Abundant and Diverse Clustered Regularly Interspaced Short Palindromic Repeat Spacers in *Clostridium difficile* Strains and Prophages Target Multiple Phage Types within This Pathogen

Katherine R. Hargreaves, a Cesar O. Flores, b Trevor D. Lawley, c Martha R. J. Clokie a

Department of Infection, Inflammation and Immunity, University of Leicester, Leicester, United Kingdom; School of Physics, Georgia Institute of Technology, Atlanta, Georgia, USA; Microbial Pathogenesis Laboratory, Wellcome Trust Sanger Institute, Hinxton, United Kingdom.

ABSTRACT *Clostridium difficile* is an important human-pathogenic bacterium causing antibiotic-associated nosocomial infections worldwide. Mobile genetic elements and bacteriophages have helped shape *C. difficile* genome evolution. In many bacteria, phage infection may be controlled by a form of bacterial immunity called the clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) system. This uses acquired short nucleotide sequences (spacers) to target homologous sequences in phage genomes. *C. difficile* carries multiple CRISPR arrays, and in this paper we examine the relationships between the host- and phage-carried elements of the system. We detected multiple matches between spacers and regions in 31 *C. difficile* phage and prophage genomes. A subset of the spacers was located in prophage-carried CRISPR arrays. The CRISPR spacer profiles generated suggest that related phages would have similar host ranges. Furthermore, we show that *C. difficile* strains of the same ribotype could either have similar or divergent CRISPR contents. Both synonymous and nonsynonymous mutations in the protospacer sequences were identified, as well as differences in the protospacer adjacent motif (PAM), which could explain how phages escape this system. This paper illustrates how the distribution and diversity of CRISPR spacers in *C. difficile*, and its prophages, could modulate phage predation for this pathogen and impact upon its evolution and pathogenicity.

IMPORTANCE *Clostridium difficile* is a significant bacterial human pathogen which undergoes continual genome evolution, resulting in the emergence of new virulent strains. Phages are major facilitators of genome evolution in other bacterial species, and we use sequence analysis-based approaches in order to examine whether the CRISPR/Cas system could control these interactions across divergent *C. difficile* strains. The presence of spacer sequences in prophages that are homologous to phage genomes raises an extra level of complexity in this predator-prey microbial system. Our results demonstrate that the impact of phage infection in this system is widespread and that the CRISPR/Cas system is likely to be an important aspect of the evolutionary dynamics in *C. difficile*.

Received 3 December 2013 Accepted 1 July 2014 Published 26 August 2014

Citation Hargreaves KR, Flores CO, Lawley TD, Clokie MRJ. 2014. Abundant and diverse clustered regularly interspaced short palindromic repeat spacers in *Clostridium difficile* strains and prophages target multiple phage types within this pathogen. mBio 5(5):e01045-13. doi:10.1128/mBio.01045-13.

Invited Editor John Paul, University of South Florida

Editor Farooq Azam, Scripps Institution of Oceanography, UCSD

Copyright © 2014 Hargreaves et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 3.0 Unported license.

Address correspondence to Martha R. J. Clokie, mrjc1@le.ac.uk.

The bacterium *Clostridium difficile* is a major nosocomial pathogen (1), which can also be carried asymptomatically (2), is present in environmental and zoonotic reservoirs (3), and can transmit between livestock and humans (4). The evolution of *C. difficile* is shaped by the acquisition and loss of mobile elements in its genome (5). A critical component of the mobilome is bacteriophages, which can mediate horizontal gene transfer (HGT) and impact upon the evolution of their hosts (6). Prophages are common within *C. difficile* genomes, and several temperate phages have been described which can also infect specific strains by following a lytic cycle (7). Either temperate or lytic phage infection could promote the HGT of novel genetic material, as exemplified by a recent demonstration of phage-mediated transduction in *C. difficile* (8). Typically, the reported lytic host ranges of *C. difficile* phages are narrow, and phages can differentially infect strains of the same ribotype (see the review by Hargreaves and Clokie [9]). The contribution that phages make to *C. difficile* evolution will clearly depend on the breadth of the hosts that they are able to infect.

The specificity of phage-host interactions is dependent on mechanisms used by bacteria to resist phage infection. One is the clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) system, which consists of arrays of short (24- to 47-bp) direct repeats (DRs) separated by variable spacer regions (26 to 72 bp) and Cas proteins. These recognize and degrade foreign DNA that is homologous to a spacer (10–15). Spacers are heritable, and can also be acquired through the incorporation of foreign DNA sequences. This means the CRISPR/Cas system is considered to be a form of adaptive immunity, and the spacer content of CRISPR arrays is a record of past infections (13). Examining spacers can provide insights into phage-host dynamics that have occurred within bacterial populations (12, 16, 17). One “cost” of an effective CRISPR/Cas system to a bacteria is that HGT is suppressed, which in turn reduces the
gain of new, potentially useful genes introduced by phages or plasmids (18). The impact of the CRISPR/Cas system in limiting interactions with phages and plasmids differs by species and has been previously described (19–21); however, relatively few species have been studied in detail, and very little has been published for *C. difficile*.

