Evidence of in vivo prophage induction during Clostridium difficile infection

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Keywords: Clostridium difficile, temperate bacteriophage, prophage, spontaneous induction

Running title: In vivo prophage induction in C. difficile
ABSTRACT

Prophages contribute to the evolution and virulence of most bacterial pathogens, but their role in *C. difficile* is unclear. Here we describe the isolation of four Myoviridae phages: φMMP01, φMMP02, φMMP03, and φMMP04, that were recovered as free viral particles in filter-sterilized stool supernatants of patients suffering from *C. difficile* infection (CDI). Furthermore, identical prophages were found in the chromosome of *C. difficile* isolated from the corresponding fecal samples. We therefore provide for the first time evidence of *in vivo* prophage induction during CDI. We completely sequenced the genomes of φMMP02 and φMMP04, and bioinformatics analyses did not reveal the presence of virulence factors, but underlined the unique character of φMMP04. We also studied the mobility of φMMP02 and φMMP04 prophages *in vitro*. Both prophages were spontaneously induced, with 4-5 log PFU/mL detected in culture supernatants of the corresponding lysogens. When lysogens were grown in the presence of sub-inhibitory concentrations of ciprofloxacin, moxifloxacin, levofloxacin, or mitomycin C, phage titers further increased, reaching 8-9 log PFU/mL in the case of φMMP04. In summary, our study highlights the extensive genetic diversity and mobility of *C. difficile* prophages. Moreover, antibiotics known to represent risk factors for CDI, such as quinolones, can stimulate prophage mobility *in vitro* and probably *in vivo* as well, which underscores their potential impact on phage-mediated horizontal gene transfer events and the evolution of *C. difficile*. 
INTRODUCTION

Clostridium difficile is the leading cause of antibiotic-associated nosocomial diarrhea in developed countries (18). Highly virulent strains such as NAP1/027 have caused severe outbreaks in North America and Europe since 2003 and are now spreading worldwide, reaching Central America, Asia, and Australia (14). C. difficile infections (CDI) are a consequence of antibiotic treatments that reduce the diversity of the intestinal microbiota (11). C. difficile is a strictly anaerobic, spore-forming, Gram-positive bacillus that causes a wide range of clinical symptoms varying from mild to severe diarrhea to fatal pseudomembranous colitis. The pathogenic potential of C. difficile lies mainly in the expression of two large exotoxins, TcdA and TcdB encoded on a pathogenicity locus (PaLoc) (26, 29). Additional virulence determinants are probably important for full virulence of this pathogen, but little is known about these factors and their importance in the development of CDI (38).

The rapid change in the epidemiology of C. difficile over the last decade has raised several concerns and the genetic basis for the evolution of this pathogen is still unclear. With the advent of next-generation sequencing, several genomes of C. difficile have been sequenced recently. The data obtained reveals that horizontal transfer of mobile genetic elements (MGE), such as conjugative transposons and prophages, likely account for the great plasticity of the C. difficile genome (23, 39, 43). For instance, 11% of the genomic DNA of strain 630 is made up of MGE, including 8 conjugative transposons and 2 functional and highly similar prophages (39). Moreover, the new epidemic NAP1/027 strain R20291 was found to have acquired 5 unique DNA regions containing phage and transposon genes, two-component systems, and
transcriptional regulators, compared with the historic NAP1/027 strain CD196 and strain 630 (43). This suggests that the acquisition of genetic material through horizontal gene transfer is important in modeling the genome of *C. difficile*.

Recent studies have highlighted the great diversity of prophages in clinical isolates of *C. difficile* (17, 36, 42), but only 5 fully characterized phages with complete genomic sequences are available in public databases. Phage φC2 (20), φCD119 (21), and φCD27 (33) are members of the *Myoviridae* family, *i.e.* phages with long non-flexible contractile tails (1), whereas phages φCD6356 (24) and φCD38-2 (40) are members of the *Siphoviridae* family, *i.e.* phages with long and flexible non-contractile tails. It is noteworthy to mention that all known phages of *C. difficile* are temperate, *i.e.* they can either adopt a lytic or a temperate lifestyle upon infection of their host. Prophages are well-known contributors in the evolution of most bacterial species, including important pathogens (10) but their role in the virulence and evolution of *C. difficile* is still highly speculative. Two recent studies have shown that *C. difficile* phages φCD119 and φCD38-2 can modulate toxin production, even if these phages do not encode identifiable virulence factors (22, 40).

