficile* isolates. The designations above the lanes are the designations of the *C. difficile* isolates analyzed by PCR ribotyping. Isolates CD5, CD6, and CD44 have identical ribotypes and correspond to hypervirulent strain NAP1/027. Lane 100-bp contained a DNA marker. (B) Comparison of HindIII DNA restriction profiles of phage DNA. DNA was purified from the lysates of mitomycin C-induced cultures of various *C. difficile* clinical isolates. The phage designations above the lanes refer to the *C. difficile* isolate designations in panel A. Lanes 1-kb contained a DNA marker.

the findings described above, we concluded that these 31 isolates represented hypervirulent strain NAP1/027 (25, 33).

Prophage induction. Addition of mitomycin C to growing cultures, including cultures of *C. difficile* strains, is well known to induce excision of prophages (22, 23, 49). Mitomycin C was added to early-log-phase cultures of representative isolates of each of the 11 PCR ribotypes. The optical density was monitored, and a sudden decrease in the OD_{600} after 3 to 5 h of growth following mitomycin C addition suggested that there was cell lysis due to the induction of prophages and possibly the release of viral particles. The latter suggestion was confirmed by TEM observations and extraction of whole phage DNA from the crude lysates. With this approach, *C. difficile* isolates CD5, CD8, CD24, CD38, CD52, and CD630 representing the six different ribotypes (Fig. 1A) were found to release complete viral particles from which DNA could be purified, whereas the isolates representing the other five ribotypes did not contain inducible prophages under the conditions tested. Interestingly, isolate CD5, which is representative of the predominant and hypervirulent strain, was found to contain an inducible prophage. Previous computational analyses by our group and other groups failed to identify a complete prophage in the genome of this strain, most likely because the sequencing project is incomplete (21). Thus, to our knowledge, this is the first report of the presence of an inducible prophage in the genome of the NAP1/027 strain.

Finally, we also noticed that some *C. difficile* isolates showed a decrease in the OD_{600}, but we could not find intact phage particles by TEM observation or isolate phage DNA from the crude lysates. Instead, numerous phage tail-like particles (PT-LPs) that were 130 by 18 nm were found (Table 2), and these particles were morphologically similar to those previously identified by Nagy and Foldes in mitomycin C-induced lysates of *C. difficile* (39). Similar PT-LPs were also observed in *Vibrio* cultures (19). It is noteworthy that the amount of these PT-LPs varied greatly from one *C. difficile* isolate to another.

TEM observations. From the six *C. difficile* isolates (with distinct PCR ribotypes) that contained inducible prophages, eight phages could be differentiated based on TEM observations (Table 2). Two distinct phage morphotypes were observed in the lysates of *C. difficile* strains CD8 and CD38, whereas only one morphotype was found in the other four lysates (CD5, CD24, CD52, and CD630).

Three phages (ϕCD5, ϕCD52, and ϕCD630) had an isometric capsid and a contractile tail (morphotype A1). These three phages were very similar in morphology and size (Table 2). Phage particles with contracted sheaths were also observed in several lysates containing these phages (data not shown). Based on criteria of the International Committee on Taxonomy of Viruses, they belong to the *Myoviridae* family (1). Our results are in agreement with a recent article showing that the inducible *C. difficile* prophages ϕCD630-1 and ϕCD630-2 belong to the *Myoviridae* family (21). However, contrary to the findings of Goh et al. (21), we observed only one morphotype in our CD630 lysates, even though two distinct phages were present as revealed by PCR and partial sequencing (data not shown). In addition, it was previously reported that phage ϕCD630-1 had a larger capsid (31.7 ± 0.7 nm versus 28.1 ± 1.3 nm) and a longer tail (62.4 ± 5.1 nm versus 39.5 ± 5.8 nm) than phage ϕCD630-2 (21). It is noteworthy that our ϕCD630 phage dimensions (capsid diameter, 62 nm; tail length, 126 nm) are significantly larger than those previously reported for these inducible prophages (21). On the other hand, our measurements are consistent with the usual size of capsids that are capable of packaging 49-kb (ϕCD630-2) and 55-kb (ϕCD630-1) genomes (16, 21).

