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Evolution of a Self-Inducible Cytolethal Distending Toxin Type V-Encoding Bacteriophage from *Escherichia coli* O157:H7 to *Shigella sonnei*

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Some *cdt* genes are located within the genome of inducible or cryptic bacteriophages, but there is little information about the mechanisms of *cdt* transfer because of the reduced number of inducible Cdt phages described. In this study, a new self-inducible *Myoviridae* Cdt phage (ΦAA91) was isolated from a nonclinical O157:H7 Shiga toxin-producing *Escherichia coli* strain and was used to lysogenize a *cdt*-negative strain of *Shigella sonnei*. We found that the phage induced from *S. sonnei* (ΦAA91-ss) was not identical to the original phage. ΦAA91-ss was used to infect a collection of 57 bacterial strains, was infectious in 59.6% of the strains, and was able to lysogenize 22.8% of them. The complete sequence of ΦAA91-ss showed a 33,628-bp genome with characteristics of a P2-like phage with the *cdt* operon located near the *cosR* site. We found an IS*21* element composed of two open reading frames inserted within the *cox* gene of the phage, causing gene truncation. Truncation of *cox* does not affect lytic induction but could contribute to phage recombination and generation of lysogens. The IS*21* element was not present in the ΦAA91 phage from *E. coli*, but it was incorporated into the phage genome after its transduction in *Shigella*. This study shows empirically the evolution of temperate bacteriophages carrying virulence genes after infecting a new host and the generation of a phage population with better lysogenic abilities that would ultimately lead to the emergence of new pathogenic strains.

Cytolethal distending toxin (Cdt) belongs to the AB_2 type of toxins that block the G_2 and early M phases during mitosis (1, 2). As a result, the cells do not divide, but since they continue to grow, they may distend up to five times their normal size before disintegration. Cdt is a virulence factor that benefits bacterial survival and enhances microbial pathogenicity (2). Cdts have an important role in the *in vivo* pathogenesis of Cdt-producing bacteria, causing severe complications, as demonstrated in mouse models (3).

The Cdt operon comprises three adjacent genes (*cdtA*, *cdtB*, and *cdtC*) that must be expressed for Cdt to initiate cellular toxicity. CdtB encodes the catalytic subunit, homologous to the mammalian DNase I, and CdtA and CdtC are binding proteins that deliver CdtB into the eukaryotic cells, with cytotoxic effects (3, 4). The complete Cdt operon has been reported in several Gram-negative bacteria, including *Escherichia coli*, *Shigella* spp., *Campylobacter* spp., *Aggregatibacter actinomycetemcomitans*, *Haemophilus ducreyi*, *Helicobacter* spp., and *Vibrio* (4–8). The Cdt subunits of a given genus show various degrees of similarity to the Cdt proteins of other genera.

The *cdt* operon is generally described as being located on the chromosome of Cdt-producing bacteria. However, in *E. coli*, five variants of Cdt have been described with different locations. Some variants are encoded by chromosomal genes (Cdt-II) (8) or by a pVir plasmid (Cdt-III) (9), and others are flanked by prophage genes, mostly bacteriophage P2 and bacteriophage lambda sequences (Cdt-I, Cdt-IV, and Cdt-V). A few studies have suggested that *cdt* genes in *E. coli* are located within prophages that could be defective (10–13) or within inducible bacteriophages (14, 15). Analyses of *cdt* flanking regions suggest that the *cdt-I* and *cdt-IV* genes have been acquired from a common ancestor by phage transduction and then evolved in their bacterial hosts (11).

Most available information about Cdt-producing strains pertains to clinical isolates. Recently, environmental strains harboring Cdt-V-encoding phages were described (14). Moreover, Cdt phages were detected in fecally polluted waters (14, 16) and showed high persistence under different disinfection processes and under natural inactivation conditions (16). Therefore, these phages may be interesting candidates for the mobilization of *cdt* genes in the environment. As with other virulence genes, the spread of *cdt* can lead to the emergence of new pathogenic strains or strains with increased virulence (17).

There is a clear relationship between Cdt production and pathogenic serotypes of Shiga toxin-producing *E. coli* (STEC) (10, 18, 19). While several studies have been conducted for other *E. coli* phages, including Stx phages, the relative frequency of the different Cdt phages in relevant pathotypes is not well defined. The information required about the mechanisms of induction of Cdt phages, their host range for lytic infection, and their ability to generate lysogens and to transduce *cdt* still is not available for Cdt phages. The limited number of inducible Cdt phages described so far represents an additional challenge for studying and understanding the role of these phages in *cdt* mobilization.

This study focuses on a new Cdt-V phage isolated from an *E. coli* O157:H7 strain from urban raw wastewater. We characterize its capacity for induction, infection, and transduction, and we present the complete phage genome sequence. Our study has revealed that the phage, during the lysogenization of a *Shigella* strain, has evolved and acquired new genes (an insertion element composed of two genes). We discuss how this genetic acquisition

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could influence the abilities of the phage to generate new lysogens and transduce the toxin gene.

MATERIALS AND METHODS

Bacterial strains, serotyping, bacteriophages, and media. The Cdt phage in this study, named ΦAA91, was detected in a Cdt-positive *E. coli* strain of serotype O157:H7 strain 91 isolated from urban raw wastewater. This phage was induced from the wild-type strain as described below and used to lysogenize a collection of strains used as hosts for lytic infection and transduction (Table 1). A lysogen of the phage ΦAA91 in *Shigella sonnei* strain 866 was obtained. To avoid confusion, here the phage induced from the *S. sonnei* lysogen is called ΦAA91-ss. Luria-Bertani (LB) broth or LB agar was used to culture the bacteria. When necessary, media were supplemented with ampicillin (100 μg/ml) (Sigma-Aldrich, Steinheim, Germany).

PCR studies. PCRs were performed using a GeneAmp PCR system 2400 (Perkin-Elmer, PE Applied Biosystems, Barcelona, Spain) with the oligonucleotides listed in Table 2. Screening for *cdt* was performed by multiplex *cdt* PCR (Table 2), and then the identity of the *cdt* variants was analyzed with the specific oligonucleotides. Purified DNA was diluted 1:20 in double-distilled water. The PCR product was analyzed by gel electrophoresis, and bands were visualized by ethidium bromide staining. When necessary, PCR products were purified using a PCR purification kit (Qiagen Inc., Valencia, CA).

Real-time qPCR. Quantification of *cdt-V* gene by real-time quantitative PCR (qPCR) was performed as previously described (14). A pGEM-T Easy vector containing a 466-bp fragment of *cdtB* obtained with conventional PCR primers Cdt-up1/Cdt-lp1 (Table 2) was used for the standard curve (14) to quantify the number of *cdt* gene copies in phage DNA by qPCR.

The qPCR assay for *cdt* was a custom TaqMan (Applied Biosystems, Spain) set of primers and probe. The forward qcdtB-UP/LP and qcdt probe with a 6-carboxyfluorescein (FAM) reporter and a nonfluorescent quencher (NFQ) were used under standard conditions in a Step One reverse transcription-PCR (RT-PCR) system (Applied Biosystems, Spain). *cdtB* genes were amplified in a 20-μl reaction mixture using TaqMan environmental real-time PCR master mix 2.0 (Applied Biosystems, Spain). The reaction mixture contained 9 μl of the DNA sample or quantified plasmid DNA. All samples were run in triplicate, along with the standards, positive and negative controls, and the number of genome copies (GC) was the average from the three replicates. A 1:10,000 dilution of positive bacterial DNA was used as a positive control.