DNA-damaging and SOS-inducing stresses are often good prophage inducers that can contribute to horizontal gene transfer in bacteria (3, 32). The phenomenon of spontaneous and antibiotic-triggered prophage induction has been described in several phage-host systems and the consequences of this phenomenon can be significant. For example, the increase in the production of Shiga toxins was shown to be tightly linked with spontaneous and quinolone_triggered...
induction of prophages in *Escherichia coli* (45, 47). Spontaneous prophage-induced lysis was associated with the release of extracellular genomic DNA from *Streptococcus pneumoniae* and increased biofilm formation (13). Prophage induction from a sub-population of bacteria can also lead to killing of competing species, thus increasing fitness of the non-induced lysogens (8, 10, 31, 41) and can also promote horizontal gene transfer among bacteria, thereby speeding up genome evolution (5, 10). CDI is a consequence of antibiotic treatments that destroy the intestinal microbiota and recent epidemic clones of *C. difficile* are resistant to numerous antibiotics, including most fluoroquinolones (37). Studying the impact of antibiotics, in particular fluoroquinolones, on horizontal gene transfer and prophage mobility during *C. difficile* infection is thus of great interest.

In this study, we report the isolation of four different *C. difficile* phages that were recovered as free viral particles in feces of patients suffering from CDI. We studied the spontaneous and antibiotic-triggered prophage induction *in vitro* to assess the mobility of these phages. Finally, the complete genomic sequence was determined for two of these phages, thus providing additional genomic data to a yet understudied group of phages.
MATERIAL AND METHODS

Bacterial strains and culture conditions
All bacterial isolates used in this work are listed in Table 1 and were kindly provided by Drs Louis Valiquette and Jacques Pépin from the Université de Sherbrooke. When required, *C. difficile* was isolated from feces of patients suffering from CDI after alcohol shock and growth on CDMN selective agar (Oxoid) supplemented with 5% sheep blood, 0.1% taurocholate and 1 mM glycine. The institutional review board of the CHUS has approved the study protocol. Bacteria were routinely grown at 37°C in an anaerobic chamber (Coy Laboratories) in brain heart infusion broth (BHI) (BD Bioscience) or TY broth (3% tryptose, 2% yeast extract, pH 7.4). All media were pre-reduced overnight under anaerobic conditions.

Bacterial DNA extraction and PCR ribotyping
Total genomic DNA was purified using the Illustra Bacterial Genomic DNA extraction kit (GE Healthcare) as described before (40). All *C. difficile* isolates were analyzed by PCR ribotyping using an Eppendorf Mastercycler with 20 ng purified genomic DNA and primers published by Bidet *et al* (7), with modifications described elsewhere (17). Band patterns were analyzed with GelComparII (Applied Maths) and the Pearson’s correlation was used for cluster analysis.

Phage enrichment and isolation
Sewage samples from two water treatment plants in Sherbrooke and human fecal samples from patients suffering from CDI collected over a one-year period were screened for the presence of free phages. Raw sewage samples (400 mL) were passed through 1.5 μm Whatman filters,
followed by another filtration on 0.45 μm EZ-Pak filters (Millipore) and then 5 mL from 3 different samples were pooled. Stool samples were homogenized in 10 mL of BHI, centrifuged at 4000 xg for 60 min at room temperature and then passed through 0.45 μm filter discs to remove bacteria. Five stool samples were then pooled before the phage enrichment procedure. For phage enrichment, fifteen _C. difficile_ isolates were use as hosts, which represented 8 different PCR ribotypes, including 3 isolates of ribotype 027 (Table 1). Enrichment was done by adding 1.25 mL of the pooled sewage sample to 1.5 mL of BHI containing 10 mM CaCl₂ and 10 mM MgCl₂ (BHIS) and a 2% (vol/vol) inoculum of an overnight _C. difficile_ culture. For enrichment from stool samples, 2.5 mL of pooled stool supernatants were combined with 2.5 mL of BHIS and 2% of an overnight _C. difficile_ culture. The next day, cultures were centrifuged for 15 min at 4000 xg and passed through 0.45 μm filter discs (Sarstedt). A second enrichment was performed in a total volume of 5 mL BHIS, using 2.5 mL of the first enrichment as phage inoculum. Finally a third enrichment step was done as above using the second enrichment broth as the phage inoculum. Culture supernatants were then filter-sterilized on 0.45 μm disks and 0.1 mL samples were added to soft agar overlays inoculated with the same strains as hosts, as described previously (40).