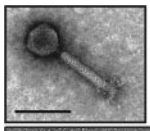
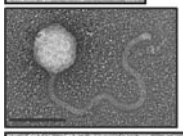
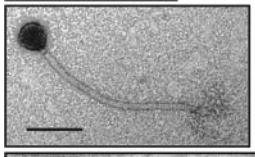
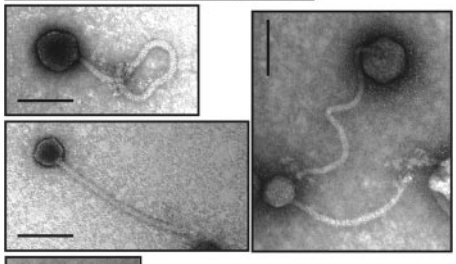
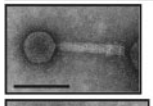
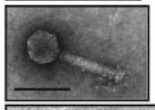
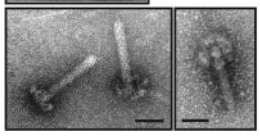
The remaining five phages (ϕCD8-1, ϕCD8-2, ϕCD24, ϕCD38-1, and ϕCD38-2) had the B1 morphotype, which corresponds to an isometric capsid and a long noncontractile tail, and thus belong to the *Siphoviridae* family (1). Interestingly, *C. difficile* strains CD8 and CD38 both released two types of *Siphoviridae* phages (Table 2). Phage ϕCD8-1 had a larger capsid than ϕCD8-2 (diameters, 90 and 46 nm, respectively) and a longer tail (432 and 338 nm, respectively). Similarly, ϕCD38-1 had a larger capsid than ϕCD38-2 (diameters, 77 and 58 nm, respectively), as well as a longer and larger tail (402 by 15 and 262 by 12 nm, respectively). ϕCD38-1 is morphologically similar to the virulent *Lactococcus lactis* phage 949 (16), although its capsid is larger than that of phage 949 (90 nm versus 70 nm). The size of ϕCD24 was midway between the sizes of the four other phages belonging to the *Siphoviridae* family; the capsid diameter was 66 nm, and the tail size was 302 by 10 nm. Detailed observation of ϕCD24 morphology revealed the presence of six fibers that were about 60 nm long at the tip of its tail (Table 2). Each of these fibers also had a globular structure at its extremity. This is the first time that such a tail appendage has been described for a *Clostridium* phage.

Phage genome comparison. Phage DNA was extracted from the crude lysates of *C. difficile* isolates representing the six PCR ribotypes (Fig. 1). The HindIII endonuclease was used to generate DNA restriction profiles, which allowed easy discrimination of the induced prophages. As shown in Fig. 1B, each unique *C. difficile* ribotype had a distinct phage DNA restriction profile. Interestingly, isolates with identical PCR ribotypes, such as CD5, CD6, and CD44 (and others not shown here), had identical phage DNA restriction profiles. The profiles obtained for lysates of CD8 and CD38 were rather complex, with numerous bands and high-molecular-mass fragments. These findings are consistent with phages with a large capsid and/or with the presence of distinct phages in the lysates.

We performed a series of DNA-DNA hybridization experiments in order to determine the relatedness of the phage genomes shown in Fig. 1B. This methodology has been found to be very effective for classifying phages of *L. lactis* (16) and *Streptococcus thermophilus* (32). Genomic DNA from each phage preparation was DIG labeled and used as a probe to detect homologous DNA sequences. Hybridization results obtained with probes for phages ϕCD5, ϕCD24, ϕCD38-1/ϕCD38-2, and ϕCD52 are shown in Fig. 2. Using the ϕCD5 and ϕCD52 probes, we noted that the three phages belonging to the *Myoviridae* family were related and that phage ϕCD52 was more similar to ϕCD630 (Fig. 2A).

