Inducible Clostridium perfringens bacteriophages \( \Phi S9 \) and \( \Phi S63 \)

Different genome structures and a fully functional \( \text{sigK} \) intervening element

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Two inducible temperate bacteriophages \( \Phi S9 \) and \( \Phi S63 \) from Clostridium perfringens were sequenced and analyzed. Isometric heads and long non-contractile tails classify \( \Phi S9 \) and \( \Phi S63 \) in the Siphoviridae family, and their genomes consist of 39,457 bp \((\Phi S9)\) and 33,609 bp \((\Phi S63)\) linear dsDNA, respectively. \( \Phi S63 \) has 3’-overlapping cohesive genome ends, whereas \( \Phi S9 \) is the first Clostridium phage featuring an experimentally proven terminal redundancy and circularly permuted genome. A total of 50 and 43 coding sequences were predicted for \( \Phi S9 \) and \( \Phi S63 \), respectively, organized into 6 distinct lifestyle-associated modules typical for temperate Siphoviruses. Putative functions could be assigned to 26 gene products of \( \Phi S9 \), and to 25 of \( \Phi S63 \). The \( \Phi S9 \) attB attachment and insertion site is located in a non-coding region upstream of a putative phosphorylase gene. Interestingly, \( \Phi S63 \) integrates into the 3’-part of \( \text{sigK} \) in \( C. \) perfringens, and represents the first functional skin-element-like phage described for this genus. With respect to possible effects of lysogeny, we did not obtain evidence that \( \Phi S9 \) may influence sporulation of a lysogenized host. In contrast, interruption of \( \text{sigK} \), a sporulation associated gene in various bacteria, by the \( \Phi S63 \) prophage insertion is more likely to affect sporulation of its carrier.

Introduction

Clostridium perfringens is an anaerobic Gram-positive spore-forming rod, frequently isolated from soil, freshwater sediments, sewage and the gastrointestinal tract of both humans and animals. It is the causative agent of food poisoning and gas gangrene in humans, and enteric diseases in these hosts. Fourteen types of toxins are known so far,

among them, \( \tau \)- (phospholipase C), \( \zeta \), \( \eta \)- and \( \iota \)-toxins are used to classify \( C. \) perfringens into five biotypes (A–E). Others include \( \theta \)-toxin (perfringolysin O), \( \kappa \)-toxin (hyaluronidase), a sporulation-associated (food-poisoning) enterotoxin (CPE), the structure of which was recently solved, and others. The TpeL-toxin, which is produced during sporulation, is another addition to that family. Phenotypic variations among the isolates, such as different toxins produced and various degrees of symptoms severity could mainly be attributed to a high degree of genomic variability, as evidenced from comparative genomic studies using three complete \( C. \) perfringens (biotype A strains) genome sequences (a food poisoning strain \( \text{S13} \), a CPE-negative gas gangrene isolate \( \text{ATCC13124} \) and a CPE-negative and gas gangrene-causing strain \( \text{SM101} \)). In addition to the variation in chromosome-encoded toxin/virulence genes, large plasmids with strain-specific genes were identified, offering insights into a wide range of environmental adaptations and virulence traits. Interestingly, no clear explanation regarding the extremely diverse sporulation efficiencies among the isolates could yet be found. Many of the above mentioned genes appear to be located in mobile elements, or are transferred via conjugational processes.

Bacterial chromosomes contain a significant proportion of prophage sequences, as mobilizable elements. For example, Streptococcus pyogenes features a genome with more than 10% phage-related sequences, and in Escherichia coli O157:H7 strain Sakai, prophage elements account for 16% of the total genome. These elements are involved in horizontal gene transfer and their characteristics offer insights into evolutionary processes of the host. In addition, prophages often encode virulence genes such as toxins, and provide an explanation for various bacterial virulence characteristics among the different strains. Thus far, only 12 \( C. \) perfringens phage sequences are available from public databases. Prophage \( \Phi 3626 \) was the first \( C. \) perfringens phage sequence published, later followed by episomal prophage \( \Phi S101 \) identified in sequenced \( C. \) perfringens genomes. Recently, sequences of phages \( \Phi C39 \) and \( \Phi CP26 \), as well as...
as \( \Phi CP9O \), \( \Phi CP13O \) and \( \Phi CP19O \) were reported.\(^{14,15} \) The Podovirus \( \Phi CPV1 \) was described as the smallest \( C. \) perfringens phage isolated so far, both in terms of particle dimensions and DNA size.\(^{16} \) Three recently described virulent podoviruses feature slightly bigger genomes of approximately 18 kb.\(^{17} \) Some trials using \( C. \) perfringens specific bacteriophages (CPAS-cocktail) to counteract necrotic enterocolitis have been published.\(^{18} \) Phage \( \Phi CP24R \) was described as a small virulent podovirus featuring an 18.92 kb genome.\(^{19} \)

We here report the sequence and analysis of temperate phages \( \Phi S9 \) (\( vB_\text{CpeS-PhiS9} \)) and \( \Phi S63 \) (\( vB_\text{CpeS-PhiS63} \)), induced from \( C. \) perfringens strains \( S9 \) and \( S63 \), respectively. We determined and compared their physical genome structures and phage integration sites. \( \Phi S9 \) was previously reported to influence sporulation of \( C. \) perfringens,\(^{20} \) which prompted us to investigate the effects of lysogenic conversion of \( C. \) perfringens by \( \Phi S9 \) and \( \Phi S63 \).