All *C. difficile* phages sequenced to date carry integrases, which suggests that they can access the temperate life cycle and are therefore not truly lytic (22–27). Although prophage infection is prevalent in *C. difficile* (28), relatively few or inductive prophages have been found that can propagate in a lytic manner on tested strains, despite large-scale screens for such phages (24, 27, 29). The mechanisms controlling these phage-host dynamics in *C. difficile* strains, despite large-scale screens for such phages (24, 27, 29).

Several studies have produced sequence data for *C. difficile* phages and prophages within *C. difficile* genomes which were included in our analysis. In order to determine the genomic diversity across *C. difficile* phages, we compared the genomes of 12 phages and 19 prophages (see Table S1 in the supplemental material). Genomes were aligned in MAUVE, which identifies locally colinear blocks (LCBs), which are shared genomic regions free from homologous recombination (Fig. 1). The presence or absence of LCBs can be used to determine patterns of evolutionary relatedness, and several conserved orthologous LCBs were identified in multiple genomes. The phages can be separated into distinct lineages and have been arranged according to genomic similarity, genome size, and particle morphology, if known (37, 38). The majority of prophages examined in this study are similar to φC2, and representatives are present in all 15 of the *C. difficile* strains examined. Four of these strains carry two predicted prophages. Strain CD630 (R012) carries two φC2-like prophages, and strain B19 (R001) carries a φC2-like prophage and one phage that appears similar to the small myovirus type. Lastly, strain M68 (R017) and strain CF5 (R017) carry φC2-like prophages that are closely related to one another. There are several instances where LCBs are shared across divergent lineages, and two were identified in all the examined sequences.

**Prophages are a source of diverse CRISPR spacers in the *C. difficile* cell.** Because the identification of CRISPR arrays on prophages in *C. difficile* is unusual, we tested how widespread and diverse they are in the 19 prophage and 12 phage genomes using CRISPRfinder (32). All of the φC2-like prophages carry multiple predicted CRISPR arrays (n = 2 to 4), with the number of spacers varying between 7 and 24 (Fig. 2). The arrays are located in the structural region of the phage genomes between the xldN gene (whose product is a predicted structural protein) and the tape measure protein (TMP) gene. In one, ppB19_1, an additional single predicted array is located in the DNA replication region. Interestingly, no CRISPR arrays were identified in the φC2-like phage genomes which had been isolated following lytic propagation. However, we detected a PCR product of the expected size of the CRISPR arrays using primers to specifically amplify the CRISPR arrays from released free phage particles (see Fig. S1 in the supplemental material). This was established by performing transmission electron microscopy (TEM) to visualize phage particles and PCR on DNase-treated phage lysates which were negative for a bacterial 16S rRNA PCR product.

Six distinct prophage-carried CRISPR arrays can be resolved based on their consensus DR sequence, spacer content, and adjacent coding DNA sequences (CDs). The strain CD630 prophages have CRISPR regions that are identical to one another, as do the strain R027 prophages; they are represented in Fig. 2 by ppCD630_1 and ppCD196, respectively. Across the prophages, there is at least one array with a conserved DR sequence, and all the DRs belong to the same family (Fig. 2), which is also present in *C. difficile* chromosomal arrays. Most spacers are unique, but eight
are present in more than one array type and in different locations within the arrays which carry them. No cas genes were identified in the prophage genomes, and it is likely that they are processed by the bacterial Cas proteins. There are several CDSs adjacent to the arrays. They encode predicted proteins with putative regulatory or DNA binding roles, for example, ORF6N (39), Bro-N (40), and ribosomal_L12 (41). These proteins may be involved in transcription of the prophage-carried CRISPR arrays.

C. difficile CRISPR spacer homology supports various host-phage interactions. To determine if the CRISPR/Cas system of C. difficile could target known phages, the 31 genomes were searched against the CRISPRdb database, containing spacers (33). In total, 758 matches between spacers and phage sequences were identified, of which 162 were identical (Fig. 3). This large number is despite there being only nine C. difficile strains in the CRISPRdb database (as of September 2013), four of which are the same ribotype and therefore likely to represent a small proportion of the total C. difficile CRISPR spacer diversity. The spacers we identified represent the minority of total spacers, with 17 to 38% in each strain. All phages and prophages have a spacer which matches to them in at least one C. difficile strain, and similarly every strain has at least one spacer with an identical match to a phage sequence. The number of spacers with matches for each phage or prophage ranges from 12 to 53 (with between 1 and 16 having identical matches) and in each strain from 38 to 300 (with identical matches ranging between 4 and 55 for each). The CRISPR profiles generated are the same for the R027 strains, with the exception of strain B11. This strain has additional spacers in CRISPR arrays on a large extrachromosomal piece of DNA ~300 kbp in size (GenBank accession no. NC_017177), and the seven spacers account for a further 30 matches (three identical) in this data set.