Greater genomic diversity was observed among the phages belonging to the *Siphoviridae* family. First, it was rather obvious that ϕCD24 was quite different from the other phages, since the ϕCD24 probe strongly hybridized mainly with the ϕCD24 genome. Limited similarity was observed between ϕCD24 and ϕCD5 (Fig. 2A), and according to the results

TABLE 2. Features of *C. difficile* temperate phages

Phage or particles	Family	Capsid diam (nm)[a]	Tail length (nm)[a]	Tail diam (nm)[a]	Electron microscopy image[b]
ϕCD5	*Myoviridae*	66	127	18	
ϕCD8-1	*Siphoviridae*	90	432	12	
ϕCD8-2	*Siphoviridae*	46	338	11	
ϕCD24	*Siphoviridae*	66	302	10	
ϕCD38-1	*Siphoviridae*	77	402	15	
ϕCD38-2	*Siphoviridae*	58	262	12	
ϕCD52	*Myoviridae*	64	125	19	
ϕCD630	*Myoviridae*	62	126	19	
PT-LPs			130	18	

[a] Means of at least 5 to 10 measurements obtained with different viral particles.
[b] The magnification is ×200,000 and the bars are 100 nm long except for PT-LPs. For PT-LPs the magnification is ×300,000 and the bars are 50 nm long.

obtained with the DIG labeling and detection methods used, DNA regions as short as 200 bp with ≥60% DNA identity could be detected across the membrane. Thus, complete genome sequencing of ϕCD24 and ϕCD5 is required to precisely determine the extent of DNA identity between these two phages.

Finally, the ϕCD38-1/ϕCD38-2 genomic probe hybridized with DNA fragments from the corresponding phage lysate and also from ϕCD8-1 and ϕCD8-2 (Fig. 2B). Since the CD8 lysate contained two phages (ϕCD8-1 and ϕCD8-2) and the ϕCD38-1/ϕCD38-2 probe contained two genomes (ϕCD38-1 and ϕCD38-2), it is not possible at this time to know which phage probe hybridized to which phage from the lysate. Nonetheless, it is clear that these siphophages are different from the other phages (e.g., ϕCD24, ϕCD5, ϕCD52, and ϕCD630).

Correlation between the type of prophage and PCR ribotype. Our TEM and restriction profile analyses suggested that there was a unique prophage for each individual PCR ribotype. This was exemplified by phages ϕCD5, ϕCD6, and ϕCD44; all three of these phages were identical in morphology (not shown) and HindIII restriction profiles (Fig. 1B and 2), and they were induced from *C. difficile* isolates with identical PCR ribotypes (Fig. 1A). We investigated a total of seven different isolates corresponding to epidemic strain NAP1/027 and found that they all released identical prophages following the addition of mitomycin C (data not shown). A similar observation was made with two isolates of the CD8 ribotype and two isolates of the CD38 ribotype (data not shown). Thus, at least in our strain collection, the PCR ribotype correlates with the inducible prophage content.