**Results**

\( \Phi S9 \) and \( \Phi S63 \) are Siphoviruses. Transmission electron microscopy revealed an icosahedral head (60.4 nm in size) and a long non-contractile tail (Figs. 1A–C) for \( \Phi S9 \), placing it into the family Siphoviridae in the order of the Caudovirales.\(^{21} \) The tail structure is rather unusual since it lacks a typical baseplate structure and features a tail fiber cover-like structure (TFC) instead (Fig. 1B, black arrows and 1C). This component was found to quite easily separate from the tail during particle preparation for EM. The tail shaft is 191 nm long and 11 nm wide, and the prominent central tail fiber itself is 46.6 nm long, 3.2 nm wide. Dimension of the TFC is approximately 102 nm long and 10 nm wide (Fig. 1C).

Phage \( \Phi S63 \) also belongs to the Siphoviridae, featuring a 170 nm tail with a diameter of 11 nm and a head of 62 nm diameter (Fig. 1D–G). In contrast to \( \Phi S9 \), the \( \Phi S63 \) tail features a classical baseplate structure (Fig. 1F and G). Putative baseplate spikes are visible at the lower end of the base plate (Fig. 1G). It is interesting to note that in all negatively stained \( \Phi S63 \) particles, very unusual and satellite bubble-like structures are present, arranged around the lower part of the tail just above the base plate, and at the upper portion of the tail just below the head-tail connector (Fig. 1D, black arrows). These structures most likely represent curled tail fibers and/or long whiskers, likely involved in the recognition of and/or interaction with the host cells surface.

Complete nucleotide sequence and genome organization of \( \Phi S9 \) and \( \Phi S63 \). The complete unit genome (not considering possible redundancy of the packaged DNA molecule) of \( \Phi S9 \) features 39,457 bp, which agrees well with the overall size predicted from restriction analysis (Figs. 2 and 3). The GC content of 28.1 mol% is identical to \( C. \) perfringens (28.1–28.4%).

![Figure 1. Transmission electron micrographs of negatively stained \( C. \) perfringens phage particles. (A–C) \( \Phi S9 \) and close-up view of the tail fiber cover-like structure. (D–G) \( \Phi S63 \) and close-up view of the tail adsorption apparatus. Scale bars in images represent 100 nm; scale bars in (F and G) could not be drawn due to size limitations.](image-url)
as determined from sequenced C. perfringens genomes; results not shown). A total of 50 open reading frames with a minimum length of 150 nt were identified in the Φ59 genome (coding capacity 92.0%) (Table 1), which is organized into distinct functional modules (Fig. 2). One putative tRNA<sub>as</sub> gene was found at nt position 10,367 to 10,439. A putative function could be assigned to 26 gene products.

The Φ563 unit genome is 33,609 bp in size, which matches very well with PFGE analysis of full-length phage DNA (data not shown), and reflects the physical size of the packaged molecule (see below). It features a GC-content of 27.5 mol%, slightly less than Φ59 and the Clostridium host strains. A total of 43 open reading frames could be annotated (89.9% of the coding capacity) (Table 1) and a putative function could be assigned to 25. The Φ563 genome is also organized in a lifestyle specific, modular fashion (Fig. 2).

Φ59 contains terminally redundant, circularly permuted genomes, and Φ563 features single-stranded overlapping DNA ends. We determined the genome structure of both phages Φ59 and Φ563. Run-off Sanger sequencing reactions with primers complementary to the ends of the Φ59 single large contig produced sequence complementary to the other end of the contig (data not shown). Ligation of Φ59 DNA prior to digestion and heat treatment (75°C for 10 min) did not alter restriction patterns (Fig. 3A). In addition, Bal31 exonuclease treatment of Φ59 DNA followed by EcoRI or NsiI digestion simultaneously decreased the intensity of all restriction fragments over time (Fig. 3B,22,23) and no specific fragment was shortened. These findings clearly indicated that Φ59 DNA represents a collection of terminally redundant and circularly permuted DNA molecules.

In contrast, when full length Φ563 DNA was subjected to pulsed field gel electrophoresis, it yielded a pattern of unit-size genomes (joined in a concatenated fashion (data not shown)), indicating the presence of self-ligating cohesive (cos) genome ends in these DNA molecules. Heating prior to electrophoresis changed the restriction pattern in a characteristic fashion (Fig. 3C), which also perfectly matched the in silico predictions. Sequencing of a PCR product generated from C. perfringens S63, using primers cos<sub>fl</sub> and cos<sub>re</sub> (Table S1), and comparison to sequence generated with the same primer pair using linear Φ563 DNA yielded the precise structure and sequence of the terminal single-stranded cos site region (Fig. 3D), featuring 3′-overhangs of 11 nt (CGCAGTGTCTA).