Multiple strains carry spacers which match to the same phage, e.g., 8/9 strains have spacers that identically match to sequences in the genomes of \(\phi H9278\) CD27. In contrast, spacers of one strain match to \(\phi H9278\) C2, which suggests less widespread predicted immunity to this phage. From a bacterial perspective, individual strains have multiple spacers which match to several phages (e.g., those in strain M120 match to all but two phages), whereas other strains have fewer spacers with matches (e.g., those in strain M68 match to only four phages). If the CRISPR/Cas system can impart immunity, our data suggest that this mechanism would result in some phages being able to infect in a generalist manner, but others would be more specialized.

Despite the occurrence of identical matches between spacers and sequences, the CRISPR/Cas system can be evaded by divergence in the PAM (42). Using the identical spacer matches detected in the analysis, the corresponding protospacer sequences in the phage and prophage genomes were located. The upstream and downstream nucleotide (nt) sequences were compared, and a putative PAM sequence was identified, CCN, but no motif was identified in the downstream sequences (see Fig. S2 in the supplemental material). Multiple PAMs have been reported in other type I-B
systems (43), and our alignment suggests that this may also be the case in *C. difficile*, as a motif of CCA was present in 46% and a motif of CCT was present in 23% of these upstream regions.

The complexity of the *C. difficile* CRISPR/Cas system is highlighted by the surprising identification of spacers with matches to their own prophage sequences. An example of this is in strain CD630, which has one chromosomally carried spacer that matches to the genomic sequence of one of its prophages, ppCD630_1. Avoidance of chromosomal self-targeting has been described in other bacterial species by mutation of the PAM sequence (44). The protospacer sequence in CD630 is identical to that of the spacer, and it may avoid recognition by the CRISPR/Cas system, as it has a divergent PAM sequence, CTA.

CRISPR arrays carried by *C. difficile* prophages may provide widespread phage resistance. The nine *C. difficile* strains in the CRISPRdb database all harbor at least one prophage with CRISPR arrays, as shown in Fig. 2. As part of our analysis, these 10 prophages were included in the searches against the CRISPRdb database in order to identify protospacers in their genomes (Fig. 2). We detected spacers in these prophage arrays which match to other phage sequences used in the analysis. For example, ppCF5_1 has four spacers, located across its arrays, which match to 16 phages (Fig. 4). For the 31 spacers with matches, 11 of the corresponding protospacer sequences are located in the TMP gene, but identical matches were also identified to protospacer sequences located in a predicted endonuclease, hypothetical proteins, and tail sheath proteins. Several more spacers were found to match in a nonidentical manner to all phage sequences except the small myoviruses, ppMMP02 and ppCF5_2, illustrating the potential immunity conferred by these prophage-carried spacers across phage lineages.

The number of shared protospacer sequences is higher in related phages than in less-related phages. In order to determine if the level of genetic similarity of phages could predict host ranges based on CRISPR/Cas immunity, we identified shared protospacer sequences between phage sequences. If host immunity is controlled by the CRISPR/Cas system, then shared protospacers...
would predict similar host ranges. We plotted the number of shared protospacers against a whole-genome blastn score in pairwise comparisons (see Fig. S3 and Tables S2 and S3 in the supplemental material). The two values positively correlate, \( R = 0.7467 \), supporting this suggestion. However, differences in host ranges between related phages can be explained by unshared spacer matches as well as sequence differences in protospacers or PAM. The resulting nucleotide differences in the phage sequences include both synonymous and nonsynonymous mutations. An example is in ppCF5_1 and ppM68_1. The endolysin genes in each prophage share a similarity of 98.40% at the nucleotide level, and each gene contains two protospacer sequences. In each case, these differ by one nucleotide; in one, this difference results in an identical match between the spacer and protospacer in ppCF5_1 and a nonidentical match in ppM68_1. Whether this confers evasion against the immune system is unknown, but when the nucleotide sequence is translated, a nonsynonymous change occurs. The amino acid sequence of the endolysin differs by 6 residues between the two prophages, 2 of which result from the protospacer sequences, and we suggest that CRISPR evasion likely impacts on the conserved endolysin gene.

**Protospacer containing CDSs by function tested relative to size and frequency.** In order to investigate whether specific genes were more frequently observed to contain protospacers than others in our analysis, the location of each protospacer was identified. Protospacers are present in genes with predicted functions that encompass all essential processes associated with a temperate phage life cycle (see Fig. S4 in the supplemental material). This includes genes encoding structural proteins, involved in the control of lysogeny, and involved in the lytic life cycle. No protospacers were identified in genes whose predicted products are potential lysozyme conversion factors, such as AbiF encoded in some phage genomes (e.g., phiC2p37 in phiC2), the agrDBC-like cassette in phiCDHM1, or the VirE protein (e.g., CD_1450 in ppCD196), but protospacers were found in genes encoding hypothetical proteins located in the predicted lysogeny conversion modules of some phage genomes, downstream of the endolysin genes and on the negative-sense orientation. Protospacers are also present in many hypothetical proteins whose functions are unknown, as well as some which are located outside predicted CDSs.

