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Research paper

ArgO145, a Stx2a prophage of a bovine O145:H- STEC strain, is closely related to phages of virulent human strains

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ABSTRACT

Shiga toxins (Stx) are the main virulence factor of a pathogroup of *Escherichia coli* strains that cause severe human diseases. These toxins are encoded in prophages (Stx prophages), and generally their expression depends on prophage induction. Several studies have reported high diversity among both Stx prophages and Stx. In particular, the toxin subtype Stx2a is associated with high virulence and HUS. Here, we report the genome of ArgO145, an inducible Stx2a prophage identified in a bovine O145:H- strain which produced high levels of Shiga toxin and Stx phage particles. The ArgO145 genome shared lambda phage organization, with recombination, regulation, replication, lysis, and head and tail structural gene regions, although some lambda genes encoding regulatory proteins could not be identified. Remarkably, some Stx2a phages of strains isolated from patients in other countries showed high similarity to ArgO145.

1. Introduction

Phages (virus that infect bacteria) represent the most abundant biological entities on earth (Hatfull and Hendrix, 2011; Suttle, 2016). They are found in very large numbers wherever their host live (Kutter and Sulakvelidze, 2004), and it is estimated that they outnumber bacteria by 10-fold (Hatfull and Hendrix, 2011). Phages play key roles in regulating the microbial balance in ecosystems, as phage lytic activity affects bacterial mortality and non-lytic activity may affect bacterial traits. Moreover, some phages play important roles in bacterial virulence, converting the host from a nonpathogenic strain to a virulent strain (Kutter and Sulakvelidze, 2004).

The phage-encoded Shiga toxin (Stx) is the main virulence factor of a pathogroup of *Escherichia coli* strains that cause severe human diseases, such as hemorrhagic colitis and hemolytic-uremic syndrome (HUS) (Karmali et al., 1985; Riley et al., 1983). These strains, named STEC (Shiga toxin producing *E. coli*) or VTEC (verocytotoxigenic *E. coli*) can carry one or more Shiga toxin-encoding genes (*stx*) in their genome. Generally, the *stx* genes are located within the phage lysis region, and expression of *stx* depends primarily on prophage induction (Wagner and Waldor, 2002; Waldor and Friedman, 2005). Several studies have shown that there is diversity in both Shiga toxin and Stx phage families. Furthermore, epidemiological data suggest associations between specific Stx subtypes and severity of human illness, being Stx2a the subtype most associated with high virulence and HUS (Chui et al., 2015; Friedrich et al., 2002; Persson et al., 2007). However, little is known about characteristics of Stx phages that may contribute to high virulence. Of particular interest is the genetic region upstream of the *stx* genes that could regulate *stx* expression. Interestingly, some different anti-terminator Q sequences have been reported, and there are studies that showed a relationship between Q allele and level of *stx* expression (Ahmad and Zurek, 2006; Lejeune et al., 2004; Steyert et al., 2012).

Surveillance data on STEC infections indicates that approximately half of HUS confirmed cases are associated with serogroup O157, and that O26, O91, O103, O111, O121 and O145 serogroups are the most frequently ones isolated from non-O157 cases (EFSA Panel, 2013; Gould et al., 2013). Particularly, O145 strains have been commonly isolated in several countries, like U.S., Canada, Germany, U.K., Spain, Italy, Denmark, Finland, Japan (FSIS, 2011), and have been associated with outbreaks in Japan, U.S., Belgium, Norway (De Schrijver et al., 2008; Kaspar et al., 2010; Taylor et al., 2013; Wahl et al., 2011). In Argentina, STEC O145:H- was firstly detected in cattle in 2001 (Padola et al., 2002). Strains belonging to this serotype were later associated with one family and one kindergarten outbreak (Rivas et al., 2010). Currently, O145:H- is the serotype that follows O157:H7 in prevalence in this country (Zotta et al., 2014).

Taking into account that cattle is the main reservoir of most STEC strains (Gyles, 2007; Persad and LeJeune, 2014), and that, to our

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knowledge, there are few studies analyzing DNA sequences of Stx phages from bovine strains (Beutin et al., 2013; Johansen et al., 2001), the aims of this study were to characterize and determine the whole genome sequence of a Stx2a prophage present in an STEC strain representing one of the first described stx_2-positive O145:H- strains isolated from cattle in Argentina, and to perform a comparative analysis against complete Stx phage sequences available in databases.

2. Materials and methods

2.1. Bacterial strains

FB5, a STEC strain producing high cytotoxicity titers on Vero cells (Krüger et al., 2011) was chosen for a deep characterization of a Stx2 phage from bovine origin. This strain had been isolated from a feedlot cow during a rectal swab screening for STEC detection in 2001–2002, and reported among the first isolates of STEC O145:H- in Argentina (Padola et al., 2002). Further characterization showed that strain FB5 is positive for stx_{2a}, *eae*γ and *ehx*A genes (Krüger et al., 2011).

