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Isolation, characterization and complete genome sequence of PhaxI: a phage of *Escherichia coli* O157 : H7

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Bacteriophages are considered as promising biological agents for the control of infectious diseases. Sequencing of their genomes can ascertain the absence of antibiotic resistance, toxin or virulence genes. The anti-O157 : H7 coliphage, PhaxI, was isolated from a sewage sample in Iran. Morphological studies by transmission electron microscopy showed that it has an icosahedral capsid of 85–86 nm and a contractile tail of 115×15 nm. PhaxI contains dsDNA composed of 156 628 nt with a G+C content of 44.5 mol% that encodes 209 putative proteins. In MS analysis of phage particles, 92 structural proteins were identified. PhaxI lyses *Escherichia coli* O157 : H7 in Luria-Bertani medium and milk, has an eclipse period of 20 min and a latent period of 40 min, and has a burst size of about 420 particles per cell. PhaxI is a member of the genus 'Viunalikevirus' of the family *Myoviridae* and is specific for *E. coli* O157 : H7.

INTRODUCTION

Escherichia coli O157 : H7, an enterohaemorrhagic *E. coli*, was identified as a human pathogen after two food-borne outbreaks in 1982 (Schroeder *et al.*, 2002; Wang *et al.*, 1996). It later caused other outbreaks not only related to meat, but also to milk, water and vegetables (O'Flynn *et al.*, 2004; Olsen *et al.*, 2002; La Ragione *et al.*, 2009; Wang *et al.*, 1996). The symptoms range from a watery or bloody diarrhoea to haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura and even death (Mao *et al.*, 2001; O'Flynn *et al.*, 2004; La Ragione *et al.*, 2009; Raya *et al.*, 2006). *E. coli* O157 : H7 is highly virulent, has a low infective dose (10–100 cells) (O'Flynn *et al.*, 2004; Raya *et al.*, 2006), a high tolerance to acidic conditions (Feng, 1995; Mao *et al.*, 2001; O'Flynn *et al.*, 2004; Wang *et al.*, 1996), drying, freezing (Mao *et al.*, 2001; Wang *et al.*, 1996), and high concentrations of NaCl (Glass *et al.*, 1992).

Antibiotic therapy at early stages of infection seems to prevent haemolytic uraemic syndrome development; however, the efficacy of antibiotic therapy is uncertain (Zhao *et al.*, 2001). Some antibiotics induce the expression of Shiga toxin genes or disrupt bacterial cell walls and cause the release of Shiga toxins (Galland *et al.*, 2001; Schroeder *et al.*, 2002 ; Zhao *et al.*, 2001).

With or without antibiotics, symptomatic and supportive treatment such as administration of morphine and acetaminophen for abdominal pain, isotonic saline or Ringer's lactate in cases of diarrhoea, and dialysis in renal involvement is vital (Bitzan, 2009).

Abbreviations: EOP, efficiency of plating; TFA, trifluoroacetic acid; TM, transmembrane; TSP, tail spike protein.

The GenBank/EMBL/DDBJ accession number for the genome sequence of PhaxI is JN673056.

One supplementary figure and three supplementary tables are available with the online version of this paper.

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The human gastrointestinal tract, especially the colon, hosts about 10^{11}–10^{12} bacteria per gram of content (Guarner & Malagelada, 2003; Mazmanian *et al.*, 2005). The gut microflora has important functions, such as the digestion of remaining carbohydrates, production of K and B group vitamins, ion absorption, intestinal cell differentiation, immune system development and protective effects against pathogens (Guarner & Malagelada, 2003; Hill, 1997; Mazmanian *et al.*, 2005). Disturbing the gut ecosystem by antibiotic therapy may also lead to other severe conditions, such as pseudomembranous colitis (Guarner & Malagelada, 2003).

It is estimated that the recent emergence of antibiotic-resistant *E. coli* O157 : H7 is due to the use of antibiotics for prophylaxis or growth promotion in food animal production (Galland *et al.*, 2001; Schroeder *et al.*, 2002; Zhao *et al.*, 2001). Antibiotic resistance is a serious complication in the treatment of infections (Mizoguchi *et al.*, 2003; O'Flynn *et al.*, 2004).

Bacteriophages (phages), or viruses of bacteria, are the most abundant biological entities in the biosphere. Their numbers have been estimated at about 10^{31}–10^{32} (Brüssow & Hendrix, 2002; Suttle, 2005). Phages occur in soil, water, and on and within human and animal bodies. Bacterial viruses were discovered by Twort in 1915 and d'Herelle in 1917 and were used worldwide in the treatment of infections until the 1930s and 1940s (Ackermann & DuBow, 1987; d'Herelle, 1917; Twort, 1915). After this period, bacteriophage therapy continued in Eastern Europe and the former Soviet Union (Petty *et al.*, 2007; Sulakvelidze *et al.*, 2001; Weinbauer, 2004). However, it was discontinued in the West after the advent of antibiotics and because of poor preparation and inappropriate application of therapeutic phages (Hanlon, 2007; Kudva *et al.*, 1999; O'Flynn *et al.*, 2004; Petty *et al.*, 2007; Weinbauer, 2004).