In this analysis, we also see that the number of times a specific gene is targeted varies, as does the number of prophages or protospacers with the same protospacers (see Fig. S4). Examples where we can see this relative bias are within the lysis and structural genes. To examine this further, we investigated the TMP and the endolysin gene and phage-encoded holin gene. An example is in phiCDHM1, with frequencies of 8.19% and 7.62%, respectively, but the relative proportions of spacers in this data set are 11.98% and 6.45%, respectively. Phylogenetic analysis of these genes and the mapping of spacer matches illustrate the conservation for each and the distribution of matches with this phage set (see Fig. S5 in the supplemental material). The overall mean distances for each gene when aligned are 0.116 and 0.776 for the endolysin and TMP genes, respectively, which suggests that sequence conservation is likely to account for bias observed in our data set.

The positions of the spacers were mapped to the translated endolysin sequences. Two are positioned in the C-terminal region of the protein and two within the amidase 3 protein domain (PF01520) in the N-terminal region. The crystal structure of this has been solved for CD27L, the endolysin of phiCD27, so it is possible to determine how the position of one spacer corresponds to amino acid residues 56 to 67. These are located in a loop extension and alpha-2 helix of the protein (45) and could explain how the function of the endolysin is retained following sequence mutation. The other spacer is homologous to one phage, and the sequence it matches to overlaps the predicted start of the CDS. This sequence is divergent to that in the other phage endolysin sequences (data not shown).

The holin gene is another highly conserved phage gene which is targeted by two spacers in strain M120. One of these matches to the holin gene of PHMM02, whereas the second matches to a sequence in the holin genes of seven prophages. Previously, the sequence similarity between the tcdE gene and phage-encoded holin gene led to the suggestion that there is a phage origin of the toxin carrying PaLoc in *C. difficile* (23). To determine if the CRISPR system differentiates between these two genes, we searched the tcdE gene from strain CD630 (CD630_06610) against the CRISPRdb. The first of these spacers has a nonidentical match to tcdE, reflecting the sequence similarity of these genes and the theory that this gene has a xenologous origin.

**DISCUSSION**

Although phage CRISPR/Cas system dynamics have been explored in other systems, little analysis has been published examining the possible role of the CRISPR/Cas system in *C. difficile*. To test the hypothesis that the *C. difficile* CRISPR/Cas system could contribute to phage infection dynamics in this species, we examined the potential for *C. difficile* spacers to target known *C. difficile* phage genomes.

The CRISPR/Cas system of *C. difficile* is shown to be diverse between strains, and in this respect it is similar to the functional systems that have been reported and studied for other species (30, 31). Our results show that multiple *C. difficile* spacers were identified that are homologous to known phage and prophage sequences (Fig. 5). They include spacers located on prophages, the extrachromosomal DNA of strain B11, and the chromosomes of all examined strains. The spacer content of CRISPR arrays can provide insights into recent and predominant phage predation; Díez-Villaseñor et al. (46) found that the most recently incorporated spacers in *Escherichia coli* had more matches to known “extant” phage genomes. In contrast, we found spacers that match to the known phages positioned throughout the bacterial or prophage CRISPR arrays. This may mean spacer acquisition does not occur primarily at the leader region or that the *C. difficile* strains examined have been more recently challenged by unknown phages or plasmids. This is pertinent when considering the potential role of phages in driving the evolution of epidemic strains.

Spacer content has been used to estimate the abundance and diversity of phage populations for specific species: examples include the M120-like phages infecting *Streptococcus* (47) and the
FIG 5 Locations of CRISPR arrays in the C. difficile cell. Multiple CRISPR arrays are in C. difficile genomes, on the bacterial chromosome, and on extrachromosomal DNA and prophages.

diversity of phages infecting Microcystis aeruginosa (16). In our data set for C. difficile, we identified multiple spacer matches to single phages, with multiple shared and distinct spacers between C. difficile strains. These observations are consistent with a scenario resulting from a model of coevolutionary dynamics of bacteria and phage populations using evolving CRISPR defense, where multiple hosts are present in a coalition and have immunity conferred by different spacers against similar viruses (48). In the model, these coalitions are dominant but fall when a newly divergent phage emerges to which no strains have immunity. When the CRISPR spacers of natural bacterial populations have been examined, genotypes were detected which had multiple specific phage immunities (16). Similarly, in our analysis, the specific patterns of matches between host spacers and phage sequences suggest there are groupings of susceptible hosts to groups of phages. However, while CRISPR analysis showed that the genetic relatedness of phages and shared protospacers were positively correlated, there were also unique protospacers between these related phages. Also, the locations of several protospacers suggest that there may be bias in this data set, as highly conserved genes are targeted by multiple spacers which could confer wide immunity. Importantly, the CRISPR system is unlikely to be the only phage resistance mechanism in C. difficile. The use of the CRISPR system to predict phage interactions is currently limited by unknown factors, such as the rates of escape mutants and of spacer acquisition. Published host range data available for C. difficile phages suggest that phage-host interactions do not depend solely on the CRISPR/Cas system (e.g., differences in absorption [49]). A host range analysis which included φCD38-2, φMMMP04, and φMMMP02, also used in the CRISPR analysis here, showed that φCD38-2 could infect strains CD196 and R20291 (50), but these strains have a spacer which identically matches to the sequence in its genome with an intact CCA motif. Although infective, the infection is reported on the lowest scoring, indicating that there may be predominantly lysogenic infection occurring. These findings suggest that this phage may have a mechanism to evade the CRISPR/Cas system. Notably, the CRISPR/Cas system has recently been found to be evaded by Pseudomonas phages that carry anti-CRISPR proteins (51).