E. coli laboratory strain DH5α was used as an indicator strain for phage quantification in double-agar-layer plaque assays and *E. coli* EDL933 strain as a positive control for phage and toxin production. Nine *E. coli* strains were selected for studies on phage infectivity and lysogenization: DH5α, HB101 and Y1090 *E. coli* K-12 derivatives; two ETEC strains, one O157 and one O145 EPEC strain, one O157 and one O145 STEC (stx_1-positive, stx_2-negative) strain.

2.2. Evaluation of Shiga toxin and phage production in strain FB5

Shiga toxin and phage production were evaluated in strain FB5 using an inducing agent (mitomycin C) and under non-inducing conditions. The EDL933 strain was also included as a positive control of Stx phage induction. Aliquots of overnight cultures in Luria Bertani (LB) broth were inoculated onto fresh LB medium and incubated at 37 °C and 180 rpm up to an optical density at 600 nm (OD_{600}) ≈ 0.2–0.3, after which each culture was subdivided into two flasks. Mitomycin C (final concentration 0.5 μg/ml) was added to one of the subcultures and the incubation was continued under the same conditions.

2.2.1. Analysis of stx_{2a} expression

The expression of stx_2 was evaluated 90 min after mitomycin C addition. Total RNA was purified from 500 μl aliquots (SV total RNA isolation system; Promega) and treated with DNase I (Roche Diagnostics GmbH) at 37 °C for 1 h. cDNA was synthesized from 1 μg total RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and diluted 1/10 for qPCR reactions. Two qPCRs were carried out for each sample on a OneStep Plus Real-Time PCR System (Applied Biosystems). Primers 2SF and 2R (de Sablet et al., 2008) were used to amplify the stx_{2a} gene, and TufAqR plus TufAqF for the *tuf*A gene (de Sablet et al., 2008). Each reaction was performed in a 20 μl reaction mix containing 10 μl 2× SYBR Green mix (FastStart Universal SYBR Green Master, Roche) and either primers 2SF and 2R at 400 nM each, or TufAqR and TufAqF at 300 nM each. Expression levels of stx_{2a} were normalized against *tuf*A.

In order to evaluate extracellular Stx2a production, supernatants from cultures at 18 h were obtained by centrifugation at 12,000 ×*g* for 10 min, diluted 1/10 in LB and analyzed with an enzyme immunoassay (EIA, Ridascreen Verotoxin, R-Biopharm) according to the manufacturer instructions.

2.2.2. Quantitative analysis of phage production

Phages were purified from cultures at 3 h post induction. Briefly, a 2.6 ml aliquot of each culture was centrifuged at 10,000 ×*g* at 4 °C for 10 min. Supernatants were centrifuged again in the same conditions and filtered through low-protein-binding 0.22 μm membrane filters (Millex-GP, Millipore).

The number of infective phage particles was determined by the double-agar-layer method. For this purpose, filtrates and corresponding tenfold serial dilutions were mixed with 500 μl of an exponential phase culture of *E. coli* DH5α (OD_{600} = 0.6–0.8) and incubated for 30 min at 37 °C with shaking (120 rpm). This suspension was then mixed with 3 ml of LB soft agar (0.75% w/v) supplemented with 9 mM $CaCl_2$ and poured onto LB agar plates. After 18 h incubation, visible lysis plaques were enumerated and phage titers were expressed as plaque forming units (pfu)/ml.

For quantitative PCR analysis of Stx2 phage particles, filtrates were treated with chloroform (1/10), vortexed and centrifuged at 10,000 ×*g* for 5 min. Phage particles were concentrated 5 times by centrifugation of those supernatants at 35,000 ×*g* for 30 min at 4 °C. To degrade any remaining bacterial DNA, phage suspensions were treated with DNase I (Roche Diagnostics GmbH) at 37 °C for 1 h. Phage DNA was purified with a commercial kit (High Pure Viral Nucleic Acid Kit, Roche) and diluted 1/100 for qPCR reactions. Amplification and detection were performed on a OneStep Plus Real-Time PCR System (Applied Biosystems) with primers 2SF and 2R as described before. It was assumed that one copy of the *stx* gene corresponds to one Stx phage particle.

2.2.3. Evaluation of Stx2a phage infectivity and generation of lysogens in E. coli strains

Filtered phage suspensions of induced strain FB5 cultures were assayed onto the nine selected *E. coli* strains mentioned above. Briefly, each *E. coli* strain was grown in 5 ml LB broth at 37 °C and 180 rpm. Five hundred microliter of exponential-phase cultures were mixed with 2 ml LB soft agar (0.75% w/v) containing 9 mM $CaCl_2$ and poured onto a LB agar plate. A drop of the phage suspension and 1/10 dilutions were spotted onto each host monolayer and incubated at 37 °C overnight.

