

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

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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 (protospacers) 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*.

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The bacterium *Clostridium difficile* is a major nosocomial pathogen (1), which can also be carried asymptotically (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 free or inducible 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* are unknown. Interestingly, an unusual situation exists in *C. difficile* in which CRISPR arrays are also found in *C. difficile* prophages (5). Transcription and processing of CRISPR RNA (crRNA) from prophage-carried arrays have been detected in *C. difficile* strain CD630 (30). If these prophage-carried arrays are fully functional CRISPR elements, they represent a mechanism for prophage elements to influence secondary phage infection and, subsequently, the extent of HGT. An example of phages impacting the CRISPR dynamics can be seen in the ICR1 phage that infects *Vibrio cholerae*. This phage has been shown to carry a functioning CRISPR/Cas system that targets a phage inhibitory chromosomal island, thus permitting infection of the host (31). The relationship between prophages and the CRISPR/Cas system is therefore not unidirectional.

In order to examine the potential significance of the CRISPR/Cas system in *C. difficile*, we used bioinformatic approaches to identify how the CRISPR/Cas system in diverse *C. difficile* strains relates to 31 phage and prophage genomes. To do this, we first examined phage and prophage genomic diversity to determine their relatedness. Second, we examined these 31 genomes for CRISPR arrays and established their DR and spacer diversity. We then searched the same phage genomes against a database of *C. difficile* spacers (including those from arrays in prophages). The locations of protospacers were identified, and the targets of the CRISPR/Cas system were examined in regard to gene function and conservation. Finally, putative protospacer adjacent motifs (PAMs) were identified using protospacer sequences from this analysis. Taken together, our analyses suggest that the CRISPR/Cas system is an important determinant of phage infection in *C. difficile*.

RESULTS

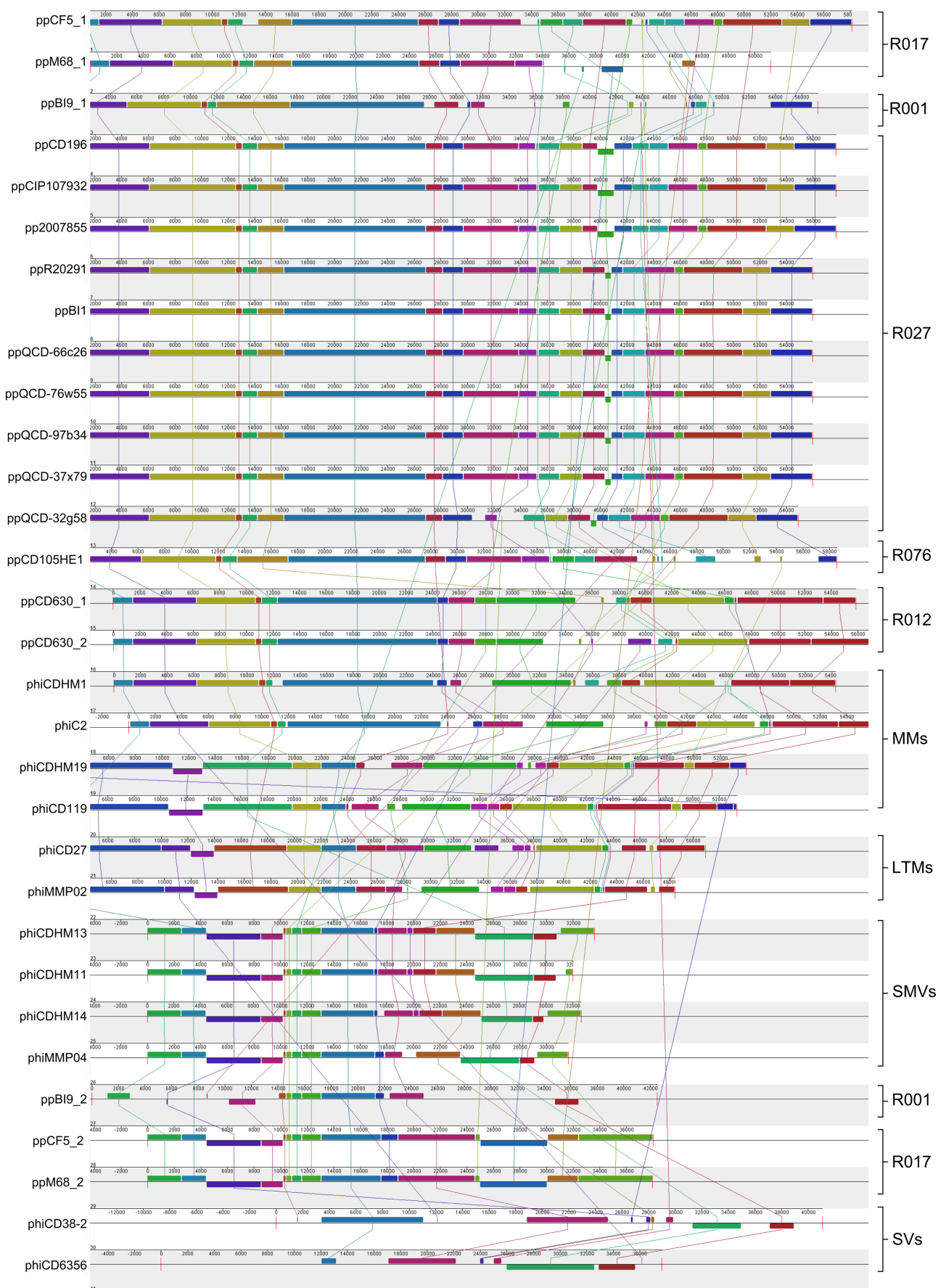
The CRISPR/Cas system in *C. difficile* strain CD630. In order to characterize the CRISPR/Cas system within *C. difficile* strains, we examined the seven strains present in the CRISPRdb database of CRISPRfinder (32, 33). These carry multiple and diverse arrays, with the total number of spacers per strain ranging from 43 to 153, as determined using the CRISPRcompar tool (34). The *C. difficile* strain CD630 carries two sets of *cas*-like genes. One set belongs to type I-B (Tneap subtype) according to the typing system of Markarova et al. (35) and is composed of *cas2* (CD2975), *cas1* (CD2976), *cas4* (CD2977), *cas3* (CD2978), *cas5* (CD2979), *cas7* (or *cst2*) (CD2980), *cas8b* (or *cst1*) (CD2981), and *cas6* (CD2982), as assigned by the Genome Properties Report from the JCVI Comprehensive Microbial Resource. These genes are near to the largest of the CD630 CRISPR arrays, NC_009089_17, which contains 19 spacers. The second set of predicted *cas* genes is distinct from the