Phage ViI and its relatives were recently classified into the genus 'Viunalikeviruses' (Adriaenssens *et al.*, 2012a). Phage ViI, the type species, has been known since 1938 and was used for phage typing (Baron *et al.*, 1955) and studied by electron microscopy (Ackermann *et al.*, 1970). Phages of Viunalikeviruses have an isometric head and a contractile tail. They share features such as genome size, mode of regulation of late transcription and mode of host recognition (Adriaenssens *et al.*, 2012a).

Today, phage therapy has again become a promising biological approach to control antibiotic-resistant infections. This is due to our increased knowledge of phage biology, the relative host-specificity of some phages, their inability to affect the normal microflora and mammalian cells (Hanlon, 2007; Petty *et al.*, 2007) and, finally, the ability of phages to grow exponentially in the presence of their specific hosts and to disappear in their absence (Carlton, 1999). Furthermore, phage genome sequencing is a reliable way to ensure the lack of virulence or antibiotic-resistance genes in phage genomes to increase safety and efficacy (Skurnik *et al.*, 2007).

This study describes a large novel anti-O157 : H7 coliphage, a member of the new genus 'Viunalikevirus'. It reports its morphology, genome sequence and some biological properties.

METHODS

Bacterial strains. Strain *E. coli* O157 : H7 B-1 was provided by the Bu Ali Reference Laboratory, Tehran, Iran, and characteristically produces purple colonies on CHROMagar O157 differential medium. Non-O157 strains form blue colonies. The MAMA-multiplex hot start PCR assay described by Cebula *et al.* (1995) was also used for verification. All strains were cultured in Luria–Bertani (LB) medium containing 1 % tryptone, 0.5 % yeast extract and 0.5 % NaCl (pH 7.4) at 37 °C, and spun at 150 r.p.m. to reach an OD_{600} of 0.4 unless mentioned otherwise.

Phage isolation. To isolate anti-*E. coli* O157 : H7 phages, 60 samples of raw lake and pond water and sewage from different locations in Iran were collected in sterile tubes during spring and summer and stored at 4 °C. Five millilitres of each sample was centrifuged at 5000 ***g*** for 15 min. The supernatants were filtered using 0.22 μm filters (Millipore), added to 5 ml of a broth culture of *E. coli* O157 : H7 (OD_{600} of 0.4), and incubated without agitation at 37 °C for 24 h. The mixtures were then centrifuged at 5000 ***g*** for 15 min, and the supernatants were filtered through 0.22 μm filters. To detect the presence of phages, 10 μl aliquots of the filtered supernatants were tested by the spot assay method on LB agar plates seeded with *E. coli* O157 : H7. After 16 h incubation at 37 °C, plates were checked for a clear zone of bacterial lysis. The supernatants containing phages were then serially diluted to obtain separate plaques, and a single plaque of each positive sample was isolated and propagated for further assays. This procedure was done three times.

Preparation and storage of phages. The isolated single plaques were inoculated into 5 ml LB medium containing *E. coli* O157 : H7 and were incubated at 37 °C without shaking for 24 h. Then, the 5 ml suspension was added to 100 ml LB medium containing *E. coli* O157 : H7 and incubated in 37 °C for another 24 h without shaking. After centrifugation and filtration, phage particles were concentrated by ultracentrifugation (Beckman L5-65) at 4 °C and 80 000 ***g*** using a swinging bucket rotor for 1 h. The pellets were resuspended in SM buffer (10 mM $MgSO_4$, 100 mM NaCl, 0.01 % gelatin and 50 mM Tris/HCl, pH 7.5) and phage concentrations of the samples were calculated by the soft agar overlay method (Adams, 1959). Mixtures of *E. coli* O157 : H7 and phage dilutions were incubated at room temperature for 5 min for phage adsorption, then inoculated into warm LB soft agar (0.5 %) and poured onto LB agar plates (1 % agar). The plates were incubated at 37 °C for 16 h and the plaques were counted. Phage stocks were stored at 4 °C.

Host range. Spot assays were done using 10 μl of phage stocks (10^9 p.f.u. ml^{-1}) on plates seeded with bacteria. Tests were done in duplicate. Four samples contained coliphages and were tested on 11 strains of *E. coli* O157 : H7 and 17 strains of non-O157 : H7 *E. coli* to determine the specificity of phages. One of these phages seemed to be O157 : H7-specific and was tested on seven strains of other members of the family *Enterobacteriaceae* (Table 1). Dilutions of phage stocks were tested in the same way on positive bacterial strains. Efficiency of plating (EOP) determinations were performed both in Tehran and in Helsinki using the soft agar overlay method and in both locations the EOP of the strain giving the highest number of plaques was set to 1, and the EOPs of other strains were compared with those. Thus, in Tehran strain B-1 and in Helsinki strain T-112514 (Table 1) gave the highest number of plaques.