Controls were performed to rule out the presence of bacterial or nonencapsidated DNA. After DNase treatment but before desencapsidation, the samples were used as templates for qPCR of *cdt*. Negative results confirmed that all nonviral DNA was eliminated from the sample and that only genes in phage DNA would be detected.

Isolation of temperate Cdt bacteriophages and preparation of phage lysates. A 10-ml culture of wild-type strain O157:H7 or of the lysogen of Cdt phage in *S. sonnei* 866 were grown from single colonies in LB broth at 37°C until the exponential growth phase, determined by an optical density at 600 nm (OD_{600}) of 0.3 measured using a spectrophotometer (Spectronic 501; Milton Roy, Belgium). For induction experiments, noninduced cultures and cultures induced with mitomycin C (0.5 μg/ml), ciprofloxacin (0.4, 1, or 4 μg/ml), or EDTA (20 mM, pH 7.2) were incubated overnight at 37°C under aerobic conditions with agitation (180 rpm) in the dark. The cultures were centrifuged at 4,000 × *g* for 10 min, and the supernatants were filtered through low-protein-binding 0.22-μm-pore-size membrane filters (Millex-GP, Millipore, Bedford, MA).

To evaluate the number of PFU of each phage suspension, each suspension was 10-fold serially diluted. One 1 ml of each dilution was mixed with 1 ml of the host strain (*E. coli* WG5) and 2.5 ml of LB soft agar, poured onto LB agar plates, and incubated as described above.

For electron microscopy and for infectivity studies, phage ΦAA91 (from the O157:H7 wild-type strain) and ΦAA91-ss (from *S. sonnei* 866

TABLE 1 Host range of phage ΦAA91-ss in a collection of *cdt*-negative strains[a]

Host	Strain or serotype	Origin[b]	*stx* variant	Lysis	Transduction
E. coli	C600	ATCC 23724		−	−
E. coli	DH5α	Invitrogen		−	−
E. coli	O157:H7	ATCC 43888		++	−
E. coli	WG5	ATCC 700078		++	+
Salmonella Typhimurium	WG49	ATCC 700730		−	−
S. sonnei	866	Clinical		++	+
S. sonnei	30673	Clinical		+	−
S. sonnei	30674	Clinical		++	+
S. sonnei	30676	Clinical		++	+
S. sonnei	30679	Clinical		++	+
S. sonnei	30682	Clinical		++	+
S. flexneri	805-F	Clinical		+	−
E. coli	O111	Clinical		+	−
E. coli	O26	Clinical		−	−
E. coli	O26	Clinical		−	−
E. coli	O157:H7	Clinical	stx_{2a}, stx_{2c}	−	−
E. coli	O4:H4	Clinical	stx_{2a}	++	−
E. coli	O15:H16	Clinical	stx_{2a}	−	−
E. coli	O174:H-	Clinical	stx_{2c}	++	−
E. coli	O104:H4	Clinical	stx_{2a}	−	−
E. coli	O146:H21	H	stx_{2d}	−	−
E. coli	O90:H-	M	stx_{2d}	−	−
E. coli	O171:H2	C	stx_{2c}	−	−
E. coli	O171:H2	C	stx_{2c}	−	−
E. coli	O181:H49	C	stx_{2a}	++	−
E. coli	O181:H20	C	stx_{2c}	+	−
E. coli	O2:H25	C	stx_{2g}	+	−
E. coli	O136:H1	C	stx_{2g}	+	+
E. coli	O90:H-	H	stx_{2d}	−	−
E. coli	ONT:H2	C	stx_{2c}, stx_{2d}	+	−
E. coli	O2:H25	C	stx_{2g}	−	−
E. coli	O1:H20	C	stx_{2a}	+	−
E. coli	O8:H31	C	stx_{2g}	−	−
E. coli	O1:H20	C	stx_{2a}	+	−
E. coli	O157:H-	C	stx_{2c}	+	−
E. coli	O22:H8	C	stx_{2c}	+	+
E. coli	O166:H21	H	stx_{2c}	−	−
E. coli	O157:H-	C	stx_{2c}	−	−
E. coli	O156:H8	C	stx_{2c}	+	+
E. coli	O1:H20	C	stx_{2a}	−	−
E. coli	O171:H2	C	stx_{2a}, stx_{2c}	+	−
E. coli	O76:H2	C	stx_{2c}	++	−
E. coli	O113:H21	C	stx_{2a}	+	+
E. coli	O100:H-	P	stx_{2e}	+	−
E. coli	O171:H2	C	stx_{2c}	−	−
E. coli	O28:H28	C	stx_{2a}	+	+
E. coli	O8:H9	H	stx_{2e}	−	−
E. coli	O181:H49	C	stx_{2a}	+	−
E. coli	O171:H2	C	stx_{2c}	−	−
E. coli	ONT:H-	C	stx_{2c}	+	−
E. coli	O2:H25	M	stx_{2g}	+	−
E. coli	ONT:H-	M	stx_{2e}	−	−
E. coli	O22:H8	C	stx_{2c}	+	+
E. coli	O157:H7	C	stx_{2c}	+	−
E. coli	O157:H7	C	stx_{2c}	+	−
E. coli	O22:H8	H	stx_{2c}	++	+
E. coli	O1:H20	H	stx_{2a}	−	−

[a] Lytic infection ability was determined by observation of lysis visualized directly on the plate and after hybridization with the *cdtB* probe: +, weak lytic spot; ++, strong lytic spot; −, negative. Transduction ability was evaluated by PCR on colonies obtained after infection with the Cdt phage.

[b] H, strain isolated form raw urban wastewater (human fecal pollution); C, strain isolated from cattle wastewater; P, strain isolated from poultry wastewater; M, strain isolated from mixed animal wastewater.

TABLE 2 Oligonucleotides used in this study[a]

