Characterization of Temperate Phages Infecting *Clostridium difficile* Isolates of Human and Animal Origins

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Clostridium difficile is a Gram-positive pathogen infecting humans and animals. Recent studies suggest that animals could represent potential reservoirs of *C. difficile* that could then transfer to humans. Temperate phages contribute to the evolution of most bacteria, for example, by promoting the transduction of virulence, fitness, and antibiotic resistance genes. In *C. difficile*, little is known about their role, mainly because suitable propagating hosts and conditions are lacking. Here we report the isolation, propagation, and preliminary characterization of nine temperate phages from animal and human *C. difficile* isolates. Prophages were induced by UV light from 58 *C. difficile* isolates of animal and human origins. Using soft agar overlays with 27 different *C. difficile* test strains, we isolated and further propagated nine temperate phages: two from horse isolates (ϕ CD481-1 and ϕ CD481-2), three from dog isolates (ϕ CD505, ϕ CD506, and ϕ CD508), and four from human isolates (ϕ CD24-2, ϕ CD111, ϕ CD146, and ϕ CD526). Two phages are members of the *Siphoviridae* family (ϕ CD111 and ϕ CD146), while the others are *Myoviridae* phages. Pulsed-field gel electrophoresis and restriction enzyme analyses showed that all of the phages had unique double-stranded DNA genomes of 30 to 60 kb. Phages induced from human *C. difficile* isolates, especially the members of the *Siphoviridae* family, had a broader host range than phages from animal *C. difficile* isolates. Nevertheless, most of the phages could infect both human and animal strains. Phage transduction of antibiotic resistance was recently reported in *C. difficile*. Our findings therefore call for further investigation of the potential risk of transduction between animal and human *C. difficile* isolates.

Clostridium difficile is currently the principal cause of antibiotic-induced infectious diarrhea and is an important nosocomial pathogen (1). This Gram-positive, spore-forming, anaerobic bacterium also causes infections in a number of animal species, especially horses, piglets, calves, and dogs (2). In humans, the clinical symptoms range from mild watery diarrhea and abdominal pain to fulminant pseudomembranous colitis and are caused by two main exotoxins, TcdA and TcdB, encoded by the pathogenicity locus of toxigenic isolates. Other virulence factors, like a binary toxin (CDT) and various cell surface proteins, possibly contribute to the overall virulence of this species as well (3). The epidemiology of *C. difficile* infections (CDI) has changed significantly over the last decade, in particular after the emergence of epidemic strains such as NAP1/BI/027, simply referred to as PCR ribotype 027 (4). This particular strain caused several outbreaks in North America and Europe, and although other PCR ribotypes, such as 014, 001, and 078, have gradually replaced 027 in Europe (5), 027 is still dominant in North America (6).

C. difficile is generally considered to be a nosocomial pathogen, but a growing number of studies suggest that asymptomatic carriage and infections contracted outside hospitals, in the community, may be more frequent than previously thought (7). In addition, some PCR ribotypes that were previously associated mainly with animal infections, like 078, are now recognized as causing significant human infections (8). This led to the hypothesis that the environment, and in particular farm animals, could represent a natural reservoir for the amplification of *C. difficile* (9). A number of *C. difficile* isolates from animals have the same PCR ribotype as human isolates (e.g., ribotype 078), which suggests that these strains can pass from one host to another and vice versa (2, 10). However, current epidemiologic and genomic data are insufficient to clearly establish that transmission of CDI can be zoonotic.

Integrated bacteriophages, i.e., prophages, often drive the evolu-

tion of bacteria and represent a major source of genetic diversity (11). Unfortunately, the importance of these prophages is often neglected in epidemiological studies with *C. difficile*, mainly because of the typing methods used, which do not take into consideration mobile genetic elements (MGE). Indeed, the most common typing methods, like PCR ribotyping (12), multilocus variable-number tandem-repeat analysis (MLVA) (13), and multilocus sequence typing (14), do not take into account the gain or loss of prophages and other MGE, which can make up as much as 10% of the whole bacterial genome (15). Pulsed-field gel electrophoresis (PFGE), which is another common *C. difficile* typing method in North America (16), can, in principle, reveal such differences, but whether these differences are due to the acquisition or loss of specific DNA fragments or the result of acquisition or loss of a particular restriction site is difficult to establish.