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Table 1. Host range of phage PhaxI

Bacterial strain	Source	Sensitivity to PhaxI (EOP)*
E. coli O157 : H7 B-1	Bu Ali Reference Laboratory, Tehran, Iran	1†
E. coli O157 : H7 DP-10	Pathobiology Department, Bacteriology Division, Tehran University of Medical Sciences, Tehran, Iran	0.98†
E. coli O157 : H7 UTMC01901	Micro-organisms Collection, University of Tehran, Tehran, Iran	0.97†
E. coli O157 : H7 ATCC 35150	Helsinki University Central Hospital, Helsinki, Finland	+‡
E. coli O157 : H7 T-112674	Helsinki, as above	0.69§
E. coli O157 : H7 T-112512	Helsinki, as above	0.99§
E. coli O157 : H7 T-112514	Helsinki, as above	1§
E. coli O111 T-5	Veterinary Microbiology Department, University of Tehran, Tehran, Iran	–
E. coli O26 T-8	Veterinary Microbiology Department, University of Tehran	–
E. coli ATCC 8739 (Crooks)	American Type Culture Collection, Manassas, VA	–
E. coli ATCC 35218 (1532)	American Type Culture Collection	–
E. coli JM107	Fermentas, Lithuania	–
E. coli EC-T1 to EC-T12 (12 isolates)	Clinical isolate, Qods Clinic, Tehran, Iran	–
Enterobacter aerogenes PTCC 1221	Persian Type Culture Collection, Tehran, Iran	–
Enterobacter fecalis EF-T1	Clinical isolate, Shariati Hospital, Tehran, Iran	–
Klebsiella pneumonia KP-T1	Clinical isolate, Shariati Hospital	–
Proteus mirablis PM-T1	Clinical isolate, Shariati Hospital	–
Salmonella typhi PTCC 1639	Persian Type Culture Collection	–
Serratia marcescens PTCC 1187	Persian Type Culture Collection	–
Shigella dysentriae SD-T1	Clinical isolate, Shariati Hospital, Tehran, Iran	–

*A dash indicates no lysis.
†Compared with *E. coli* O157 : H7 B-1.
‡No reliable result.
§Compared with *E. coli* O157 : H7 T-112514.

Electron microscopy. A high-titre (10^9 p.f.u. ml^{-1}) lysate was sedimented for 60 min at 25 000 ***g*** in a Beckman JE-21 high-speed centrifuge using a JA-18.1 fixed-angle rotor and was washed twice under the same conditions in neutral ammonium acetate. Phages were then deposited on carbon-coated Formvar films on copper grids, stained with 2 % sodium phosphotungstate (pH 7) and examined in a Philips EM300 electron microscope. Magnification was monitored with phage T4 tails (Ackermann, 2009a).

Proteomics analysis of structural proteins. About 10^{11} phage particles were suspended in SDS-PAGE loading buffer (2 % SDS, 10 % glycerol, 2.5 % β-mercaptoethanol, 0.0025 % bromophenol blue and 6.25 mM Tris/HCl, pH 6.8), boiled for 10 min, and then loaded onto a 12 % polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R250 (Merck) for 5 min, destained with destaining solution (30 % methanol and 10 % acetic acid in distilled water), and photographed on a light box.

Twenty micrograms of the twice ultracentrifuged phage particles were tryptic digested to peptides in the presence of 1 M urea after reduction and alkylation with DTT and iodoacetamide, respectively. Tryptic peptide digests were quenched with 10 % Trifluoroacetic acid (TFA), concentrated and purified by reversed-phase chromatography columns (C18 material, eluted with 90 % CH_3CN, 0.1 % TFA) (Varjosalo *et al.*, 2013). The dried peptides were reconstituted (2 % CH_3CN, 0.1 % TFA) and the MS analysis was performed on an Orbitrap Elite ETD mass spectrometer (Thermo Scientific), using Xcalibur version 2.7.1, coupled to an Thermo Scientific nLCII nanoflow HPLC system. Peak extraction and subsequent protein identification was achieved using Proteome Discoverer software (Thermo Scientific). Calibrated peak files were searched against the phage sequences (accession no. JN673056) by a SEQUEST search engine. Error tolerances on the precursor and fragment ions were ± 15 p.p.m. and ± 0.6 Da, respectively. For peptide identification, a stringent cut-off (0.5 % false discovery rate) was used.

Genomic characterization. The PhaxI genome was extracted from phage particles using a High Pure Viral Nucleic Acid kit (Roche) according to the manufacturer's protocol. The extracted genome was treated with DNase I, RNase A, S1 nuclease and restriction endonucleases (*Bam*HI, *Pvu*II, *Eco*RI, *Xho*I, *Nde*I, *Sma*I, *Hin*dIII, *Eco*RV; Roche) according to the supplier's recommendation, and run on a 0.7 % agarose gel.

Sequencing. Twenty decamer random primers (Saeidnia *et al.*, 2009) were synthesized by Alpha DNA. Random PCRs were carried out using Pwo DNA polymerase (Roche) containing different mixtures of these primers (0.8 pM each) and 50 ng genomic DNA in a total volume of 50 µl. The amplification was carried out using 30 amplification cycles (94 °C for 30 s, 38 °C +1 °C every 10 s for 4 min, 72 °C for 3 min). The PCR products were then purified using the PCR product purification kit (Roche) according to the manufacturer's protocol (Fig. S1 available in *Microbiology* Online) and were cloned into the *Sma*I site of pUC19. The obtained clones were used as templates for sequencing (Applied Biosystems 3730xl sequencer at Macrogen). Finally, 51 gaps between the obtained contigs were filled in by primer walking.

Annotation. Annotation was performed using Glimmer 2.02 (Delcher *et al.*, 1999), which searched the sequence for ORFs.

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RBSfinder (Suzek *et al.*, 2001) was used to search for ribosome-binding sites. Gene prediction was also done using GeneMarkS (Besemer *et al.*, 2001) and ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Similarities were compared using the BLASTP algorithm available in NCBI. The program TMHMM v. 2.0 was used to determine transmembrane domains (Krogh *et al.*, 2001).