**Phage purification and amplification**

Phages obtained after the enrichment procedure were purified from single isolated phage plaques using three successive rounds of soft agar overlays as described before (40). Phage titers of ≥ 10⁹ plaque-forming units/mL (PFU/mL) were routinely obtained with this method.
Creation of lysogens and prophage induction

C. difficile lysogens carrying φMMP02 or φMMP04 prophages were created by spreading dilutions of phage-sensitive C. difficile cultures on soft agar overlays containing phages (10^8 PFU/mL), as described previously (40). Five potentially phage-immune colonies were picked and re-streaked 3 times on BHI agar plates without phage to purify the lysogens. The presence of the integrated prophage was confirmed by Southern blot hybridization using lysogenic bacterial DNA and a digoxigenin (DIG)-labeled whole phage DNA probe (17). Alternatively PCR with phage-specific primers was used. The functionality of the integrated prophage was verified by treating lysogens with UV light (302 nm) or mitomycin C (3µg/mL), followed by phage DNA purification and restriction analysis, as described previously (40).

MIC determination

Minimal inhibitory concentrations (MIC) for ciprofloxacin (CIP), moxifloxacin (MXF) and levofloxacin (LVX) were determined in 96-wells plates. Briefly, antibiotics were serially diluted in 96-wells plates in a final volume of 0.1 mL. An equal volume of a bacterial culture at an optical density at 600nm of 0.3 (OD_600 = 0.3) was added in each well. Plates were incubated under anaerobic conditions at 37ºC and the OD_600 was monitored every ten minutes during 16 hours using a PowerWave XS microplate reader (BioTek Instruments).

Prophage induction by quinolones

Lysogenic bacteria were grown on BHI soft agar plates containing either φMMP02 or φMMP04 to ensure that bacteria were still phage-immune and carrying the prophages. A single colony was picked and grown overnight in TY broth. Cultures were then washed by centrifugation in TY
broth in order to eliminate any free phage particle that could have induced spontaneously and 0.1 mL of washed bacteria was used to inoculate 10 mL of fresh TY broth. When the OD$_{600}$ reached 0.15, 0.5X MIC and lower concentrations of antibiotics were added and the OD$_{600}$ was monitored for a total of 8 hours. Mitomycin C was used as a control for prophage induction (17). Aliquots (1 mL) from each induction assay were then centrifuged at 14,000 x $g$ for 1 minute to remove bacterial cells and the supernatants were stored at 4°C. Phage titers were determined by soft agar overlays containing the sensitive host strain as described earlier. A control without antibiotic was also run in parallel to determine the level of spontaneous prophage induction. At least three independent assays were performed and the mean ±. Student’s T test and one-way ANOVA analyses were performed with Prism 5.04 (GraphPad) to determine whether antibiotics stimulated prophage induction compared to untreated controls. The level of statistical significance was set to $P < 0.05$.

**Transmission electron microscopy (TEM)**

Phage particles were washed in ammonium acetate and fixed onto 400-mesh Formvar/carbon-coated copper grids (Cederlane Laboratories) and negatively stained with 2% uranyl acetate (Cederlane Laboratories) as described before (17). Phage particles were observed with a Hitachi H-7500 transmission electron microscope operating at 60 kV and pictures were taken with a 10 megapixel digital camera (Hammamatsu) controlled with the AMT software (Advanced Microscopy Techniques).
Phage DNA purification, restriction analysis and Southern hybridization

A rapid phenol/chloroform protocol was used for small-scale phage DNA purification from crude lysates (34), and the Lambda Maxi-DNA purification kit (Qiagen) was used for large-scale preparations. Restriction enzyme analysis of whole phage DNA was done as described elsewhere (40) and southern hybridization was carried out using DIG-labeled whole phage DNA probes (17).

Phage genome sequencing and bioinformatics analysis

Complete phage genome sequencing was performed on a 454 GS-FLX sequencer system (Roche) at the Génome Québec Innovation Center of McGill University (Montréal, QC, Canada). Single contigs were obtained for both phages and additional sequencing was performed directly on purified phage DNA with specific primers on an ABI 3730xl sequencer (Applied Biosystems) at the genomic platform of the CHUL research center (Québec, QC, Canada). Additional sequence assembly was done using the Gap v4.10 application of the Staden package v1.6.0. Some editing was also done using BioEdit v7.0.5.3 and Artemis 13.0. Putative open reading frames (ORFs) ≥ 30 amino acids were predicted using GeneMark.hmm for Prokaryotes v2.8 (28). All predicted ORFs were translated into proteins using the standard ATG initiation codon or the alternative codons GTG and TTG, based on the presence of a suitable ribosome-binding site. The predicted proteins were compared with the BLASTp tools of the NCBI (4) and ACLAME (27) databases. Identification of conserved domains was performed through searches in the CDD database (NCBI) and InterProScan analyses (46).
Nucleotide sequence accession number

The complete genome sequences of phages \( \phi \text{MMP02} \) and \( \phi \text{MMP04} \) have been deposited in GenBank under the accession numbers JX145341 and JX145342, respectively.