Small portions of drop area (with or without visible lysis) were picked and streaked onto an agar plate by a sterile inoculation loop. After overnight incubation at 37 °C for 18 h, a loop of confluent bacterial growth zone and 20–30 single colonies were suspended in sterile water and screened by PCR for stx_2. Three stx_2-positive colonies from each host were subcultivated three times on LB agar plates. One stable lysogen was tested for its ability to produce infective phages. For this purpose, one single lysogenized colony was cultured in LB medium at 37 °C and 120 rpm 18 h. Overnight cultures were inoculated in fresh LB and treated as described above for viable phage counting. Additionally, lysis plaques were PCR screened for stx_2. Stx2a production was tested in overnight supernatants of mitomycin C-induced cultures.

2.3. Genomic DNA extraction and sequencing

Bacterial DNA was extracted from an overnight culture of strain FB5 with the Ultraclean Microbial DNA isolation kit (MoBio). The DNA concentration and purity were measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and the Qubit double-stranded DNA (dsDNA) HS and BR assay kits (Life Technologies). One nanogram of bacterial DNA was used for library preparation. The DNA library was prepared using the Nextera XT library preparation kit with the Nextera XT v2 index kit (Illumina). Subsequently, the library was sequenced on a MiSeq sequencer, using the MiSeq reagent kit v2 generating 250-bp paired-end reads (Illumina). Sequencing was aimed at a coverage of at least 60-fold. MiSeq data were processed with MiSeq control software v2.4.0.4 and MiSeq Reporter v2.4 (Illumina). Quality trimming of reads was performed with CLC Genomics Workbench 7.0.4 (Qiagen) using a minimum Phred (Q) score of 28 (Kluytmans-Van Den Bergh et al., 2016). In addition, a mate-pair DNA library was prepared using the Mate Pair Library Prep Kit v2 (Illumina) according to the manufacturer's instructions followed by running on the Miseq for generating 100-bp reads.

2.4. Bacterial genome assembly and phage genome detection

Reads from mate pair sequencing were combined with those from paired-end sequencing and were *de novo* assembled using CLC Genomics Workbench. The genome of STEC strain FB5 was analyzed with BLASTN (https://blast.ncbi.nlm.nih.gov/) using the stx_{2a} sequence from the phage 933W (AF125520) as query sequence to identify contigs of interest and with PHAge Search Tool Enhanced Release (PHASTER) web server (http://phaster.ca) to identify prophage genomes (Arndt et al., 2016).

2.5. Genome annotation and comparative analyses

The sequence of the identified stx_{2a}-encoding prophage, named ArgO145, was analyzed with Open Reading Frame (ORF) Finder (https://www.ncbi.nlm.nih.gov/orffinder) to search for potential protein encoding segments using the following settings: minimal ORF length of 150 bp, a standard genetic code and "ATG" and alternative initiation codons. Results from ORF finder were combined with the coding sequences detected by PHASTER. The translated amino acid sequences were examined with SMART BLAST (https://blast.ncbi.nlm.nih.gov/smartblast/?LINK_LOC=BlastHomeLink) to search and identify putative proteins.

BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare selected ORFs and the complete predicted genome of the ArgO145 against sequences in the database. Prophage genomes showing the best matches to ArgO145 were further aligned and visualized with Easyfig 2.2.2 (Sullivan et al., 2011). In addition, the prophage genome of ArgO145 was compared with selected prophages of O157 and non-O157 STEC strains using BRIG (BLAST Ring Image Generator).

2.6. Accession number

The complete nucleotide sequence of the prophage ArgO145 was deposited in GenBank under accession number KY914478.

3. Results

Strain FB5, one of the first isolates of STEC O145 in Argentina, was selected to study a Stx phage of a bovine strain. Comparative qPCR analysis showed that stx_{2a} expression levels in strain FB5 cultured in untreated conditions are similar to those observed in *E. coli* EDL933 reference strain. In addition, in both strains the treatment with mitomycin C incremented the stx_{2a} mRNA levels approximately 100-fold (Fig. 1 A). High extracellular Stx production was confirmed by enzyme immunoassay, as results from strain FB5 overnight supernatants were classified strongly positive (+4) according to the kit classification.

The ability of strain FB5 to produce Stx2a phages was also assessed for mitomycin C treated and untreated cultures. By double-agar-layer method, titers of 8 10^3 and 9 10^6 pfu/ml were observed for supernatants of untreated und treated cultures, respectively. Lysis plaques were assayed by hybridization, which revealed that all plaques belonged to Stx2 phages. Mitomycin C induction was also evidenced by qPCR results that indicated two order of magnitude higher levels of Stx2a phage particles in treated cultures relative to the levels seen with spontaneous induction (Fig. 1 B). In agreement with the results of Stx toxin production, strain FB5 produced Stx2a phage levels similar than those of EDL933.

Infectivity of Stx phages induced from strain FB5 was tested on agar monolayers containing diverse selected *E. coli* strains. Drops of undiluted and 1/10 diluted strain FB5 filtrates produced confluent lysis and/or individual plaques on K-12 derivative *E. coli* strains (Fig. 1 C). Presence of Stx2 phages in drop areas were confirmed by PCR. The spot test was negative for the remaining *E. coli* strains.