Bioinformatic analyses and relationship of ΦS9 and Φ563 to other phages. Only few similarities were found among Φ59 and Φ563, and also to other C. perfringens prophages. The two apparently unrelated viruses feature significant similarities only in the lysogenic control region (integrase and represor), and the endolysin enzymes. However, proteins of both phages feature several homologies to Siphoviruses of other Firmicutes, such as Listeria, Streptococcus and Bacillus,24–26 as well as to (often cryptic) prophages identified in the genomes of these organisms. Some of the Φ59 structural genes show sequence homology to Brachorhizus phage BL3 (e.g., gp38),27 while some of the early genes feature homologies to Listeria phages A118, A006 and A500.27,28

All predicted gene products encoded by the two phages and putative functional assignments are listed in Tables S2 and S3. A phylogenetic tree of large terminase subunit amino acid sequences can serve as measure of similarity in DNA packaging strategy and relatedness between phages.29 A tree generated of

Table 1. Synopsis of published Clostridium perfringens phages

| Name   | Family   | Genomic size (Mb) | ORFs | Dimensions (nm) | Genome structure | Integration (proct/lifestyle) | Reference                      |
|--------|----------|-------------------|------|-----------------|-----------------|-------------------------------|--------------------------------|
| Φ59    | Siphoviridae | 39.46             | 50   | 60 × 190        | c.p., t.r.      | noncoding, temperate          | This work                      |
| Φ563   | Siphoviridae | 33.61             | 43   | 62 × 170        | 3′ cos          | sige, temperate                | This work                      |
| S63    | Siphoviridae | 33.51             | 50   | 55 × 170        | 3′ cos          | good, temperate               | Zimmer et al. 2000             |
| S9     | Siphoviridae | 39.59             | 62   | 57 × 100        | unknown         | n.d.                          | Oakley et al. 2011             |
| S130   | Siphoviridae | 38.33             | 66   | 57 × 100        | unknown         | n.d.                          | Oakley et al. 2011             |
| S269   | Siphoviridae | 39.19             | 62   | 57 × 100        | c.p., t.r. (putative) | n.d.                          | Seal et al. 2011, Oakley et al. 2011 |
| S340   | Siphoviridae | 38.31             | 65   | 57 × 100        | n.d.                          | n.d.                          | Seal et al. 2011, Oakley et al. 2011 |
| S390   | Siphoviridae | 38.75             | 62   | 57 × 100        | c.p., t.r. (putative) | n.d.                          | Oakley et al. 2011             |
| S101   | Siphoviridae | 38.09             | 54   | n.d.             | n.d.              | temperate                      | Myers et al. 2006              |
| CP4    | Podoviridae | 18.92             | 22   | 44              | inverted t.r. (possible terminal protein) | none (virulent) | Morales et al. 2011 |
| CPV1   | Podoviridae | 16.75             | 22   | (42 × 23) × 37  | inverted t.r. (predicted) | none (virulent) | Voloshantsev et al. 2011 |
| CPV4   | Podoviridae | 17.97             | 26   | 40–42 × 35–38   | inverted t.r. (predicted) | none (virulent) | Voloshantsev et al. 2012 |
| CP2    | Podoviridae | 18.08             | 27   | 40–42 × 35–38   | inverted t.r. (predicted) | none (virulent) | Voloshantsev et al. 2012 |
| CP7R   | Podoviridae | 18.40             | 28   | 40–42 × 35–38   | inverted t.r. (predicted) | none (virulent) | Voloshantsev et al. 2012 |

n.d., not determined; c.p., circularly permuted; t.r., terminally redundant.
109 terminase sequences (Fig. S1) placed WS101 with WC3626 in close relation to WS63 in the branch of 3’ cos-phages. WS9 and WCS9O cluster in the headful packaging branch and phages WCP9O, WCP13O, WCP26F and WCP34O form an own branch in the tree. These findings confirm experimentally evaluated packaging strategies and overall relatedness of the phages. No terminase sequences were available for phages WCP7R, WCP24R, WCVPV4 and WZP2 and none could be predicted by homology searches.

The WS9 attB lies in an intergenic region, whereas WS63 inserts into sigK. Because genome sequences of C. perfringens strains S9 and S63 have not been available, the insertion sites used by WS9 and WS63 were identified by inverse PCR from self-ligated C. perfringens S9 or S63 genomic DNA fragments (Fig. 4), and comparison to non-lysogenic host DNA. In the case of WS9, the sequence matched a region located next to a different prophage-like element (referred to as W13124). Phage WS9 integrates into the non-coding intergenic sequence, 541 nt downstream of a gene encoding a putative phosphorylase, and 157 nt upstream of a hypothetical protein. The core sequence of WS9 integration is TTACATATTTG (Fig. 4A), which is similar in length to those used by Clostridium phages WC3626 (12 bp), FC2 (11 bp) and FC3119 (14 bp).13,27,28

The same approach was used to identify the insertion site for WS63 in C. perfringens S63 (Fig. 4B). The GTAATGAAAT 10 nt core of the attB sequence is located at nt position 427 from the 5’ end and nt 265 from the 3’ end, and the insertion region features significant homology to sigK from C. perfringens S13,7 ATCC 131246 and SM101.6

C. perfringens strain ATCC 13124 harbors two prophages. In the course of our in silico analyses, we identified region 1088991–1128198 (corresponding to CPF_0926–CPF_0977) of strain ATCC131247 as a putative 39,208 bp prophage genome, which was designated W13124. Surprisingly, a putative W13124 integrase (CPF_0926) was found 100% identical to the WS9 integrase. Moreover, the beginning and end of the W13124 attP sequence matched the WS9 attP, and overall good sequence homologies were found between the two phages. Sequence alignment with WS63 indicated another putative prophage sequence in ATCC 13124 (termed W13124_2), located on a genomic island in between positions 1783746 to 1820131 of the ATCC 13124 genome.6 The putative W13124_2 genome is 36,385 bp in size, and this prophage sequence is also flanked by the attachment site used by WS63.