RESULTS

Phage isolation

Our initial goal was to isolate strictly lytic ("virulent") phages in raw sewage samples and feces from CDI patients using an enrichment protocol. A total of 30 sewage samples and 59 stool samples were processed and only 6 stool samples contained free phage particles capable of infecting the \( C. \text{difficile} \) isolates we selected. Phage plaques were detected on isolates CD19, CD73, and CD117 that represent three different PCR ribotypes (Table 1). Six phages have been isolated from independent stool samples, 3 of which had identical HindIII DNA restriction profiles. Hence, they were considered as identical phages and only one of them, \( \phi \text{MMP02} \), was further studied (Fig. 1). Overall, 4 phages had unique HindIII restriction profiles and were thus considered to be different. Phage \( \phi \text{MMP01} \) was isolated on strain CD19, \( \phi \text{MMP02} \) and \( \phi \text{MMP03} \) on strain CD117 and \( \phi \text{MMP04} \) on strain CD73. Phage particles were observed in TEM and they all had an isometric head with a diameter of 58-70 nm connected by a neck to a sheathed tail of 106-248 nm long and about 20 nm wide (Table 2). Some particles with contracted sheaths were observed in the lysates (data not shown) and based on our observations,
these phages would be classified as members of the *Myoviridae* family of the order *Caudovirales* (1).

We verified whether the 15 *C. difficile* isolates that we used in the enrichment protocol contained endogenous prophages corresponding to those that we isolated from fecal samples. As shown in Fig. 2, southern hybridizations with whole phage DNA probes corresponding to φMMP01, φMMP02, φMMP03, and φMMP04 confirmed that the *C. difficile* sensitive isolates CD19, CD73 and CD117 did not carry these prophages, although CD19 carried a somewhat similar prophage but with a different restriction profile. Thus, the phages that we isolated had not been induced from *C. difficile* isolates used in the course of our enrichment and screening protocol, but were truly free phage particles present in the stool samples. We attempted to detect the phage particles by direct plating of fecal supernatants on indicator strains without prior enrichment, but the titers were below the limit of detection.

**Phage lifestyle**

In order to determine whether φMMP01, φMMP02, φMMP03, and φMMP04 were virulent or temperate, we infected the sensitive hosts CD19, CD73, and CD117 at a high MOI with the corresponding phages and screened for lysogens. Several colonies were obtained and southern hybridization assays on the extracted genomic DNA using whole phage DNA probes were performed. As shown in Fig. 2, prophages with restriction profiles corresponding to each infecting phage were found in the chromosome of the CD19, CD73 and CD117 lysogens but not in the parental uninfected isolates. A few minor differences were observed between the restriction profiles of the purified phages and the lysogens, which is the consequence of
integration of the phage DNA into the bacterial chromosome. Additional UV and mitomycin C treatments were done on the lysogens to confirm the functionality of the prophages (data not shown). Our results confirmed that φMMP01, φMMP02, φMMP03, and φMMP04 are all temperate phages.

Since free temperate phages were isolated directly from stool samples, we deduced that they had probably been released from indigenous *C. difficile* cells during infection. To demonstrate that, we isolated *C. difficile* from the phage-positive stool samples and looked for the presence of the corresponding prophage by southern blot hybridization. We could not recover *C. difficile* from the stool sample containing φMMP01 due to loss of the initial sample but as predicted, isolates CD343, CD368, and CD380 carried a prophage corresponding to φMMP02, φMMP03, and φMMP04, respectively (Fig. 2). This confirmed that prophages had been induced and released by *C. difficile* during infection. To our knowledge, this is the first report of such *in vivo* prophage induction by *C. difficile*.

**Influence of antibiotics on prophage induction**

Prophage induction from lysogens was assessed *in vitro* in the presence of three common quinolones. We focused our analysis on φMMP02 and φMMP04 because we only determined the genomic sequence of these two phages (see below). The MIC for CIP, MXF and LVX was determined on lysogenic isolates CD343 and CD380, which correspond to the naturally occurring clinical isolates purified from phage-positive stools and that carry φMMP02 and φMMP04, respectively. The MIC was also determined on CD408 and CD412 that were obtained upon lysogenization of strains CD117 and CD73 with φMMP02 and φMMP04, respectively.
(Table 1). Bacteria were grown in the presence of sub-MIC concentrations (≤0.5X-MIC) of antibiotics and phage titers were determined in culture supernatants after 8 h of growth. As shown in Fig. 3, φMMP02 and φMMP04 spontaneously induced and initiated a lytic cycle, leading to the release of ~4 to 5.5-log PFU/mL after 8 h of growth (white bars). In addition, spontaneous induction of φMMP04 from the naturally occurring CD380 lysogen led to a phage titer ~1.5-log higher than the laboratory-generated CD412 lysogen, suggesting a greater stability in the later strain. On the contrary, spontaneous induction of φMMP02 was similar in both CD343 and CD408 lysogens with titers of ~5x10⁴ PFU/mL (Fig. 3A, B).