Production of stx_2 lysogens was successful with each of the three K-12 derivative strains tested. However, stx_2 lysogens were more

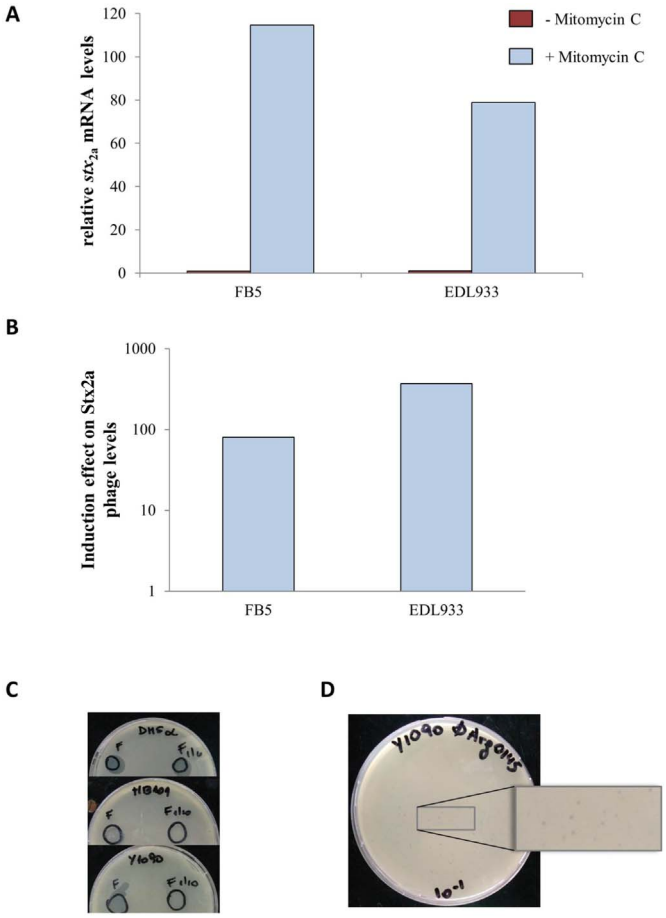

Fig. 1. A) stx_{2a} expression by qPCR. Levels of stx_{2a} mRNA were normalized against *tuf*A and expressed relative to stx_{2a} mRNA levels of untreated EDL933. B) The effect of mitomycin C on phage production was measured by qPCR. y axis, logarithmic scale of relative increase calculated as the ratio of stx_{2a} gene copies in supernatants of cultures treated with mitomycin C (0.5 µg/ml) to those in supernatants of untreated cultures. C) Infection and lytic effect of ArgO145 phage on DH5α, Y1090 and HB101 *E. coli* strains. F and F1/10 correspond to drops of undiluted and 1/10 diluted filtered phage suspensions of strain FB5 culture, respectively. D) ArgO145 plaques formed on double layer agar plates with DH5α as host strain. The image corresponds to quantification of ArgO145 present in 100 µl tenfold diluted supernatant of an untreated Y1090 lysogen culture.

frequently detected and showed to be more stable using Y1090 as recipient strain. One stable Y1090 stx_2 lysogen was evaluated for phage and toxin production. Infective Stx phages were detected in both treated and untreated cultures (Fig. 1 D), and titers were in the same order of magnitude that those observed in the strain FB5 cultures. In addition, high extracellular Stx production was detected in supernatants of an overnight culture of mitomycin C treated Y1090 stx_2 lysogen (classified +4, strongly positive according to the kit classification).

The whole genome of STEC strain FB5 was sequenced and contigs were analyzed to identify the Stx2a encoding prophage. Only one stx_2 copy was detected in strain FB5 genome. A first analysis using data from the *de novo* assembly of paired-end reads did not allow detection of a complete Stx prophage genome within a single contig (data not shown). However, this was solved by combining data from two sequencing runs, *i.e.*, data obtained from sequencing a paired-end and a mate-paired library. A predicted prophage carrying stx_{2a} was identified in a 279 kbp contig. This prophage was named ArgO145. In addition to ArgO145, other 13 non-Stx prophage regions were identified in strain FB5 genome by analysis with PHASTER (3 regions classified as intact phages, 7 incomplete and 3 questionable according to the completeness score).

Table 1
Main genomic features of ArgO145 prophage.