Several putative prophages can be identified by bioinformatic analyses of *C. difficile* whole-genome sequences available in public repositories. Several prophages and phage tail-like particles have also been induced from *C. difficile* lysogens by using UV, mitomycin C, and other antibiotics, and some of these phages have been partially characterized by electron microscopy, PFGE, and restriction profiling (17–22). Most phages are members of the *Myoviridae* family, i.e., phages with nonflexible and contractile tails,

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whereas very few are members of the *Siphoviridae* family, i.e., phages with long, flexible, and noncontractile tails (23). So far, only seven temperate phages infecting *C. difficile* have been characterized in more detail, including whole-genome sequencing: ϕ CD119 (24), ϕ C2 (25), ϕ CD27 (26), ϕ MMP02, ϕ MMP04 (27), ϕ CD38-2 (28), and ϕ CD6356 (29). The latter two phages are members of the *Siphoviridae* family, whereas the others are members of the *Myoviridae* family.

The role of prophages in the evolution and virulence of most bacterial pathogens has been extensively studied (for reviews, see references 30 and 31), but in *C. difficile*, their exact contribution is still unclear. So far, bioinformatic analyses have failed to identify virulence factors in the available phage genomes, but nevertheless, two studies have reported that ϕ CD119 and ϕ CD38-2 can repress and induce toxin production, respectively (28, 32). In the case of ϕ CD119, a phage repressor was shown to bind to the promoter region within the pathogenicity locus, thereby repressing toxin production (32). However, this is only what we know about the possible role of prophages in this species. One likely reason for such limited data and the small number of completely characterized phages is the lack of suitable bacterial hosts and conditions to isolate and propagate these phages.

To gain insight into the biology and genomics of temperate phages infecting *C. difficile*, we sought to induce prophages from a number of *C. difficile* lysogens of various origins, including human and animal isolates, and to screen for suitable hosts to replicate and further characterize them. Using this strategy, we isolated and propagated to high titers nine new temperate phages. We characterized these phages by electron microscopy, PFGE, and DNA restriction profiling, and we determined their host ranges by using a panel of *C. difficile* isolates.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The human *C. difficile* isolates used in this study were kind gifts from collaborators (for details, see Table 1) or were isolated from fecal samples with the approval of the institutional review board of the Centre Hospitalier Universitaire de Sherbrooke (CHUS) (33). All animal and meat *C. difficile* isolates were obtained from Scott Weese of the University of Guelph (Guelph, Ontario, Canada). Animal isolates were from clinical cases. Two isolates were purified from effluents from water treatment plants in Sherbrooke, and one isolate was purified from our hospital sewer (27). Bacteria were routinely grown at 37°C in an anaerobic chamber (Coy Laboratories) in prereduced brain heart infusion (BHI) broth (BD Biosciences) or TY broth (3% tryptose, 2% yeast extract, pH 7.4).

Bacterial DNA extraction and PCR ribotyping. Bacteria were grown overnight in BHI broth, and genomic DNA was extracted with the bacteria genomicPrep kit (GE Healthcare Canada). Capillary-based PCR ribotyping was performed on the RNomic platform of the Laboratoire de Génomique Fonctionnelle de l'Université de Sherbrooke (<http://lgfus.ca/public/>). Briefly, amplifications were done in a final volume of 10 μ l with primers CD16S-1F and CD23S-2R (12), 1 $\times$ PCR buffer, 200 μ M deoxynucleoside triphosphates, 1.5 mM MgCl₂, 0.6 μ M each primer, 0.2 U of Platinum Taq DNA polymerase (Invitrogen), and 20 ng of purified genomic DNA. The cycling conditions were initial incubation for 2 min at 95°C, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The amplification was completed by a 2-min incubation at 72°C. The amplified products were analyzed by automated chip-based microcapillary electrophoresis on a Caliper LC-90 instrument (Caliper Life Sciences). The chromatograms generated by the Caliper software were converted into band profiles that were exported as tagged image file format images and integrated into the GelCompar II database (Applied Maths)

for cluster analysis. Reference strains of known ribotypes were included in our panel, and the corresponding ribotypes were assigned accordingly. All other isolates were assigned ribotype designations according to our internal database (designations beginning with the letter N).

