Pathogens and Disease, 74, 2016, ftw037

doi: 10.1093/femspd/ftw037
Advance Access Publication Date: 23 April 2016
Research Article

RESEARCH ARTICLE

Phage-mediated Shiga toxin (Stx) horizontal gene transfer and expression in non-Shiga toxigenic *Enterobacter* and *Escherichia coli* strains

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One sentence summary: Transduction of non-toxigenic strains with Stx phages.
Editor: Nicholas Carbonetti

ABSTRACT

Enterobacter cloacae M12X01451 strain recently identified from a clinical specimen produces a new Stx1 subtype (Stx1e) that was not neutralized by existing anti-Stx1 monoclonal antibodies. Acquisition of *stx* by *Ent. cloacae* is rare and origin/stability of stx_{1e} in M12X01451 is not known. In this study, we confirmed the ability of Stx1a- and Stx1e-converting phages from an *Escherichia coli* O157:H7 strain RM8530 and M12X01451 respectively to infect several *E. coli* and *Ent. cloacae* strains. stx_{1e} was detected in 97.5% and 72.5% of progenies of strains lysogenized by stx_{1e} phage after 10 (T_{10}) and 20 (T_{20}) subcultures, versus 65% and 17.5% for stx_{1a} gene. Infection of M12X01451 and RM8530 with each other's phages generated double lysogens containing both phages. stx_{1a} was lost after T_{10}, whereas the stx_{1e} was maintained even after T_{20} in M12X01451 lysogens. In RM8530 lysogens, the acquired stx_{1e} was retained with no mutations, but 20% of stx_{1a} was lost after T_{20}. ELISA and western blot analyses demonstrated that Stx1e was produced in all strains lysogenized by stx_{1e} phage; however, Stx1a was not detected in any lysogenized strain. The study results highlight the potential risks of emerging Stx-producing strains via bacteriophages either in the human gastrointestinal tract or in food production environments, which are matters of great concern and may have serious impacts on human health.

Keywords: ELISA; *Enterobacter cloacae*; *Escherichia coli* O157; horizontal gene transfer; Stx-converting bacteriophages; transduction

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are major foodborne pathogens, capable of causing human diseases ranging from diarrhea to life-threatening complications such as hemolytic–uremic syndrome (McAllister *et al.* 2016). Virulence of STEC strains and their ability to cause severe diseases are linked to the activity of Shiga toxins (Stxs) (Smith *et al.* 2012; Bloch *et al.* 2015) encoded by Stx-converting bacteriophages (Stx phages) (Strauch *et al.* 2008). These lambdoid temperate phages (Allison 2007; Łoś, Łoś and Węgrzyn 2011) can encode either stx_1 or stx_2 of which there are four and seven subtypes, respectively (Zhang *et al.* 2002; Mauro and Koudelka 2011; Scheutz *et al.* 2012).

Stx-converting phages can lyse their STEC hosts (Chibani-Chennoufi *et al.* 2004) in the gastrointestinal tract (GIT) and transfer *stx* genes from cell to cell via transduction,

Received: 4 March 2016; **Accepted:** 18 April 2016
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representing a significant mechanism of horizontal gene transfer (HGT), and is most likely the cause of *stx* genes dissemination among a multitude of STEC serotypes (Gyles and Boerlin 2013). Following infection with a Stx-converting phage, the integrated *stx* genes either remain silent in the lysogens (Paul and Weinbauer 2010) or are expressed at low levels (Węgrzyn, Licznerska and Węgrzyn 2012). Upon induction by DNA damaging agents (such as mitomycin C or UV light), or by other factors that provoke the bacterial S.O.S. response, excision of prophage DNA from the host chromosome and initiation of the lytic cycle take place (Węgrzyn and Węgrzyn 2005; Imamovic and Muniesa 2012), followed by host cell lysis, expression of Stx (Allison *et al.* 2003) and release of phage particles that can transduce the *stx* genes to other bacteria (Tóth *et al.* 2003). Production of both Stx1 and Stx2 has been shown to increase upon induction of the Stx-encoding bacteriophages *in vitro* (Zhang *et al.* 2000). On the other hand, Stx1 has been demonstrated to be iron regulated, but iron regulation seems to have no impact on the expression of stx_2 (Pacheco and Sperandio 2012).