Discussion

The high degree of genomic variation and phenotypic diversities among bacteria appears to be mediated by mobile elements such as prophages and transposons. The ability of these elements to insert into the bacterial genome can alter the host's genetic makeup, leading to changes in its phenotypic traits. In the case of C. perfringens, the identification of two putative prophages, W13124 and W13124_2, further supports the hypothesis that these mobile elements play a crucial role in the evolution and adaptation of this bacterium. The close relationship of these prophages to other known phages, as indicated by the phylogenetic analysis, suggests a shared evolutionary history. Understanding the mechanisms by which these mobile elements integrate into the bacterial genome and the specific roles they play in bacterial physiology is essential for the development of targeted antibacterial strategies.
as conjugative plasmids, transposons and insertion elements. Although lysogenic conversion was established for numerous bacterial species including Clostridia, it has not yet been observed for C. perfringens, possibly due to a lack of data regarding temperate C. perfringens phage.

We here describe the two heterogeneous Siphoviridae WS9 and WS63. Compared with the other studied C. perfringens phages, WS9 and WS63 feature significantly larger head diameter and tail length. Interestingly, both also feature unusual tail-associated appendices, which probably assume functions comparable to tail fibers and whiskers. WS9 possess the second-largest genome of all known C. perfringens phages, and has been shown to represent a collection of terminally redundant and circularly permuted DNA molecules. In contrast, WS63 features identical unit-length genomes with cohesive ends, similar to WS6326. Most of the sequence-based similarities exist to proteins of other (putative) prophages infecting members of the Firmicutes, namely Streptococcus, Lactococcus, Bacillus, Staphylococcus, Listeria, Brochothrix and other Clostridium species (Tables S2 and S3). Altogether, these findings clearly indicate horizontal gene transfer among the ancestors of the bacterial host and their mobile genome element, i.e., the prophages. Likewise, the surprisingly few homologies between WS9 and WS63, and to other known C. perfringens phages can be explained by divergent evolution of these phages from a distant ancestor. Interestingly, WS9 and WS63 feature a virtually identical endolysin (95.3% amino acid identity), which has most likely been acquired by a more recent horizontal gene exchange. Also, the endolysin of WS63 is 98% identical to the murein hydrolase of the episomal C. perfringens phage WS63101, strongly suggesting a modular exchange of
Functional units. Altogether, the significant heterogeneity among *C. perfringens* phages emphasizes the need for more sequences in order to obtain a better overview of this probably large and diverse group of viruses infecting and interacting with an important pathogen.

Homology searches with *WS9* sequences identified prophage *WS13124* in the genome of *C. perfringens* ATCC 13124. Based on significant homology over wide areas of the genome, the two phages appear to have a common origin, and are clearly different from *WS63* and *WS3626*. Similarities of *WS9* and *WS13124* include almost identical integrases and repressors, tail structural components, the holin-endolysin dual lysis module, and the major capsid protein. *WS13124* is inserted within the largest genomic island (243 kb) of the host bacterium, which also contains genes responsible for iron transport, fucose utilization, and glycolytic activities,6 enabling this strain to exploit various environments.6 Interestingly, it also contains the sporulation-related genes *cotJB* and *cotJC*, as well as some putative virulence factors such as a sialidase located near the right arm of *WS13124*. Whether prophage *WS13124* is able to mobilize these closely positioned genes by either a faulty phage excision or generalized transduction is, however, speculative and needs more investigation.

Another putative prophage *WS13124_2* in the ATCC 13124 genome6 was identified using sequence alignment with the *WS63* genome, sharing extensive sequence similarity among most of the structural proteins. The lack of homology in lysogeny control or DNA replication proteins suggested a more distinct evolution of these two phage sequences. However, it remains to be determined if *WS13124_2* is a functional virus, in contrast to the frequent occurrence of defective or cryptic remnants of inserted phage.

Lysogenic conversion may result from expression of genes located on an inserted phage genome,10,29 or by integration and disruption of coding sequence.22,27 The putative effect of *C. perfringens* phage *WS9* on sporulation of its lysogenized host has been subject of discussion over many years. Stewart and Johnson (1977) claimed that curing of *WS9* from *C. perfringens* strain S9 delayed sporulation, while a re-lysogenized strain S9CR restored the sporulation competent phenotype.20 This suggested possible lysogenic conversion of *C. perfringens* by *WS9*. Unfortunately, we were unable to confirm this hypothesis, i.e.,
we found no indication for lyogenic conversion of C. perfringens by phage Φ59. The presence or absence of prophage Φ59 in the C. perfringens strain S9 genome did not significantly influence the onset of production of heat-resistant spores or the total number of spores produced under experimental conditions similar to those published previously29 (Kim K.-P., unpublished data).