Strand	Start	Stop	Predicted function
	1	25	*att*L site
+	220	1,389	Integrase
−	1,555	1,373	DNA-binding protein[a]
−	2,845	2,219	Adenine methylase
+	4,382	4,588	Toxin-antitoxin system, antitoxin component
+	4,594	4,893	Toxin-antitoxin system, toxin component
−	5,804	5,439	HNH endonuclease
−	6,024	5,806	Sugar acetyltransferase inhibitor
−	10,004	9,126	NinC
−	13,216	12,527	Exonuclease
−	14,163	13,213	RecT
−	14,989	14,777	Kil
−	15,707	15,060	Phage regulatory protein, Rha family
−	17,522	16,568	Type II restriction enzyme BsuBI[b]
−	18,988	17,519	Modification methylase BsuBI
−	19,796	19,083	Repressor CI
+	19,892	20,095	Regulatory protein Cro
+	20,998	22,518	Helicase
+	22,508	23,479	DNA primase
+	23,936	24,499	NinG
+	24,496	24,690	NinH
+	24,683	25,117	Antiterminator Q
+	25,366	25,518	DNA methylase
+	25,559	25,634	tRNA Met-CAT
+	25,644	25,720	tRNA Arg-TCG
+	25,734	25,810	tRNA Arg-TCT
+	25,901	26,860	Shiga-like toxin II subunit A
+	26,872	27,141	Shiga-like toxin II subunit B
+	27,627	29,564	Esterase
+	30,242	30,457	Holin
+	30,462	30,995	Lysozyme
+	31,270	31,839	Antirepressor
+	31,996	32,460	Rz
−	32,785	32,492	Bor
+	33,194	34,000	Small subunit terminase
+	33,981	35,687	Large subunit terminase
+	35,687	37,831	Portal protein
+	39,020	40,234	Major capsid protein
+	42,408	44,321	Tail fiber protein
+	44,323	44,592	Tail fiber protein
+	46,835	48,103	Tail fiber protein
+	48,118	48,396	Outer membrane protein
+	49,110	49,844	Outer membrane protein Lom precursor
+	51,884	52,135	Bacteriocin
	61,996	62,020	*att*R site

[a] The predicted protein shows a good alignment (56/60 aa) with the excisionase of the Stx2 converting phage vB_EcoP_24B (ADN68389.1).

[b] This region shows high nucleotide identity (954/955) with those from PA2, PA8, Stx2a_F765 and VT2phi272. However, an additional C results in a frameshift and in-frame stops in the coding sequence.

Analysis of the genome of the ArgO145 prophage from *att*L to *att*R showed a 62,020 bp sequence with a GC content of 50.0%. Examination of the chromosomal flanking sequences revealed that ArgO145 lies within the tRNA-Arg-CCT gene (*arg*W), which appears to remain intact. Sequence analysis predicted 83 genes. Forty-three of the gene predicted products showed high identity to annotated proteins and tRNAs reported in other phages. Putative products are listed in Table 1.

At first glance, the ArgO145 genome structure seems to follow the lambda phage organization. In the genome, ORFs for predicted repressor protein CI, regulatory protein Cro, antiterminator Q, and lysis, head and tail gene regions were detected. However, other lambda-like genes encoding regulatory proteins could not be identified.

Like in most Stx phages, the *stx* gene was detected downstream *Q*. Directly downstream the *stx* gene, ArgO145 encodes a predicted 645 bp esterase with a 99% identical translated sequence to that of the 933 W bacteriophage (designed locus 933Wp42), that could facilitate STEC colonization and maintenance in the gut (Nübling et al., 2014).

ArgO145 also carries several genes probably associated with phage persistence in the host, some of them useful to circumvent host defense mechanisms. In addition, a toxin-antitoxin system, and three predicted DNA methyltransferases were identified in this prophage. Particularly, it carried genes encoding a Type II restriction-modification *Bsu*BI/*Pst*I system, with a 99% identity with a previously reported methyltransferase encoded by a Stx phage of a HUS-linked *E. coli* O104:H4 (Fang et al., 2012).

Whole genome comparison of ArgO145 against sequence databases revealed matches with several Stx phages. Comparative analyses of these phage genomes showed that the best matches (95% sequence coverage with 99% of identity) corresponded to phages PA2, PA8, Stx2a_F765 and VT2phi272 (phage accession numbers KP682371, KP682374, AP012534.1 and HQ424691.1, respectively), which were detected in O157:H7 strains isolated from humans (Ogura et al., 2015; Yin et al., 2015; Zhang et al., 2007). Fig. 2 shows sequence alignments of ArgO145 with these Stx2a phages. The sequence of ArgO145 appeared to be shorter than the four mentioned phages. Main differences were related to the absence of the IS629 insertion element in ArgO145 and to the sequence between 4.6 and 7.8 kbp in ArgO145. In this region, ArgO145 encodes a putative toxin, a putative endonuclease and some hypothetical proteins not found in the other four phages, but lacks some genes present in the others.