Codon usage and regulatory elements. Aragorn and tRNAscan-SE were used to predict tRNA genes (Laslett & Canback, 2004; Schattner *et al.*, 2005). To find Rho-independent terminators, TransTerm (Ermolaeva *et al.*, 2000) and FindTerm (SoftBerry) were used. The program PHIRE was also used to find phage regulatory elements (Lavigne *et al.*, 2004). The genomic map was prepared using CGView (Grant & Stothard, 2008) and WebLogo v.2.8.2 was used to create a sequence logo of PHIRE analysis results (Crooks *et al.*, 2004). To find consensus sequences for promoters, extractUpStreamDNA (http://lfz.corefacility.ca/extractUpStreamDNA/) was used to extract 100 bp of 5′ upstream and the data were submitted for MEME analysis (Adriaenssens *et al.*, 2012a; Bailey & Elkan, 1994).

Sequence accession number. The genome sequence of PhaxI was deposited in NCBI (GenBank accession number JN673056).

Phage infection experiments in LB medium and milk, and one-step growth. An m.o.i. of 10^3 of phage was used to determine the effect of phage on *E. coli* O157 : H7 growth in two different media. Briefly, 50 ml of LB broth containing 3×10^3 c.f.u. *E. coli* O157 : H7 ml^{-1} was mixed with phages at a concentration of 3×10^6 p.f.u. ml^{-1}. Phage-free and cell-free cultures were used as controls. The infected cultures and controls were incubated at 37 °C without shaking for 2 h. The test was repeated under shaking at 150 r.p.m. Sampling was done at 30 min intervals, and viable cell counts were calculated by plating on LB agar in triplicate. The procedure was also carried out using sterilized milk (Mihan Company) instead of LB broth to observe the effect of PhaxI in a natural medium.

For determination of one-step growth, *E. coli* O157 : H7 was inoculated into 10 ml LB broth medium and incubated at 37 °C with shaking to reach an OD_{600} of 0.3; it was then centrifuged at 4 °C and 5000 ***g*** for 10 min. The pellet was resuspended in 1 ml LB and infected with PhaxI at an m.o.i. of 0.1. After 5 min incubation at room temperature for phage adsorption, the mixture was centrifuged at 4 °C and 10 000 ***g*** for 4 min. The supernatant containing unadsorbed phage particles was discarded, and the cell pellet was washed twice with LB medium and immediately resuspended in 10 ml warm LB (37 °C). A dilution of 1 : 1000 was then incubated at 37 °C, and samples were taken at various intervals. The samples were divided into two parts. One of them was titrated immediately, and the second part was treated with 2 % chloroform, shaken briefly, set aside for 30 min at room temperature and centrifuged. The aqueous portion was titrated to determine intracellular and free phage concentrations. Burst size was determined by dividing the number of liberated phage particles by the number of infected bacterial cells.

RESULTS

Isolation of PhaxI

Sixty samples of lake and pond water and raw sewage were screened for the presence of *E. coli* O157 : H7 phages. Four were positive for coliphages; however, host range studies on non-O157 *Enterobacteriaceae* showed that only one phage was specific for *E. coli* O157 : H7. This was isolated from a sample of Shahr-e-Rey (Tehran, Iran) sewage and named PhaxI. The phage is virulent and forms clear plaques of 0.05 cm diameter on lawns of *E. coli* O157 : H7 B-1 (0.5 % agar). Table 1 shows the host range of PhaxI. The phage appears to be O157 : H7-specific, lysing all tested strains of this serotype, but none of the other assayed enterobacteria. EOP is indicated in Table 1. Interestingly, the *E. coli* O157 : H7 reference strain ATCC 35150 was fully sensitive to PhaxI in the drop titration test, but with soft agar plating, it did not produce reliable results apparently due to heat shock-induced activation of a prophage.

Morphology

Under transmission electron microscopy, PhaxI showed an isometric head of 85–86 nm between opposite apices, a neck provided with collar of 17 × 7 nm and a contractile tail of 115 × 15 nm in the extended state. Heads were icosahedra, as ascertained by the observation of both hexagonal and pentagonal capsids. Contracted tail sheaths measured 30 × 23 nm (Fig. 1a). Tails showed transverse striations and 3–4 terminal prongs of about 12 × 3 nm which unfolded into an intricate fibrous network. The overall morphology of Viunalikeviruses is so characteristic that they can be instantaneously identified by electron microscopy alone.

Structural proteins

Thirteen protein bands were observed in a Coomassie-stained SDS polyacrylamide gel. As in many tailed phages, four strong protein bands with a size range from 26 to 50 kDa were present (Fig. 1b, bands A–D). In addition, at least nine weaker bands could be distinguished (Fig. 1b, bands 1–9). When twice ultracentrifuged phage particles were subjected to proteomics analysis by MS, 92 proteins were identified with high confidence (Table S1). Altogether 38 proteins had 10–67 identified tryptic peptides, 34 proteins had 5–9, and 19 proteins had 3 or 4 identified tryptic peptides (Table S1). Also included in the table is Orf183, which had only two identified peptides but a very high score. Peptide coverage was from 15 to 88 %; for some very long polypeptides, it was below 10 %.