The integration site of Φ59 is different from the Φ5620,21 and Φ563 attachment sites, and lies in an intergenic region upstream of a gene encoding a putative membrane protein of unknown function (BC190/C925 in ATCC 13124, and downstream of a putative phosphorlase-encoding gene.

In contrast, phage Φ563 integrates into a B. subtilis sigK-like gene, which encodes a RNA polymerase sigma factor involved in the late stage of spore formation. SigK directs the Stage IV to Stage V transition, i.e., the spore coat formation in the sporulation phase. SigK was shown to be required for the production of exosporium.33 Previous studies demonstrated that Φ563 integration removers the pro-sequence in B. subtilis and is created by splicing and the excision of a sigK intervening sequence (skin element). Interruption of sigK by this prophage-like sequences has been reported not only for B. subtilis (skin36) but also for C. difficile (skin35) and C. tetani (skin37). It should be noted that C. perfringens strain S9 lacks a complete gene of sigK; however, the presence of a skin element has been described in C. perfringens. Phage Φ563 integrates in the opposite direction of sigK, similar to the situation in B. subtilis and C. tetani,37 but different to C. difficile.35 There also seems to be some variety regarding the exact insertion locus; while the integration sites of Φ563, skin35 and skin37 are at a similar location within the coding sequence, skin36 is located in a different region of sigK.36 It was found that a specific recombinase can excise the B. subtilis skin element from sigK.38 Our findings also demonstrate precise excision, resulting in reconstitution of native sigK (Fig. 4).

Although these observations point to an important role of this insertion element for control of sigK function and a potential influence on sporulation, while it was reported that, insertion is not required for sporulation in B. subtilis,36 it is needed in C. difficile. A possible explanation is a missing sigK pro-sequence in C. difficile, which lacks an N-terminal position that needs to be cleared in order to activate SigK.21 sigK of strain S63 is not different from other C. perfringens strains (the pro-sequence is present), similar to the situation in B. subtilis.20,21 This would suggest that their interruption might not be strictly required for successful sporulation of the host cell. However, the sequenced C. perfringens strains do not contain a putative SpolIVF homolog, which is necessary to remove the pro-sequence in B. subtilis.20 A reliable sporulation model for strain S63 is not available, and the precise sporulation phenotype of the Φ563 sigK integration remains to be elucidated.

Materials and Methods

Bacterial strains and growth. C. perfringens strains used in this study included S13,13,23 S9,17 C. tetani 236213 and ATCC 13124. Strains were anaerobically grown in TGY medium (3%, tryptone peptone; 2%, glucose; 1%, yeast extract; 0.1% cysteine, pH 7.4) at 37°C in a flexible vinyl glove chamber (Coy Laboratories), containing a 96% N2 and 2% H2 atmosphere, E. coli DH5α MCR and XL1-blue MRF+ (Invitrogen) were grown in Luria-Bertani medium (LB) (1% tryptone peptone; 1% NaCl, 0.5% yeast extract) at 37°C. If required, media were supplemented with ampicillin (100 μg/ml) or tetracycline (18 μg/ml).

UV induction and preparation of Φ59 and Φ563 stocks. To induce temperate phages Φ59 and Φ563, C. perfringens S9 and S63 were grown to exponential growth phase, and exposed to UV light (254 nm) in a UV-500 Crosslinker (Amersham) for a strain at 2 J/cm2. An equal volume of TGY medium was added to the culture, and bacteria were incubated for 2 h at 37°C, followed by centrifugation (14,000 ×g, 5 min) and filter-sterilization (0.2-μm pore size). Serially diluted phage-containing lysates were mixed with C. perfringens strain S13 indicator cells, and plated using soft-agar overlays.37 After overnight incubation, distinct plaques were picked and eluted with SM buffer (50 mM TRIS-HCl (pH 7.5), 100 mM NaCl, 8 mM MgSO4). The procedure was repeated twice. Initial stocks of Φ59 or Φ563 were prepared by plating the single plaque eluates onto C. perfringens S13, and elution of the entire soft agar layer with SM buffer. Cell debris was removed by centrifugation, and the phage suspension was filter-sterilized and stored at 4°C.

Propagation and purification of phages. For phage Φ59, exponentially growing cells of C. perfringens strain S13 in both culture were infected with Φ59 at a multiplicity of infection (MOI) of 1, and incubated for 8 h at 37°C. Phage Φ563 was propagated by the agar overlay method and removed by eluting the phage particles with 4 ml SM-buffer per plate. Following centrifugation of the lysates at 6,000 ×g for 10 min, 8% (w/v), polyethylene glycol (PEG, MW 8,000) and 0.5 M NaCl were added to the supernatant and incubated overnight at 4°C. After centrifugation (10,000 ×g, 10 min), the supernatant was removed and precipitated phage particles resuspended in SM buffer, followed by step CeCl3 density gradient centrifugation (76,000 ×g, 18 h, L-60 Ultracentrifuge, Beckman) as previously described.37 Finally, virus particles were removed and dialyzed against SM buffer (pore size 50,000 Da, Spectrum) overnight at 4°C.