Prophage induction. Prophages were induced from *C. difficile* isolates by UV irradiation (302 nm) (28). Briefly, aliquots of overnight cultures were diluted 10⁻¹, 10⁻², and 10⁻³ and further spotted onto the surface of a BHI agar plate and bacteria were allowed to grow in an anaerobic atmosphere for 4 h. Bacteria were then exposed to UV light (302 nm) for 10 s with a transilluminator and overlaid with molten top agar containing a log-phase culture of a *C. difficile* test strain (optical density at 600 nm of $\sim$ 0.3 to 0.5) (27, 34). After overnight incubation under anaerobic conditions, a clearing zone in the bacterial lawn above a given spot was indicative of phage induction and further propagation on the test strain. These zones of lysis were collected with a pipette tip and transferred into BHI broth to allow phages to diffuse out of the agar plug. Aliquots were then diluted and plated again on the same sensitive host to isolate single phage plaques, and two additional rounds of soft agar overlaying were performed to purify the phages. After the last round, the purified phages were amplified in BHI broth supplemented with 10 mM MgCl₂, 10 mM CaCl₂, and the sensitive host to allow high titers to be reached ($\sim$ 1 $\times$ 10⁹ PFU/ml). The lysates were then passed through a 0.45- μ m filter and stored at 4°C until further DNA purification and analysis (28).

Transmission electron microscopy (TEM). Phage particles were washed in ammonium acetate buffer as described before (17) and transferred onto 400-mesh Formvar/carbon-coated copper grids (Cedarlane Laboratories). After negative staining with 2% uranyl acetate (Cedarlane Laboratories), phage particles were observed on a Hitachi H-7500 transmission electron microscope operating at 60 kV and pictures were taken with a 10-megapixel digital camera (Hamamatsu) controlled with the AMT software (Advanced Microscopy Techniques). The average sizes of viral capsids and tails were determined from five different images of isolated phage particles.

Phage DNA purification, PFGE, and restriction analysis. Phage DNA purification was done by a rapid phenol-chloroform protocol starting with 5 ml of crude lysate (35). For PFGE analysis, undigested DNA was heated at 75°C for 10 min before being loaded onto a 1% SeaKem Gold agarose gel and run in 0.5 $\times$ TBE buffer (Tris-borate-EDTA, pH 8.0) in a CHEF-DR-II apparatus (Bio-Rad Laboratories). The migration conditions were 15 h at 14°C and 6 V/cm with a pulse ramp of 5 to 13 s. The gel was stained with ethidium bromide, and DNA bands were visualized under UV light and photographed with the ImageQuant IQ300 gel documentation system (GE Healthcare). Restriction enzyme analysis of whole phage DNA was done with HindIII (High Fidelity; New England BioLabs) as described before (28).

Host range analysis. Five-microliter volumes of undiluted and 10⁻²-diluted phage lysates (10⁷ to 10⁹ PFU/ml) were deposited on top of soft agar overlays containing different log-phase cultures of *C. difficile* test strains. Additional phages previously isolated by our group, i.e., ϕ CD38-2 (28), ϕ CD52 (17), ϕ MMP01, ϕ MMP02, ϕ MMP03, and ϕ MMP04 (27), were also included to complete the list. A total of 47 test strains of various origins (humans, animals, the environment) and PCR ribotypes were tested (see Table 1). The intensities of the clearing zones were recorded, and only isolates that were sensitive to at least one phage are reported (see Table 2).

RESULTS

Prophage induction and phage isolation. In order to gain insight into the diversity of temperate phages of *C. difficile* infecting animals, we analyzed a set of *C. difficile* isolates from different animal species. We also included *C. difficile* from meat products, as well as a number of human isolates (Table 1). Using a combination of UV induction and soft agar overlays, we performed both prophage induction and host screening simultaneously. We induced 58 isolates, of which 9 were from horses, 10 were from calves, 9 were