Similarly, a chromosomal spacer in strain CD630 is identical to the sequence of one of its prophages, ppCD630_1. The control of prophage insertion and excision in Pseudomonas aeruginosa has been previously suggested to occur, as this species also has spacers which match to temperate rather than virulent phage genomes (52, 53). However, the targeting of an established prophage has been shown in E. coli mutants to be highly lethal (54). In our example of a chromosomal spacer matching an established prophage, it appears likely to avoid recognition via a mutation in its PAM, which is known to avoid chromosomal self-targeting (44). Other sequence differences in specific regions of the protospacer, such as the seed regions identified in E. coli, can also interfere with the CRISPR/Cas pathway (55). Mutations in the protospacers located outside the seed region do not inhibit CRISPR spacer recognition, and if this is the case also in C. difficile, it would alter the predicted interactions and warrant future research.

This analysis has also shown that several related prophages carry CRISPR arrays which are diverse in composition. Their presence may be the result of chromosomal scattering of the CRISPR arrays, and subsequent loss and gain of spacers occurred. A partial transposase gene is located upstream of the CRISPR array regions, which supports the theory that these have been transferred via one or more HGT events. Homologs of genes adjacent to the CRISPR arrays are present in some of the other C. difficile phage genomes, for example, phiC2p19 in dC2. Although in prophage genomes, the CRISPR arrays may be transferred, as the CD630 prophages can also access the lytic pathway (23), and we show that the prophage of C. difficile CD105HE1 was released spontaneously and retains the CRISPR arrays in its genome. This is consistent with the report from a metagenomic study of the human virome that found that CRISPR arrays are present in DNA sequence data from free viral particles, suggesting that there is the potential for exchange of arrays via HGT in the human gut microbial population (56). In this study and a subsequent study, the researchers report the detection of virus-carried spacers targeting viral sequences, specifically, from a temperate phage infecting Ruminococcus bromii (57).

Whether the prophage CRISPR arrays function to confer immunity has not been established, but in strain CD630, both prophage arrays are transcribed, and the processing of the pre-crRNA was detected in arrays 15 and 16 (on ppCD630_2) (30). Our analysis found that the spacer content could be highly divergent between prophages, suggesting that continued spacer acquisition and loss have occurred. Typically, spacers are acquired from the leader region, but HGT and other mechanisms of incorporation occur in some CRISPR/Cas systems (31, 58–60). While CRISPR array evolution is thought to be primarily rapid, the spacer contents of the R027 prophages are identical despite originating from isolates obtained over decadal timescales (61), which agrees with a slower reported rate of CRISPR change in E. coli (19, 21). Mechanisms for the loss of CRISPR spacers are not fully understood but have been observed following a pathogenic host shift in Mycoplasma gallisepticum (62). Similarly, CRISPR arrays in prophages from an environmental isolate (CD105HE1) (this study) and an isolate from asymptomatic human (CF5) (63) carry more spacers than those in prophages from clinical strains, which have an average 38% fewer spacers. The numbers of CRISPR spacers in surviving populations of E. coli have been found to increase during phage infection but decrease during instances where lateral gene transfer was beneficial in an in vitro test using antibiotic selection.
and a plasmid carrying the resistance gene (64). The different numbers of arrays and spacers between prophages, and between strains of \textit{C. difficile}, may have arisen following different selection pressures in the natural and clinical environments.

Our findings suggest that specific prophages in \textit{C. difficile} could confer immunity to invading phages via the spacers in their CRISPR arrays. This is important to consider, as phage infection in this species has been found to influence bacterial physiology, such as toxin production (26, 65–68), and is also being explored as novel therapeutics (68). Prophage carriage in the ribotypes examined show how they could influence phage susceptibility, as the R027 strains carry similar prophages with identical CRISPR spacers; but in contrast, two strains belonging to R017 carry prophages with distinct CRISPR contents. Differential carriage of prophages could explain differences in phage susceptibility in specific ribotype groups and presents a highly novel facet of the CRISPR immune system in phage-phage wars. We suggest that an advantage to retaining prophages in the highly lysogenized \textit{C. difficile} may be due to the fact that some are a source of spacers. Further work will evaluate the activity of this CRISPR/Cas system.

**MATERIALS AND METHODS**

Whole-genome alignments to assess phage and prophage diversity. The genomes of 31 phages and prophages were used in the analysis, as they represent morphologically diverse types (including medium myoviruses, long-tailed myoviruses, small myoviruses and siphoviruses, and prophages in epidemic and nonepidemic, recent and historical strains) (see Table S1 in the supplemental material). Prophage sequences were predicted using PHAST (69). CDSs were predicted using FGNEVES (Softberry Inc., United States) and annotated based on results of searches against the online Pfam database (70) and the NCBI nt/nr database using blastp accessed at http://www.ncbi.nlm.nih.gov/Blast.cgi. Genome sequences were visualized using Artemis Genome Browser (71), and prophage sequences were rearranged to start with the terminase genes. Whole-genome alignment was performed using MAUVE v2.3.1 (37) by using the progressiveMAUVE algorithm (38).