Phages play an integral role in the evolution of new foodborne pathogens (Brabban, Hite and Callaway 2005). Available evidence suggests that STEC O157:H7 has evolved from the enteropathogenic *E. coli* strain O55:H7 by the acquisition of two prophages encoding Stx1 and Stx2 (Ohnishi *et al.* 2002; Wick *et al.* 2005; Tóth *et al.* 2016). Several studies reported a limited host range for individual Stx phages (Saunders *et al.* 2001; Kelly, Vespermann and Bolton 2009). Stx phages can infect commensal *E. coli* (Wagner, Acheson and Waldor 1999; Muniesa *et al.* 2003; Gamage, McGannon and Weiss 2004), and convert Stx-negative *E. coli* strains *in vivo* as well as in natural environments (Gamage *et al.* 2003). Acquisition of *stx* by hosts other than *E. coli* (such as *Acinetobacter haemolyticus*, *Aeromonas* sp., *Citrobacter freundii*, *Enterobacter cloacae* (*Ent. cloacae*) and *E. albertii*) through Stx phages has been infrequently reported (Schmidt *et al.* 1993; Alperi and Figuera 2010; Ooka *et al.* 2012). However, the wide distribution of Stx1 and Stx2 variants in different bacteria indicates that these phages possess the ability of transmitting *stx* genes throughout members of Enterobacteriaceae.

Ent. cloacae is a part of the normal flora of the human GIT and is frequently found in environmental samples (Krzymińska *et al.* 2009). Although it is a fairly common nosocomial pathogen, there are few studies examining its pathogenic potential beyond opportunistic infections (Davin-Regli and Jean-Marie 2015). In contradiction to the fact that most *Enterobacter* members are often considered as typical commensals, the number of epidemiologic studies involving these microorganisms has been increasing (Mezzatesta, Gona and Stefani 2012), reflecting the potential of *Ent. cloacae* strains as emerging pathogens. An emergent Stx variant named Stx1e was recently identified in an *Ent. cloacae* M12X01451 strain (Probert, McQuaid and Schrader 2014) isolated from a patient with a mild diarrheal illness. This new toxin is the most divergent subtype of the Stx1, and has only limited reactivity with the available commercial anti-Stx1 antibodies, therefore representing a challenge for detection. Because of *Ent. cloacae*'s reputation as a nosocomial pathogen, its clinical relevance in human pathogenicity, especially in gastrointestinal infections, has not yet been addressed.

More epidemiological studies have focused on stx_2-encoding phages rather than stx_1-encoding phages, since stx_2-harboring strains are typically associated with more severe clinical outcomes (Ethelberg *et al.* 2004; Orth *et al.* 2007; Kawano *et al.* 2008). The ability of Stx-encoding phages from clinical EHEC O26 and EHEC O157 isolates to transduce the laboratory strain *E. coli* K-12, commensal *E. coli* and EPEC of various origins within the GIT has been demonstrated *in vivo* (Cornick *et al.* 2006). However, there are limited studies concerning the potential of Stx1-encoding phages to infect other non-pathogenic or pathogenic strains. Incorporation of stx_{1e} in particular, which is difficult to detect by current immunoassays, into established human pathogens could present diagnostic complications. Since the stx_{1e} operon could be disseminated via phage to host-adapted strains, we investigated the capability of the stx_{1e}-encoding prophage to lysogenize other *E. coli* or *E. cloacae* strains. Lysogenization was compared to that by a prophage induced from an *E. coli* O157:H7 clinical strain, encoding for another stx_1 subtype. Differences in *stx* gene(s) acquisition, stability and Stx expression were evaluated at the time of infection and after multiple subcultivation steps. The described experimental set up mimics to an extent the clinical scenario of harmless commensal organisms colonizing the GIT environment, together with other *stx*-carrying pathogenic strains. Under conditions of extended antibiotic treatment, such co-existence may result in an effective conversion of the harmless organisms into new strains with pathogenic potential, which may contribute to bacterial pathogenesis and pose high risks to human health.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strains used in this study are listed in Table 1. The stx_{1e}-positive *Ent. cloacae* M12X01451 strain kindly provided by

Table 1. Bacterial strains used in this study.

Bacterial strains	stx gene	Serotype	stx_{1a} spot-test	stx_{1e} spot test	Source	Reference
Donor strains						
Enterobacter cloacae M12X01451 (harboring stx_{1e} phage)	stx_{1e}	–	+	–	Clinical sample	Probert, McQuaid and Schrader (2014)
Escherichia coli RM8530 (harboring stx_{1a} phage)	stx_{1a}	O157:H7	–	+	CDC 2886-75	Kyle *et al.* (2012)
Host (recipient) strains						
Enterobacter cloacae 13047	–	–	+	+	ATCC	–
Escherichia coli 25922	–	–	–	–	ATCC	–
Escherichia coli DH5α	–	–	+	+	Invitrogen	This work
Escherichia coli K-12 (29425)	–	–	–	–	ATCC	Skinner *et al.* (2013)
Escherichia coli 465–97	–	O157:H7	+	+	USDA-FSIS	USDA (2012)