Treatment of C. difficile lysogens carrying φMMP02 with different sub-MIC concentrations of CIP, MXF, LVX, or with MC had little effect on prophage induction (Fig. 3A,B). A slight increase in phage titer was observed at some concentrations, but these differences were not statistically significant after one-way ANOVA analysis. The only exception was observed with 16 µg/mL CIP (0.25X MIC) where a statistically significant increase in phage titer was noted (7.5x10⁵ vs 4.5x10⁴ PFU/mL, P<0.01). These results suggest that induction of the φMMP02 prophage by quinolones and MC is not very efficient, at least under the conditions tested.

On the contrary, the φMMP04 prophage was more sensitive to treatment with MXF, LVX and MC with phage titers 2 to 4-log higher than the untreated controls in both lysogens tested (Fig. 3C,D). For example, the highest phage titers obtained with the CD402 lysogen were 8.1x10⁷, 5.1x10⁶ and 3.1x10⁸ PFU/mL after treatment with MXF, LVX and MC, respectively, whereas the untreated control released 8.3x10³ PFU/mL (Fig. 3C). For the CD380 lysogen, treatment with
CIP, MXF, LVX and MC led to the release of 1.7x10^8, 7.4x10^8, 3.3x10^8, and 5.4x10^8 PFU/mL, respectively, as opposed to a phage titer of 4x10^5 PFU/mL in the untreated control (Fig. 3D). Interestingly, treatment with CIP stimulated prophage induction in the wild type CD380 lysogen (Fig. 3D) but not in the laboratory-generated CD402 lysogen (Fig. 3C). Taken together, our results suggest that stability of φMMP02 and φMMP04 prophages is similar, but φMMP04 is significantly less stable in the presence of antibiotics.

Whole genome comparison
We assessed the overall genome similarity of phage φMMP01, φMMP02, φMMP03, and φMMP04 by southern blot hybridization with whole phage probes (Fig. 4). We compared the four phages with each other, and also with other phages from our collection (17). Hybridization with a φMMP02 probe revealed significant DNA similarity with φCD52, but limited similarity with φMMP01 and φMMP03, whereas hybridization with a φMMP03 probe revealed extensive similarity with φMMP01, φCD52, φCD630-2, and φCD24-2. The 4 φMMP phages were also found to be genetically distant from φCD38-2, a Siphoviridae phage that we described previously (data not shown) (40). Phage φMMP04 seemed to be very different from the 3 other φMMP phages, suggesting that this phage is genetically unique relative to our collection of isolates. In brief, φMMP01, φMMP02 and φMMP03 were similar to other known Myoviridae phages, but φMMP04 seemed genetically unique.
Genome sequencing

The low similarity observed by southern hybridizations between φMMP02, φMMP04 and the other phages prompted us to determine their whole genome sequence. The complete genome of φMMP02 was determined after 454 sequencing and assembly of 51,685 reads with an average length of 317 bp. Sequencing of φMMP04 also resulted in a single contig of 31,662 bp assembled from 23,416 reads (average length of 318 bp). For both phages, additional PCR and direct sequencing on purified phage DNA confirmed the completeness of the genomes. Phage φMMP02 is composed of a double-stranded DNA of 48,396 bp with an average G+C content of 29.6%, while φMMP04 genome is much smaller with 31,674 bp of double-stranded DNA and a G+C content of 30.0%. To our knowledge, this is the smallest C. difficile phage genome sequence reported so far.