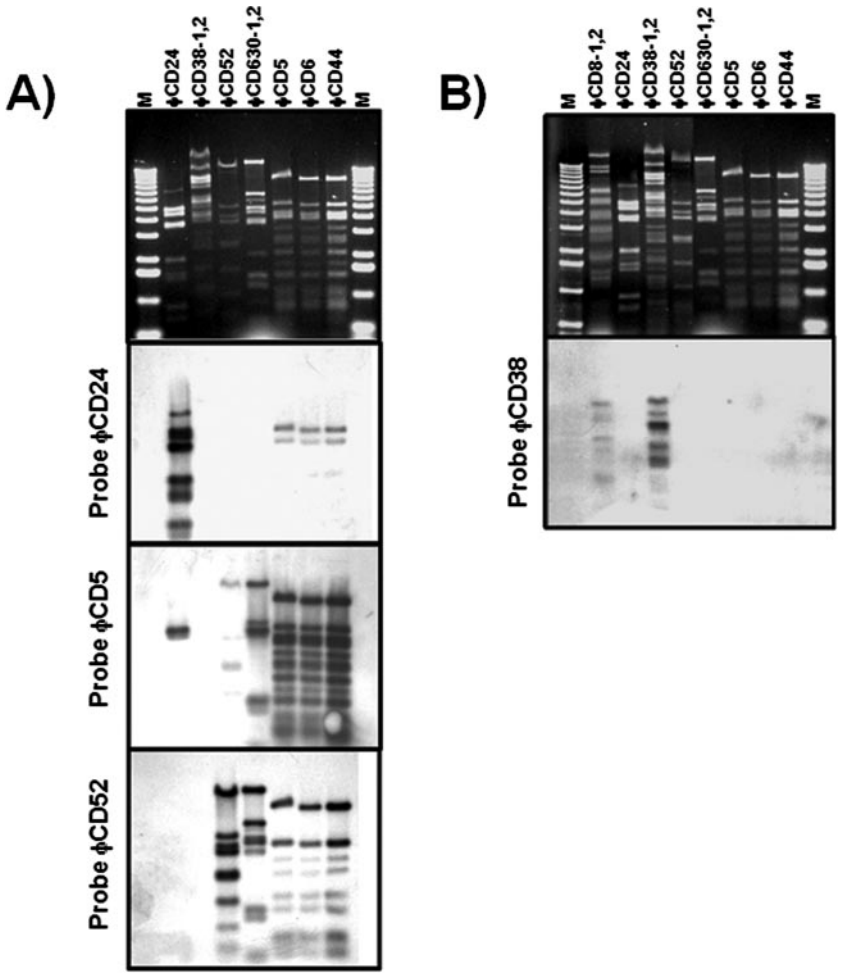


FIG. 2. Southern hybridization analysis of phage DNA. (A) An ethidium bromide-stained gel containing HindIII-digested genomic phage DNA is at the top. Autoradiographs of Southern blots are below this gel, and the corresponding phage probes are indicated on the left. The phage designations above the lanes refer to the *C. difficile* isolates used for mitomycin C induction. (B) Same procedure as in panel A, but a different agarose gel which included φCD8 DNA was used. Lanes M contained a 1-kbp DNA marker.

Screening for potential toxin genes within *C. difficile* genomes. To test whether our phages could potentially encode toxins or toxin regulatory proteins related to those encoded in the PaLoc, we performed a series of DNA-DNA hybridizations using probes for *tcdA*, *tcdB*, *tcdC*, *tcdE*, and *tcdR*. The probes for *tcdA* and *tcdB* were amplified from the genome of strain CD630 and covered a portion of the nonrepeating region of both toxin genes corresponding to nucleotide positions 910 to 1423 for *tcdA* and nucleotide positions 1089 to 1589 for *tcdB*. We also included a probe for *cdtA*, one of the two genes encoding the binary toxin. All probes were PCR amplified and labeled with DIG-11-dUTP; the PCR primers used to generate the probes are listed in Table 1. Genomic DNA from strain CD630 was used as the template for all PCR probes except the probe for *cdtA*. Indeed, *cdtA* and *cdtB* were found to be highly altered pseudogenes in the genome of strain CD630 (47). Thus, for amplification of *cdtA*, we used DNA from strain CD6, corresponding to the hypervirulent strain, which was previously shown to encode the binary toxin (33). Primers were designed based on the available genomic sequence of strain QCD-32g58 (accession number AAML00000000). Although all our probes allowed easy detection of the corresponding genes in the genomic DNA of our *C. difficile* isolates, no signal could be detected with our phage genomes, despite extended autoradiography exposures (data not shown). It is worth noting that in these experiments the number of copies of each phage genome was significantly greater than the number of copies of whole bacterial DNA. Hence, if a positive signal was detected with bacterial DNA but not with phage DNA, we concluded that the phages do not contain genes homologous to genes in the PaLoc or to the *cdtA* gene encoding the binary toxin.

Indicator strains. We tried to identify *C. difficile* strains that could be used as indicator strains to propagate the induced prophages. Ten microliters of each lysate was deposited on soft agar overlays containing 500 μl of mid-log-phase cultures (OD_{600}, 0.5 to 0.8) of each *C. difficile* isolate from our collection. Plates were incubated anaerobically for 48 h. Unfortunately, none of the 45 *C. difficile* isolates available allowed lytic amplification of the phages under the conditions tested. The difficulty of finding proper indicator strains has been reported previously by several authors, although a limited number of indicator strains have been found for other *C. difficile* phages,

including ϕCD630-1 and ϕCD630-2 (21, 23, 34). We are currently screening a larger collection of *C. difficile* isolates using different experimental conditions in order to identify such strains.