Dr William Probert at the Richmond Department of Public Health (Richmond, CA) and the stx_{1a}-positive *E. coli* O157:H7 RM8530 strain (CDC 2886-75) were used as the donor strains for the phage transduction experiments. The *Ent. cloaceae* type strain (ATCC 13047), the *E. coli* strain ATCC 25922 and the *E. coli* K-12 strain ATCC 29425 obtained from the American Type Culture Collection, together with other *stx*-negative strains (Table 1), served as recipients for lysogenization experiments (unless otherwise stated) and were used as negative controls in PCR analyses. Strains were routinely grown overnight in Luria–Bertani (LB) broth at 37°C with shaking at 150 rpm or on LB agar plates. Stock cultures of each strain were prepared by centrifugation (8000 × g, 10 min), pellets were suspended in 1 mL of fresh LB, 0.5 mL portions were transferred to 2 mL beads-containing cryogenic vials (Corning) and stored at –80°C for further use. Purity of *E. coli* O157 strains was routinely checked by testing their ability to ferment sorbitol on Sorbitol MacConkey agar (SMAC; Oxoid), followed by the detection of *rbfE* gene using the latex agglutination kit (Oxoid DRYSPOT *E. coli* O157, Oxoid, Ltd, Basingstoke, UK), according to the manufacturer's instructions.

Prior to infection experiments, the absence of *stx* in *Ent. cloacae* 13047, *E. coli* DH5α and *E. coli* 465–97 and the presence of stx_{1a} in *E. coli* RM8530 and stx_{1e} in *Ent. cloacae* M12X01451 were verified by colony PCR. Positive bands characteristic of stx_{1a} (amplicon size of 477 bp) and stx_{1e} (amplicon size of 250 bp) genes were detected (Fig. S1, Supporting Information).

Induction and purification of *stx* phages

Ent. cloacae strain M120X1451 (harboring stx_{1e}-encoding prophage) and *E. coli* O157:H7 strain RM8530 (harboring stx_{1a}-encoding prophage) were grown from frozen cryogenic vial stocks, streaked on LB agar plates and grown from single colonies (Strauch, Schaudinn and Beutin 2004) overnight at 37°C in LB broth. LB broth was inoculated with 0.1% (v/v) of each strain's overnight culture, and incubated at 37°C with shaking (150 rpm) for 3 h until the early exponential phase ($\sim OD_{600} = 0.05$, ca. $\sim$ 6 log CFU mL^{-1}). At this point, phage induction was carried out by adding mitomycin C to a final concentration of 50 ng mL^{-1} to the culture bottles, which were further incubated for 16–18 h at 37°C with shaking (150 rpm) in the dark, followed by centrifugation at 14 000 × g for 20 min at 4°C, to separate the cells and other debris from phage particles. Cell-free supernatants (CFSs) were filter sterilized using 0.2 μm sterile EMD Millipore Millex filters (Thermo Fisher Scientific, Pittsburgh, PA), and treated with 2 μL of DNase (10 U mL^{-1}; Sigma-Aldrich) for 1 h at 37°C to digest possible genomic DNA contamination. Phage particles in both DNase-treated CFSs were precipitated by adding 1:6 volume of PEG/NaCl solution (20% polyethylene-glycol-8000, 2.5 M NaCl), and incubating overnight at 4°C (Lunder *et al.* 2008). Resulting phage precipitates were separated by centrifugation as described above, suspended in 2 mL of SM buffer (100 mM NaCl, 10 mM $MgSO_4$; 50 mM Tris-Cl, pH 7.5) to which two to three drops of chloroform were added, and stored at 4°C for further use (Sambrook and Russell 2001). Aliquots of the purified lysates were used to amplify the *stx* gene by PCR to confirm the prophage induction.

Phage enumeration

Phage titers were determined by the soft-agar overlay assay (Velandia *et al.* 2012) with slight modifications. A total of 100 μL of filtered media containing either stx_{1e}-carrying or stx_{1a}-carrying phages was diluted 10-fold in SM buffer, 500 μL aliquots of each dilution were mixed with 100 μL of $CaCl_2$ (0.1 M final concentration) and 100 μL of exponential-phase culture of *Ent. cloacae* 13047 (for enumeration of stx_{1e} phage) and *E. coli* DH5α (for enumeration of stx_{1a} phage) was added to the phage mixture. The phage-host cell mixtures were left to stand at 37°C for 30 min to allow phage particle adsorption (Muniesa *et al.* 2004; Muniesa, Serra-Moreno and Jofre 2004), added to test tubes containing 3 mL of molten soft LB agar (0.7% agarose w/v), mixed by phage style mixing and then poured in duplicates over prewarmed LB agar plates. Plates were left for 15 min at room temperature (RT) to obtain solidified overlays, inverted and incubated overnight at 37°C until the appearance of plaques. Plates prepared simultaneously with overlay mixtures either lacking the phage lysate dilutions or the exponential-phase cultures of host strains were used as negative controls. Plaques were enumerated on each plate and phage titers were expressed as plaque-forming units per milliliter (PFU mL^{-1}) of each phage-containing lysate (Anderson *et al.* 2011).