Based on sequence similarity searches 50 proteins were hypothetical and from the remaining 42 proteins 17 were clearly annotated as structural proteins. Surprisingly many proteins were predicted to be involved in DNA processing. A few unexpected proteins were identified, such as DNA ligase (Orf42) and thymidylate synthase (Orf58), that should be soluble cytoplasmic enzymes. However, both of these were identified with high confidence (13 and 16 tryptic peptides, respectively), and therefore are likely to be packaged into the phage particles. In addition, a few proteins that were identified by sequence similarity as structural proteins, such as gp25 base plate wedge protein and gp14 neck protein, were not confidently detected in the MS analysis.

Genome characterization and sequence analysis

The PhaxI genome could not be digested by any of the tested restriction endonucleases, RNase A or S1 nuclease;

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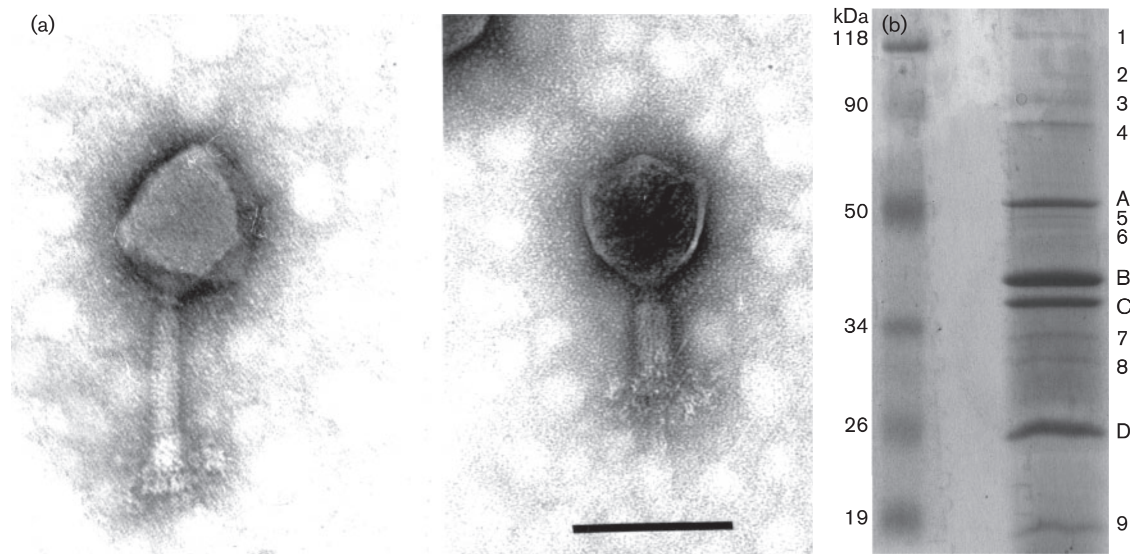

Fig. 1. Electron micrographs of PhaxI and SDS-PAGE of PhaxI structural proteins. (a) Phage particle with non-contracted (left) and contracted tail (right). Phosphotungstate, final magnification 297 000×. Bar, 100 nm. (b) PhaxI structural proteins were separated by 12 % SDS polyacrylamide gel and stained with Coomassie blue. The sizes of protein markers are indicated on the left. A pre-stained protein size marker from Fermentas was used.

however, it was totally digested by DNase I (data not shown). After sequencing, it was concluded that the genome has several cutting sites for all assayed restriction endonucleases except *Sma*I. Sequencing of the PhaxI genome yielded a linear genome map of 156 628 bp with a G+C content of 44.5 mol% (Fig. 2a). Final sequence coverage was about 6× and the genome is circularly permuted.

Sequence analysis revealed 209 ORFs occupying 144 453 bp (92.2 %) of the genome on both strands. The frequencies of start codons are 98.1 % for ATG, 1.4 % for GTG and 0.5 % for TTG.

tRNAs, codon usage and regulatory elements

Four and five tRNA genes clustering at map position 136 000–138 000 were identified using tRNAscan-SE and Aragorn, respectively: tRNA methionine (anticodon: CAT), asparagine (GTT), serine (GCT) and two others, glycine (GCC) and proline (AGG), that were predicted to carry introns. Comparison of codon usage in *E. coli* O157 and PhaxI shows that *E. coli* uses CUG for L more frequently than other codons while PhaxI uses more UUG. Other differences in codon usage are: GUG for V in *E. coli* and GUU in PhaxI, CCG for P in *E. coli* and CCA in PhaxI, CAG for Q in *E. coli* and CAA in PhaxI, CGU for R in *E. coli* and AGA in PhaxI, and GGU for G in *E. coli* and GGA in PhaxI (Table S2). Forty-six terminators were identified in the genome (Table S3). Terminator 19 248 is related to an ORF on the reverse strand (complement of 19 351–19 806), but this ORF has no equivalent in public databases, and therefore the ORFs on the positive strand (19 198–19 605, 19 606–19 824) which have similarity equivalents in public databases, were considered for annotation. Terminator 25 396 is located within the 3′ end of Orf39. In fact, Orf38 and Orf39 are encoded on opposite strands and their stop codons are actually adjacent to each other. This is a strange situation; usually such genes are separated by tens of base pairs. Some other sequences were found using PHIRE with the consensus sequence of ttgtCATgATgtatttCCTtctttt (Fig. 2b). Most of the sequences acquired by PHIRE are intergenic in relation to the predicted genes. It is not possible to predict their possible function. Similar results are reported for *Salmonella* phage *φ*SH19 (Hooton *et al.*, 2011).