Electron microscopy. Purified phage particles were negatively stained with either 2% uranyl acetate, or 2% Na-phosphotungstic acid, or 2% ammonium molybdate.37 Samples were observed in a Philips CM100 transmission electron microscope at 100 kV acceleration voltage (FEI Company), equipped with a TVIPS Fastscan CCD camera (Tietz Systems), or in a Tecnai G2 Spirit electron microscope at 120 kV equipped with an EAGLE CCD camera (FEI Company).

Cloning, nucleotide sequencing, and genome analysis. Phage genomic DNA was prepared by pronase K (Fermentas) treatment of purified phage particles, and subsequent organic extraction as described elsewhere.24 Genomic shotgun libraries of Φ59 and Φ563 were constructed as previously described.23,25 Briefly, partial restriction digestion (Topo9001) (New England Biolabs) or complete digestion with HindIII (Fermentas) or TaqI (Fermentas) were performed, fragments of 1 to 2.5 kb in length were separated on agarose gels (0.8%), eluted using QIAquick Gel...
Identification of attB and attP. Identification of $\Phi 59$ attB and attP was performed as previously described,6,7 using two divergent primers inv3a and inv10 (Table S1), derived from the 3' end of the putative $\Phi 59$ integrase (int). The $\Phi 59$ template DNA was first digested with TaqI (Fermentas), and self-ligated using T4 DNA ligase (New England Biolabs). The PCR product was cloned into the pGEMT-easy TA vector (Promega), yielding $\Phi 59$ attB fragments. For confirmation of prophage presence or absence in the identified locus, PCR with $S9attBP-3$ and $S9attBP1-1$ was performed on $\Phi 59$ attP-3 (homologous to $C.\ perfringens$ ATCC 13124 sequence facing the integration site) were used to confirm the prophage location. For confirmation of prophage presence or absence in the identified locus, PCR with $S9attBP-3$ and $S9attBP1-1$ was performed on genomic DNA of the $\Phi 59$ lysogen, and of a $\Phi 59$ cured strain ($\Phi 59\Phi 59$) (Fig. 4).

A similar strategy was used to identify the attB and attP of phage $S63$. After digestion of $S63$ DNA with MboI (Fermentas) and self-ligation, inverse PCR was performed using primers orf23_fw, orf23_rev, both homologous to downstream sequence of the putative $S63$ integrase gene. Using alignments with sequence obtained by inverse PCR with primer pair int_fw and int_rev corresponding to the phage integrase, the transition point from phage to host DNA was identified. Results were confirmed by sequencing of PCR products generated with primer combinations $S63\_att25 + orf23\_fw$ and $S63\_att24 + int\_fw$, as well as sigK_upstr and sigK_dstr on $C.\ perfringens$ lysozyme DNA (Fig. 4).

Bioinformatic analyses. CLC Genomics Workbench Version 5.1 (CLC, Aarhus, Denmark) was used for analysis of nucleotide (nt) and amino acid (aa) sequences. The BLAST algorithms8 were used for similarity searches in the non-redundant protein and nucleotide sequence databases available through the NCBI website (http://www.ncbi.nlm.nih.gov). HHPred (http://toolkit.tuebingen.mpg.de/hhpred) was used for additional homology and structure predictions. The integrated ChetaW algorithm of CLC Genomics Workbench was used for multiple sequence alignments and comparisons.9 InterProScan (http://www.ebi.ac.uk/InterProScan/) was used to identify conserved domains in translated Orfs, and TMHMM protein analysis software (version 2.0) was used to predict transmembrane domains.10 Putative tRNA genes were identified using tRNAScan SE.11

Nucleotide sequence accession numbers. The DNA sequences reported here appear in GenBank under accession number AY082689 ($\Phi 59$), JQ660954 ($S63$) and JQ660953 (partial sequence of sigK gene of strain $S63$).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Material
Supplemental materials may be found at:
www.landesbioscience.com/journals/bacteriophage/article/21363