Preparation of high titer phage stock solutions

For the infection and transduction experiments, high titers of stx_{1e} and stx_{1a} purified phage-containing lysates were prepared according to the method of Allison *et al.* (2003). *Enterobacter cloacae* 13047 and *E. coli* 465–97 were grown in LB as previously described to an OD_{600} of $\sim$0.05, stx_{1e}- and stx_{1a}-phage lysates were then added to the corresponding cultures at a multiplicity of infection (MOI) of 0.5. The infected cultures were further incubated overnight at 37°C with shaking (150 rpm), and the corresponding phages were recovered from the resulting supernatants after the removal of whole cells and cellular debris by centrifugation at 14 000 × g (4°C, 20 min) followed by filter sterilization. For further use, the phage stock solutions containing stx_{1e} and stx_{1a} phages were stored at 4°C with a few drops of chloroform as previously stated.

Host range and infectivity of stx_1 phages

The spot test (Holguín *et al.* 2015) was employed to test the susceptibility of host strains to infection by the stx_{1e} and stx_{1a} phages with slight modifications. A total of 500 μL aliquots of the early exponential phase ($OD_{600} \sim 0.05$) cultures of each host strain (Table 1) were mixed with 100 μL of $CaCl_2$ (0.02 M final concentration) and 3 mL molten soft LB agar, poured onto LB agar plates, and allowed to solidify at RT. A total of 15 μL aliquots of each phage stock solution (containing stx_{1e} and stx_{1a} phages) were spotted onto the bacterial overlays, dried for 15–20 min. Plates were incubated at 37°C for 24 h. Results were considered as positive for each stx_1-phage stock solution if clear lysis zones were observed (Carlson 2005).

Sensitivity of host strains to both *stx* phages was further assessed by the optical density (OD) method (Muniesa *et al.* 2003) with some modifications. Each host strain was grown in 5 mL of LB at 37°C with shaking until the early exponential phase, then mixed with each stx_1-phage stock solution (MOI = 0.5) and 100 μL of 1 M $CaCl_2$ (0.02 M final concentration) as previously described. Cultures were incubated at 37°C, and growth/cell lysis of infected cultures was monitored hourly at OD_{600} for 5 h. A total of 1 mL aliquots of each infected culture were centrifuged at 6000 × g for 10 min at 4°C, washed to remove loosely attached phage particles as previously described, suspended in 1 mL of fresh LB broth, serially diluted in 0.1% sterile PW and the surviving populations were estimated by colony count on LB agar plates (expressed as $\log_{10}$ colony-forming units per mL; CFU mL^{-1}). OD

measurements and colony counts were performed in duplicate, results were averaged and compared to those of control cultures (prepared in a similar manner but without the stx_1-phage stock solution). The spot and the OD tests were repeated at least three times for each strain grown in the presence of each phage stock solution ($n = 3$).

Phage-mediated transduction and generation of lysogens

All *stx*-negative host strains (Table 1) with an exception of *E. coli* ATCC 25922 and *E. coli* K-12 strains were lysogenized by each *stx*-phage stock solution according to the methods of Muniesa *et al.* (2004) and Bielaszewska *et al.* (2007) with some modifications. Host strains were grown at 37°C from stock cryogenic vials in 3 mL of LB broth until the early exponential phase, from which 100 μL aliquots were transferred to 3 mL of LB broth tubes containing 100 μL of 1 M $CaCl_2$ (0.02 M final concentration) and 500 μL of each phage stock, and incubated overnight at 37°C without shaking. Infected cells were recovered by centrifugation at 6000 × g (20 min, 4°C), and cell pellets were washed three times with phosphate buffer saline (PBS) and one time with 0.1 M Glycine-HCl (pH 2.3) to remove non-specifically bound phage particles (Maaß *et al.* 2014), and avoid false *stx*-positive PCR results. The washed pellets were then suspended in 3 mL of LB broth, serially 10-fold diluted in 0.1% sterile PW, plated onto LB plates and incubated overnight at 37°C. At least 10 well-isolated colonies (designated as T_0 colonies) from duplicate plates of each host strain were screened by PCR for the presence of stx_{1e} and/or stx_{1a} genes. T_0 colonies were maintained on LB plates, stored at 4°C and subcultured on LB agar 24 h prior experimental analysis.