DISCUSSION

So far, only two prophages of *C. difficile*, ϕCD119 and ϕC2, have been studied at the molecular level (21–23). Hence, our knowledge of the genetic diversity of these phages and their possible role in the biology of *C. difficile* is still very limited. Here, we describe morphological characterization and the DNA relatedness of eight other temperate phages of *C. difficile*.

Ribotyping of *C. difficile* is based on PCR amplification of the 16S-23S spacer region within the rRNA gene cluster. The number of alleles and the length of the spacer region vary from one strain to another, which results in a specific banding pattern upon gel electrophoresis of the PCR products (5, 53). Considering the conserved nature of ribosomal gene sequences and the mobility of phage elements (24), we initially thought that the ribotype may not be indicative of the prophage content. Moreover, bacteria tend to gradually delete from their genomes DNA sequences that represent a threat to their survival (30). However, our observations suggest that there is a correlation between the PCR ribotype and specific prophages. For each PCR ribotype, we found one prophage with a unique restriction profile. In the case of hypervirulent strain NAP1/027, seven different isolates, all collected in the province of Québec during the outbreak in 2003 and 2004, were shown to contain identical prophages. We cannot exclude the possibility that identical restriction profiles could in fact be slightly different due to small insertions or deletions or to point mutations. Nonetheless, to our knowledge, the association between ribotyping and prophage content is reported for the first time here and could be valuable for selecting strains to study their prophage contents. Since over 150 different *C. difficile* ribotypes have been described so far (27), there could potentially be many different prophages.

Electron microscopy observations revealed the presence of two phage morphotypes in our lysates, morphotypes A1 and B1 (1). Three of the phages (ϕCD5, ϕCD52, and ϕCD630) had the A1 morphotype, which is a characteristic of the members of the *Myoviridae* family. The morphology and size of these myophages fell in the morphology and size ranges of previously described *C. difficile* myophages, like phage 56 (34) and ϕCD119 (23), as well as ϕC2, ϕC5, and ϕC8 (21, 22). Nagy and Foldes also reported TEM observations for six similar myophages in both toxin-positive and toxin-negative *C. difficile* clinical isolates (39). The Southern hybridization data reported here revealed that phages ϕCD5, ϕCD52, and ϕCD630 are closely related as they share significant DNA similarity. Likewise, Goh et al. (22) showed that the highly related phages ϕC2 and ϕC5 (*Myoviridae*) were also related to the prophage ϕC8, but to a lesser extent. Comparative bioinformatics analyses of the genomes of *C. difficile* prophages ϕC2 and ϕCD119, as well as the genomes of ϕCD630-1 and ϕCD630-2 (all *Myoviridae* phages), also confirmed this relatedness (21–23). In fact, it has been suggested that the prophages found in the genome of strain CD630 are a mosaic of ϕC2 and ϕCD119 (23). Based on our DNA hybridization experiments, we deduced that phages ϕCD5 and ϕCD52 described in this study are similar to ϕCD630 and hence are genetically related to phages ϕC2, ϕC5, and probably ϕC8 described by Goh et al. (21, 22). Together, the *Myoviridae* prophages isolated so far from *C. difficile* make up a genetically closely related group.

Five prophages (ϕCD8-1, ϕCD8-2, ϕCD24, ϕCD38-1, and ϕCD38-2) had the B1 morphotype and belonged to the *Siphoviridae* family. Phage ϕCD24 is very similar in size and morphology to *C. difficile* phages 41 (34) and ϕC6 (22) and to one phage identified by Nagy and Foldes in *C. difficile* strain 25 (39). Nagy and Foldes also isolated two siphophages with tails that were 300 and 370 nm long, which is in the size range of the tails of phages ϕCD8-1, ϕCD8-2, and ϕCD38-1 (338 to 432 nm), but the capsid sizes were smaller than the capsid sizes of the phages described here (39). Previous Southern hybridization experiments showed that the siphophage ϕC6 was only distantly related to *Myoviridae* phages (22). Likewise, we showed that phage ϕCD24 is distantly related to the myophages characterized in this study. The other *Siphoviridae* phages (ϕCD8-1, ϕCD8-2, ϕCD38-1, and ϕCD38-2) reported here are also different from the other phages as no or only very limited DNA similarity was detected in Southern hybridization experiments. For example, no hybridization signal could be detected between phage ϕCD24 and ϕCD38-1 or ϕCD38-2. Thus, these phages constitute two genetically distinct groups of *Siphoviridae* phages. Whole-genome sequencing of these genetically unrelated phages should provide valuable data on the genetic diversity of *C. difficile* temperate phages.