ArgO145 was also aligned with a selection of Stx2a prophages present in O157 and non-O157 strains. The comparison revealed a similar gene organization with differences concentrated mostly in the early transcribed region (Fig. 3). Interestingly, regions encoding the integrase and NinC were highly similar to those of the RM13514 phage from an O145:H28 isolate (Cooper et al., 2014) (Fig. 3 B). Alignment of the *stx* and flanking genes revealed sequence conservation among the analyzed phages with the highest similarity in the stx_{2a} sequences, which encode the same amino acid sequences. ArgO145 differed in the region located at ~45 kbp encoding tail fiber proteins with that of phage P13374 from strain CB13374 and that of the Stx phage from 2011C-3493, both O104:H4 enteroaggregative hemorrhagic *E. coli* (EAHEC) strains isolated during the German outbreak in 2011 (Ahmed et al., 2012; Beutin et al., 2012) (Fig. 3 B). But in fact, this region was similar among ArgO145 and the phages carried by the other two non-O157 strains. The predicted sequence for a tail fiber protein (encoded in the region between 42,408–44,321 bp) showed 96% identity with that of the Stx2a prophage from the clinical O145:H28 isolate RM13514 and also a high identity (ranging from 98 to 93%) with those reported for short tailed Stx phages recognizing the adsorption target BamA (Smith et al., 2007).

4. Discussion

The Stx phages encode and regulate Shiga toxin production, which is considered the main virulence factor of STEC strains (Tyler et al., 2013; Wagner et al., 2001). The family of Stx phages is a heterogeneous group, whose differences in relation to Shiga toxin production and to other phage characteristics that contribute to STEC virulence are poorly understood.

Genetic and phylogenetic analyses of STEC strains propose that some strains isolated from bovines and food have the potential to cause severe illness (Feng et al., 2017; Feng et al., 2014; Krüger et al., 2015). In agreement with these observations, previous studies in our laboratory suggest that Stx phages from some bovine strains are able to be induced to similar levels than those from clinical isolates (Krüger et al., 2011; Lenzi et al., 2016). To contribute to the understanding of the role of Stx phages in HUS epidemiology, we performed a deep characterization of a Stx2a phage carried by an O145 STEC isolated from cattle. This phage, the only Stx phage carried by strain FB5 according to genome sequencing, was named ArgO145. Evaluations on phage production and phage infectivity confirmed that ArgO145 is an inducible phage able to produce lytic and lysogenic infection.