Genomic organization and comparative analysis

GeneMark.hmm analysis was performed on both φMMP02 and φMMP04 genomes (28). Seventy-six and 51 putative open reading frames (ORFs) of ≥ 30 amino acids were found in φMMP02 and φMMP04, respectively. Comparison against NCBI and ACLAME databases enabled us to assign a putative function to 36 of the 76 ORFs (47%) in φMMP02, and 24 of the 51 ORFs (47%) in φMMP04 (Supplementary Table S1 and Table S2). The overall genome organization of both phages appeared to be classical, with clusters of genes coding for distinct functional modules (Fig. 5). It is noteworthy to mention that virulence factors or toxin genes were not found in the two genomes using the bioinformatics approach. Protein comparison against public databases and the 5 C. difficile phage genome sequences currently available
revealed the great extent of similarity between phage φMMP02 and previously characterized Myoviridae phages. For example, the whole DNA packaging, structural, and lysis modules (ORFs 1-34) encode proteins highly similar and for the most part unique to phage φCD27 (Fig. 5, upper panel). However, some tail proteins as well as the holin and endolysin (ORFs 22-34) were also similar to phage φC2, φCD119, and prophages from C. difficile strain 630 and a putative prophage from strain ATCC 43255. In fact, besides φCD27, the prophage in strain ATCC 43255 was the most similar to φMMP02 with 30 ORFs showing >71% protein identity. Some divergence was observed in ORFs 24-28 between φMMP02 and φCD27, suggesting that these tail proteins are probably involved in host specificity.

In the case of φMMP04, little similarity was observed with previously characterized C. difficile phage genomes over the packaging, capsid and part of the tail modules (ORFs 1-13, Fig. 5, lower panel). However, similarity was observed with proteins found in Clostridium hiranonis, Clostridium cellulovorans and Clostridium botulinum. As well, a prophage with extensive similarity to φMMP04 was identified in a draft genome of a C. difficile strain (Accession number AGAB01000039). The whole packaging, head and tail structural modules as well as the lysis cassette (ORFs 1-26) were highly similar at the protein level (>81% identity). As for φMMP02, some of the tail fiber proteins (ORFs 20-22) diverged between φMMP04 and the other phages, suggesting that these proteins may also be responsible for host specificity. Some of the tail proteins (ORFs 9-26) were also similar to proteins from phages φCD119 and prophages from strain 630. We found several ORFs with significant similarity with proteins from two
Siphoviridae phages, φCD6356 and φCD38-2 (Fig. 5) and the similarity was concentrated downstream the lysis cassette (ORF 26).

Lysogeny module
Phage φMMP02 has a lysogeny module delimited by the endolysin gene on the left side (orf34) and a set of phage repressors and regulators on the right side (orfs 42-48). A similar organization was also reported in other Myoviridae phages infecting C. difficile (20, 21, 33). However, a lysogeny module could not be clearly identified in φMMP04: an integrase gene (orf47) and a putative phage repressor (orf37) were found interspersed between other DNA replication and regulation genes (Fig. 5). This type of organization was also reported in the temperate Siphoviridae phage φCD38-2 (40). In brief, the similarity observed at the protein level between φMMP04, φCD38-2, and φCD6356 in the DNA replication / gene regulation and lysogeny modules suggests that φMMP04 is somewhat related to Siphoviridae phages.

DISCUSSION
We report the isolation and characterization of four phages infecting C. difficile: φMMP01, φMMP02, φMMP03, and φMMP04. TEM observations revealed that they are morphologically similar to other Myoviridae phages recently described in C. difficile (17, 20, 21, 33, 36, 42). These phages were isolated as free viral particles in feces from patients infected by C. difficile and identical prophages were found in the chromosome of the C. difficile strains present in the corresponding fecal samples. We demonstrated that the phages were also able to lysogenize other
laboratory strains of *C. difficile*, confirming their temperate lifestyle. We therefore conclude that the φMMP phages were spontaneously induced from *C. difficile in vivo*. To our knowledge, this is the first report providing evidence of *in vivo* prophage induction during *C. difficile* infection.