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

Strain ^a	Origin	Ribotype	Source	Strain ^a	Origin	Ribotype	Source
CD3	Human	014	J. Pépin	CD482	Horse	N003	S. Weese
CD19	Human	N007	J. Pépin	CD483	Horse	015	S. Weese
CD24	Human	078	J. Pépin	CD484	Calf	002	S. Weese
CD62	Human	001	J. Pépin	CD485	Calf	N003	S. Weese
CD71	Human	N010	J. Pépin	CD486	Calf	017	S. Weese
CD73	Human	N002	J. Pépin	CD487	Calf	017	S. Weese
CD77	Human	N001	J. Pépin	CD488	Calf	078	S. Weese
CD93	Human	012	J. Pépin	CD489	Calf	N003	S. Weese
CD105	Human	N003	L. Valiquette	CD490	Calf	002	S. Weese
CD107	Human	078	L. Valiquette	CD491	Calf	N003	S. Weese
CD111	Human	027	L. Valiquette	CD492	Calf	NA ^d	S. Weese
CD112	Human	N002	L. Valiquette	CD493	Calf	078	S. Weese
CD117	Human	014	L. Valiquette	CD494	Pig	078	S. Weese
CD118	Human	N006	L. Valiquette	CD495	Pig	078	S. Weese
CD124	Human	N011	L. Valiquette	CD496	Pig	078	S. Weese
CD125	Human	N010	L. Valiquette	CD498	Pig	037	S. Weese
CD132	Human	N002	L. Valiquette	CD499	Pig	078	S. Weese
CD137	Human	N005	L. Valiquette	CD500	Pig	NA	S. Weese
CD146	Human	027	L. Valiquette	CD501	Pig	NA	S. Weese
CD186	Human	N003	L. Valiquette	CD502	Pig	078	S. Weese
CD192	Human	N004	L. Valiquette	CD503	Pig	NA	S. Weese
CD211 (ATCC 9689)	Human	001	ATCC	CD504	Dog	015	S. Weese
CD272 (CD630)	Human	012	J. Parkhill	CD505	Dog	014	S. Weese
CD273 (VPI 10463)	Human	037	ATCC	CD506	Dog	N050	S. Weese
CD274	Human	027	L. Valiquette	CD507	Dog	N003	S. Weese
CD316	Water ^b	N011		CD508	Dog	014	S. Weese
CD326	Water ^b	078		CD510	Dog	NA	S. Weese
CD337	Sewage ^c	N002		CD511	Dog	N054	S. Weese
CD383	Human	027	L. Valiquette	CD513	Dog	NA	S. Weese
CD384	Human	N012	L. Valiquette	CD514	Meat	NA	S. Weese
CD386	Human	027	L. Valiquette	CD515	Meat	N003	S. Weese
CD390	Human	027	L. Valiquette	CD516	Meat	NA	S. Weese
CD398	Human	N012	L. Valiquette	CD518	Meat	017	S. Weese
CD419 (R20291)	Human	027	T. Lawley	CD519	Meat	015	S. Weese
CD420 (CD196)	Human	027	T. Lawley	CD520	Meat	002	S. Weese
CD422 (CF5)	Human	017	T. Lawley	CD521	Meat	027	S. Weese
CD424 (M120)	Human	078	T. Lawley	CD522	Meat	NA	S. Weese
CD425 (BI9)	Human	001	T. Lawley	CD523	Meat	015	S. Weese
CD426 (Liv022)	Human	106	T. Lawley	CD524	Human	001	S. Weese
CD427 (Liv024)	Human	001	T. Lawley	CD525	Human	017	S. Weese
CD428 (TL174)	Human	015	T. Lawley	CD526	Human	014	S. Weese
CD429 (TL176)	Human	014	T. Lawley	CD527	Human	N054	S. Weese
CD430 (TL178)	Human	002	T. Lawley	CD528	Human	N002	S. Weese
CD431 (CD305)	Human	023	T. Lawley	CD529	Human	001	S. Weese
CD474	Horse	N052	S. Weese	CD530	Human	027	S. Weese
CD475	Horse	N002	S. Weese	CD531	Human	027	S. Weese
CD476	Horse	N002	S. Weese	CD532	Human	002	S. Weese
CD478	Horse	014	S. Weese	CD533	Human	027	S. Weese
CD479	Horse	015	S. Weese	CD540	Human	N051	C. Bergeron
CD480	Horse	027	S. Weese	CD544	Human	N053	C. Bergeron
CD481	Horse	014	S. Weese				

^a Alternative names are in parentheses. Shaded isolates were used as potential hosts in the first screening, and those in boldface were induced with UV.^b Isolated from water effluent samples from a water treatment plant in Sherbrooke, Québec, Canada.^c Sewage from CHUS.^d NA, not available.

from pigs, 8 were from dogs, 9 were from meat, and 13 were from humans (Table 1). After testing 27 different *C. difficile* isolates as potential hosts in soft agar, we ended up with nine new phages that could be further propagated well in broth culture, five of which were of animal origin (Table 2). Horse isolate CD481 released two

different phages named ϕ CD481-1 and ϕ CD481-2. Three dog isolates released phages ϕ CD505, ϕ CD506, and ϕ CD508, whereas four human isolates released phages ϕ CD526, ϕ CD24-2, ϕ CD111, and ϕ CD146. Note that by using mitomycin C, we had previously induced the *Siphoviridae* phage ϕ CD24-1 from isolate