References
1. Petit S, Gilbert MR, Poggi MI. Clostridium perfringens toxiroxin and genetics. Trends Microbiol 1999; 7: 184-190. PMID:10400308. http://dx.doi.org/10.1016/S0966-842X(99)01439-8
2. Briggs DC, Naylor CE, Smedley JG, 3rd, Lukoyanova ML, Gans S, Awdeh ZL, et al. Structures of the food-disease enterotoxins: Pore-forming toxins from Clostridium perfringens. J Mol Biol 2011; 415:33-49. PMID:21306505. http://dx.doi.org/10.1016/j.jmb.2011.07.046
3. Kitadokoro K, Nishimura K, Kamitani S, Fukui-Nakamura S, Yamashita A, Shiba T, et al. Complete genome sequence of the neurotropic enterotoxin-producing Clostridium perfringens strain. J Bacteriol 2011; 193:10742-55. PMID:21848993. http://dx.doi.org/10.1128/JB.01211-11
4. Aminov R, Novo T, Ochla E, Stanina M. A novel toxin homologue in $C.\ enterotoxigenes$ found in out-of-group type-C isolates. J Microbiol 2007; 153:139-206. PMID:17727975. http://dx.doi.org/10.1051/jom:200602287.0
5. Pandeo-Salip D, Sarker N, Sarker MR. Clostridium perfringens iota toxin is expressed during colonization. Microbiol Peplin 2011; 51:846-9. PMID:21380165. http://dx.doi.org/10.1016/j.miba.2011.05.012
6. Myers GS, Bank DA, Chenke JK, Rendell J, Sehidi R, Dohme RT, et al. Novel genomic variability in strains of the enteric-bacterium pathogenic $C.\ perfringens$. Genome Res 2006; 16:1531-40. PMID:16424845. http://dx.doi.org/10.1101/gr.5328106
7. Shimizu T, Ohnishi K, Hayakawa M, Ohtsuka K, Yamasaki A, Ishihara T, et al. Complete genome sequence of Clostridium perfringens, an anaerobic food-borne pathogen. Proc Natl Acad Sci U S A 2008; 105:9956-61. PMID:18673984. http://dx.doi.org/10.1073/pnas.0801234105
8. Bannan TL, Tong WL, Balch D, Dian D, Road JJ. Functional identification of conserved and replication regions of the tetracycline resistance plasmid (pCCR) from Clostridium perfringens. J Bacteriol 2006; 188: 4942-51. PMID:16798202. http://dx.doi.org/10.1128/JB.188.15.1585-1598.2006
9. Bannan TL, Balch D, Bubains KD, et al. Hoff JS, Minamata M, BD, et al. Genome sequence of a nontype M3 strain of group A Streptococcus phage-encoded toxins, the high-virulence phenotype, and clone emergence. Proc Natl Acad Sci U S A 2002; 99:1073-83. PMID:11822386. http://dx.doi.org/10.1073/pnas.013422099
10. Hayashi T, Makino K, Ohnishi M, Kurokawa K, Ishii T, Niinosaka Y, et al. Complete sequence of Clostridium perfringens 63 (JQ660939), the closest relative of the tetracycline resistance plasmid in Clostridium perfringens type A. New food-borne human gastrointestinal disease isolates. J Bacteriol 2006; 188: 1585-98. PMID:16424846. http://dx.doi.org/10.1128/JB.188.4.1585-1598.2006
11. Bannan TL, Balch D, Bubains KD, et al. Hoff JS, Minamata M, BD, et al. Genome sequence of a nontype M3 strain of group A Streptococcus phage-encoded toxins, the high-virulence phenotype, and clone emergence. Proc Natl Acad Sci U S A 2002; 99:1073-83. PMID:11822386. http://dx.doi.org/10.1073/pnas.013422099

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21. Ackermann HW. Tailed bacteriophages: the order Picovirinae. In: Bacteriophages, 2nd ed. Academic Press, 1973.

22. Klausing J, Knappe J, Reinhardt R, Delong M, Zinov M, et al. The normally redundant, non-permitted gene of bacillus subtilis AS1-1 a model for the SPO1-like recombination of phage lysogens. J Bacteriol 2008; 190:1575-85. PMID: 18357646. http://dx.doi.org/10.1128/JB.00441-08

23. Diener J, Klausing J, Reinhardt R, Scholz T, Blau V, Zimmer M, et al. Comparative genome analysis of Clostridium difficile bacteriophages reveals extensive horizontal gene transfer, coordinated transcriptional regulation, and novel prophage insertion sites. Nat Biotechnol 2009; 27:976-80. PMID: 19479702. http://dx.doi.org/10.1038/nbt.1514

24. Chain J, Donker-De Pauw E, Van der Heyden A, De Smedt S, et al. The permuted genome of Listeria monocytogenes. J Bacteriol 2008; 190:4289-94. PMID: 18484356. http://dx.doi.org/10.1128/JB.00892-08

25. Klausing J, Reinhardt R, Skovgaard T, Scholz T, et al. Genome-scale transcriptome analysis reveals unusual transcriptional regulation in Clostridium difficile bacteriophages. J Bacteriol 2009; 191:7206-15. PMID: 19169167. http://dx.doi.org/10.1128/JB.01041-09

26. Klenk PH, Rehman M, Skowronek A, Balows A, et al. The generalized transducing bacteriophage phiC2 and comparisons to phiCD119 and complete genome sequence of Clostridium difficile. J Bacteriol 2003; 185:676-85. PMID: 12775872. http://dx.doi.org/10.1128/JB.185.3.676-85.2003

27. Miller RW, Skinner EJ, Sulakvelidze A, Mathis GF, et al. Comparative genomics of Clostridium difficile requires disruption of the sigmaK gene. Mol Microbiol 2003; 48:811-21. PMID: 12756846. http://dx.doi.org/10.1046/j.1365-2958.2003.03662.x

28. Schicklmaier P, Schmieger H, Pedulla ML, et al. The mosaic genomes and utilize unique prophage insertion strategy. J Bacteriol 2005; 187:1091-104. PMID: 15756406. http://dx.doi.org/10.1128/JB.187.3.1091-1094.2005

29. Sakaguchi Y, Hayashi T, Kurokawa K, Nakayama K, et al. The Bacillus subtilis sporulation recombinase gene. Genes Dev 2000; 14:1640-51. PMID: 10949803. http://dx.doi.org/10.1101/gad.800802

30. Paredes CJ, Alsaker KV, Papoutsakis ET. A comparative analysis of Corynebacterium glutamicum Type A and Type B beta-lactamase genes. J Bacteriol 2001; 183:4421-30. PMID: 11379456. http://dx.doi.org/10.1128/JB.183.13.4421-4430.2001

31. Sonenshein AL, Haraldsen JD, Dupuy B. RNA polymerase and alternative sigma factors. In: Handbook on Clostridia. Boca Raton: CRC Press, 1999. 15-93.