described types and has genes homologous to *cas3* (CD2451), *cas5* (CD2452), and *cas6* (CD2455) but lacks a homolog of *cas1*, which is a nearly universal component of the *cas* system. The DR sequences from strain CD630 arrays are predicted to be folded, and a comparison of the DR sequences to those in Kunin et al. (36) shows they cluster closest to groups 4 (folded bacterial) and 11 (unfolded bacterial).

Whole-phage-genome analysis supports subgrouping according to particle morphology. 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 ϕ C2, 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 ϕ C2-like prophages, and strain BI9 (R001) carries a ϕ C2-like prophage and one phage that appears similar to the small myovirus type. Lastly, strain M68 (R017) and strain CF5 (R017) carry ϕ C2-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 ϕ C2-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 *xkdN* gene (whose product is a predicted structural protein) and the tape measure protein (TMP) gene. In one, ppBI9_1, an additional single predicted array is located in the DNA replication region. Interestingly, no CRISPR arrays were identified in the ϕ C2-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 (CDSs). 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



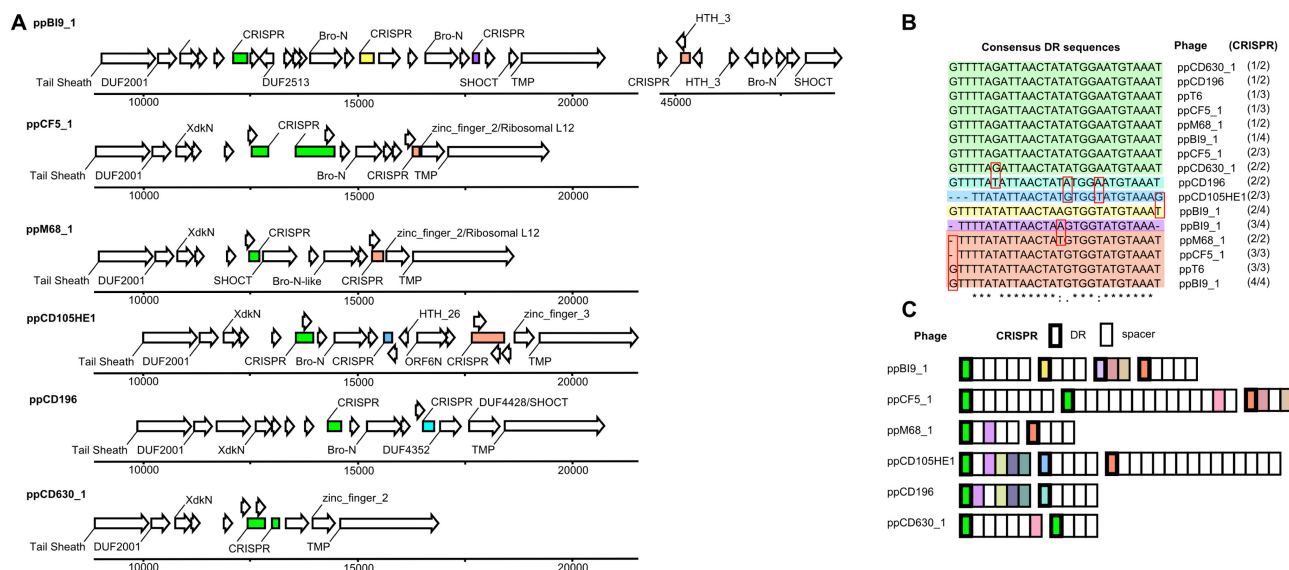


FIG 2 Locations of CRISPR arrays on prophage genomes and spacer content. (A) The CRISPR arrays (boxes) and adjacent genes (arrows showing orientation) were mapped to each prophage genome (scale in bp). Gene annotations were assigned following identification of protein domains in Pfam and by performing blastp searches against the NCBI nr/nt database. The consensus DR sequences of the arrays are color coded. Spacer content of the arrays revealed that ppCD196 represents all the R027 prophages, as they have identical CRISPR contents. ppCD630_1 also represents the CRISPR content of ppCD630_2, as both are identical. (B) Alignment of the consensus DR from each CRISPR array identified with single-nucleotide polymorphisms (SNPs; red boxes). Color coding reflects conserved groups. (C) The spacer content for each CRISPR array is shown with the corresponding DR with use of color to show identical sequences. White indicates spacers which are unique.

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 gener-

ated 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 ϕ CD27. In contrast, spacers of one strain match to ϕ 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

FIG 1 Whole-genome alignment of 31 phages and prophages infecting *C. difficile* displays genomic lineages and homologous recombination occurring between phage subtypes. Alignments were performed in MAUVE using the progressiveMAUVE algorithm (36). Colored blocks indicate regions free from homologous recombination, and lines to colored blocks show homologous regions in other genomes. The phages have been grouped according to morphology if known; medium myoviruses (MMs), long-tailed myoviruses (LTMs), small myoviruses (SMVs) and siphoviruses (SVs), and prophages, by the ribotype of their lysogen and genomic similarity to the phage genomes.

