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***In vivo* lysogenization of a *Clostridium difficile* bacteriophage Φ CD119**

Revathi Govind^{1,2,*}, Joe A. Fralick², and Rial D. Rolfe²

¹Division of Biology, Kansas State University, Manhattan, KS 66506, U.S.A.

²Department of Microbiology and Immunology, Texas Tech University Health Sciences Center, Lubbock, TX 79430, U.S.A.

Abstract

Clostridium difficile is a nosocomial pathogen identified as the cause of antibiotic associated diarrhea and colitis. In this study, we have documented the lysogeny of a *C. difficile* bacteriophage in hamsters during *C. difficile* infection. The lysogens isolated from the hamsters were toxin typed and their phage integration site was confirmed by PCR. Through toxin ELISA it was found that the toxin production in the *in vivo* isolated lysogens was affected due to Φ CD119 lysogenization as in the case of *in vitro* isolated Φ CD119 lysogens. Together our findings indicate that a bacteriophage can lysogenize its *C. difficile* host even during the infection process and highlights the importance of lysogeny of *C. difficile* phages as an evolutionary adaptation for survival.

Keywords

Clostridium difficile; Bacteriophage; Lysogenization

1. Introduction

C. difficile has been recognized as an enteric pathogen causing disease almost exclusively in the presence of antibiotic exposure [1-3]. The spectrum of illness caused by *C. difficile* ranges in severity from asymptomatic carriage to life-threatening pseudomembranous colitis, fulminant colitis, and death [4, 5]. Infection begins with a disruption of normal colonic bacterial flora due to the antibiotic treatment; this disruption promotes the colonization of *C. difficile* and the production of toxins, A (an enterotoxin) and B (a cytotoxin) that cause colonic injury and inflammation [6, 7]. The *tcdA* and *tcdB* genes are located on the chromosome within a region known as the pathogenicity locus (PaLoc)[8]. In addition to *tcdA* and *tcdB*, PaLoc encodes three additional genes designated *tcdR*, *tcdE* and *tcdC* which encode an alternative sigma factor, TcdR[9], a putative holin, TcdE[10], and an anti-sigma factor, TcdC[11], respectively. The expression of *tcdA* and *tcdB* are positively regulated by TcdR[9] and negatively regulated by TcdC[12]. In *C. difficile*, it has been shown that pathogenicity locus (PaLoc) genes share homology with phage genes, *tcdE* with phage holins[10], *tcdA* to a *C. tetani* phage Φ CT2 gene [13] and *tcdC*, with ORF 22 of

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*Corresponding author: Revathi Govind, Division of Biology, Kansas State University, Manhattan, KS 66506, U.S.A., Tel: (785) 587-0379; Fax: (785) 587-6653; rgovind@ksu.edu.

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Lactobacillus casei phage A2 [14]. In a previous study we have sequenced a *C. difficile* temperate phage Φ CD119 [15] and have characterized the role of its repressor protein (RepR) on *C. difficile* toxin gene expression [16]. It was found that the expression of RepR protein in *C. difficile* Φ CD119 lysogens decreased the toxin production by controlling the transcription of *tcdR*, the positive regulator [16]. Since the Φ CD119 lysogeny was found to down regulate the major virulence factors in *C. difficile*, in this study we have tested the ability of Φ CD119 to lysogenize its *C. difficile* host strain during the infection in a mammalian host. We have found that Φ CD119 can lysogenize its host even during the infection and the *in vivo* isolated lysogens were also found to produce less toxins similar to the *in vitro* isolated Φ CD119 lysogens.

2. Materials and Methods

2.1. Bacterial strains, growth conditions, Phage propagation and purification

The *C. difficile* strains F10, F2 and F6 are the lysogens of bacteriophages Φ CD119, Φ CD001 and Φ CD006 respectively (table 1). Along with F10, F2 and F6 strains, the bacteriophages host *C. difficile* strain 602 were obtained from Rosanna Dei, Università degli Studi di Firenze, Italy. The *C. difficile* strains were stored in chopped meat broth (Carr Scarborough Microbiologicals, Inc., Decatur, GA) at room temperature. When required, the broth cultures were sub-cultured onto Brain Heart Infusion (BHI) agar and incubated anaerobically at 37 °C. Bacteriophage were induced from their lysogens as described by Mahony et al., [17, 18], propagated and purified according to methods described previously [15].

