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A *Clostridioides difficile* bacteriophage genome encodes functional binary toxin-associated genes

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ABSTRACT

Pathogenic clostridia typically produce toxins as virulence factors which cause severe diseases in both humans and animals. Whereas many clostridia like e.g., *Clostridium perfringens*, *Clostridium botulinum* or *Clostridium tetani* were shown to contain toxin-encoding plasmids, only toxin genes located on the chromosome were detected in *Clostridioides difficile* so far. In this study, we determined, annotated, and analyzed the complete genome of the bacteriophage phiSemix9P1 using single-molecule real-time sequencing technology (SMRT). To our knowledge, this represents the first *C. difficile*-associated bacteriophage genome that carries a complete functional binary toxin locus in its genome.

1. Introduction

Spore-forming pathogenic clostridia typically produce toxins as virulence factors which can cause severe diseases of both humans and animals. These toxins are often located on the chromosome, but in some cases they were also found to be encoded on plasmids, e.g., in *Clostridium perfringens* (Adams et al., 2014; Gurjar et al., 2010; Li et al., 2007, 2013; Miyamoto et al., 2006, 2008, 2011; Popoff and Bouvet, 2009; Rood, 1998, 2004; Sayeed et al., 2007, 2010), *Clostridium botulinum* (Adams et al., 2014; Hill et al., 2007; Hill and Smith, 2013), *Clostridium tetani* (Adams et al., 2014; Brüggemann et al., 2003; Finn et al., 1984; Popp et al., 2012) or *Paeniclostridium sordellii* (formerly *Clostridium sordellii*) (Couchman et al., 2015; Sasi Jyothsna et al., 2016).

In contrast, only little is known about the presence and functions of extrachromosomal elements in *Clostridioides difficile* (formerly *Clostridium difficile*) (Lawson et al., 2016). Plasmids of sizes between 4.5 and 75 kb were identified in a few isolates of *C. difficile* (Muldrow et al., 1982). The model strain 630 harbors a cryptic plasmid pCD630 which encodes eleven open reading frames associated with phage genes (Sebaihia et al., 2006; Monot et al., 2011). This plasmid got subsequently lost in this strain (Riedel et al., 2015a; Dannheim et al., 2017). The plasmid pCD630 was not reported to occur in the erythromycin-sensitive derivative *C. difficile* strain 630Δ*erm* (Hussain et al., 2005; Van Eijk et al., 2015), but was detected during a recent resequencing study of the same strain 630Δ*erm* (Hussain et al., 2005; Dannheim et al., 2017). Another well-characterized *C. difficile* plasmid is the 6.8 kb plasmid pCD6 isolated from *C. difficile* strain CD6. In contrast to pCD630 (Sebaihia et al., 2006; Dannheim et al., 2017) the plasmid pCD6 encodes a *repA* plasmid replication gene (Purdy et al., 2002). In addition to plasmids, very few studies reported extrachromosomal bacteriophage sequences in *C. difficile*: Strain DSM 1296^T was found to encode two extrachromosomal elements associated with bacteriophages (Riedel et al., 2015b; Wittmann et al., 2015), and in strain BI1 two putative extrachromosomal bacteriophage-associated elements were detected (He et al., 2010).

In comparison to other clostridia no extrachromosomal element encoding toxin genes was found in *C. difficile* so far. In this study, we determined, annotated, and analyzed the complete genome of the *C. difficile* bacteriophage phiSemix9P1 using single-molecule real-time (SMRT) sequencing technology. To our knowledge, this represents the first *C. difficile* associated bacteriophage genome

Abbreviations: CdtLoc, *C. difficile* binary toxin locus.