Genome characterization and nucleotide sequence comparison of

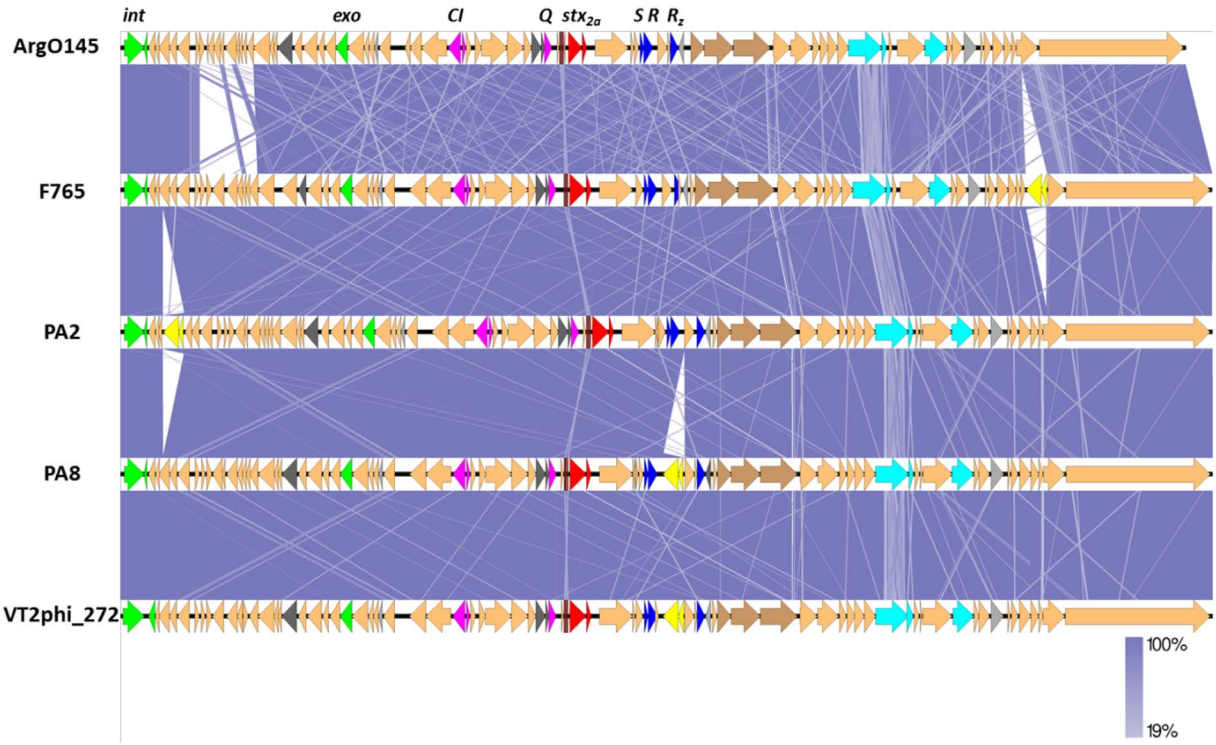

Fig. 2. Whole genome alignments of ArgO145 and four closely related Stx2a prophages.
Arrows represent ORFs. The violet gradients (dark to pale) represent the sequence identity (from 100 to 19%) between ArgO145 and the other phages. Red indicates stx_{2a} subunits. In addition, some genes encoding for putative lambda-like products were highlighted in colors: green indicate genes for integrase, excisionase and exonuclease; dark pink for regulatory elements CI, Cro and Q, magenta for tRNA, blue for lysis proteins S, R and Rz; brown for terminase and portal protein, light blue for tail proteins, grey for some accessory proteins (Nin, Bor, Lom and Kil). Yellow arrows indicate the location of IS*629*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

bacteriophage genomes provide valuable information about common and diverse characteristics among Stx phages and identification of possible factors that affect bacterial fitness and/or virulence. The development of new technologies facilitates bacterial whole-genome sequencing, including prophage sequences. However, the short read technology, and the common presence of other prophages (like in strain FB5) are factors that hinder the full *de novo* assembly of Stx phage genomes from bacterial sequences (Yin et al., 2015). In the present

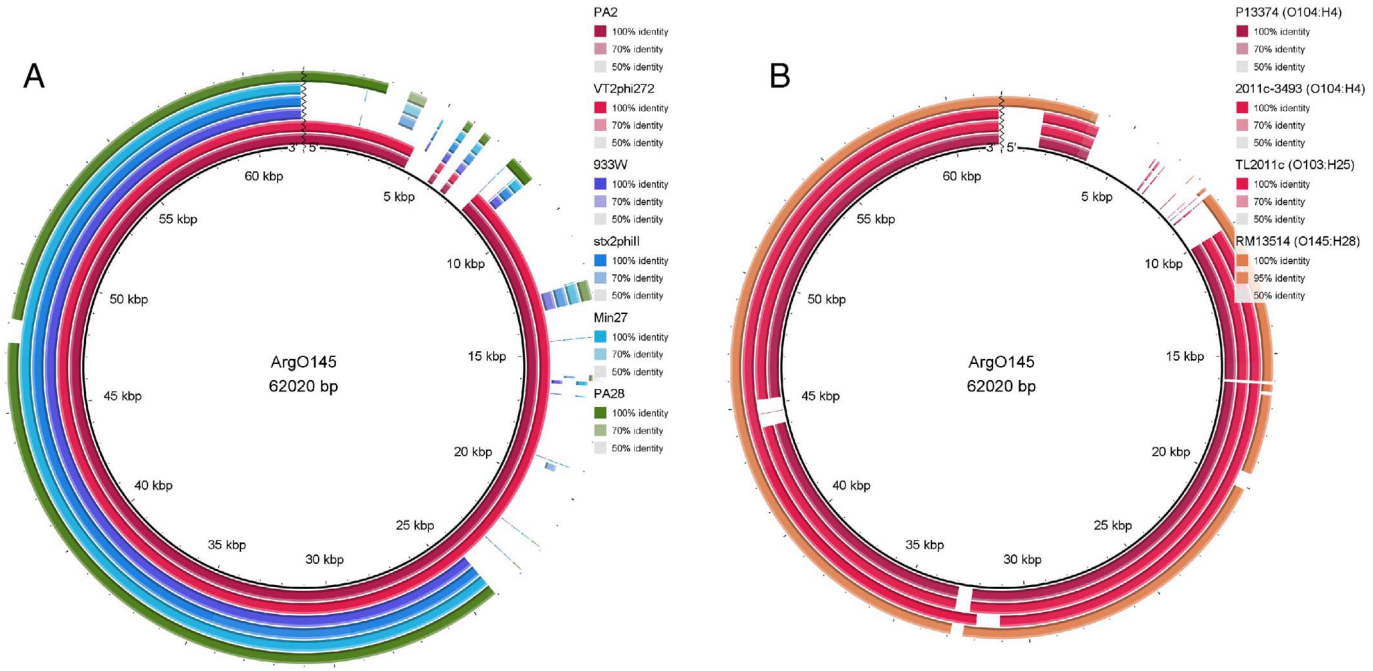

Fig. 3. Relationship of ArgO145 to other Stx2a phages carried by O157 (A) and non-O157 strains (B). The gradients (dark, pale, and white) of each color represent the sequence identity (from 100 to 0%) between ArgO145 and the other Stx2a phages.
Prophage sequences were retrieved from the NCBI and correspond to accession numbers KP682371 (PA2), HQ424691.1 (VT2phi_272), AF125520.1 (933 W), AP005154.1 (stx2-phiII), EU311208 (Min27), KP682381.1 (PA28), HE664024.1 (P13374), CP003289.1 (2011c-3493), JQ011318.1 (TL-2011c), CP006027.1 (RM13514).

study, the complete genome of ArgO145 could be obtained in a single contig by combining data from two sequencing runs. Interestingly, Stx2a prophages of O157:H7 STEC strains isolated from humans in the U.S. (2006–2008), Japan (1995) and Canada (1995) showed a remarkable similarity to ArgO145 genome (Ogura et al., 2015; Yin et al., 2015; Zhang et al., 2007). In addition, the integration site of ArgO145, *arg*W, has been also reported as integration site for Stx2 phages in O104:H4, O157:H7 (lineage I/II), O111:H and O145:H28 strains (Cooper et al., 2014; Laing et al., 2012).