One of the most intriguing findings of this study was the presence of PT-LPs in all our *C. difficile* lysates. Induction of some *C. difficile* isolates led to the production of large amounts of these PT-LPs, which resemble contractile tails from *Myoviridae* phages. Nagy and Foldes also reported detection of highly similar PT-LPs in 7 of 18 strains induced with mitomycin C (39). Several other examples of bacteria producing PT-LPs have been reported, including the R-type pyocin of *Pseudomonas aeruginosa* (31) and the PT-LPs of *Budvicia aquatica* (50), *Pragia fontium* (50), and *Photorhabdus luminescens* (18), as well as the PT-LPs of mitomycin C-induced cultures of *Vibrio* sp. (19). The latter PT-LPs are of particular interest as they possess flower-like appendages similar to those observed in this study (19). Some of these PT-LPs are known to have bactericidal activity (58), although such activity remains to be demonstrated for the PT-LPs of *C. difficile*. According to a current hypothesis, genes encoding PT-LPs were derived from partially deleted prophages and they provide competitive advantages to their hosts (11). For example, the pyocin of *P. aeruginosa* was derived from an ancestor of phage P2 (*Myoviridae*) (41). Interestingly, in some cases, the different amounts of released PT-LPs depended on the host's physiological status and growth environment (19). Whether modifying the growth conditions of *C. difficile* strains influences or does not influence the production of these PT-LPs remains to be determined. Nonetheless, the presence of the PT-LPs in numerous *C. difficile* isolates suggests that they may have a functional role in the biology of this pathogen.

Previous studies have revealed some degree of similarity between phage genes and *tcdA*, *tcdB*, *tcdC*, and particularly *tcdE* in the PaLoc of *C. difficile* (20, 21, 23, 55). This finding is supported by the results of Southern blot and PCR experi-

ments with whole phage DNA, functional assays in *E. coli*, and bioinformatics analyses (20, 55). Our experiments using whole phage DNA and probes against *tcdA*, *tcdB*, *tcdC*, *tcdE*, *tcdR*, and the binary toxin gene *cdtA* failed to detect any significant similarity between these bacterial genes and the induced prophages. Although we didn't find any DNA similarity, this result does not exclude the possibility that these phages could impact the expression of the toxins, as observed by Goh et al. (20), or could contribute somehow to the virulence of *C. difficile*. In fact, the identification of virulence factors in phage genomes is not always straightforward. A good example of unsuspected virulence factors was reported recently for *Streptococcus mitis* (37). The authors found that the prophage tail proteins PblA and PblB were released upon induction from a subset of the bacterial population. Unexpectedly, subsequent attachment of PblA and PblB to the surface of intact surrounding *S. mitis* cells was shown to increase the virulence of this pathogen in an animal model of endocarditis (37).

In summary, this study provides new data regarding the prophage diversity within *C. difficile* strains. Our results suggest that particular prophages are associated with specific PCR ribotypes. Over 150 different *C. difficile* ribotypes have been reported; it will be interesting to assess if this is representative of the prophage diversity within *C. difficile*. Also, our data enabled us to distinguish at least three genetically distinct phage groups. The first group is composed of genetically related *Myoviridae* phages, including ϕCD5, ϕCD52, ϕCD630-1, ϕCD630-2, ϕC2, ϕC5, ϕC8, and ϕCD119. The other two groups, composed of *Siphoviridae* phages, are more heterogeneous. The siphophage ϕCD24 is the only member of the second group, whereas the third group includes the *Siphoviridae* phages ϕCD8-1, ϕCD8-2, ϕCD38-1, and ϕCD38-2. Finally, an inducible prophage was found within the genome of the hypervirulent strain NAP1/027 (QCD-32g58), which was shown to be responsible for outbreaks in North America and Europe. We failed to detect any DNA similarity between this phage and genes in the PaLoc or encoding the binary toxin. Thus, whether this prophage increases the fitness of its host remains to be determined.

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