PCR analyses

PCR assays were conducted to detect the presence/absence of stx_{1e} and stx_{1a} in bacterial genomic DNA, colonies of each lysogenized recipient, phage plaques and the stx_{1a}- and stx_{1e}-phage stock solutions as previously described (Skinner *et al.* 2013) with a few modifications. Single colonies and phage plaques were suspended in 1 mL of DNase-free water in sterile eppendorf tubes, heated to 95°C for 10 min and centrifuged, from which 2 μL was used as a template for PCR amplification. For purified phage stock solutions and genomic DNA, 2 μL were directly heated to 95°C for 10 min, and used as templates for PCR amplifications. PCR amplifications were performed using a Biorad T100 Thermal cycler, in 25 μL volume with 12.5 μL Taq-Green master mix (Promega, Madoson, WI), 9.5 μL DNase-free water and 1 μL (from a 20 μM stock) of either stx_{1e}-specific or stx_{1a}-specific primers (forward, 5′-TATTGATGATTTTCAGGGGG-3′; reverse, 5′-GCGTAAATTGTCAAACCGTT-3′ and forward, 5′-CCTTTCCAGGTACAACAGCGGTT-3′; reverse, 5′-GGAAACTCATC AGATGCCATTCTGG-3′ respectively) (Scheutz *et al.* 2012; Skinner *et al.* 2016). The PCR program used was as follows: 95°C denaturation for 1 min, 32 cycles of 94°C denaturation for 30 s, 65°C annealing for 30 s, 68°C extension for 75 s and a final step of 68°C extension for 7 min. A 5 μL portion of each amplified product was resolved by electrophoresis on 1.0% (w/v) agarose gel, stained with gel red, visualized using a UV transilluminator at a wavelength of 420 nm and documented with a Gel Doc 2000 Fluorescent Imaging System (U:Genius, UK).

Stability of *stx* in lysogenized strains

Ten colonies resulting from each phage-infected host strain (T_0) that were confirmed *stx* positive by PCR were grown overnight at 37°C in LB broth, where equal aliquots from each culture were combined, and subcultured daily (0.1% v/v) in 10 mL of fresh LB at 37°C for 10 days. Subcultures of the tenth day were serially diluted 10-fold in 0.1% sterile PW, spread on LB agar plates and incubated overnight at 37°C, where 10 colonies (designated as T_{10} colonies) were tested for the presence of *stx* genes by PCR. Ten *stx*-positive colonies were grown overnight in LB broth and equal aliquots from each culture were combined and subcultured daily (0.1% v/v) for 10 additional days, spread on LB agar plates, and again 10 colonies (designated as T_{20} colonies) were examined for *stx* by colony PCR. The stability of stx_{1e} or stx_{1a} genes in each lysogenized host strain was assessed based on the percentage of *stx*-positive colonies (Bielaszewska *et al.* 2007). The wild-type donor strains (originally harboring the *stx*-encoding prophages) and the *stx*-negative host strains (Table 1) were used as positive and negative controls respectively in all PCR experiments.

Evaluation of Stx1 production by bacteria using enzyme-linked immunosorbent assays (ELISAs)

Sandwich ELISAs were performed as previously described (He *et al.* 2016) with some modifications. Briefly, plates were coated with monoclonal antibody (mAb) Stx1e-2 or Stx1a-1 (100 μL well^{-1}, 5 μg mL^{-1} in PBS), and incubated overnight at 4°C after washing and blocking steps (Skinner *et al.* 2016). Bacterial culture supernatants were added (80 μL well^{-1}) to each well and incubated for 1 h at RT, followed by six washes with TBST. Stx1 polyclonal antibody (200 ng mL^{-1} in blocking solution) was then added (80 μL well^{-1}), incubated for 1 h at RT and plates were again washed six times with TBST. HRP-conjugated goat anti-rabbit IgG (1:10 000; Promega) in blocking solution was added to the plates and incubated for 1 h at RT, followed by six washes with TBST. Finally, TMB substrate (Sigma-Aldrich, St. Louis, MO) was added (80 μL well^{-1}), and the reaction was stopped after 10 min with 80 μL well^{-1} of 0.3 N HCL. The ELISA signals (A_{450}) were measured using a Victor 3 plate reader (Perkin-Elmer, Shelton, CT). All ELISAs were repeated at least twice. Results were considered Stx1 positive when ELISA signal-to-noise ratio (s/n) was $\geq$3 and Stx1 negative when s/n $<$ 3.