The search for strictly lytic phages, *i.e.* those that can only infect and kill their host by lysis, has become very attractive over the last years because of their potential usefulness as therapeutic agents (2). Nobody has ever reported the isolation of such phages active against *C. difficile* and all phages known to infect this species are temperate (15, 19, 21, 24, 30, 33, 33, 35, 36, 40, 42). Using only 15 different *C. difficile* test strains, we were able to detect free phages in 10% of the fecal samples tested. Considering the very narrow host spectrum of *C. difficile* phages in general, we could probably detect more phages if additional test strains were used. Therefore, *in vivo* prophage induction appears to occur frequently during CDI. On the contrary, free phage particles could not be isolated in sewage samples. In fact, due to extreme oxygen sensitivity, most live cells of *C. difficile* are expected to be in their spore form outside the mammalian gut, and are thus insensitive to phage infection. Propagation of a virulent phage under these conditions should therefore be very unlikely. Conversely, bacteria are metabolically active during infection and are thus susceptible to phage attacks. In this context, a temperate lifestyle with controlled spontaneous prophage induction from a subset of the bacterial population seems to be a better strategy guaranteeing phage survival and dissemination than a strictly lytic lifestyle. Such a strategy seems to be the one adopted by phages infecting *S. pyogenes* (9, 16).
In an effort to gain insight into the genetics of *C. difficile* phages, we determined the complete genome sequence of φMMP02 and φMMP04. No virulence factors or toxin genes could be readily inferred from bioinformatics analyses, which seems to be so far a common feature of this group of phages (20, 21, 24, 33, 39, 40). Nevertheless, recent studies suggest that even in the absence of identifiable virulence factors or toxin genes, φCD119 and φCD38-2 prophages can influence toxin production in *C. difficile* (22, 40). Our comparative genome analyses also further demonstrate the mosaic nature and the great genetic diversity of this group of phages, with φMMP02 and φMMP04 forming distinct phage families based on their head and tail structural components. Moreover, the presence of an atypical regulatory / lysogeny module and similarity with phages φCD6356 and φCD38-2 also suggest that φMMP04 might have arisen from a past recombination event between a *Myoviridae* and a *Siphoviridae* phage. This is the first example of such an unusual genomic organization among *C. difficile* phages but only two *Siphoviridae* phage genomes have been sequenced so far, φCD6356 (24) and φCD38-2 (40). As we gain more sequences of *Siphoviridae* phages in the future, we might observe other examples of cross family recombination events.

Under laboratory conditions, φMMP02 and φMMP04 lysogens spontaneously released 4 to 5-log PFU/mL after 8 h of incubation, thus suggesting a certain degree of instability among *C. difficile* prophages. Similar phage titers have also been reported after growing lysogenic staphylococcal strains under normal laboratory conditions (31). Some years ago, Mahony *et al* reported the spontaneous release of two bacteriophages after *in vitro* screening of 94 *C. difficile* isolates (30). Goh *et al* also noticed that 72% of their laboratory-generated lysogens carrying either φC2, φC6...
or φC8 spontaneously lost their prophage upon storage at -70°C while no stable lysogen of φC5 could be obtained (19). The biological impact of spontaneous prophage induction on the virulence and lifestyle is well established in other bacteria such as *Staphylococcus aureus*, *S. pneumoniae*, *S. pyogenes*, or *E. coli* (6, 12, 13, 25), but the importance of this phenomenon in *C. difficile* has yet to be demonstrated.

Ciprofloxacin and beta-lactams were shown to promote transfer of pathogenicity islands (SaPIs) in *S. aureus* (31, 44) and norfloxacin was recently used to induce several prophages from *C. difficile* isolates (36, 42). We have shown that φMMP04 was particularly unstable compared to φMMP02 in the presence of ciprofloxacin, moxifloxacin and levofloxacin and phage titers several orders of magnitude above the titers of non-induced cultures were observed. Our *in vitro* results strongly suggest that antibiotics such as fluoroquinolones, known to represent risk factors for CDI (37), could also increase the frequency of prophage induction *in vivo* and, potentially, the frequency of phage-mediated gene transfer during CDI. Antibiotic pressure is an important driving force in bacterial evolution and the extensive use of antibiotics that induce CDI and that stimulate prophage mobility at the same time could provide an ideal background for rapid evolution of *C. difficile* and its associated phages. Further genetic and molecular studies will be needed to better grasp the impact of prophages on the lifestyle and evolution of *C. difficile*.

**ACKNOWLEDGEMENTS**

This work was supported by a discovery grant from the Natural Sciences and Engineering Research Council of Canada (NSERC), by a seed grant from the Canadian Institutes of Health
Research (CIHR), and by the Centre de Recherche Clinique Étienne-Le Bel. LCF is the holder of a Junior 2 research scholar from the Fonds de la Recherche du Québec - Santé (FRQ-S).

LEGENDS OF FIGURES

**Figure 1.** HindIII restriction profiles of isolated phages. Ethidium bromide-stained gel showing \(\phi\)MMP01 (lane 1), \(\phi\)MMP02 (lane 2), \(\phi\)MMP03 (lane 3), and \(\phi\)MMP04 (lane 4). Lane M: DNA Logic Ladder.

**Figure 2.** Prophage detection by southern blot hybridization with whole phage DNA probes. DIG-labeled whole phage DNA probes (\(\phi\)MMP1-4) were used to confirm the presence of corresponding prophages in wild type strains and laboratory-generated lysogens. A) Ethidium bromide-stained gel of HindIII-digested bacterial genomic DNA and purified phage DNA (\(\phi\)MMP1-4). B) Southern blot hybridization of the gel shown in A); the phage probes used is indicated below the corresponding panels. M: Dig-labeled Lambda HindIII DNA marker (NEB).