The consensus sequence for promoters is TTCAAT[N14]TATAAT and CTAAATAcCcc (Adriaenssens *et al.*, 2012a). The latter sequence is similar to T4 late promoter TATAAATA. This motif acts as a recognition site for host RNA polymerase in T4 by the means of gp55. And gp33 is another factor which binds to RNA polymerase for late transcription (Geiduschek & Kassavetis, 2010; Miller *et al.*, 2003). Orf67 and Orf100 in PhaxI encode gp33 and gp55, respectively. Fifteen sequences were found for the first consensus and 28 for the second (allowing one mismatch).

Lysis

Similarly to phages vB_EcoM_CBA120 and phiSboM-AG3, no holin or lysin genes were directly identified in the PhaxI genome (Anany *et al.*, 2011; Kutter *et al.*, 2011). Class I holins possess 95 aa or more with three transmembrane

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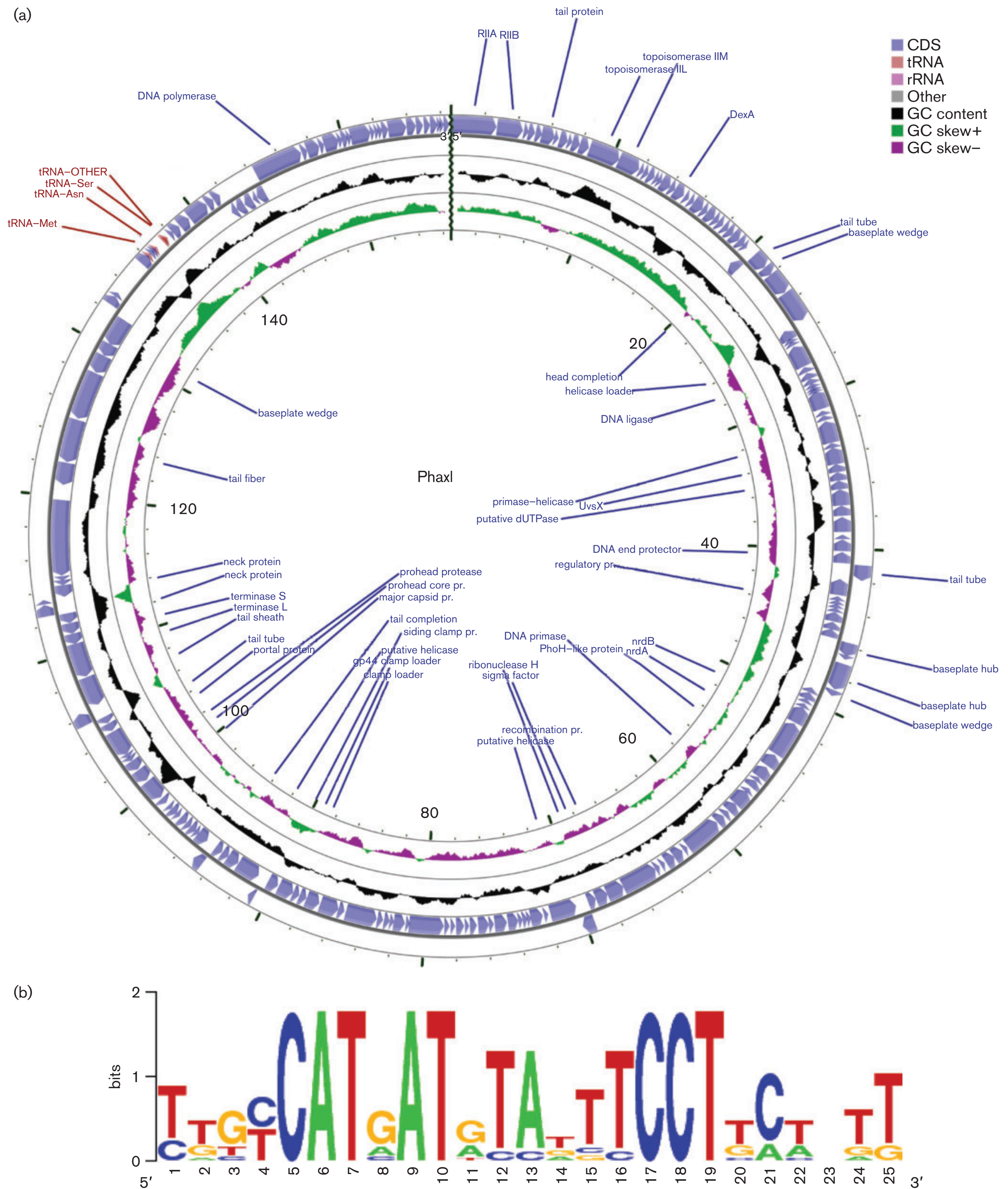

(TM) domains and class II holins have 65–95 residues with two TM domains (Young *et al.*, 2000). We identified five ORFs that could encode class II holins, i.e. ORFs 9, 69, 106, 194 and 196. They all had two predicted TM domains and the sizes of the predicted proteins were 63, 98, 69, 67 and 61 aa, respectively. Although lysin genes were not found, Orf84 encodes a protein of 264 aa, which belongs to the PG_binding_1 superfamily. This is a peptidoglycan binding

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Fig. 2. Genomic map of PhaxI created by CGView and sequence logo of PhaxI regulatory elements recognized by PHIRE. (a) The circle with green and violet peaks represents GC skews and the circle with black peaks shows GC contents. The outermost circle indicates genes on the forward strand, while the second circle represents genes on the reverse strand. tRNA genes are indicated in red. (b) Seven sequences were characterized by PHIRE in the PhaxI genome. The consensus sequence of these regulatory elements was constructed by WebLogo.

protein and shares 85 % identity with a putative endolysin in LIMEstone1.