TABLE 2 Characteristics of isolated phages and their hosts

Natural host	Origin	Ribotype ^a	Phage	Family	Propagating host ^b	Origin	Ribotype ^a	Reference
CD481	Horse	014	φCD481-1	Myoviridae	CD515	Meat	N003	This study
CD481	Horse	014	φCD481-2	Myoviridae	CD515	Meat	N003	This study
CD505	Dog	014	φCD505	Myoviridae	CD117	Human	014	This study
CD506	Dog	N050	φCD506	Myoviridae	CD493	Calf	078	This study
CD508	Dog	014	φCD508	Myoviridae	CD117	Human	014	This study
CD526	Human	014	φCD526	Myoviridae	CD117	Human	014	This study
CD24	Human	078	φCD24-2	Myoviridae	CD19	Human	N007	This study
CD111	Human	027	φCD111	Siphoviridae	CD274	Human	027	This study
CD146	Human	027	φCD146	Siphoviridae	CD274	Human	027	This study
CD38	Human	N010	φCD38-2	Siphoviridae	CD274	Human	027	28
CD52	Human	N055	φCD52	Myoviridae	CD24	Human	078	17
NA ^c	Human	NA	φMMP01	Myoviridae	CD19	Human	N007	27
CD343	Human	014	φMMP02	Myoviridae	CD117	Human	014	27
CD368	Human	014	φMMP03	Myoviridae	CD117	Human	014	27
CD380	Human	002	φMMP04	Myoviridae	CD73	Human	N002	27

^a Ribotype designations starting with the letter N were assigned arbitrarily on the basis of our internal database; the other ribotypes correspond to reference strains.

^b Propagating hosts are *C. difficile* isolates routinely used to propagate the corresponding phages.

^c NA, not available.

CD24, which is different from phage φCD24-2 isolated here (17). This suggests that using different inducing conditions with the same isolate can release different phages. Of note, five of the nine newly isolated phages originated from ribotype 014 isolates of both human and animal origins. If we include the other phages that we previously described (27), a total of seven phages originated from this ribotype. Likewise, five of the sensitive hosts routinely used for propagation of these phages are also of ribotype 014. This suggests that prophage diversity within ribotype 014 is important, which might result from a natural susceptibility of this particular ribotype to multiple infecting phages.

Phage morphology. TEM revealed that newly isolated phages φCD111 and φCD146 had long and flexible tails reminiscent of

members of the *Siphoviridae* family within the order *Caudovirales* (23). All of the other phages had long, straight, and thick striated tails, suggesting contractile tails. Therefore, these phages would be members of the *Myoviridae* family (Fig. 1). We determined the sizes of the phage particles, and the capsid diameters ranged from 47.3 ± 1.8 nm for φCD481-1 to 64.3 ± 1.9 nm for φCD505. Tail lengths ranged from 92.3 ± 2.5 nm for φCD481-1 to 283.5 ± 3.9 nm for φCD111. These values fall within the range of other phages described previously (17, 21, 24, 26–29).

Genome size and restriction profiling. The total genomic DNA was extracted from the isolated phages and separated by PFGE to get estimates of the genome sizes. All of the phages had double-stranded DNA genomes ranging in size between ~30 and

FIG 1 Phage morphology as observed by TEM after negative staining with uranyl acetate. The sizes of the capsids and tails were measured on five different particles, and the average values ± the standard deviations are reported below each phage. Note that all of the phages are not displayed on the same scale. The black bar represents 100 nm.

FIG 2 PFGE analysis of the genomic DNA of isolated phages. Undigested purified phage DNA was separated by PFGE and stained with ethidium bromide. The sizes of the mass marker fragments are indicated on the left.

~60 kb, which is in the range of most phages previously described in *C. difficile*. Phages ϕCD506 and ϕCD481-1 had the smallest genomes at ~30 kb, whereas ϕCD24-2 and ϕCD526 had the largest genomes at ~55 to 60 kb (Fig. 2). The HindIII restriction profiles of the different phages were compared, and as shown in Fig. 3, all of the phages had distinct profiles, confirming that they are all different.