**Figure 3.** Effect of antibiotics on \(\phi\)MMP02 and \(\phi\)MMP04 induction. Prophage induction was assessed after 8h of growth in the presence of various sub-MIC concentrations of ciprofloxacin (CIP), moxifloxacin (MXF), or levofloxacin (LVX). Mitomycin C (MC) was used as positive control of induction. A) and C), induction from the laboratory-generated lysogens. B) and D), induction from the wild type lysogens. Differences in phage titers were analyzed by One-way
ANOVA followed by a Dunnett’s post test using the non-induced control (NI) as the comparator. Statistical significance: *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \).

**Figure 4.** Whole phage genome comparison using southern blot hybridization. Purified phage DNA from different temperate phages in our collection was compared using DIG-labeled whole phage genomes as probes. Phages \( \phi \)CD38-2, \( \phi \)CD24-1, \( \phi \)CD111 and \( \phi \)CD29 are *Siphoviridae* phages whereas all the others are *Myoviridae* phages. M: Dig-labeled Lambda HindIII DNA marker (NEB).

**Figure 5.** Genome organization of \( \phi \)MMP02 and \( \phi \)MMP04. Arrows indicate the predicted ORFs and their respective orientation. Putative functions inferred from bioinformatics analyses are indicated below the ORFs. Functional modules were assigned with regard to gene annotation and whole genomic organization and a color code was assigned as follows: yellow, DNA packaging; red, capsid morphogenesis; blue, tail morphogenesis; grey, lysis; orange, lysogeny; green, DNA replication, transcription and gene regulation. The dot matrices above and below the genomic maps show the degree of protein identity observed among \( \phi \)MMP02, \( \phi \)MMP04, and other known phages using BLASTp analysis.
REFERENCES


pyogenes to Tox(+) with Lysogenic Streptococci or free phage. Infect Immun 71:3782–3786.


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Table 1. *C. difficile* isolates used in this study

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>PCR Ribotype</th>
<th>Comments</th>
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<td>CD19</td>
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<td>Naturally occurring lysogens b</td>
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<td>CD343</td>
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<td>CD368</td>
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<td>Clinical isolate carrying φMMP03 b</td>
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<td>CD380</td>
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<td>Laboratory-generated lysogens c</td>
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<td>036</td>
<td>CD19 isolate lysogenized with φMMP01</td>
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<td>CD117 isolate lysogenized with φMMP02</td>
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<td>023</td>
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<tr>
<td>CD412</td>
<td>012</td>
<td>CD73 isolate lysogenized with φMMP04</td>
</tr>
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</table>

a PCR ribotype 027 represents the current BI/NAP1/027 epidemic clone and the other ribotype numbers were given arbitrarily according to our internal database.

b Naturally occurring lysogens were isolated from stool samples that contained the indicated free phages.

c Laboratory-generated lysogens were obtained upon stable infection with the indicated phage.
### Table 2. Morphological characteristics of isolated phages

<table>
<thead>
<tr>
<th>Phage</th>
<th>Family</th>
<th>Capsid diameter (nm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tail length (nm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TEM picture&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
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<td>φMMP01</td>
<td>Myoviridae</td>
<td>67 ± 3</td>
<td>133 ± 2</td>
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<td>φMMP02</td>
<td>Myoviridae</td>
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<td>248 ± 10</td>
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<tr>
<td>φMMP03</td>
<td>Myoviridae</td>
<td>70 ± 4</td>
<td>135 ± 7</td>
<td><img src="#" alt="TEM picture" /></td>
</tr>
<tr>
<td>φMMP04</td>
<td>Myoviridae</td>
<td>58 ± 1</td>
<td>106 ± 3</td>
<td><img src="#" alt="TEM picture" /></td>
</tr>
</tbody>
</table>

<sup>a</sup> Means of 5 measurements obtained with different viral particles

<sup>b</sup> The black bars represent 100 nm
EtBr-stained gel

Whole phage probe

φMMP01
φMMP02
φMMP03
φMMP04

CD19
CD407
φMMP01
CD117
CD408
CD343
φMMP02
CD117
CD411
CD368
φMMP03
CD73
CD412
CD380
φMMP04
Dig-labeled whole phage DNA probes

φMMP03  φMMP02  EtBr-stained gel

φMMP01  φMMP02  φMMP03  φMMP04  φCD52  φCD53-2  φCD35-2  φCD24-1  φCD24-2  φCD111  φCD29