\text{\textit{C. difficile} CRISPR array analysis and protospacer identification in phage and prophage genomes.} The same set of phages and prophages were searched for CRISPR arrays using CRISPRfinder (32) and against the CRISPRdb database (33). DR and gene alignments were performed using Clustal Omega (72). A relative measure of phage genetic relatedness was calculated from the blastn results generated from pairwise comparison of genomes using Double Act v2 accessed at http://www.hpa-bioinfotools.org.uk/ipse/double_act.html#. The score value was defined as the total number of bases aligned in sequences that had \(\geq80\%\) identity and were \(\geq20\,nt\) in length. Shared protospacer analysis used unique identical and nonidentical matches. Data analysis and correlation tests were performed in Microsoft Office Excel and MiniTab. Protospacer adjacent motifs (PAMS) were identified from comparison of 10-nt upstream and down-stream sequences for 52 unique protospacers which had a perfect match to spacers using WebLogo (73, 74). Cas genes in strain CD630 were searched for CRISPR arrays using CRISPRfinder (32) and against the online Pfam database (70) and the NCBI nt/nr database using blastp accessed at http://www.ncbi.nlm.nih.gov/Blast.cgi. Genome sequences were visualized using Artemis Genome Browser (71), and prophage sequences were rearranged to start with the terminase genes. Whole-genome alignment was performed using MAUVE v2.3.1 (37) by using the progressiveMAUVE algorithm (38).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl?doi=10.1128/mBio.01045-13/-/DCSupplemental.

Figure S1, PDF file, 0.3 MB.
Figure S2, TIF file, 6.7 MB.
Figure S3, TIF file, 0.2 MB.
Figure S4, TIF file, 41.3 MB.
Figure S5, TIF file, 0.6 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, XLSX file, 0.1 MB.
Table S4, XLSX file, 0.1 MB.
Table S5, XLSX file, 0.1 MB.

**ACKNOWLEDGMENTS**

This work was supported by an MRC Centenary Fellowship awarded to K.R.H. and an MRC New Investigator award (G0700855) awarded to M.R.J.C. C.O.F. is supported by a CONACyT (Mexico) graduate fellowship.

We thank Julie Pratt for her useful comments on the manuscript. We also thank Joshua Weitz for his useful discussion, comments, and expert advice when we were writing the manuscript.

**REFERENCES**

1. Bouza E. 2012. Consequences of \textit{Clostridium difficile} infection: understanding the healthcare burden. Clin. Microbiol. Infect. 185–12. http://dx.doi.org/10.1111/j.1469-0691.2012.03862.x.

2. Hall IC, O’Toole E. 1993. Intestinal flora in newborn infants: with a description of a new pathogenic anaerobe, \textit{Bacillus difficile}. Am. J. Dis. Child. 49:390–402. http://dx.doi.org/10.1001/archpedi.1935.01970020105010.

3. al Saif N, Brazier JS. 1996. The distribution of \textit{Clostridium difficile} in the environment of South Wales. J. Med. Microbiol. 45:133–137. http://dx.doi.org/10.1099/00222815-45-2-133.

4. He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, Connor TR, Harris SR, Fairley D, Bamford KB, D’Arc S, Brazier J, Brown D, Coia JE, Douce G, Gerding D, Kim HJ, Koh TH, Kato H, Senoh M, Louie T, Michell S, Butt E, Peacock SJ, Brown NM, Riley T, Songer G, Wilcox M, Pirmohamed M, Kuijper E, Hawkey P, Wren BW, Dougan G, Parkhill J, Lawley TD. 2013. Emergence and global spread of epidemic healthcare-associated \textit{Clostridium difficile}. Nat. Genet. 45:109–113. http://dx.doi.org/10.1038/ng.2478.
71. Goh S, Ong P, Song K, Riley T, Chang B. 2008. Molecular characterization of a Clostridium difficile bacteriophage and its cloned biologically active endolysin. J. Bacteriol. 190:6734–6740. http://dx.doi.org/10.1128/JB.00686-08.

24. Horgan M, O’Sullivan O, Coffey A, Fitzgerald GF, van Sinderen D, McAluliffe O, Ross RP. 2010. Genome analysis of the Clostridium difficile phage PhIC6356, a temperate phage of the Siphoviridae family. Gene 455:33–43. http://dx.doi.org/10.1016/j.gene.2009.10.067.

5. Sebastian M, Wren BW, Mullaney P, Fairweather NF, Minton N, Stabler R, Thomson NR, Roberts AP, Cerdeño-Tárraga AM, Wang H, Holden MT, Wright A, Churcher C, Quail MA, Baker S, Bason N, Brooks K, Chillingworth T, Cronin A, Davis P, Dowd I, Fraser A, Feltwell T, Hance Z, Holroyd S, Jagels K, Moule S, Mungall K, Price C, Rabinowitsch E, Sharp S, Simmonds M, Stevens K, Unwin L, Whithead S, Dupuy B, Duran G, Barrell B. 2004. The multi-resistant human pathogen Clostridium difficile has a highly mobile, mosaic genome. Nat. Genet. 38:787–786. http://dx.doi.org/10.1038/ng1830.