Phage infection in LB medium and milk, and one-step growth curve

Growth curves of phage-free and infected cultures with and without shaking are shown for LB medium (Fig. 3a) and milk (Fig. 3b). Under these conditions, all bacteria were killed after 60 min in LB medium and 90 min in milk. There was no significant difference between killing times in shaken and non-shaken samples.

As determined by a one-step growth experiment (Fig. 3c), the eclipse and latency periods were 20 and 40 min, respectively. Mean burst size was about 420 (SD=22) particles per cell.

DISCUSSION

Phage PhaxI is a typical member of the family *Myoviridae* of tailed phages and the order *Caudovirales* (Ackermann, 2009b) and belongs in every respect, whether morphological, genomic or otherwise, to the recently defined genus 'Viunalikevirus'(Adriaenssens *et al.*, 2012a).

Viruses of this genus have genome sizes of 152–153 kb and capsid diameters of about 90 nm. They resemble in this respect T4-like phages, which are among the larger members of the *Caudovirales* (Ackermann, 2009b). They are characterized by a system of tail fibres which undergo conformational changes and unfold from simple prongs into an umbrella-like structure. These phages occur in enterobacteria, *Acinetobacter* and *Rhizobium*.

Most phages of this group have four tail spike proteins (TSPs); however, PhaxI has three TSPs encoded by ORFs 167, 168 and 169. Another exception is phage LIMEstone1, which has only one complete and a truncated TSP (Adriaenssens *et al.*, 2012a).

As indicated, the genome of PhaxI is a DNA molecule which was digested by DNase I, but not by RNase A. As S1 nuclease could not digest the genome, it is double-stranded. The genome was resistant to several restriction endonucleases, which suggests the presence of modified nucleotides. Furthermore, Orf58 in the PhaxI genome is a putative thymidylate synthase, probably a hydroxymethyl uracil transferase. This thymidylate synthase occurs in most Viunalikeviruses, indicating that phages of this group incorporate HMdU instead of thymine (Adriaenssens *et al.*, 2012a). Orf31 is also a putative dCMP deaminase. This indirect

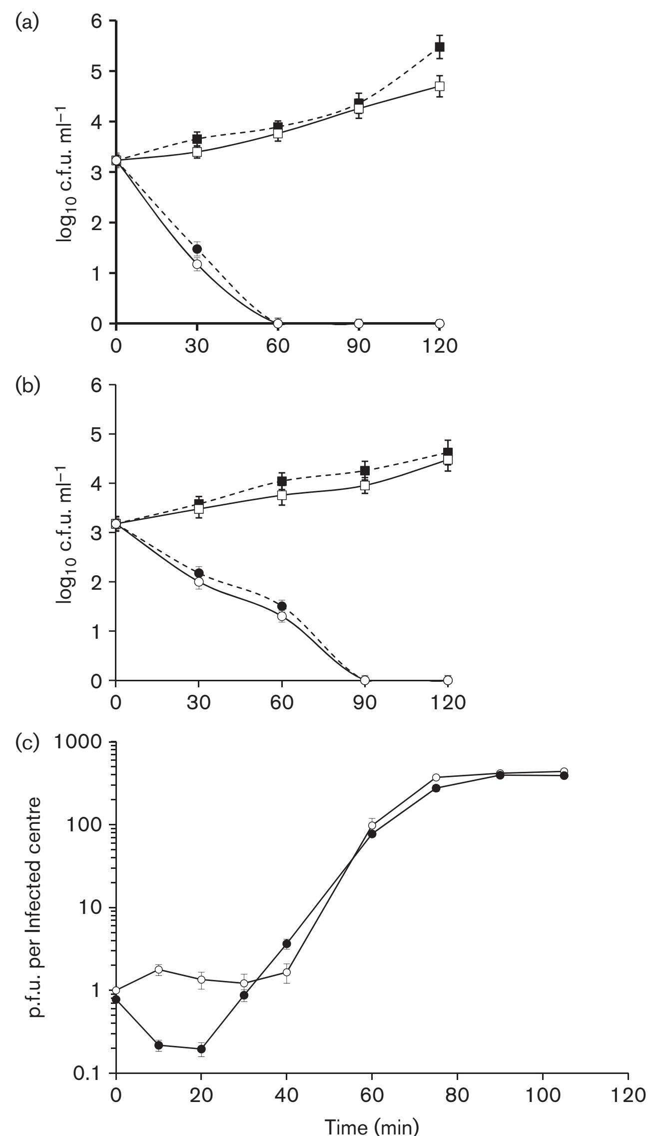

Fig. 3. Effect of PhaxI on *E. coli* O157 : H7 in LB broth (a) and milk (b) at an m.o.i. of 10^3 and one-step growth curve of PhaxI in LB broth (c). Phages were added to cultured *E. coli* O157 : H7 B-1 at an m.o.i. of 10^3 and incubated at 37 °C. Samples were taken at 30 min intervals. (■) Bacterial control with shaking, (□) bacterial control without shaking, (●) phage-infected with shaking, (○) phage-infected without shaking. For one-step growth experiment (c), *E. coli* O157 : H7 B-1 was used as host and incubated at 37 °C. Phage was added at an m.o.i. of 0.1. Samples were taken at various times. Non-treated (○) and chloroform-treated (●) samples. The eclipse and latent times are 20 and 40 min, respectively, and the burst size is about 420 particles per cell.