Western immunoblot analysis

Western blots were conducted as previously described (He *et al.* 2016). Briefly, pure Stx1e toxoid and bacterial culture supernatants (7 μL) were denatured at 72°C for 10 min in 1X Nu-Page LDS loading buffer, and then separated on a 4%–12% NuPAGE Novex Bis-Tris mini gel (Invitrogen, Carlsbad, CA). The proteins were transferred to a PVDF membrane (pore size, 0.45 μm), blocked with 2% ECL Prime blocking agent (GE Healthcare, UK) in PBST and washed three times with PBST. Stx1e-specific mAb, Stx1e-3 (Skinner *et al.* 2016), was diluted to 1 μg mL^{-1} in blocking solution and incubated with the blots for 1 h at RT. After washing three times, the blots were incubated with HRP-conjugated goat anti-mouse IgG (Promega) at 5 ng mL^{-1} for 1 h at RT. The blots were developed using Lumigen TMA-6 (Lumigen, Southfield, MI) substrate and visualized using a FluorChem HD2 (Alpha Innotech, San Leandro, CA).

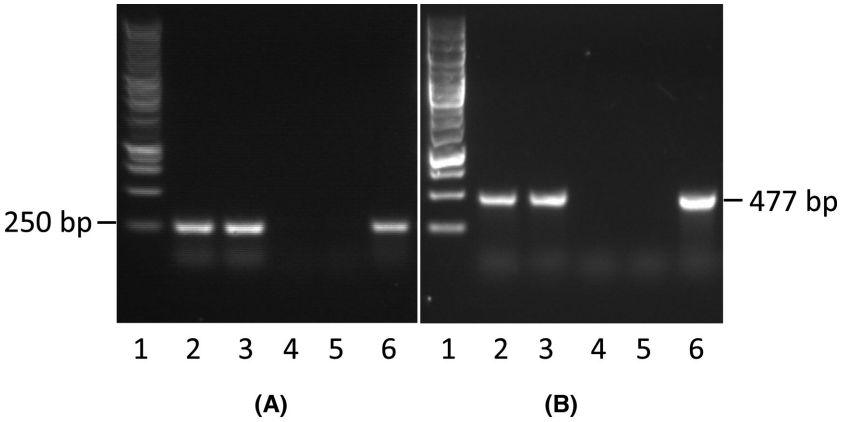

Figure 1. Detection of phage-encoded *stx* genes by PCR analysis. (**A**) Lane 1, DNA ladder; lanes 2–3, PCR products of stx_{1e} from DNase-treated supernatants of *Ent. cloacae* M12X01451 under non-inducing (lane 2) and inducing (lane 3) conditions; lane 4, stx_{1e} PCR products from DNase-treated supernatants of *Ent. cloacae* 13047 under inducing conditions; lanes 5–6, stx_{1e} PCR products from *Ent. cloacae* M12X01451 genomic DNA with (lane 5) and without (lane 6) DNase treatment. (**B**) Lane 1, DNA ladder; lanes 2–3, stx_{1a} PCR products from DNase-treated supernatants of RM8530 under non-inducing (lane 2) and inducing (lane 3) conditions; lanes 4, stx_{1a} PCR products from DNase-treated supernatants of *E. coli* 465–97 under inducing conditions; lane 5–6, stx_{1a} PCR products from RM8530 genomic DNA with (lane 5) and without (lane 6) DNase treatment. The sizes of the stx_{1e} and stx_{1a} PCR products are indicated at the left and right side of the figure.

Sequencing of stx_{1e}

The full stx_{1e} operon in *E. coli* O157:H7 strain (RM8530) lysogenized by the stx_{1e}-phage stock solution was sequenced. Genomic DNA was prepared using 5 mL cultures derived from 10 colonies of the lysogenized strain which had been subcultured 20 times using a DNA Stool Mini kit (Qiagen, Valencia, CA). The stx_{1e} operon was analyzed by PCR using Phusion polymerase (New England Biolabs), Stx1e-A-F1 (Stx1e-A-F1: GGAATTCATATGATGATATTGATGATTTTCAGGG and Stx1e-B-R1 (Stx1e-B-R1: GTGGTGCTCGAGGCGAAAAATCACCTCAC) primers and genomic DNA (diluted 1:100) as a template. The resulting PCR products were then gel extracted (Qiagen) and cloned into the pCR 4.0 vector using a Zero-Blunt cloning kit (Qiagen). Plasmid DNA was extracted from 35 colonies (from 10 cloning plates), and the stx_{1e} operon was sequenced using M13-F and M13-R primers (Elim Biopharmaceuticals, Hayward, CA).