Siefert JL. 2009. Defining the mobilome. Methods Mol. Biol. 532:13–27. http://dx.doi.org/10.1007/978-1-60327-853-9_2.

2. Goh S, Riley TV, Chang BJ. 2005. Isolation and characterization of temperate bacteriophages of Clostridium difficile. Appl. Environ. Microbiol. 71:1029–1033. http://dx.doi.org/10.1128/AEM.71.1079-1083.2005.

10. Jansen R, Embden JD, Gruastra-WV, Schouts M. 2002. Identification of genes that are associated with DNA repeats in prokaryotes. Mol. Microbiol. 43:1565–1575. http://dx.doi.org/10.1046/j.1365-2958.2002.02839.x.

14. Goh SH, Hussain H, Chang B, Emmett W, Riley TV, Mullaney P. 2013. Phage dC2 mediates transduction of Tn6215, encoding erythromycin resistance, between Clostridium difficile strains. mBio 4(6):e00840-13. http://dx.doi.org/10.1128/mBio.00840-13.

19. Barrangou R, Horvath P, Doyle M, Klaenhammer T. 2008. CRISPR—a widespread system for antiviral defense in prokaryotes. Science 321:64–68. http://dx.doi.org/10.1126/science.1160250.

22. Govind R, Fralick JA, Rolfe RD. 2006. Genomic organization and molecular characterization of Clostridium difficile bacteriophage Phi CD119. J. Bacteriol. 188:2568–2577. http://dx.doi.org/10.1128/JB.188.7.2568-2577.2006.

20. Mayer MJ, Narbad A, Gasson MJ. 2008. Molecular characterization of a Clostridium difficile bacteriophage and its cloned biologically active endolysin. J. Bacteriol. 190:6734–6740. http://dx.doi.org/10.1128/JB.00686-08.

23. Shah SA, Erdmann S, Mojica FJ, Garrett RA. 2013. Protospacer recogn.
nition motifs: mixed identities and functional diversity. RNA Biol. 10: 891–899. http://dx.doi.org/10.4161/rna.23764.

44. Vercoe RB, Chang JT, Dy RL, Taylor C, Grivistood T, Clulow JS, Richter C, Przybielski R, Pitman AR, Fineman PC. 2013. Cytotoxic chromosomal targeting by CRISPR/Cas systems can reshape bacterial genomes and expel or replace pathogens from islands. PLoS Genet. 9:e1003545. http://dx.doi.org/10.1371/journal.pgen.1003545.

45. Mayer MJ, Garefalaki V, Spoerl R, Narbad A, Meijers R. 2011. Structure-based modification of a Clostridium difficile-targeting endolysin affects activity and host range. J. Bacteriol. 193:5477–5486. http://dx.doi.org/10.1128/JB.00439-11.

46. Diez-Villaseñor C, Almendros C, García-Martínez J, Mojica FJ. 2010. Diversity of CRISPR loci in Escherichia coli. Microbiology 156:1351–1361. http://dx.doi.org/10.1099/mic.0.036046-0.

47. van der Ploeg JF. 2009. Analysis of CRISPR in Streptococcus mutans suggests frequent occurrence of acquired immunity against infection by M102-like bacteriophages. Microbiology 155:1966–1976. http://dx.doi.org/10.1099/mic.0.027508-0.

48. Childs LM, Held NL, Young MJ, Whitaker RJ, Weitz JS. 2014. Dynamics of CRISPR bacteria and phages at the interface of Lamarck and Darwin. Mol. Microbiol. 89:615–629.

49. Ramesh V, Fralick JA, Rolfe RD. 1999. Prevention of Clostridium difficile-induced ileoceleitis with bacteriophage. Anaerobe 5:69–78. http://dx.doi.org/10.1006/anae.1999.0192.

50. Essov C, Blouin Y, Loukou G, Cablanmian A, Lathro S, Kutter E, Thien HV, Vergnaud G, Pourcel C. 2013. The susceptibility of Pseudomonas aeruginosa strains from cystic fibrosis patients to bacteriophages. PLoS One 8:e60575. http://dx.doi.org/10.1371/journal.pone.0060575.

51. Bondy-Denomy J, Pawluk A, Maxwell KL, Davidson AR. 2013. Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. Nature 493:429–432. http://dx.doi.org/10.1038/nature11723.

52. Essov C, Blouin Y, Loukou G, Cablanmian A, Lathro S, Kutter E, Thien HV, Vergnaud G, Pourcel C. 2013. The susceptibility of Pseudomonas aeruginosa strains from cystic fibrosis patients to bacteriophages. PLoS One 8:e60575. http://dx.doi.org/10.1371/journal.pone.0060575.