PCR target and primer	Sequence	Size (bp)	Reference or source
Multiplex *cdt*			
CDT-up1	GAAAGTAAATGGAATATAAATGTCCG	466	10
CDT-up2	GAAAATAAATGGAACACACATGTCCG		10
CDT-lp1	AAATCACCAAGAATCATCCAGTTA		10
CDT-lp2	AAATCTCCTGCAATCATCCAGTTA		10
qPCR *cdt*			
qcdtB-F	AGGCCGATGAAGTGTTTGTTCTT	69	14
qcdtB-R	CAATCCGTATGCCAAGCAATGG		14
qcdt-probe	6-FAM-CCGCCCACCTTGCCTT- MGBNFQ		14
cdt-VA			
c338f	AGCATTAAATAAAAGCACGA	1329	52
c2135r r	TACTTGCTGTGGTCTGCTAT		52
cdt-VB			
c1309f	AGCACCCGCAGTATCTTTGA	1363	52
c2166r	AGCCTCTTTTATCGTCTGGA		52
cdt-VC			
P105	GTCAACGAACATTAGATTAT	748	52
c2767r	ATGGTCATGCTTTGTTATAT		52
Integration site left			
attP1	ACAGCGATACATCGTGAAGC	654	This study
attB1	TCATCGGTGTTGGAGATATCA		This study
Integration site right			
attP2	GCAATATAATCGCACTGCAA	585	This study
attB2	GAGACAATGCATCGCCTTGT		This study
cox			
Cox up	ATGGAAGTCAATGACTATGT	273	This study
Cox lp	TCACAACCCCATCCACAAAAG		This study
IS*21* within *cox*			
IS21rev	TGCACCGTTGCCCGGCAACC		This study
IS21fw	AGGCATCAGCGCGGTATGGA		This study
IS*21*			
IS21up	GGTTGCCGGGCAACGGTGCA	949	This study
IS21lp	TCCATACCGCGCTGATGCCT		This study
stx_2			
stx2-a	GCGGTTTTATTTGCATTAGC	115	53
stx2-b	TCCCGTCAACCTTCACTGTA		53
stx_{2c}			
stx2c-a	GCGGTTTTATTTGCATTAGT	124	53
stx2c-b	AGTACTCTTTTCCGGCCACT		53
stx_{2d}			
stx2d-a	GGTAAAATTGAGTTCTCTAAGTAT	175	53
stx2d-b	CAGCAAATCCTGAACCTGACG		53
stx_{2e}			
stx2e-a	ATGAAGTGTATATTGTTAAAGTGGA	303	53
stx2e-b	AGCCACATATAAATTATTTCGT		53
stx_{2g}			
stx2g-a	GTTATATTTCTGTGGATATC	572	54
stx2g-b	GAATAACCGCTACAGTA		54

[a] MGBNFQ, minor groove binder and nonfluorescent quencher.

lysogen) were isolated from single plaques of lysis obtained from *E. coli* WG5. Plaques isolated from 5 to 10 wells were removed with a wire loop, and each was suspended in 500 μl of LB. Suspensions were filtered through low-protein-binding 0.22-μm-pore-size membrane filters (Millex-GP, Millipore, Bedford, MA).

Isolation of phage DNA. The phage suspensions were treated with DNase (100 U/ml; Sigma-Aldrich, Spain). After digestion, DNase was heat inactivated (10 min at 80°C). Phage DNA was isolated from the phage lysates by proteinase K digestion and phenol-chloroform extraction as described previously (20). Purified DNA was eluted in a final volume of 50 μl of Tris-EDTA buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and evaluated by agarose (0.8%) gel electrophoresis. The bands were viewed following ethidium bromide staining. The concentration and purity of the DNA extracted was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE).

Preparation of DIG-labeled *cdtB*-specific gene probe. A 466-bp fragment corresponding to *cdtB* resulting from amplification with primers Cdt-up1/Cdt-lp1 (Table 2) was labeled with digoxigenin (DIG) by incorporating DIG-11-deoxyuridine-triphosphate (Roche Diagnostics, Barcelona, Spain) during PCR as described previously (21) and was used as a probe. The probe was used for hybridization of phages and bacteria by plaque and colony blot assays as previously described (14). Hybridization was performed at 64°C according to standard procedures (20). Stringent hybridization was achieved with the DIG-DNA labeling and detection kit (Roche Diagnostics, Barcelona, Spain) according to the manufacturer's instructions.

Infectivity of Cdt phages. To evaluate the ability of phages ΦAA91 and ΦAA91-ss to infect bacteria, a 10-μl drop of phage suspension, prepared from a single plaque as described above, was spotted onto a monolayer of the host strains tested (Table 1). The monolayer was prepared with 1 ml of log-phase culture (OD_{600} of 0.3) of each host strain, mixed with 2.5 ml of LB soft agar (LB broth with 0.7% agar), poured onto LB agar plates, and incubated at 37°C overnight. After incubation, the plaques were transferred to a nylon membrane (Hybond N+; Amersham Pharmacia Biotech, Spain) and hybridized with the *cdtB*-specific probe.

Generation of *cdt* lysogens. The bacterial growth from the area of lysis generated by a drop of phage suspension on the agar monolayer of the different host strains (Table 1) was harvested in 1 ml of Ringer 1/4, 10-fold diluted, plated on LB agar, and incubated at 37°C for 18 h. Colonies obtained were evaluated for the presence of *cdt* by colony hybridization assay (14) using the DIG-labeled *cdtB* probe and confirmed by PCR.

Electron microscopy. Phages ΦAA91 and ΦAA91-ss were propagated from a single plaque of lysis obtained from *E. coli* WG5 as described above. The phage suspensions obtained were 100-fold concentrated by means of protein concentrations (100-kDa Amicon Ultra centrifugal filter units; Millipore, Bedford, MA). Both phage suspensions were DNase treated as described above and purified by CsCl centrifugation (20). The easily visible gray band in which the bacteriophages were expected (20, 22), corresponding to a density of 1.45 ± 0.02 g/ml, was collected and dialyzed to remove the CsCl. A drop of phage suspension was deposited on copper grids with carbon-coated Formvar films and stained with 2% KOH phosphotungstic acid (pH 7.2) for 2.5 min. Samples were examined in a Jeol 1010 transmission electron microscope (JEOL USA, Inc.) operating at 80 kV.

Sequencing of Cdt phage DNA. The ΦAA91-ss phage was purified from an induced culture of the *S. sonnei* lysogen. Phages were purified as described above and concentrated by protein concentrators (Amicon Ultra centrifugal filter units; Millipore, Bedford, MA). The phages present were further purified by CsCl centrifugation as described above. Contaminating bacterial DNA and RNA were removed by additional treatment with DNase (0.3 μg/ml) and RNase (0.3 μg/ml), and the mixtures were incubated at 37°C for 1 h. Phage DNA was extracted by proteinase K digestion and phenol-chloroform precipitation to a final concentration of 1 μg/μl. The purity and integrity of the DNA were confirmed by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE) and 0.8% agarose gel electrophoresis.

Genome sequencing was performed using an Ion Torrent personal genome machine (PGM) (Life Technologies). Libraries were generated using 0.5 μg of the genomic DNA and an Ion Xpress Plus fragment library kit comprising the Ion Shear chemistry according to the user guide. After