32. Schicklmaier P, Schmieger H, Pedulla ML, et al. The Bacillus subtilis sigmaK gene. Mol Microbiol 2003; 48:811-21. PMID: 12756846. http://dx.doi.org/10.1046/j.1365-2958.2003.03662.x

33. Kunkel B, Losick R, Stragier P. The Bacillus subtilis sporulation transcription factor. Science 1989; 243:1170-3. PMID: 2669694. http://dx.doi.org/10.1126/science.243.4884.1170

34. Zinkernagel RM, Ohashi PS. The cellular immune response. Annu Rev Immunol 2004; 22:347-77. PMID: 15304561. http://dx.doi.org/10.1146/annurev.immunol.22.021703.144443

35. Sakaguchi Y, Okazaki Y, Ogasawara K, et al. The Bacillus subtilis sporulation sigma factor precursor Pro-sigma K in the absence of other sigE gene products. J Bacteriol 1995; 177:1082-5. PMID: 7864057. http://dx.doi.org/10.1128/JB.177.3.1082-1085.1995

36. Moser GI, Kirkland MN. The use of transposon Tn5 phage P22 for large-scale virus purification. Virology 1970; 40:734-5. PMID: 508228. http://dx.doi.org/10.1016/0042-6822(70)90218-7

37. Noller FH. Three-dimensional structure of ribosomes. Annu Rev Biophys Chem 1976; 7:293-311. PMID: 960749. http://dx.doi.org/10.1146/annurev.bi.07.020176.001531

38. Mechanic CA, Bosisio DH, Costanza ME, et al. A comparative genomics approach reveals expansion of the tRNA gene repertoire. Proc Natl Acad Sci U S A 2008; 105:2487-72. 1073/PNAS.0505503102

39. Weil MA, de Lamballerie X, Mailhot C, et al. Large-scale virus purification. Virology 1970; 40:740-5. PMID: 508229. http://dx.doi.org/10.1016/0042-6822(70)90219-9

40. Yamamoto KR, Alberts BM, Benzinger R, Lawhorne L, Troger G. Rapid bacteriophage salivation in the presence of polyethylene glycol and in application to largescale virus purification. Virology 1970; 40:736-40. PMID: 508230. http://dx.doi.org/10.1016/0042-6822(70)90220-5

41. Butin AE, Weis JJ, Leson PG, et al. Generation of a composite Mu array that allows the expression of an inverse polymerase chain reaction. Proc Natl Acad Sci U S A 1985; 82:1792-7. PMID: 2890728. http://dx.doi.org/10.1073/pnas.82.5.1792

42. Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press, 2001.

43. Osbourn H, Golub AL, Hart DL. Genetic applications of a tRNA polymerase phosphoesterase. Gene 1988; 68:201-5. PMID: 3379838. http://dx.doi.org/10.1016/0378-1119(88)90115-5

44. Larkin MA, Blackshields G, Brown NP, Chenna R, et al. Clustal X version 2.0. Bioinformatics 2007; 23:2947-54. 17472-7; PMID:17846036; http://dx.doi.org/10.1093/bioinformatics/btm401

45. Clustal W and Clustal X version 2.0. Bioinformatics 2007; 23:3389-402; PMID:7622(88)90246-X

46. Broch T, Jorgensen J, Givskov M, et al. Randomised sampling of naturally occurring enteric cerebrosides isolated from human faeces. Carbohydr Res 2005; 340:53-61. PMID:15848396. http://dx.doi.org/10.1016/j.carres.2004.10.005

47. Miller RW, Skinner EJ, Sulakvelidze A, Mathis Gf, et al. Comparative genomics of Clostridium difficile requires disruption of the sigmaK gene. Mol Microbiol 2003; 48:811-21. PMID:12756846. http://dx.doi.org/10.1046/j.1365-2958.2003.03662.x

48. Blumenthal T. Tailored bacteriophages the under considered alternative. Adv Virus Res 1998; 50:1-29. PMID:9706187. http://dx.doi.org/10.1016/S0065-2664(08)50002-1

49. Ackermann HW. Tailed bacteriophages and lysogenic conversion. Adv Virus Res 1977; 20:99-105. PMID:2101976. http://dx.doi.org/10.1016/0065-2664(77)90003-5

50. Klausing J, Knappe J, Reinhardt R, Delong M, Zinov M, et al. The normally redundant, non-permitted gene of bacillus subtilis AS1-1 a model for the SPO1-like recombination of phage lysogens. J Bacteriol 2008; 190:1575-85. PMID: 18357646. http://dx.doi.org/10.1128/JB.00441-08