Host range analysis. We selected a panel of 47 isolates from various sources: 9 from animals, 1 from meat, and 3 from the environment. The other 34 isolates were of human origin. The selected panel comprised major PCR ribotypes like 001, 014, 027, and 078, as well as other ribotypes (Table 1). The isolates were used as potential sensitive hosts in spot tests with phage lysates (10^7 to 10^9 PFU/ml), and the results are reported in Table 3. The *C. difficile* isolates that were not susceptible to any of the phages tested included CD124, CD137, CD146, CD272 (CD630), CD422, CD424, CD431, CD474, CD494, and CD506. Phages of animal and human origins were capable of infecting both human and animal isolates. However, the most striking observation was that phages originating from human isolates generally had the broadest host spectrum. For example, ϕCD38-2, ϕCD146, ϕCD24-2,

TABLE 3 Host range analysis of isolated phages^a

Strain	Origin	Animal phages					Human phages									
		ϕCD481-1	ϕCD481-2	ϕCD505	ϕCD506	ϕCD508	ϕCD526	ϕMMP01	ϕMMP02	ϕMMP03	ϕMMP04	ϕCD24-2	ϕCD38-2	ϕCD52	ϕCD111	ϕCD146
CD19	Human			+			+++				+	+++				+
CD24	Human										+			++		
CD73	Human		+								+++	+	+	+		
CD77	Human												+		+	+++
CD93	Human							+			+	+				
CD105	Human										+		+			+
CD111	Human												+			+
CD117	Human	+		++		+++	++	+	+++	+		+				
CD118	Human	+	+													
CD125	Human							+				+				+
CD192	Human												+		++	+++
CD211	Human							++			+				+	++
CD273	Human							+				+			+	
CD274	Human												+++		+	+++
CD316	Human						+				++	+				+++
CD326	Human			+				++			+++					
CD337	Human	+++	+								+		+			+
CD383	Human												++			+++
CD384	Human							+++			+++	+	+++		+	+++
CD398	Human											+	+			
CD419	Human	+											+			+++
CD420	Human												+		+	+++
CD425	Human							+				+	+		+	
CD426	Human				+			+	+			+			+	
CD427	Human							+			+	+	+	+	+	+
CD428	Human									+						
CD429	Human							+	+		+					
CD430	Human				+						+		+			+
CD540	Human												+		+	+
CD544	Human							+				+				
CD475	Horse												+++		+	+
CD481	Horse			++					++				+++			
CD490	Calf					+					+		+		+	+
CD493	Calf				+++							+				
CD505	Dog				+	+			+		+		+			
CD511	Dog			+				+	+			+	+			
CD515	Meat	++	+						+		+		+		+	+
Nb sensitive isolates		5	4	5	1	4	4	14	2	2	12	15	24	4	14	18
Phage family		M	M	M	M	M	M	M	M	M	M	M	S	M	S	S

^a Isolate sensitivity (indicated as follows: +, sensitive; ++, moderately sensitive; +++, very sensitive) is based on the intensity of the clearing zones. The total number of sensitive isolates is indicated at the bottom. Phage families: M, *Myoviridae*; S, *Siphoviridae*.

and ϕCD111 infected, respectively, 24 (51%), 18 (38%), 15 (32%), and 14 (30%) different isolates out of the 47 tested. This is in contrast to animal phages, which generally infected fewer strains, on average, between one (2%; ϕCD506) and five (11%; ϕCD481-1) isolates. It is also worth noting that *Siphoviridae* phages ϕCD38-2, ϕCD111, and ϕCD146 were among those with the broadest host spectra, including epidemic isolates of ribotype 027 (Table 3).

DISCUSSION

In this work, we describe the isolation of nine temperate phages infecting the enteric pathogen *C. difficile*. We were able to further propagate them to high titers and characterize them by TEM, PFGE, restriction profiling, and host range analysis. Although several prophages have been induced from clinical and environmental *C. difficile* isolates (17–21), there is a dearth of genomic and functional data on this group of phages and one main reason for that is the lack of suitable propagating hosts and conditions, which are essential for further characterization. It is also worth mentioning that, to our knowledge, strictly lytic (i.e., virulent) phages in-

FIG 3 Restriction enzyme analysis of isolated phages. Purified phage DNA was digested with HindIII, and fragments were separated on a 0.8% agarose gel and then stained with ethidium bromide (the inverted image is presented). The sizes of selected bands from the mass marker are indicated on the left.