2.2. Testing *in vivo* phage lysogenization

Experiments were performed to determine if the phage Φ CD119 could lysogenize in a animal host as observed *in vitro*. Since, the hamster model of *C. difficile* disease is a well-established model for the study of toxin-induced antibiotic-associated diarrhea, we have tested whether the intestinal tract of hamsters could favor the Φ CD119 lysogenization during a *C. difficile* infection. Syrian golden hamsters (70 to 80g) were used for the *C. difficile* challenge. Fecal pellets were collected from all the hamsters, homogenized in 1 mL saline and were examined for *C. difficile* by plating in CCFA (Cycloserine Cefoxitin Fructose Agar) to ensure the animals did not harbor indigenous *C. difficile*.

Six hamsters were divided into two groups and all the hamsters in groups 1 and 2 were administered with 1 ml of clindamycin (3 mg/100 g body weight) intragastrically under ether anesthesia. Twenty-four hours after clindamycin treatment, the gastric acidity of the hamsters in both groups was neutralized with 1 mL 1M bicarbonate buffer administered intragastrically. Immediately following bicarbonate neutralization all the hamsters were challenged with 1 ml *C. difficile* 602 (10^3 CFU/ml) intragastrically. The hamsters in group 1 received 1 ml bacteriophage suspension (10^8 PFU) an hour after the *C. difficile* challenge and were given the number 1 Φ , 2 Φ and 3 Φ . Hamsters numbered 1C, 2C and 3C in groups 2 were not administered with bacteriophage and served as controls. Animals were observed for morbidity and mortality. Fecal pellets collected every day and the cecal contents from dead hamsters were processed for *C. difficile* counts as described previously [19]. The bacterial titer was presented as colony forming units (CFU) per gram of feces or cecal content. From each sample twenty individual colonies were randomly picked and were examined for their susceptibility to the lytic activity of Φ CD119 from which the proportion (percentage) of phage susceptible *C. difficile* strains was calculated. *C. difficile* isolates subjected to phage typing were tested for their susceptibility to phages Φ CD119, Φ CD001 and Φ CD006.

2.3. Confirmation of Φ CD119 lysogenization by Southern blot hybridization

Phage Φ CD119 DNA and the chromosomal DNA from the *C. difficile* strains was digested with *HindIII* restriction enzyme and separated on a 0.8% agarose gel by electrophoresis. The separated DNA was then transferred to a positively charged IMMOBILON-NY+ nylon membrane (Millipore, Bedford, MA) by the capillary transfer method [20]. Three *HindIII* digested Φ CD119 fragments (Figure 1B) were gel extracted and were radiolabeled with ^{32}P using the Random prime kit (Roche Applied Sciences). The membrane containing the transferred DNA was hybridized with radio-labeled probe as described previously [20] and the ^{32}P was detected by exposing the membrane on a X-ray film.

2.4. Detecting phage Φ CD119 integration site through PCR

Integration site of Φ CD119 was characterized to be present in between genes CD2693 and CD2694 [15] in the *C. difficile* 602 genome. Hence, forward primer IntF (5' GAAGAATAACAATTTATATGTGTTAT3') that anneal with the 3'prime end of gene CD2693 was used along with the reverse primer IntR (5' GTAACAATCTTAGATACATAAAATTGC3') that anneal in *int* gene of Φ CD119 was used to verify the integration site in the isolated lysogens.