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evidence for the presence of modified nucleotides needs to be confirmed by DNA hydrolysis and chromatography.

Previous studies resulted in isolating several lytic O157 : H7 coliphages such as PP01, e11/2, e4/1c, CEV1, KH1, KH4, KH5, SH1, AKFV33, rv5, wV8, AR1, SFP10 and CBA120 (Kudva *et al.*, 1999; Kutter *et al.*, 2011; Liao *et al.*, 2011; Morita *et al.*, 2002; Niu *et al.*, 2012; O'Flynn *et al.*, 2004; Park *et al.*, 2012; Raya *et al.*, 2006; Sheng *et al.*, 2006; Villegas *et al.*, 2009). Some of them lyse both *E. coli* O157 : H7 and other pathogens of the family *Enterobacteriaceae*. For example, AR1 is active on *Shigella dysenteriae* and *Salmonella enterica* and SFP10 lyses *Salmonella typhimurium* (Liao *et al.*, 2011; Park *et al.*, 2012). From the O157 phages, AKFV33, Rv5, wV8, AR1, SFP10 and CBA120 are completely sequenced (GenBank accession numbers NC_017969.1, NC_011041.1, NC_012749.1, AP011113.1, HQ259103.1 and JN593240.1, respectively). Analysis of the PhaxI genome via BLASTN shows a high similarity to Viunalike phages, namely *Salmonella* phages ViI (Pickard *et al.*, 2010), SFP10 (Park *et al.*, 2012) and PhiSH19 (Hooton *et al.*, 2011), *Escherichia* phage vB_EcoM_CBA120 (Kutter *et al.*, 2011), *Shigella* phage phiSboM-AG3 (Anany *et al.*, 2011), and *Dickeya* phage vB_DsoM_LIMEstone1 (Adriaenssens *et al.*, 2012b) (GenBank accession numbers FQ312032.1, HQ259103.1, JN126049.1, JN593240.1, FJ373894.1 and HE600015.1, respectively).

PhaxI has its own replication machinery. Its genome encodes a DNA polymerase, primase, helicase, ligase, primase–helicase, loader of helicase, clamp loader, sliding clamp, ssDNA-binding protein and topoisomerase subunits. All these genes are present in the genomes of the other six members of the genus 'Viunalikevirus' (Adriaenssens *et al.*, 2012b; Anany *et al.*, 2011; Hooton *et al.*, 2011; Kutter *et al.*, 2011; Park *et al.*, 2012). These proteins have highest similarities with proteins of phages SFP10, CBA120 and PhiSH19, and lowest with AG3 and LIMEstone1.

The phage does not encode its own RNA polymerase. Therefore, it is likely that the intergenic regulatory elements identified by PHIRE analysis (Fig. 2b) might function as targets for the phage-specific sigma factor. For late transcription, there are two sigma factors in the PhaxI genome: Orf67 and Orf100. Orf68 is also a putative transcriptional regulatory protein of the CxxC_CxxC_SSSS superfamily. FmdB is a regulator family in this entry which seems to be a DNA-binding protein (Wyborn *et al.*, 1996). Orf68 may act similarly to FmdB in binding to DNA.

Although PhaxI contains five tRNA genes, it does not appear that those genes enhance the translation or compensate for the gaps between host and phage codon usage. This is also seen in other members of the genus Viunalikevirus which possess 1–6 tRNA genes (Adriaenssens *et al.*, 2012a).

MS analysis of the phage particle proteins identified confidently 92 proteins and most of the sequence-predicted structural proteins were identified in the analysis. The number of phage particle-associated proteins is much higher than predicted based on SDS-PAGE analysis. Similar findings have been reported for other large bacteriophages such as ΦKZ and ΦR1-37 (Skurnik *et al.*, 2012; Thomas *et al.*, 2012).

During the phage infection experiment, PhaxI had a significant effect on *E. coli* O157 : H7 in LB and milk medium and eliminated bacteria completely in both media. Longer bacterial killing times in milk could be due to the presence of other interacting proteins, components and ions which can affect adsorption of phage to the host. From the O157 phages, several such as CEV1, CEV2, KH1, SH1, e11/2 and e4/1c were tested in animals (Raya *et al.*, 2006, 2011; Rivas *et al.*, 2010; Sheng *et al.*, 2006).

Burst size of PhaxI is about 420 particles per cell. Burst sizes of AR1, CEV1, CEV2, AKFV33, CBA120 and SFP10 are 34, 150, 350, 350, 440 and 100 p.f.u. per cell, respectively (Kutter *et al.*, 2011; Niu *et al.*, 2012; Park *et al.*, 2012; Raya *et al.*, 2006, 2011; Ronner & Cliver, 1990).

In conclusion, PhaxI is a virulent phage that multiplies extremely well and has no toxin genes, integrase-excisionase and antibiotic-resistance genes. As no host DNA was found during sequencing, transduction assays were not performed. This phage is thus a suitable agent for phage therapy or food preservation.

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