fecting *C. difficile* have not been described yet. Therefore, these new phages provide a good start for further study of their functional role in *C. difficile*. Five of these new phages were isolated from *C. difficile* that infected animals, specifically, horses (ϕ CD481-1 and ϕ CD481-2) and dogs (ϕ CD505 ϕ CD506, and ϕ CD508). Morphological analysis revealed that the five phages from animal *C. difficile* isolates, in addition to two other phages induced from human isolates (ϕ CD24-2 and ϕ CD526), were members of the *Myoviridae* family, whereas two other phages from human isolates (ϕ CD111 and ϕ CD146) were members of the *Siphoviridae* family. Most of the phages characterized so far, like ϕ CD119 (24), ϕ C2 (25), ϕ CD27 (26), ϕ MMP02, and ϕ MMP04 (27), are members of the *Myoviridae* family, and only two *Siphoviridae* phages have been fully characterized and sequenced, namely, ϕ CD38-2 (28) and ϕ CD6356 (29). Of note, ϕ CD38-2 was shown to stimulate toxin production in *C. difficile* (28) and it will be interesting to determine if these new *Siphoviridae* phages can have similar impacts, which could give us clues about a possible mechanism. The phages described herein have double-stranded DNA genomes of a size similar to that of other phages described before in *C. difficile*.

The epidemiology of *C. difficile* has changed rapidly over the last decade (36), and the exact reasons for such a rapid evolution are still not fully understood. Recent studies have estimated the mutation rate of *C. difficile* to be about a single nucleotide variation per genome per year (37), which could seem marginal on a 10- to 20-year time scale. However, this is when only the core genome is considered. In fact, other factors could have contributed to the rapid evolution of *C. difficile*, like MGE. Indeed, whole-genome sequencing and DNA microarray analyses revealed that prophages and MGE contribute to the genetic variability and genome evolution of *C. difficile* (15, 38, 39). For example, the complete genome sequence of historical strain CD196 isolated in 1985 was compared with that of a recent epidemic strain (R20291) isolated in England in 2006. Interestingly, although both strains are classified as PCR ribotype 027, five new genetic regions containing 234 genes were identified in the recent R20291 clone. Among others, phage-related genes and transposons were identified in these new regions, suggesting that horizontal gene transfer contributed to the evolution of strain R20291 (39).

It is worth mentioning that seven of the phages used in our study were induced from ribotype 014 lysogens and that this particular ribotype was also sensitive to several phages as well. The fact that we isolated and propagated several different phages on this particular ribotype could have important implications. Indeed, along with ribotypes 027 and 078, 014 is one of the most prevalent ribotypes in the world (it was reported in at least 19 countries), with a prevalence ranging between 16 and 34% in North America and Europe (40–43). This ribotype seems to be prevalent in cats and dogs as well (44). Ribotype 014 isolates are generally associated with nonsevere CDI cases in humans (40), they are TcdA⁺ TcdB⁺ CDT[−] (45), and resistance to clindamycin is highly common, as well as resistance to ceftriaxone and tetracycline to a lesser extent (46). The genome sequences of two ribotype 014 *C. difficile* strains (E14 and T6) have been recently released (47). Although only contig scaffolds are available at the moment, a quick search for prophage genes suggested the presence of at least one possibly complete prophage in each strain. The high degree of sequence similarity and synteny also suggested that the two prophages are highly related. Therefore, ribotype 014 offers a good

background for *C. difficile* phages to propagate and disseminate, thereby increasing the likelihood of lysogenic conversion, phage-mediated horizontal gene transfer, and phage-phage recombination events. Prophage acquisition has long been known to contribute to the emergence of new epidemic and highly virulent bacterial lineages, such as the *Escherichia coli* O157:H7 strain that acquired two different prophages carrying the Shiga toxins (30). In 2011, a new *E. coli* O104:H4 strain caused an outbreak in Germany and genome sequencing revealed the acquisition of a Shiga toxin-encoding prophage and a plasmid carrying diverse virulence genes (48). Current data on *C. difficile* suggest that prophages are very diverse (17, 19, 20) and that some of them contribute in different ways to the fitness and virulence of this species, for example, by modulating toxin production (28, 32, 49). This underlines the importance of considering prophages and other MGE in the study of *C. difficile* evolution. Unfortunately, PCR ribotyping, one of the methods most frequently used to study the epidemiology of *C. difficile*, does not account for their presence.