2.5. Toxin typing of Φ CD119 lysogens

Toxin typing of all Φ CD119 lysogens was performed as described by Rupik et al., [21]. Different parts of the PaLoc were amplified using the chromosomal DNA prepared from the Φ CD119 lysogens and the PaLoc specific primers. The fragment A3 was amplified using primer A3C 5'-TATTGATAGCACCTGATTTATATACAAG-3' and A4N 5'-TTATCAAACATATATTTTAGCCATATATC-3'. For amplification of the PL2 fragment the primers Lok3 (5'-TTTACCAGAAAAAGTAGCTTTAA-3') and PrimexB(5'-ACATATTCATCTTCTTGAGTACGA-3') (Figure 2B) were used. To obtain restriction site polymorphisms, each fragment was cut with a set of restriction enzymes. Fragment PL2 was digested with *NsiI* and A3 was cut with *EcoRI*. Fragments were visualized on 1.5% agarose, and the length polymorphisms of cut amplicons were checked.

2.6. Toxins assay

Toxins A and B were measured using the Enzyme linked Immunoassay (ELISA) kit from Meridian Diagnostics Inc., Cincinnati, Ohio. Bacterial cultures were harvested at different time points from *C. difficile* strains by centrifugation and were resuspended in Tris buffer (0.05 M Tris-HCl, pH 7.5). The cytosolic contents of the harvested cells were obtained by sonication followed by brief centrifugation. Equal amounts of cytosolic proteins were taken and their relative toxin A content was determined using the ELISA kit according to the manufacturer's directions. Tris buffer (0.05 M Tris-HCl, pH 7.5) alone was used as a negative control.

3. Results

3.1. *In vivo* lysogenization of phage CD119 in hamsters during infection

The ability of the phage Φ CD119 to lysogenize *C. difficile* in the gut of a mammalian host was tested by feeding hamsters with *C. difficile* followed by the bacteriophage. All the hamsters in the control group, that received *C. difficile* alone, died within 48 hours. The survival days were extended for 5 days in the group that received the Φ CD119 after *C. difficile* challenge. In both groups the *C. difficile* strains isolated from their fecal pellets that were collected a day after phage treatment were 100% sensitive to Φ CD119 (Table 2). All the bacterial strains isolated from the control group remained sensitive to bacteriophage throughout the study. However, in the group that received phage after a day we could

observe a shift in the population of phage sensitive to resistant bacteria. Nearly 80% of the *C. difficile* strain isolated on the second and third day was resistant to phage and all are resistant from the fourth day (Table 2). All the hamsters that received phage were dead on the fifth day after receiving *C. difficile* and phage. Upon autopsy it was found that all hamsters had diarrhea and their ceca were hemorrhagic. The bacteria isolated from each hamster carry the animals' identification number. Two bacterial isolates from each group were then checked for the lysogenization of Φ CD119 by probing their chromosomal DNA with ^{32}P labeled phage DNA fragment. *C. difficile* strains 602, F2, F6 and F10 were also included in the Southern blot analysis. The strains 602 and F10 served as negative and positive controls respectively (Figure 1A). A strong hybridization signal of CD119 phage DNA could be detected in isolates 1 Φ and 2 Φ and confirmed the lysogenization of Φ CD119 in them (Figure 1A). The prophage induced from lysogens 1 Φ and 2 Φ could infect *C. difficile* strains F2, F6, 602 but not F10, further confirms that strains 1 Φ and 2 Φ are lysogenized with phage Φ CD119.

3.2. Toxin typing of lysogenized bacteria

The *C. difficile* 602 is a host strain for bacteriophages, Φ CD119, Φ CD001 and Φ CD006. The lysogens isolated from the hamsters were tested for their susceptibility to phages Φ CD001 and Φ CD006 including Φ CD119. The lysogens were resistant to phage Φ CD119 as mentioned earlier and susceptible to phages Φ CD001 and Φ CD006. These results indicated that the strains isolated from the hamsters are derived from the *C. difficile* 602 strain. For further confirmation we have toxin typed the parent 602 strain along with all the *in vivo* isolated lysogens. An *in vitro* isolated lysogen 602A [16] was also toxin typed and served as a positive control in the protocol. Part of the PaLoc was PCR amplified (Figure 2B) from the isolated lysogens and restriction analysis was performed on the amplicons as described by Rupnik et al. [21]. The fragment A3 covers the C terminal region of the toxin A gene (*tcdA*) and the PL2 fragment contains the *tcdR* gene along with its upstream. Variations in the sequences were observed within this region among different strains [21]. Hence we chose to analyze this region to compare the isolated lysogens. The amplified A3 and the PL2 fragments were 3.1 Kb and 2.0 Kb in size respectively, for the parent 602 strain, as well as for the lysogens (Figure 2A). Restriction digestion of these PCR amplicons gave a similar pattern for the parent strain as well as for all the lysogens. The phage typing and the toxin typing results confirmed that the lysogens isolated from the hamsters were indeed derived from the 602 strain.