Statistical analysis

Statistical analysis of the OD method's data was performed using IBM-SPSS software v. 20.0 (SPSS Inc., Chicago, IL, 207 USA). A one-way analysis of variance and post hoc least significant difference were used for the analysis of normally distributed data. Significance differences between measurement means were accepted at $P < 0.05$ level. Results are presented as mean ± standard deviation (SD) of readings from three independent experiments ($n = 3$).

RESULTS

Confirmation of the presence of *stx* temperate bacteriophages in host strains

To confirm that stx_{1a} and stx_{1e} genes in RM8530 and M12X01451 strains were prophage encoded, DNA from representative phage plaques and phage stock solutions prepared using these strains were used as templates for PCR amplification of the stx_{1a} and stx_{1e} genes, where both genes were detected (Fig. S1A and B, Supporting Information, lanes 7 and 8).

To ensure that these PCR products were not amplified from contaminated genomic DNA, supernatants of M12X0145 and RM8530 cultures were treated with DNase before PCR analysis. Figure 1 shows that PCR products (stx_{1a} and stx_{1e}) were obtained from both M12X0145 and RM8530 strains with or without mitomycin C treatment, suggesting that some phage particles were spontaneously induced by the two host strains. Formation of stable lysogens in these two strains was also examined by PCR using genomic DNA as templates. PCR products (stx_{1e} and stx_{1a}) were detected using genomic DNA of M12X01451 and RM8530 strains without DNase treatment, confirming the incorporation of the phages into the hosts' genomic DNA. No PCR products were obtained using genomic DNA when treated with DNase, which confirms that the genomic DNA used for PCR was not contaminated with the *stx* phages.

Infectivity of the stx_{1a} and stx_{1e} phages to different host strains

The spot test and the OD method were conducted to assess the ability of the stx_{1a} and stx_{1e} phages to infect a range of host strains (Table 1). Small turbid plaques were observed on overlay plates of *Ent. cloacae* 13047, *E. coli* DH5α, *E. coli* 465–97 and RM8530 spotted with stx_{1e}-phage stock solution, and overlay plates of M12X01451, and 13047 strains, *E. coli* DH5α and *E. coli* 465–97 spotted with stx_{1a}-phage stock solution. No lytic areas were observed in plates seeded with cultures of the two donor strains spotted with their own phage stocks, or *E. coli* K-12, or *E. coli* ATCC 25922 strains spotted with either *stx*-phage stocks. The two latter strains were therefore excluded from further investigations.

The infectivity of stx_{1a}- and stx_{1e}-phage stock solutions to a range of host strains was additionally confirmed based on the final ODs and the corresponding final population counts of each infected culture after 5 h of incubation (Fig. 2). Negative control cultures of each host strain that did not receive either *stx*-phage stock solution had OD values ranging from 0.7 to 0.8 (~9 log CFU mL^{-1}) after 5 h of incubation, significantly higher than those of cultures infected with the *stx* phages ($P < 0.05$). The susceptibility of host strains to the two phage stock solutions varied significantly, and host strains grown in the stx_{1e}-phage stock solution recorded generally lower final ODs than those grown in the stx_{1a}-phage stock solution ($P < 0.05$). Based on their final culture ODs, phage-infected cultures were subjectively classified