53. Cady KC, Bondy-Denomy J, Heussler GE, Davidson AR, O'Toole GA. 2012. The CRISPR/Cas adaptive immune system of Pseudomonas aeruginosa mediates resistance to naturally occurring and engineered phages. J. Bacteriol. 194:5728–5738. http://dx.doi.org/10.1128/JB.01184-12.

54. Edgar R, Qimron U. 2010. The Escherichia coli CRISPR system protects from lambda lysogenization, lysogens, and prophage induction. J. Bacteriol. 192:6291–6294. http://dx.doi.org/10.1128/JB.00644-10.

55. Semenova E, Jore MM, Datsenko KA, Semenova A, Westra ER, Wanner B, van der Oost J, Neron A, Fortier L-C. 2012. Ultrafast evolution and loss of CRISPRs following a host shift in a novel wildlife pathogen, Mycoplasma gallisepticum. PLoS Genet. 8:e1002511. http://dx.doi.org/10.1371/journal.pgen.1002511.

56. Stabler RA, Gerding DN, Songer JG, Drudy D, Brazier JS, Trinh HT, Witney AA, Hinds J, Wren BW. 2006. Comparative phylogenomics of Clostridium difficile reveals clade specificity and microevolution of hyper-virulent strains. J. Bacteriol. 188:7297–7305. http://dx.doi.org/10.1128/JB.00464-06.

57. Jiang W, Maniv I, Arain F, Wang Y, Levin BR, Marraffini LA. 2013. Dealing with the evolutionary downside of CRISPR immunity: bacteria and beneficial plasmids. PLoS Genet. 9:e1003844. http://dx.doi.org/10.1371/journal.pgen.1003844.

58. Goh S, Chang BJ, Riley TV. 2005. Effect of phage infection on toxin production by Clostridium difficile. J. Med. Microbiol. 54:129–135. http://dx.doi.org/10.1099/jmm.0.45821-0.

59. Govind R, Vediyaplan G, Rolfe RD, Dupuy B, Fralick JA. 2009. Bacteriophage-mediated toxin gene regulation in Clostridium difficile. J. Virol. 83:12073–12085. http://dx.doi.org/10.1128/JVI.01125-09.

60. Meeder E, Mayer MJ, Gasson MJ, Steverding D, Carding SR, Narbad A. 2010. Bacteriophage treatment significantly reduces viable Clostridium difficile and prevents toxin production in an in vitro model system. Anaerobe 16:549–554. http://dx.doi.org/10.1016/j.anaerobe.2010.08.006.

61. Meeder E, Mayer MJ, Steverding D, Carding SR, Narbad A. 2013. Evaluation of bacteriophage therapy to control Clostridium difficile and toxin production in an in vitro human colon model system. Anaerobe 22:25–30. http://dx.doi.org/10.1016/j.anaerobe.2013.05.001.

62. Zhou Y, Liang Y, Lynch KH, Dennis JJ, Wishart DS. 2011. PHAST: a fast phage search tool. Nucleic Acids Res. 39:W347–W352. http://dx.doi.org/10.1093/nar/gkr3125.

63. Punta M, Coggill PC, Eberhardt RY, Mistri J, Tate J, Bourneff G, Pang N, Forslund K, Cerq G, Clements J, Heger A, Holm L, Sonhammer EL, Eddy S, Bateman A, Finn RD. 2012. The Pfam protein database. Nucleic Acids Res. 40:D290–D301. http://dx.doi.org/10.1093/nar/gkr717.

64. Rutherford K, Parkhill J, Crook J, Horsnell C, Rice P, Rajandream MA, Barrell B. 2000. Artemis: sequence visualization and annotation. Bioinformatics 16:944–945. http://dx.doi.org/10.1093/bioinformatics/16.10.944.

65. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7:539. http://dx.doi.org/10.1038/msb.2011.75.

66. Crooks GE, Hon G, Chandonia JM, Brenner SE. 2004. WebLogo: a sequence logo generator. Genome Res. 14:1188–1190. http://dx.doi.org/10.1101/gr.40904.

67. Schneider TD, Stephens RM. 1990. Sequence logos: a new way to display consensus sequences. Nucleic Acids Res. 18:6097–6100. http://dx.doi.org/10.1093/nar/18.20.6097.

68. Kumar S, Nei M, Dudley J, Tamura K. 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief. Bioinform. 9:299–306. http://dx.doi.org/10.1093/bib/bbn017.

69. Bioso A, Gagnon IN, BourjeSI J, Fineman PC, Brown CM. 2013. CRISPRTarget: bioinformatic prediction and analysis of crRNAs targets. RNA Biol. 10:817–827. http://dx.doi.org/10.4161/rna.24046.

70. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis, version 6.0. Mol. Biol. Evol. 30:2725–2729. http://dx.doi.org/10.1093/molbev/msu197.

71. Rozen S, Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132:365–386.

72. Hargreaves KR, Colvin HV, Patel KV, Clocie J, Clocie MR. 2013. Genetically diverse Clostridium difficile strains harbouring abundant prophages in an estuarine environment. Appl. Environ. Microbiol. 79:6236–6243. http://dx.doi.org/10.1128/AEM.01849-13.