3.3. Identification of Φ CD119 integration site through PCR

In *C. difficile* strain 602 the phage Φ CD119 was found to be integrated in the chromosome in between genes CD2693 and CD2694 [15]. We have analyzed all the lysogens isolated from the hamsters for their Φ CD119 integration site in *C. difficile* chromosome. PCR was performed using primers designed from gene CD2693 and the Φ CD119 *int* gene that encodes for integrase. We amplified the 430bps sized expected PCR product that spans the Φ CD119 integration site from the *in vivo* isolated lysogens (Figure 3). The *in vitro* lysogen 602A was used as positive control and 602 parent strain served as a negative control for PCR reactions. The resulted PCR products were sequenced and were verified as the intergenic region between CD2693 and CD2694 as reported earlier [15].

3.4. Toxin production of lysogens

Previously we have shown that *in vitro* lysogenization of Φ CD119 could modulate toxin production in *C. difficile* [16]. We measured the toxins production in the *in vivo* isolated lysogens and found that they also produce less toxins similar to the *in vitro* isolated lysogens. Cytosolic proteins from the lysogens and the parent 602 strain were harvested from overnight cultures grown in BHI medium and the relative amounts of toxins A and B

were determined by ELISA. Tris buffer used for the protein preparation was used as a negative control in the ELISA tests and it recorded zero absorbance. All the three *in vivo* isolated Φ CD119 lysogens produced less toxins than the parent strain (Figure 4).

4. Discussion

The lytic and lysogenic lifestyles of bacteriophages have long been recognized. The term lysogeny refers to the ability of a host cell and the virus to enter a latent stage where they grow together and virus is functionally hidden in the genome as prophages giving little or no detriment to the bacterial host. All *C. difficile* bacteriophages characterized so far are lysogenic in nature [15, 17, 22-25] and DNA sequences of *C. difficile* strains also revealed prophages as part of the highly mosaic genomes where nearly 11% is made of mobile genetic elements [26-28] indicating the co-evolution of *C. difficile* phages with its host. In the genus *Clostridium*, several toxin-encoding genes are located on nonintegrative lysogenic phages and lysogenic phages [29, 30]. The PaLoc carrying toxins encoding genes in *C. difficile* also shows homology to phage genes suggesting its possible phage origin [10, 13, 16, 31]. In a previous study we have showed that a *C. difficile* phage Φ CD119 could down regulate the toxin production in its host strain [16]. Following this observation we have asked the question; can Φ CD119 lysogenize its *C. difficile* host strain *in vivo* during the *C. difficile* infection in a mammalian host? The results from this study suggest that Φ CD119 indeed can lysogenize its host *in vivo* during an infection and these *in vivo* isolated lysogens produced lesser toxins similar to the *in vitro* isolated Φ CD119 lysogens [16].

The lysogeny cycle often increased the potential of the phage survival. The choice between lytic and lysogeny lifestyles is determined to the great extent by the metabolic health of the host bacteria which in turn depends on the growth environment. The role of nutrient availability in the induction of lysogens was studied by Miller and Ripp using *Pseudomonasaeruginosa* and its infecting phage F116 [32]. Increased percent of lysogeny by F116 was observed during the nutrient limited conditions. Another study by John Mittler suggested that in variable environments bacteriophages that choose lysogeny are often favored over those with low probability of establishing lysogeny [33]. Hence, it was suggested that lysogeny is an evolutionary adaptation of bacteriophages for their survival in changing environments [33]. During a bacterial infection, the host often deprives the microorganisms of nutrients as mechanism to control the multiplication of the pathogen. In our study we have isolated lysogens of Φ CD119 phage from hamsters that received *C. difficile* followed by bacteriophage Φ CD119. We assume that the unfavorable growth condition faced by *C. difficile* through various host defenses could have favored the lysogeny of Φ CD119 phage *in vivo*.