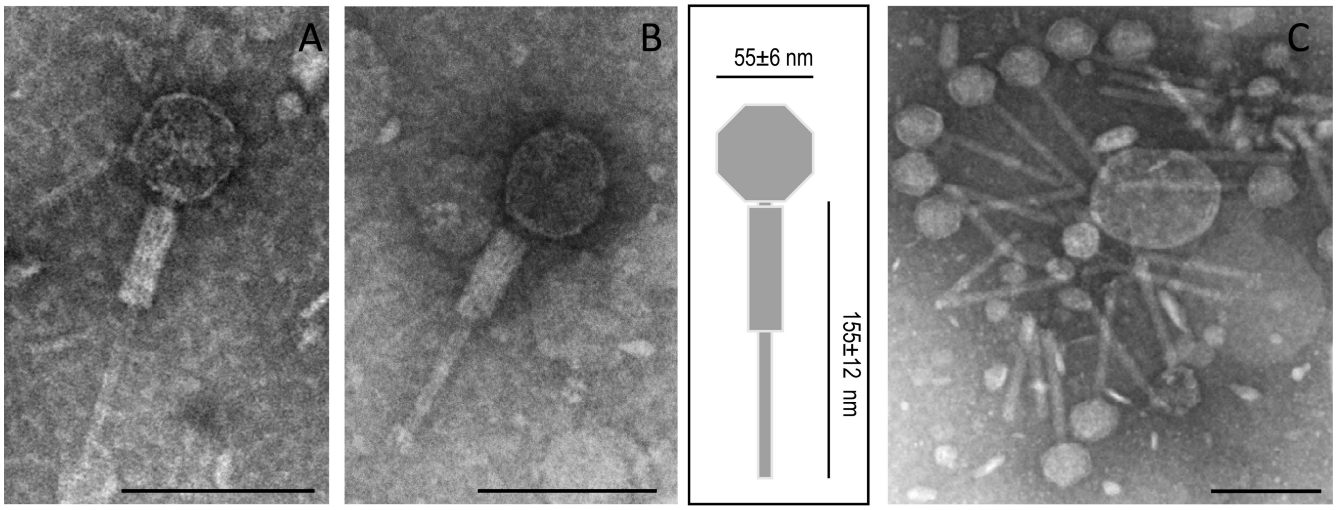

FIG 1 Electron micrographs of phage ΦAA91 (A) and phage ΦAA91-ss (B), corresponding to the *Myoviridae* morphological type. (A and B) Single phage showing the tail with contracted sheath and diagram indicating the size of capsid and tail (in nm). (C) Group of phage ΦAA91-ss attached to particles showing the tails noncontracted. Bar, 100 nm.

dilution of each library, 4.5×10^8 molecules were used as the templates for clonal amplification on Ion Sphere particles during the emulsion PCR according to the Ion Xpress template 200 kit manual (Life Technologies). The amplification was loaded onto an Ion 316 chip and subsequently sequenced using 105 sequencing cycles according to the Ion Sequencing 200 kit user guide. Approximately 105 sequencing cycles resulted in an average reading length of 182 nucleotides.

Assembly of the sequence was accomplished using CLC Genomic Workbench 6 software. The phage DNA-predicted open reading frames (ORFs) were identified using the GeneMark gene prediction program (http://exon.gatech.edu) (23) and ORF Finder (www.ncbi.nlm.nih.gov). These predictions were annotated by comparison to the nonredundant database using BLASTN from the NCBI (www.ncbi.nlm.nih.gov) and the European Bioinformatics Institute (UniProtKB/Swiss-Prot). MAUVE software (24) was used to compare ΦAA91-ss and other P2 and Cdt phages.

Nucleotide sequence accession number. The nucleotide sequence of the phage ΦAA91-ss genome of this study was submitted to the GenBank database library and assigned accession number KF322032.

RESULTS

Detection and isolation of Cdt phage ΦAA91. Bacteriophage ΦAA91 was isolated from the *cdt*-positive O157:H7 *E. coli* STEC strain that also harbors an inducible Stx phage. In order to characterize the Cdt phage without interference from the Stx phage, it was necessary to mobilize this phage to another susceptible host (14). For this, we used *S. sonnei* strain 866, which did not show the presence of inducible phages under the conditions tested. Following the common procedures for temperate phage induction, phage ΦAA91 was isolated after mitomycin C induction of an LB culture of the wild-type strain and purified. Dilutions of the phage suspension were plated on an agar monolayer of the *E. coli* WG5 strain. Lytic plaques formed by phage ΦAA91 on *S. sonnei* were clearly lytic and measured 1.2 ± 0.2 mm in diameter.

Assays for the generation of ΦAA91 lysogens in several *E. coli* and *Shigella* strains (Table 1) were unsuccessful, except for one lysogen obtained with *S. sonnei* 866. The *cdt*-positive *S. sonnei* 866 lysogen was confirmed by PCR. The lysogen was subcultured three times to confirm its stability, and our observations indicated that no losses of the phage were observed after subcultivation. Sequencing of the *cdt* variant confirmed that this phage was carrying the *cdt-V* variant of the gene.

Morphological characterization of phages ΦAA91 and ΦAA91-ss. The morphology of phage ΦAA91 obtained from O157:H7 strains and of phage ΦAA91-ss obtained from *S. sonnei* lysogen were examined using a phage suspension obtained after propagating a single lytic plaque and CsCl purification. Both phages had identical *Myoviridae* morphology (22), with a capsid measuring 55 ± 6 nm in diameter and a contractile tail of 155 ± 12 nm (Fig. 1).

Induction of phage ΦAA91-ss. To determine whether *cdt* was located in the genome of an inducible prophage, infectious phages and viral genomes were counted without induction and after induction from *S. sonnei* lysogen with mitomycin C, ciprofloxacin, and EDTA at various times. Induction was also measured by a reduction of the OD_{600} of the bacterial culture, which indicates activation of the lytic cycle of induced prophages (Fig. 2).

The infectious particles or GC of the phage showed no significant increase ($P > 0.05$ by analysis of variance [ANOVA]) between the noninduced lysogenic strain culture and the culture treated with the inducing agents. After 18 h, a significantly ($P < 0.05$) lower OD_{600} was observed in the culture treated with ciprofloxacin (Fig. 2, upper table). However, this reduction was probably not due to the induction of ΦAA91-ss, as observed after evaluation of the number of infectious phages by plaque assay (Fig. 2, left) or after evaluation of *cdt* GC in phage DNA (Fig. 2, right).

These results indicated that phage ΦAA91-ss is not inducible through the previously described methods; rather, it appears to be self-inducible. Reduction of OD_{600} after ciprofloxacin exposure could be attributable to a decrease in the number of cells by the antimicrobial agent, since it was used at a high concentration. Lower concentrations of ciprofloxacin tested (0.4 and 1 μg/ml) did not show induction of ΦAA91-ss. Subsequent evaluation of the number of ΦAA91 particles from the wild-type *E. coli* O157:H7 strain confirmed that, as observed for the *Shigella* lyso-

	Optical density (λ = 600 nm)			
	Uninduced	Mitomycin C	Ciprofloxacin	EDTA
0h	0.500	0.500	0.500	0.500
2h	1.181	1.060	1.032	1.042
4h	1.381	1.275	1.205	1.300
6h	1.557	1.466	1.182	1.501
o/n	1.545	1.437	0.633	1.497

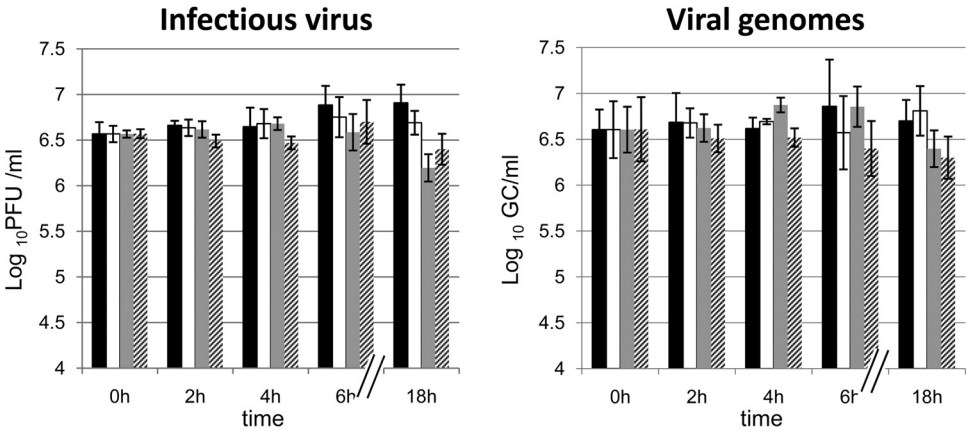

FIG 2 Induction experiments of phage ΦAA91-ss using mitomycin, ciprofloxacin, and EDTA. Monitoring of induction was performed by spectrophotometry at a wavelength of 600 nm (upper table), infectiveness on *E. coli* WG5 in $\log_{10}$ PFU/ml (chart on the left), and evaluation of phage genomes by qPCR in $\log_{10}$GC/ml (chart on the right).

gen, the number of phages did not differ significantly between noninduced and induced cultures (data not shown).