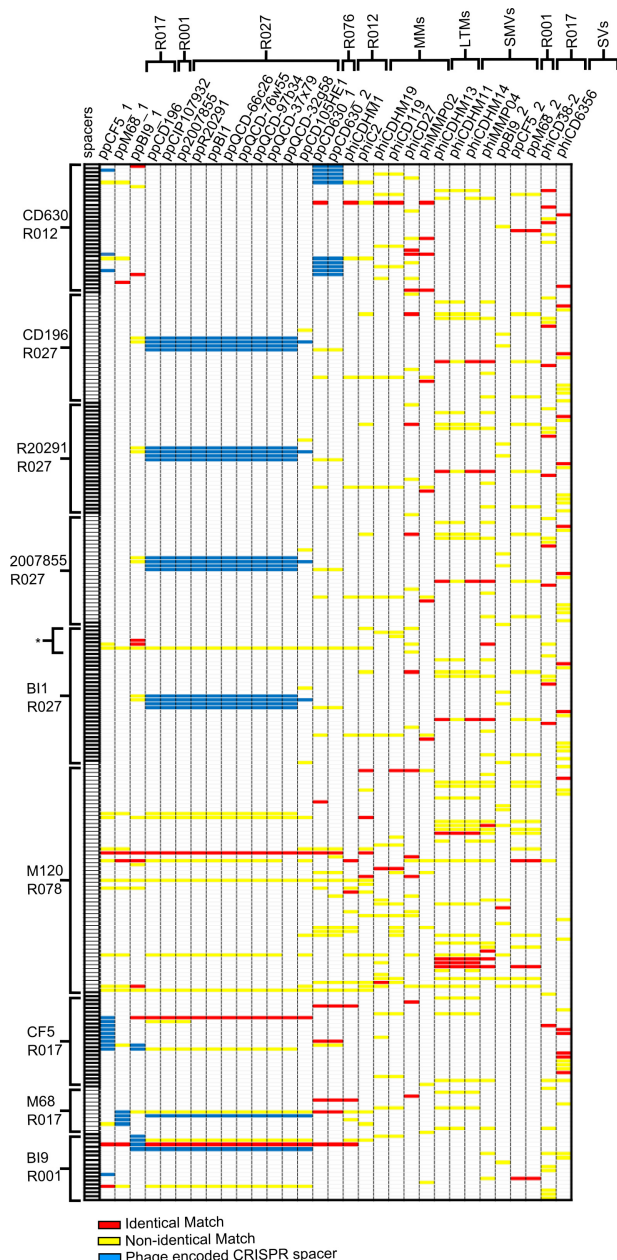


FIG 3 CRISPR profiles of 31 phages and 9 strains indicating homologous matches between spacers and genome sequences. All significantly scoring matches (E value of >0.005) between spacers and phage sequences are shown following searches using CRISPRfinder (see Table S5 for sequences). Spacers (in rows) are in numerical order for each *C. difficile* strain and include only those which had significant matches to the searched phage sequences (not all spacers in the strains). Of the nine strains, four belong to R027 and two to R017. Asterisk indicates spacers carried on strain BI1 extrachromosomal DNA. Phage and prophages (columns) are arranged according to their order in the MAUVE alignment. Solid red boxes represent identical matches between spacer and genome sequence, yellow boxes represent nonidentical matches, and blue boxes are prophage CRISPR spacers.

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 high-

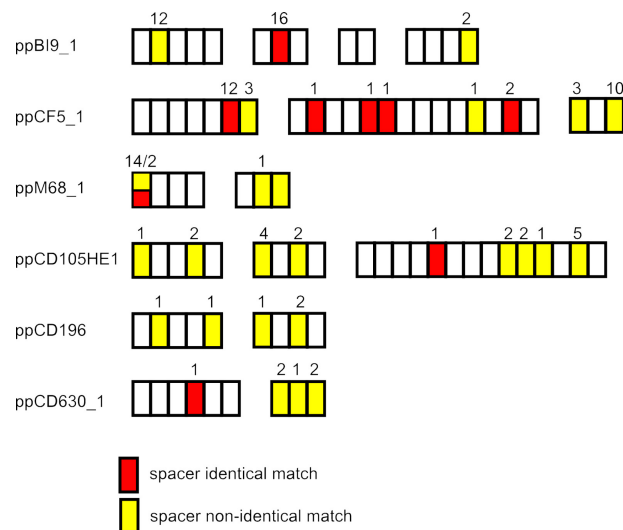


FIG 4 Prophage-carried spacers match to *C. difficile* phage and prophage genomes. The spacers from the multiple CRISPR arrays identified in the legends to Fig. 2 and 3 are shown with respective matches to the 31 phage and prophage genomes. They include identical and nonidentical matches, as well as those matching to multiple sequences and those matching to single sequences. The distributions of spacers with matches to the searched genomes are throughout the arrays.

lighted 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, Φ MMP02 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 lysogenic conversion factors, such as AbiF encoded in some of the phage genomes (e.g., phiC2p37 in ϕ C2), 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 phages or prophages 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, both of which are present in all the examined genomes. We identified 23 unique spacers across all the strains which are homologous to sequences in TMP genes; the majority of spacers match only to a single phage's copy of the gene. In contrast, four spacers in two *C. difficile* strains, M120 and BI1, match to phage and prophage endolysin genes, with 23, 20, 5, and 1 matches across the panel examined. The different number of spacers identified for each gene may result from gene length and/or PAM content. To test this, we found 192 instances of either CCA or CCT across the 2,343-nt TMP gene and 62 in the 813-nt endolysin gene of 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 ϕ CD27, 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 Φ MMP02, whereas the second matches to a sequence in the holin genes of seven phages. 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 BI1, 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