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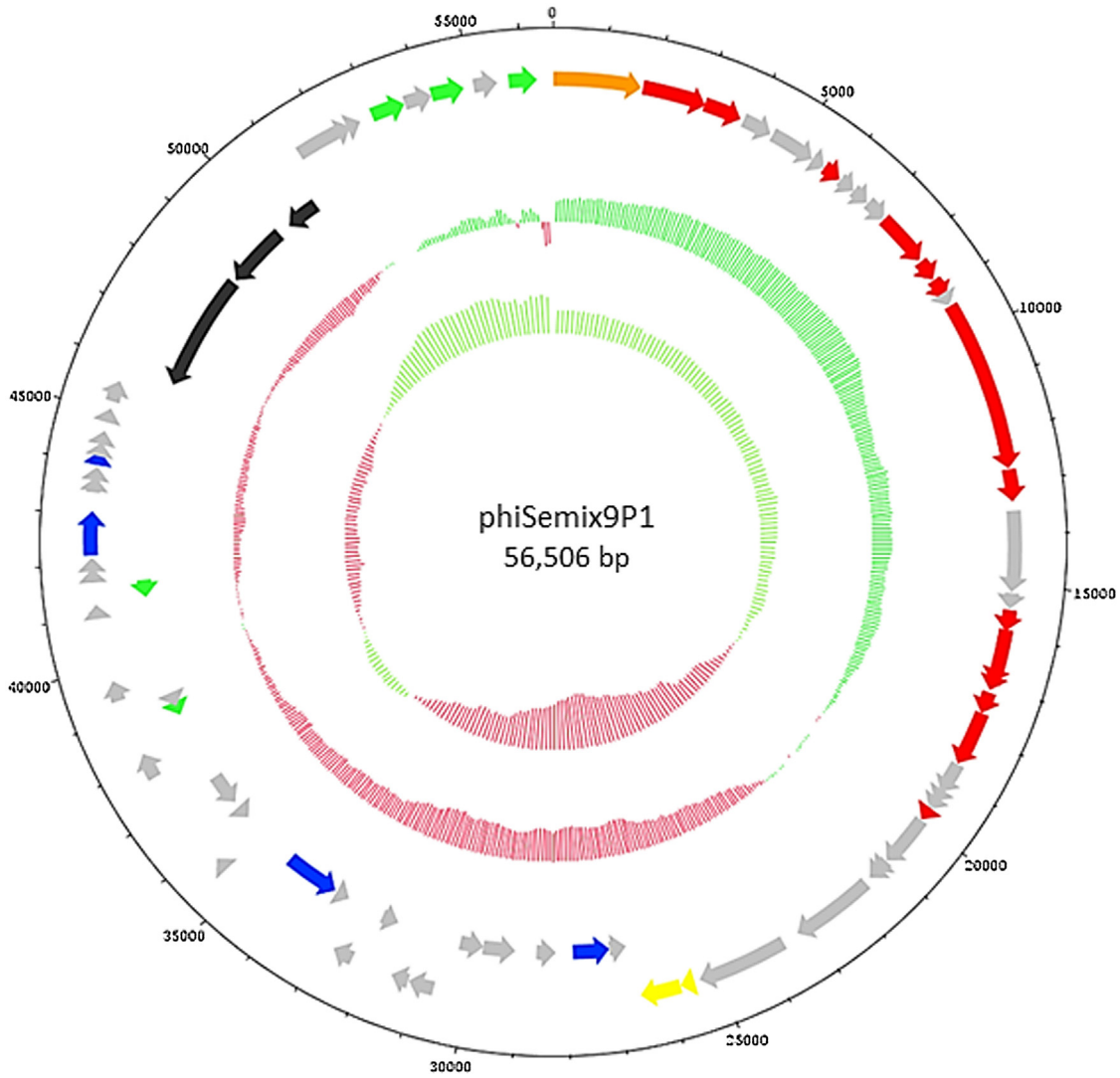

Fig. 1. Circular representation of the phiSemix9P1 genome. Outer ring represents the sequence in nucleotides (bp). The second and the third ring (from outside) show genes encoded on each the forward and reverse strand. Genes were colored according to their putative function: CdtLoc, black; DNA packaging, orange; head and tail structure, red; DNA replication, blue; host cell lysis, yellow; recombination and regulation, green. The next two rings show GC skew as well as GC content (inner ring).

which encodes a complete functional toxin locus (CdtLoc) suggesting a putative mobile character of *C. difficile* toxins.

2. Materials and methods

2.1. Strain isolation

For isolation of the environmental strain *C. difficile* Semix9 from soil, the sample was aggraded in H_2O, and prefiltered through a folded filter (Whatman, Pittsburgh, USA). 50 ml of the filtrate were then filtered through a 0.45 μm cellulose nitrate filter (Sartorius, Göttingen, Germany). The latter filter was incubated in 70% ethanol for 35 min. Finally, the filter was anaerobically incubated on chromogenic agar (chromID™ *C. difficile*, BioMérieux, Paris, France) at 37 °C overnight. Strain Semix9 was verified as *C. difficile* using a Maldi Biotyper (Bruker Daltonics, Bremen, Germany) as well as by 16S rRNA sequencing using universal primers.

2.2. DNA and RNA extraction

For genome sequencing and expression analysis, strain Semix9 was cultivated anaerobically in Wilkins-Chalgren Anaerobe Broth (Oxoid, Basingstoke, UK) at 37 °C and harvested by centrifugation (3000 rpm, 10 min) at exponential or late exponential phase.

Genomic DNA was extracted from cell pellets using the Genomic-tip 100/G Kit (Qiagen, Venlo, Netherlands) according to the instructions of the manufacturer with the following modification. Directly after 1 h of lysis with lysozyme, EDTA (0.5 M, pH 8.0) was added at a final concentration of 20 mM, followed by incubation with proteinase K overnight.

RNA was isolated from cell pellets of cultures that had not been induced, or been in parallel induced with mitomycin C, using the method of Lueders et al. (2004). Remaining genomic DNA was removed through twice subsequent digestion steps with DNase I (Fermentas, Thermo Fisher Scientific, Waltham, USA) lasting for 30 min and 1 h according to the instructions of the manufacturer, with the following modifications. After inactivation with EDTA, 3 M sodium acetate and isopropanol were added to the RNA solution followed by an incubation on ice for 1 h. Finally, the solution was centrifuged for 5 min at 20.000 × *g* and at room temperature. The pellet was washed with 70% ethanol, centrifuged again, dried and dissolved in water. For final RNA clean-up the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) as well as the TURBO DNA-free Kit (Ambion, Thermo Fisher Scientific, Waltham, USA) was employed according to instructions of the manufacturer.