Host range of phages ΦAA91 and ΦAA91-ss. Each phage, isolated from a single plaque, was used to infect a collection of 57 strains, including one *Salmonella* strain, one *Shigella flexneri* strain, five *S. sonnei* strains, several *E. coli* laboratory strains, and wild-type STEC strains of different serotypes. Phages ΦAA91 and ΦAA91-ss had the same host range and caused clear lysis in 34 of the strains (Table 1). From these, phage ΦAA91 generated only one lysogen in *S. sonnei* 866. In contrast, phage ΦAA91-ss generated lysogens in 13 strains of different bacterial genera (Table 1).

Sequence of phage ΦAA91-ss. Phage ΦAA91-ss has a genome of 33,628 bp. Annotation of the phage genome showed multiple characteristics of P2 bacteriophages, although it also displayed some differences. The sequence is presented as a linear structure terminating with the cohesive ends (Table 3 and Fig. 3) and starting with *cosL* (left cohesive end). The first identified ORF is a capsid portal protein and is followed by a set of head synthesis proteins. Downstream of the capsid genes are the genes related to lysis (holin, lysozyme, and lysins A and B) (Table 3 and Fig. 3). The next set of genes corresponds to those proteins involved in tail and baseplate synthesis. Downstream are the genes involved in lysogeny (*int*, *C*, and expected *cox*). The phage attachment site (*attP*) (Fig. 3) is located between *ogr* (a positive regulatory factor required for P2 late gene transcription) (25) and the gene encoding integrase (*int*). The product of gene *C* is the repressor that regulates the lysogenic state. The next expected gene would be *cox*, encoding a multifunctional protein of 91 amino acids (26). However, in phage ΦAA91-ss, the *cox* gene is truncated by insertion of a long element, IS*21*, composed of two ORFs that encode a transposase and a transposition protein, respectively. We will devote more attention to this element in the next section. The sequence continues with ORFs identified as DNA replication proteins, particularly gene *A*, encoding gpA, which initiates replication by inducing a single-stranded cut at the origin of replication (*ori*) (5′-GCGCCTCGGAGTCCTGTCAA-3′) (Fig. 3) (27). Finally, we found the *cdt* operons (*C*, *B*, and *A*), which appeared downstream from gene *A* and upstream from *cosR*, at the right cohesive end of the phage genome.

Determination of IS*21* in the host strains. Many insertion elements have been identified in *Shigella* and *E. coli* located on a plasmid or in the chromosome. To determine whether the presence of IS*21* in phage ΦAA91-ss was a product of rearrangements after lysogenizing the *S. sonnei* strain 866 or whether it was originally present in the phage in the wild-type O157:H7 *E. coli* strain, we used PCR to evaluate the fragment between the first part of *cox* and the IS*21* element and the fragment comprising the last fragment of IS*21* and the last *cox* fragment. In addition, we investigated whether the element is maintained in some *S. sonnei* strains of our collection in which the phage ΦAA91-ss generated new lysogens (Table 1). We amplified both segments using the primers described in Table 2 (Fig. 4). If the IS*21* element is present within *cox*, amplification using the combination of primers cox-up and S21rev would result in an amplimer of 610 bp, while the combination of primers IS*21*fw and cox-lp would result in a fragment of 890 bp. Amplification of both regions confirmed the location of the IS*21* element within *cox* in phage ΦAA91-ss from the *S. sonnei* 866 lysogen as well as from other *S. sonnei* lysogens (30676 and 30682; Table 1), as shown in Fig. 4. However, when looking at the

TABLE 3 Annotation of ORFs detected in the phage ΦAA91-ss genome

ORF	Position (bp)	Size (bp)	Description	% Cover query[a]	% Identity[a]	Organism	NCBI protein accession no.
	1–19	19	*cosL*; left cohesive end				
1	189–1223	1,035	Gene *Q*; capsid portal	100	100	*E. coli* prophage	WP_000038198.1
2	1223–2995	1,773	Gene *P*; large terminase subunit	100	100	*Yersinia* phage	NP_839851.1
3	3169–4023	855	Gene *O*; capsid scaffold	100	99	Prophage P2 in *E. coli*	YP_002391908.1
4	4082–5155	1,074	Gene *N*; major capsid protein precursor	100	100	Prophage P2 in *E. coli*	WP_001248540.1
5	5159–5902	744	Gene *M*; small terminase subunit	100	100	*E. coli* prophage	WP_000203437.1
6	6002–6511	510	Gene *L*; capsid completion protein	100	100	*E. coli* prophage	YP_001746272.1
7	6511–6714	204	Gene *X*; essential tail protein	100	100	P2-like prophage in *E. coli*	YP_005277372.1
8	6718–6999	282	Gene *Y*; holin	100	100	Phage P2	NP_046764.1
9	6999–7496	498	Gene *K*; lysozyme	100	100	*E. coli* prophage	WP_000123122.1
10	7511–7936	426	Lysin A	100	100	*E. coli* prophage	WP_000736580.1
11	7924–8349	426	Lysin B	100	100	*E. coli* prophage	WP_000040681.1
12	8454–8924	471	Gene *R*; essential tail completion protein	100	100	Phage P2	WP_001367960.1
13	8917–9369	453	Gene *S*; essential tail completion protein	100	100	*Yersinia* phage	NP_839862.1
14	9436–10071	636	Gene *V*; baseplate protein	100	100	Phage P2	WP_001093710.1
15	10068–10415	348	Gene *W*; putative baseplate protein	100	100	*E. coli* prophage	YP_002391893.1
16	10420–11328	909	Gene *J*; baseplate assembly	100	99	*E. coli* prophage	WP_001121491.1
17	11321–11851	531	Gene *I*; baseplate assembly	100	100	Phage P2	NP_046774.1
18	11862–13940	2,079	Gene *H*; tail fiber	100	100	*E. coli* prophage	WP_000104689.1
19	13944–14471	528	Gene *G*; putative tail fiber assembly	100	100	*E. coli* prophage	WP_001164103.1
20	14571–15677	1,107	Hypothetical protein	100	100	*E. coli*	WP_000382496.1
21	15979–17169	1,191	Gene *FI*; major tail sheath	100	100	*E. coli* prophage	WP_001286731.1
22	17182–17700	519	Gene *FII*; major tail tube	100	100	Phage P2	NP_046779.1
23	17757–18032	276	Gene *E*; tail protein	100	100	*E. coli* Mu-like prophage	WP_001031311.1
24	18177–20624	2,448	Gene *T*; tail length determinator	100	100	*E. coli* prophage	WP_000069913.1
25	20639–21118	480	Gene *U*; tail protein	100	100	Phage P2	WP_000978923.1
26	21118–22281	1,164	Gene *D*; phage late control	100	100	*E. coli* prophage	WP_001704948.1
27	22362–22580	219	*ogr*; activator of late transcription; Att phage integration site	100	100	*E. coli*	WP_000280530.1
28	22853–23863	1,011	Integrase	100	100	*E. coli* phage HP1	WP_001336827.1
29	23903–24196	294	Gene *C*; immunity control	100	100	*E. coli* prophage	YP_005280060.1
	24349–24471	123	Truncated *cox* gene				
30	24553–25725	1,173	IstA; IS*21* transposase	100	100	*S. sonnei*	YP_309050.1
31	25725–26522	798	IstB; IS*21* transposition protein	100	100	*S. sonnei*	YP_309051.1
	26581–26758	177	Truncated *cox* gene				
32	26922–27428	507	Gene *B*; DNA replication protein	100	100	*E. coli* prophage	WP_000426197.1
33	27492–27716	225	Hypothetical protein (similar to phage P2 ORF 80)	100	100	*E. coli*	YP_001746256.1
34	27716–28018	303	Hypothetical protein (similar to phage P2 ORF 81)	100	100	*E. coli*	WP_001277965.1
35	28018–28242	225	Zinc finger protein (similar to phage P2 ORF 82)	100	100	Phage P2	NP_046793.1
36	28239–28514	276	Replication initiation protein (similar to phage P2 ORF 83)	100	100	*E. coli* prophage	YP_852002.1
37	28504–30801	2,298	Gene A; DNA replication	100	100	*E. coli* prophage	WP_000268595.1
38	30877–31422	546	Cdt-V C operon	100	100	*E. coli*	WP_000825552.1
39	31437–32246	810	Cdt-V B operon	100	100	*E. coli* Vir plasmid	YP_003034080.1
40	32243–33019	777	Cdt-V A operon	100	100	*E. coli*	WP_001284793.1
	33609–33628	19	*cosR*; cohesive end				