Earlier studies on sequences of Stx phages described 5 gene clusters related to recombination, early regulation, replication, lysis, and head and tail structural gene regions (Miyamoto et al., 1999). However, it has been described that Stx phages are mosaic, with not all regions carrying genes conserved to the same degree. This is in line with the modular theory of evolution of lambdoid phages, which states that one region can be exchanged for another through recombination among phages infecting the same host (Brussow and Kutter, 2005). The results of comparative genomic analyses of ArgO145 with other Stx2a phages are in agreement with recent findings that highlight a divergence among Stx phage sequences (from O157 strains) in recombination and early regulation regions with more conserved lysis and structural gene regions (Yin et al., 2015).

In particular, ArgO145 showed some differences with lambda and other Stx2a phages, like 933W, in genes associated to lysogeny control. Of the CI-CII-CIII circuitry generally present in lambdoid phages (Casjens and Hendrix, 2015), only the *cI* gene was detected in ArgO145. Noticeably, both the *cII* gene, whose product is considered to be a central player in establishing lysogeny in lambda, and *cIII*, whose product controls stability of CII protein (Casjens and Hendrix, 2015), were not found. Similarly, other authors could not identify these two genes in Stx2a phages from an O104:H4 EAHEC strain of the 2011 German outbreak (Beutin et al., 2012, from an O111:H2 EAHEC strain (Grande et al., 2014), and two O157:H7 STEC strains (Yin et al., 2015). The *exo-xis* region, that was proposed to participate in the regulation of lysogenization and the promotion of phage induction, also differs from those described in 933W and $\Phi 24_b$ prophages (Bloch et al., 2013; Licznerska et al., 2016). Interestingly, despite the particular characteristics of early regulatory genes in ArgO145, phage titers were similar for strains EDL933 and FB5, both in basal and induced conditions. It is worth to note that other factors encoded in the host, including the presence of other prophages, can also affect phage induction and toxin production (Yin et al., 2015).

In a recent study about Stx2a phages of STEC O157:H7 strains, Yin et al. (2015) identified three distinct clusters of phage sequence types (PSTs), that were designated PST1, PST2 and PST3, mostly differing in regions predicted to encode the phages' early regulatory and replication genes. These authors found that the PST2 cluster (which included PA2 and PA8 phages) was the most related to Stx2a phages from highly virulent non-O157 strains, including O104:H4 and O103:H2 strains. Interestingly, phylogenetic analysis showed that ArgO145 also clustered with this type (data not shown).

Among Stx phages of non-O157 strains those carried by the emerging O104:H4 EAHEC strains have been more extensively characterized (Ahmed et al., 2012; Beutin et al., 2012; Grande et al., 2014). A 900 bp sequence including a tail fiber gene was identified as uniquely associated with phages of EAHEC (Grande et al., 2014). In accordance, this sequence was not found in ArgO145.

Most Stx phages carry genes not required for core lambdoid phage replication and life cycle control, which have a hypothetical or unknown function (Smith et al., 2012). Moreover, recent studies showed that Stx phages may encode other factors that have an impact on their bacterial host (Su et al., 2010; Tozzoli et al., 2014; Veses-Garcia et al., 2015; Xu et al., 2012).

It is known that phages and phage resistance mechanisms have key roles in regulating bacterial populations (Labrie et al., 2010). It is suggested that methylases encoded by bacteriophages have functions similar to that of methylases found in bacterial cells (Murphy et al., 2013). In addition to protection from host restriction endonucleases, other effects of methylases have been proposed, such as maintenance of lysogeny, the packaging of phage DNA and controlling bacterial genes. Interestingly, the methyltransferase-endonuclease system detected in ArgO145 showed high similarity to that identified by Fang et al. (2012), which was shown to induce changes in the expression of many bacterial genes and pathways.

5. Conclusions

To our knowledge, this is the first report describing the genome of an inducible and infective Stx2a phage detected in a bovine STEC strain. Since Stx phages are highly diverse, it is remarkably that some Stx2a phages of strains isolated from patients in other countries showed high similarity to ArgO145. Noticeably, the highest sequence identity was found with phages from O157:H7 strains. The characteristics of ArgO145 reinforce the conception that, considering phage characteristics, cattle can carry STEC strains potentially pathogenic for humans.

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