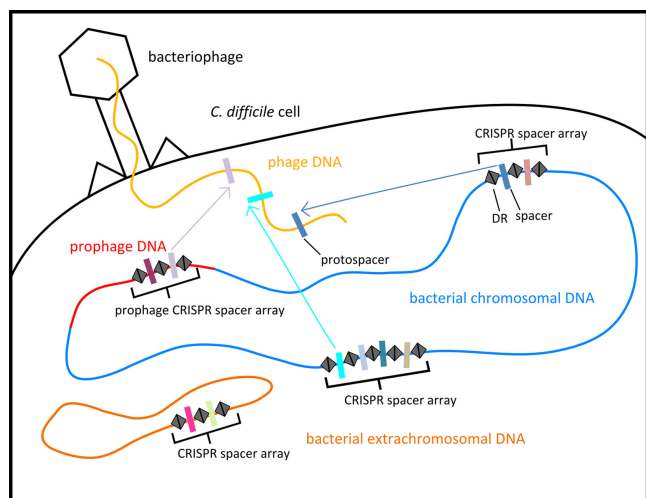


FIG 5 Locations of CRISPR arrays in the *C. difficile* cell. Multiple CRISPR arrays are in *C. difficile* genomes, on the bacterial chromosome, and on extra-chromosomal DNA and prophages.

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 ϕ CD38-2, Φ MMP04, and Φ MMP02, also used in the CRISPR analysis here, showed that ϕ CD38-2 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, ϕ C2p19 in ϕ C2. 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 on 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 *C. difficile*, may have arisen following different selection pressures in the natural and clinical environments.

Our findings suggest that specific prophages in *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 *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 FGENESV (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).

***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-bioinfotool.org.uk/pise/double_act.html#. The score value was defined as the total number of bases aligned in sequences that had $\geq 80\%$ identity and were ≥ 20 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 downstream sequences for 52 unique protospacers which had a perfect match to spacers using WebLogo (73, 74). Cas genes in strain CD630 were identified using the Genome Properties search tool (accessed 31 January 2014 at http://cmr.jcvi.org/tigr-scripts/CMR/shared/MakeFrontPages.cgi?page=genome_property). RNA structure was predicted using Vienna RNAalifold (accessed at <http://rna.tbi.univie.ac.at/cgi-bin/RNAalifold.cgi>). DR sequence alignment using MUSCLE and unweighted pair group method with arithmetic mean (UPGMA) analysis was performed in MEGA v5.2 (75). Matches to the spacers in arrays in ppCD105HE1 were identified using CRISPRTarget (76). Overall mean composite distances were calculated for the nucleotide sequences of the tail tape measure (TMP) and endolysin genes (excluding partial CDSs) in MEGA v6.06 (77). Sequences were first aligned using MUSCLE, and the distance estimation was performed using the Poisson model with uniform rates and pairwise deletions. Maximum likelihood analysis was performed

at the amino acid level using the Jones-Taylor-Thornton (JTT) model with gamma distribution and 500 interactions for bootstrapping.

Release of ppCD105HE1 particles and PCR-based detection of CRISPR arrays. Release of phage particles was assessed using TEM from culture lysates using methods described previously (28). Primers targeting each of the three CRISPR arrays were designed using Primer3 v0.4.0 (78) (see Table S2 in the supplemental material for the oligonucleotide sequences). Lysates of strain CD105HE1 grown overnight in brain heart infusion broth (BHI; Oxoid, United Kingdom) were centrifuged at $3,398 \times g$ for 10 min, and the supernatant was filtered through a 0.22- μ m-pore-size filter (Millipore, United Kingdom). The lysate was then treated with TurboDNase (Life Technologies, United Kingdom) according to the manufacturer's guidelines. Contamination with bacterial DNA was detected using primers targeting the 16S rRNA gene as described previously (79). Uncontaminated lysate was then used as the template in PCRs with each of the CRISPR array primer sets. Reactions were performed in 25 μ l, containing template DNA, 0.6 mM forward and reverse primers, 0.25 mM deoxynucleoside triphosphates (dNTPs), 3 mM $MgCl_2$, $1 \times$ PCR buffer, and 0.5 U of BioTaq polymerase (Bioline, United Kingdom) under conditions of 95°C for 5 min, 30 cycles of 95°C for 45 s, 48°C for 45 s, and 72°C for 60 s, with a final extension step of 5 min at 72°C. Products were separated using 110 V for 30 min on a 1% agarose Tris-acetate-EDTA (TAE) gel stained using ethidium bromide. Product size estimates were based on a 1-kbp GeneRuler ladder (Thermo Scientific, United Kingdom).

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.

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