[a] Percentage of coverage and the closest identity to proteins as described in the NCBI databank.

original *E. coli* O157:H7 strain 91, where phage ΦAA91 was present, we found that *cox* appeared intact.

PCR showed that the IS*21* element was present in the *S. sonnei* strains but not in the wild-type O157:H7 strain, although phage ΦAA91 was present, confirming that incorporation of IS*21* occurred after lysogenization in *S. sonnei*.

To evaluate the percentage of phages that could harbor the IS*21* element, phage ΦAA91, induced from *E. coli* O157:H7 strain 91, and phage ΦAA91-ss, from *S. sonnei* 866 lysogen, were plated using *E. coli* WG5 as the host strain (negative for IS*21*). Plates containing between 20 and 50 plaques were used to isolate phages from the plaques of lysis. Phage DNA was extracted from the

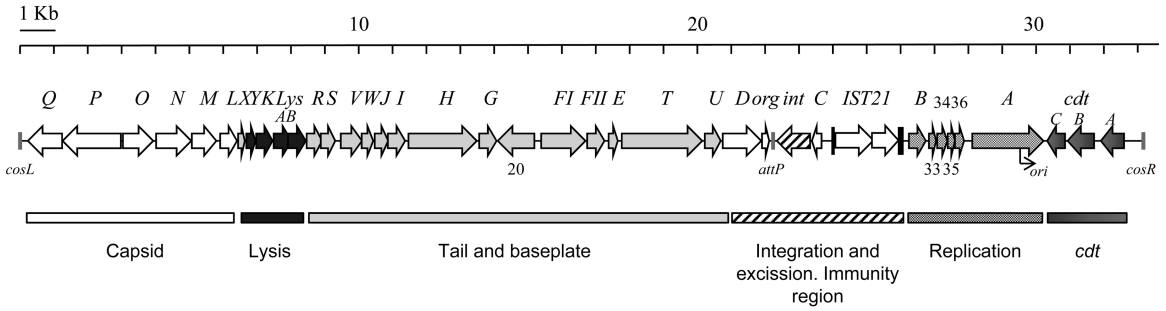

FIG 3 Genetic map of phage ΦAA91-ss. Relevant phage genes, identified by comparison to other P2-like phages, are indicated. Arrows show gene size, orientation, and context. Those ORFs without defined function are shown by ORF number. The scale (in kb) is indicated in the upper part of the figure. The origin of replication (*ori*), the cohesive ends (*cosR* and *cosL*), and the phage attachment site (*attP*) are indicated.

plaques, and the presence of IS*21* within *cox* was evaluated by PCR amplification of the IS*21* left and right insertion sites and those with intact *cox* gene by using primers cox-up/lp. The results of phage ΦAA91 induced from O157:H7 showed that 100% of plaques had the intact *cox* gene and that the IS*21* element was absent. Results of plaques of ΦAA91-ss phages induced from *S. sonnei* 866 lysogen showed that 86.1% (±3.2%) of the plaques contained IS*21* inserted within *cox*, while only 13.9% (±3.2%) presented intact *cox* and no IS*21* element. The higher proportion of phages carrying IS*21* is consistent with the sequence obtained. Phages induced from lysogens in *S. sonnei* strains 30676 and 30682 with phage ΦAA91-ss showed 100% of the plaques of lysis with the IS*21* element and *cox* truncated, suggesting that lysogens were obtained by incorporation of ΦAA91-ss.

Determination of sequence similarity between ΦAA91 and ΦAA91-ss. Using the ΦAA91-ss sequence as the template, we designed PCR primers that allowed the amplification of 30 consecutive fragments of ca. 1,200 bp each (data not shown). Each fragment amplified overlapped the previous and the following fragment, covering the whole length of the genome of phage ΦAA91-ss (33,628 bp). These primers were used to amplify the ΦAA91 DNA obtained from a single plaque, and ΦAA91-ss DNA was used as a control.

The sizes of the 30 fragments obtained were identical for both phages, except in three fragments (23, 24, and 25) (data not shown), in which at least one of the primers annealed with the IS*21* fragment; therefore, no amplimers were obtained with phage ΦAA91 DNA, but they were obtained with ΦAA91-ss. Although minor variations in the sequence cannot be excluded, these results strongly indicated that compared to the genetic organization of the ΦAA91 genome, the ΦAA91-ss genome has been modified only in the *cox* gene by the incorporation of IS*21*.