Like humans, different animals can also be colonized and infected by *C. difficile* and epidemiological studies based on PCR ribotyping suggest that strains of the same type can infect both humans and animals. For example, ribotype 078 was historically associated mainly with animals, but it was found to be increasingly associated with human infections in recent years (8, 38, 50–52). This led to the hypothesis that animals could represent a potential reservoir for the amplification of *C. difficile* that can then pass on to humans and cause disease. Along the same line, spores of *C. difficile* have been isolated in ground meat (53, 54), which further supports the general idea that livestock could represent a potential source of *C. difficile* contamination. However, drawing conclusions about the similarity of animal and human isolates on the basis of PCR ribotyping alone is risky. A case in point is a study by Zidaric et al. that demonstrated that multiple strains of the same PCR ribotype and isolated at the same cattle farm could be differentiated by PFGE, sporulation characteristics, antibiotic susceptibility, and tetracycline resistance determinants (45). Likewise, in the present study, we isolated different prophages from different isolates of the same PCR ribotype, 014. A similar situation was also reported in ribotype 027 isolates (18). Therefore, care should be taken when using PCR ribotyping alone in epidemiological studies, and when possible, another more discriminatory technique, like MLVA or PFGE, should be used in parallel to differentiate subtypes. Phage typing with phage-specific PCR primers or host range analysis or detection of conjugative transposons by PCR could also complete the epidemiological portrait. Construction of a comprehensive list of the various MGE in *C. difficile* would therefore be highly useful. Yet, whole-genome sequencing seems to be the most powerful approach because it allows the detection of all MGE and genomic variations. However, although the cost of whole-genome sequencing has significantly decreased in recent years, this approach is still not accessible to all laboratories.

Our *C. difficile* phage host range analysis revealed some interesting data. For example, the three *Siphoviridae* phages ϕ CD38-2, ϕ CD111, and ϕ CD146 were the ones with the lowest specificity; i.e., they infected the largest set of isolates. Moreover, they infected major epidemic isolates of PCR ribotypes 001, 014, 027, and 106. Also of particular interest, ϕ CD111 and ϕ CD146 were both isolated from lysogenic 027 clinical isolates and yet they could infect other 027 isolates as well. Together, this suggests that 027 isolates carry different and unrelated functional prophages, as reported

before (18), and that they have the potential to reinfect multiple and clinically relevant strains of *C. difficile*. Furthermore, 027 isolates seem to have different phage susceptibility profiles, which could reflect the expression of different phage receptors at the bacterial surface, the presence of different prophages that provide phage-specific immunity, the presence of different CRISPR spacers, or any other antiphage mechanism(s) (55). Also of note, the host ranges of ϕ CD38-2 and ϕ CD146 seemed to be more closely related than that of ϕ CD111. Further genome sequencing of ϕ CD111 and ϕ CD146 and comparison with the sequence of ϕ CD38-2 will eventually provide clues about the phage genes responsible for host specificity.

Phages from animal strains infected fewer isolates from our panel than did phages from human isolates, such as ϕ CD38-2, ϕ CD146, and ϕ CD24-2. This observation could possibly be biased by the fact that we included fewer animal isolates in our host range experiment (9 animal isolates versus 34 human isolates). Therefore, we cannot exclude the possibility that some phage specificity for animal or human isolates exists. Nevertheless, phages from animal strains were able to infect both human and animal isolates. The possibility of *C. difficile* transmission from animals to humans was proposed (56). The apparent lack of phage specificity for animal or human isolates, at least in the phages described in this work, suggests that prophages released from *C. difficile* lysogens of animal origin could, in principle, reinfect human isolates as well. The massive use of antibiotics as growth promoters in livestock creates a huge selective pressure and a perfect environment for the development and spread of antibiotic resistance (57). Our observations could therefore have important implications, since Goh et al. recently reported that phage ϕ C2 could promote the transduction of Tn6215-encoded erythromycin resistance between *C. difficile* isolates (58). Modi et al. also reported that antibiotic exposure of mice promoted the enrichment and further transduction of phage-encoded antibiotic resistance among gut bacteria (59). Although we did not try to generate lysogens with all of the newly isolated phages, we created such lysogens by introducing ϕ CD111 and ϕ CD146 into CD419 (strain R20291 of ribotype 027), and ϕ CD481-2 was introduced into CD515 (a ribotype N003 strain isolated from meat). It will be important in future studies to better assess the contribution of temperate phages to transduction and the spread of antibiotic resistance in *C. difficile*. Our new panel of temperate phages will provide new opportunities to address this question and to better understand their role in the evolution and virulence of this important pathogen.

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