The evolution of lysogeny has allowed the creation of a symbiotic relationship between phage and host bacteria. Through lysogeny in many instances the host's phenotype is altered by the addition or loss of various characteristics due to expression of genes that are part of the prophage genome. We have observed a reduced toxin production in the *in vitro* isolated Φ CD119 lysogens when compared to the parent strain. The regulation was carried out through the phage encoded repressor protein, RepR, which controlled *tcdR* [9], the major positive regulator of toxin genes [16]. We have tested the toxin production in the *in vivo* isolated lysogens and found that their toxin production is also affected by Φ CD119 lysogenization. The *in vivo* lysogens were isolated from hamsters whose mortality was due to the infection by lysogens. Hence, although the toxin production was affected moderately due the Φ CD119 lysogenization, it didn't prevent the *C. difficile* strain from causing disease in its host. Challenging the hamsters using the Φ CD119 lysogens directly would help us to determine its relative virulence over the parent *C. difficile* 602 strain and such study is currently under progress.

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Figure 1.

A. Southern hybridization to confirm the presence of Φ CD119 in isolated lysogens. (A) Ethidium bromide-stained gel with *HindIII*-digested DNA. (B) Southern hybridization with a *HindIII*-digested Φ CD119 DNA fragment as a probe (see Materials and Methods). The strains 1 Φ , 2 Φ and 1C, 2C were isolated phage received hamsters and from the control group respectively. The *HindIII*-digested Φ CD119 DNA served as a positive control and 602 served as negative control. B. *HindIII* restriction map of phage Φ CD119. Black arrows indicate a single *Hind III* recognition site and the white arrows indicate multiple *HindIII* sites close to each other. Sizes of the major fragments generated by the digest are marked. Fragments used as probes are highlighted in grey.

Figure 2.

A. Restriction fragment length polymorphisms of the pathogenicity locus (PaLoc) regions amplified from *C. difficile* strains. The PCR amplicons A3 and PL2 represents the *tcdA* C terminal and the *tcdR* region in the PaLoc respectively. The A3 was digested with *EcoRI* and the PL2 was digested with *NsiI* for analysis. **B.** Schematic diagram of PaLoc. Positions of fragments A3 and PL2 and the primers used for their amplification are marked.

Figure 3.

A. Schematic representation of the Φ CD119 integration site in *C. difficile* genome. The relative position of primers IntF and IntR are marked by arrows. **B.** Confirming the integration site of Φ CD119 in lysogens by PCR using primers IntF and IntR.

Figure 4.

Toxins titers, expressed as absorbance units were determined by ELISA. Toxin A production in overnight cultures of Φ CD119 *in vivo* lysogens and *in vitro* lysogens were compared to parental strain. The ELISA signal was recorded as absorbance at 450 nm relative to the background signal, and the data shown are the mean plus standard error of three replicative samples.

Table 1*C. difficile* strains used.

<i>C. difficile</i> Strains	Sensitivity to Φ CD119,	Sensitivity to Φ 1 and Φ 6	Reference #
F10 (Φ CD119Lysogen)	R	S	[15]
F2 (Φ 1Lysogen)	S	R to Φ 1, S to Φ 6	[15]
F6 (Φ 6Lysogen)	R	S to Φ 1, R to Φ 6	[15]
602	S	S	[15]
1 Φ (Φ CD119lysogen)	R	S	This study
2 Φ (Φ CD119lysogen)	R	S	This study
3 Φ (Φ CD119lysogen)	R	S	This study
1c	S	S	This study
2c	S	S	This study
3c	S	S	This study
602A (Φ CD119lysogen)	R	S	16

Hamster # 1C 2C 3C 1 Φ 2 Φ 3 Φ

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