Homology with other P2-like phages. Comparison of the ΦAA91-ss phage genome to those of other P2 and Cdt phages showed different similarities. The most similar was the *Enterobacteriaceae* P2 phage carrying *cdt-V* (KC618326.1) (12); 79% of the sequence was 98% identical. The second most similar phage, with a similarity of 97% in 75% of its genome, was phage WPhi (AY135739.1) of *E. coli*. Finally, phage LC-413 (AY251033.1) of *Yersinia* showed 73% coverage with 96% similarity, and phage P2 (AF063097.1) of *E. coli* showed 69% coverage with 100% similarity in the covered sequence. Some bacterial genomes also showed

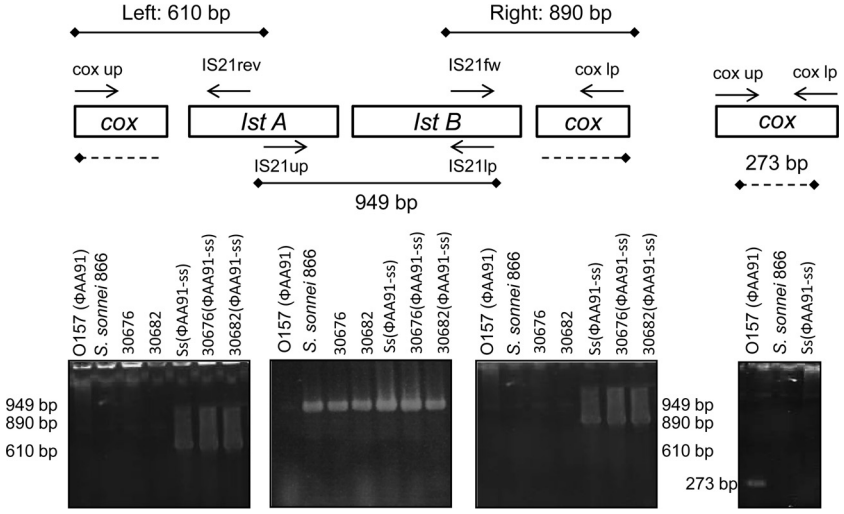

FIG 4 Amplimers generated for detection of IS*21* element in phages and host strains. Insertion of IS*21* in *cox* was detected from its left and right side by the combination of IS*21*/cox primers. The element was detected in *Shigella* (results of *S. sonnei* strains 866, 30676, and 30682 are shown here) but not in the wild-type O157:H7 *E. coli* strain. A nontruncated *cox* gene was observed only in the wild-type O157:H7 *E. coli* strain.

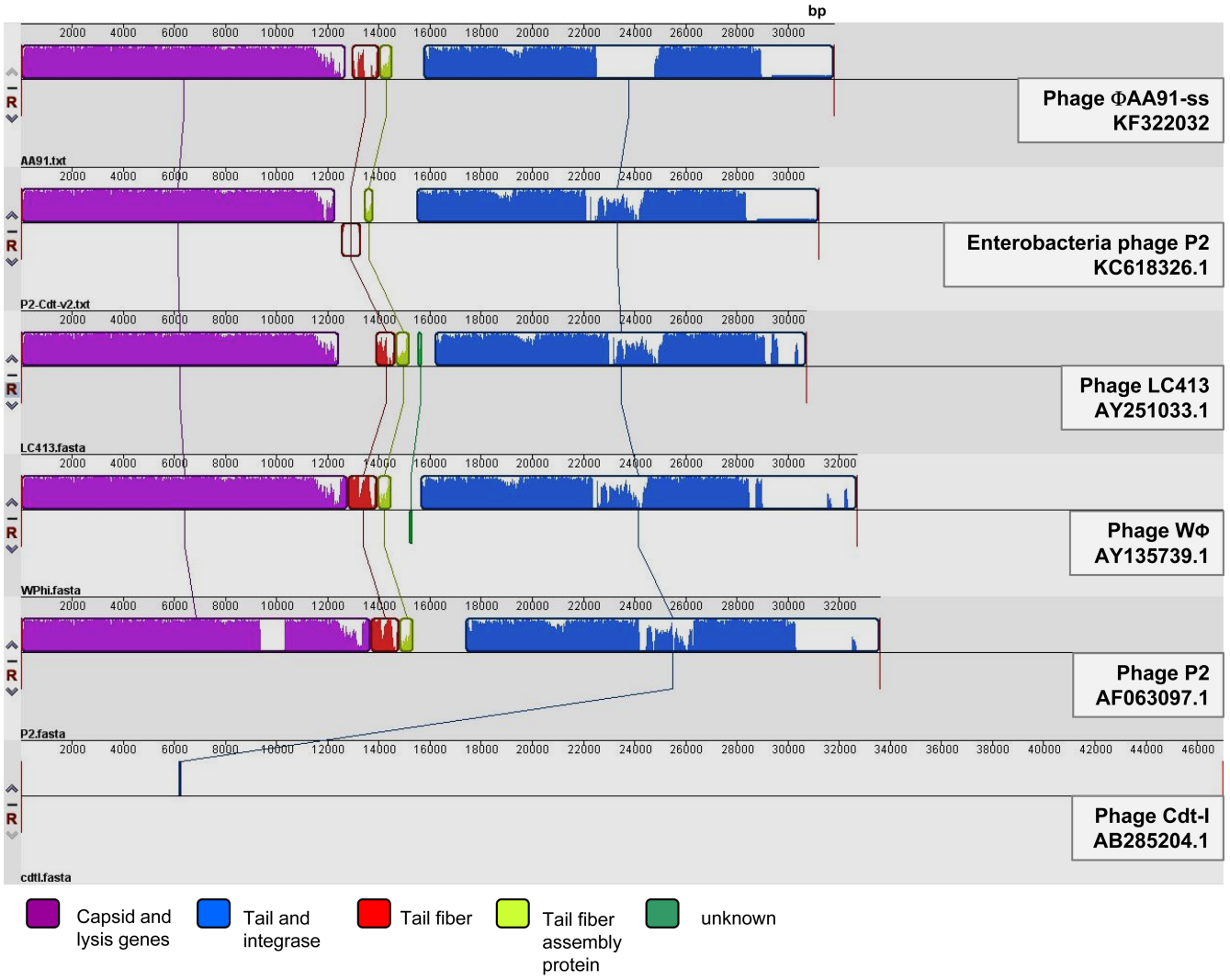

FIG 5 MAUVE analysis comparison of Cdt phage ΦAA91-ss to P2-like phages (P2, LC413, and WΦ) and genomes of Cdt phages (P2-Cdt-V and Cdt-I).

similarity to the ΦAA91-ss phage genome. The most similar, with 84% of coverage with 100% identity, was *S. sonnei* strain 53G (HE616528.1). However, this sequence has not been annotated, hence it is not known whether this strain harbors a prophage. However, it does lack *cdt*, since the area of coverage did not include the *cdt* operon. A similarity of 99% in 83% of the sequence was observed for *E. coli* strain SMS-3-5 (CP000970.1), and 99% identity in 75% of the sequence was found with other *E. coli* W genomes (CP002185.1, CP002967.1, CP002516.1, and CP002970.1), all of them harboring a P2-like phage in which the *cdt* operon is absent. Other phages or bacterial strains showed lower coverage.

Progressive MAUVE analysis (24) (Fig. 5) allowed comparison of phage ΦAA91-ss to complete sequences of P2-like phages showing similarity and to the two Cdt phages in the databases: phage encoding Cdt-I (15) and a P2 cryptic prophage encoding Cdt-V (12) (accession number KC618326.1). The results show that despite the differences in their sequences, all phages shared regions in their genome's structure, with the exception of phage encoding Cdt-I, which has a completely different genome. In the remaining phages, two main structures were observed, one comprising capsid genes and the genes involved in lysis (holins and lysins) and a second set comprising genes involved in the phage tail and the integrases. Some other regions encoding tail fiber or tail assembly proteins presented greater degrees of variability than the previously mentioned regions (Fig. 5).

Insertion site of phage ΦAA91-ss. The *att* site of phage ΦAA91-ss is located between the *ogr* and *int* genes (Fig. 3). Its integration into the *S. sonnei* 866 lysogen was determined by designing primers attP1 and attP2 in both genes of phage DNA (Table 1) and combining these with primers attB1 and attB2 located in the *fieF* and *cpxP* genes, which were previously described as the insertion site of P2-like phages in *E. coli* W, *E. coli* K KO11FL (28), and *S. sonnei* 53G (accession number HE616528.1). Amplification of attP1/B1 and attB2/P2 showed amplimers of 654 and 585 bp, respectively, corresponding to the expected size and confirming that the phage was inserted into this bacterial site. The *S. sonnei* 866 strain used as a control did not show amplification at both sites. Moreover, ΦAA91-ss also is inserted in the same locus in lysogens of *S. sonnei* strains 30676 and 30682. Controls of bacterial

strains without the phage were negative. Additionally, we obtained the same PCR results when testing phage ΦAA91 insertion in the *E. coli* O157:H7 wild-type strain. Thus, we concluded that ΦAA91 and ΦAA91-ss are inserted in the same locus in their respective host strains.

DISCUSSION

P2-like prophages seem to be quite common in *E. coli* but also seem to be distributed among other proteobacteria of the gamma subgroup (29). The genomes of phages HP1 and HP2 in *Haemophilus influenzae* (30), ΦCTX in *Pseudomonas aeruginosa* (31), and K139 in *Vibrio cholerae* (32) have all been sequenced and shown to be P2-like with respect to genome organization as well as nucleotide sequence. Phages PSP3 in *Salmonella enterica* serovar Potsdam (33) and SopEΦ in *Salmonella enterica* serovar Typhimurium (34) are also P2-like. Bacterial genome sequencing projects have revealed additional P2-like prophages, for example, Sp13 in the enterohemorrhagic *Escherichia coli* O157:H7 strain isolated during the Sakai outbreak in Japan (35).

In our previous study (14), *cdt-V* was found in an inducible Cdt bacteriophage. The presence of *cdt* in phages, although not always inducible, has been reported (11) and is supported by the P2 sequences (12) or lambdoid phage sequences (15) found in the flanking regions of *cdt* in several bacterial isolates. In this study, a new Cdt-V phage, named ΦAA91, was used to lysogenize *S. sonnei* strain 866, since this strain gave good results in studies with Cdt phages (14) and also had previously shown the capacity to generate lysogens of temperate Stx bacteriophages (21, 36). No lysogens of ΦAA91 were obtained with any of the other laboratory strains assayed. Since the phage induced from the *S. sonnei* strain was shown to be different from the phage induced from the *E. coli* wild type, it was renamed ΦAA91-ss.

Phages ΦAA91 and ΦAA91-ss are self-inducible, or at least lead to no increase in the number of infectious viruses or viral genome copies when using common phage-inducing agents (37–40). This can be attributed to the fact that these phages belong to the P2-like family of phages. In P2 phages, the product of gene *C*, which is responsible for lysogeny, cannot be cleaved by the RecA protease (41, 42). For this reason, this gene is not affected by UV or other inducing agents, causing derepression of the lysogenic state. Therefore, P2-like phages do not promote their lytic pathway when using one of the common inducing agents; rather, they maintain the same levels of induction in the absence of these agents. This lack of inducibility was previously observed for another phage harboring Cdt (phage Φ125) (14).

The remarkable difference between the genomes of phages ΦAA91 and ΦAA91-ss is the IS*21* insertion element. Insertion elements are numerous in *E. coli* and *Shigella* genomes and are capable of causing many kinds of DNA rearrangements (43). Deletions as well as translocations and inversions are likely the result of the copious numbers of insertion sequence elements in bacterial chromosomes. This element is inserted within the *cox* gene, encoding Cox, which is responsible for inhibiting phage integration and activating phage DNA excision. In P2 phages, by leading to excision of P2 DNA out of the host chromosome, Cox is directly involved in the site-specific recombination event (44). Early expression of *cox* ensures that at least a few copies of the P2 genome are synthesized free of the host chromosome (44). However, a mutation in *cox* will produce a phage that is as efficient as or more efficient than the parental strain in the generation of lysogens. This phage would be able to recombine with higher frequency (5 to 20 times), at least in the *att* region pathway (45). Successful generation of lysogens with ΦAA91-ss in other hosts also could be attributable to its potentially higher recombination ability after *cox* truncation compared to the original phage, ΦAA91. This could not be properly assayed, since with the original phage, only one *S. sonnei* lysogen of ΦAA91 was obtained, and the phage induced from this strain (ΦAA91-ss) already carried IS*21*.

IS*21* has not been found in the wild-type *E. coli* O157:H7 strain containing phage ΦAA91; therefore, it must have been incorporated into the phage genome during lysogenization of the *Shigella* strain in which this element is present and subsequently is maintained in the phage, since it was found in some lysogens generated *a posteriori* with phage ΦAA91-ss. The IS*21* element has been reported to be located close to a *cdt-III* gene in a pVir plasmid in *E. coli* (46) but is present in a different position and with the opposite orientation. IS*21* has also been found in P2-like phage ΦCTX in *Pseudomonas aeruginosa* (31) but without the *cdt* operon. The insertion of IS*21* during the generation of *Shigella* lysogens provides experimental evidence of the evolution of phages after the lysogenization of new hosts, as widely reported, mostly by comparison of different phage genomes (47, 48).

The genes encoding Cdt were found inserted into a region of the phage (named TO) that has been identified as a hot spot for the insertion of foreign genes (49). The gene content of the TO region varies extensively among P2-like prophages found in *E. coli* (49, 50). A previous sequence of a prophage harboring the *cdt* operon indicates the same insertion site of the genes (12).

Phages ΦAA91 and ΦAA91-ss showed the morphology of *Myoviridae* and, like other P2-like phages, are transducing phages (51). In our previous studies with another Cdt-V phage, phage Φ125 (14), the phage showed *Siphoviridae* morphology, and no phages with *Myoviridae* morphology were observed. *Siphoviridae* morphology has also been reported for the Cdt-1Φ phage described by Asakura et al. (15). However, the *cdt-V* operon in phage Φ125 was flanked by a bacteriophage region showing homology with a fragment of a P2 phage. Similarly, homology with P2 in the flanking regions of the *cdt-V* cluster has been widely reported (11, 12, 18). Whether these previously reported phages belonged to the P2 family or were the product of recombination of these and other phages of the *Siphoviridae* group remains unknown. On the contrary, descriptions of the *cdt-I* and *cdt-IV* flanking sequences indicate that these are not always related to P2 phage genes (11, 15). In fact, variability among Cdt phages is not surprising considering the demonstrated ability of phages to interchange different fragments of diverse origin mediated by both homologous and nonhomologous recombination (48). The presence of P2-like phages in different genera (*Vibrio*, *Salmonella*, and *E. coli*, for example) is also coincident with the wide distribution of *cdt* genes in all of these genera.

The phage in this study is a good example that shows not only the ability of phages to generate new strains, in this case by the incorporation of *cdt*, but also the evolution of phages when moving from one strain to another, which can cause the generation of phage variants able to convert strains in a more successful way. It is of clinical importance to gain more insights into the *in vivo* dissemination of virulence factors by means of bacteriophages in enteric infections, particularly within the intestinal tract, where phages can spread through a wide range of hosts and cause the emergence of new pathogenic bacteria.

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