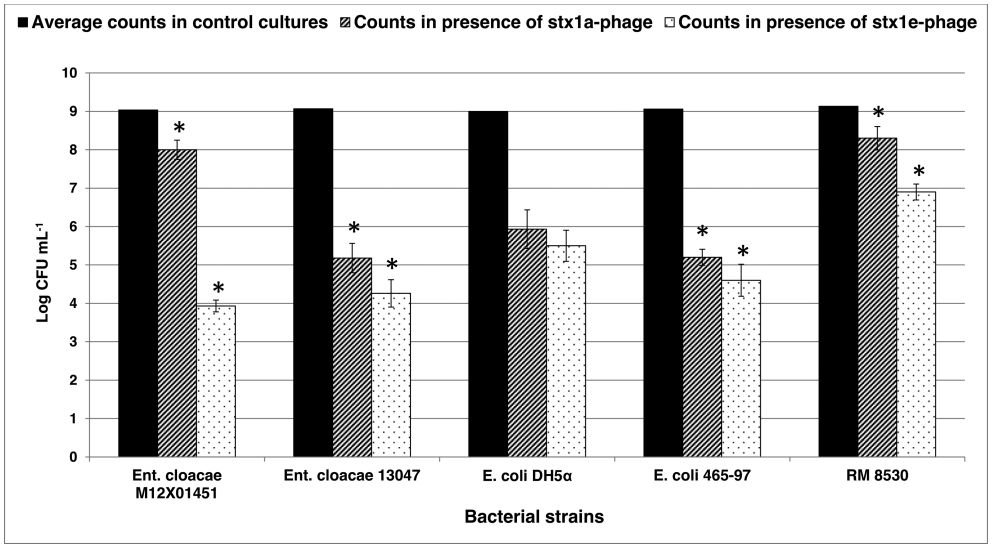

Figure 2. Susceptibility of different bacterial strains to lysis by stx_{1a} and stx_{1e} phages. Each host strain was grown from a single colony in LB at 37°C with shaking to an OD_{600} of approximately 0.05 (~6 log CFU mL^{-1}), then mixed with 100 μL of $CaCl_2$ and each phage stock solution (MOI = 0.5). Growth/cell lysis of each infected culture was monitored spectroscopically (OD_{600}) every hour for up to 5 h without the presence of inducing agent. The corresponding phage-free cultures were grown to serve as controls. Y-error bars represent the standard error of the mean population counts of host strains from three independent experiments ($n = 3$). An asterisk denotes statistically different values ($P < 0.05$) of average population counts of each strain grown in the presence of each phage lysate.

into highly susceptible (final ODs < 0.1, including *Ent. cloacae* 13047 and *E. coli* 465–97), intermediately susceptible (final ODs between 0.2 and 0.3, including *E. coli* DH5α) and non-susceptible (final OD > 0.3, including RM8530) host strains.

Enterobacter cloacae M12X01451 was the most susceptible strain to its original phage stock solution (stx_{1e} positive), where the infected cultures had final population counts of 3.93 log CFU mL^{-1} (more than 5 log units of reduction). In contrast, the growth reduction of the RM8530 strain in the presence of stx_{1e} phage was significantly ($P < 0.05$) less (approximately 2 log units of reduction). *Enterobacter cloacae* 13047 and *E. coli* 465–97 strains were highly susceptible to both stx_{1a}- and stx_{1e}-phage stock solutions, as indicated by the sharp drop in their culture ODs compared to their controls, and their corresponding CFU mL^{-1} (Fig. 2). Susceptibility of *E. coli* DH5α to stx_{1a}- and stx_{1e}-phage stock solutions (5.93 and 5.5 log CFU mL^{-1}, respectively) was statistically similar ($P > 0.05$), although both infected cultures exhibited significantly lower ODs and population counts compared to the control culture (not infected by either *stx* phage).

Stability of the *stx* phages in lysogenized strains

According to the spot test and the OD experiments, the stx_{1a} phage carried by RM8530 and the stx_{1e} phage carried by M12X01451 were able to lysogenize the selected host strains with different incidences. To check if stable lysogens were produced, the presence of *stx* phages was examined by PCR amplification of the *stx* genes using 10 colonies randomly picked from the culture plates immediately after phage infection (T_0), after 10 (T_{10}) and 20 (T_{20}) generations. Table 2 indicates that the stx_{1a} and stx_{1e} phages were able to lysogenize all bacterial strains listed, and that stx_{1e} gene was stably maintained in almost all cells of lysogenized strains up to 20 subcultivation steps, except for *E. coli* DH5α, in which the stx_{1e} gene was lost in all colonies examined after T_{20}.

The stx_{1a} gene in hosts lysogenized by the stx_{1a} phage was not as stable compared to the stx_{1e} gene, especially in the host M12X01451 (originally carries the stx_{1e} prophage), where the stx_{1a} gene was completely lost after T_{10}. In contrast, stx_{1a} was still retained in lysogens of *Ent. cloacae* 13047 strain up to 10 subcultures, but 40% of colonies lost the gene at the T_{20} (Table 2). Subculturing of *E. coli* DH5α and *E. coli* 465–97 lysogenized by stx_{1a} phage for 10 days resulted in 30% (7/10) and 10% (9/10) stx_{1a} loss, and the loss increased to 90% and 100% respectively after 10 additional subcultivation steps (Table 2).

Lysogenization of *Ent. cloacae* M12X01451 and RM8530 donor strains (originally carrying stx_{1e} and stx_{1a} prophages) by stx_{1a} and stx_{1e} phages respectively resulted in their acquisition of an additional *stx* phage, hence the production of lysogens with two *stx*-harboring prophages. To examine the effect of acquiring a new phage on the stability of the existing phage in the lysogenized bacterial cells, the newly formed double lysogens were tested by the colony PCR for the presence of their original *stx* gene at T_{20}. stx_{1e} gene was stably maintained in all T_{20} colonies of *Ent. cloacae* 12X01451 strain lysogenized by the stx_{1a} phage, whereas 20% of T_{20} colonies lost the stx_{1a} gene originally carried by the RM8530 strain after infection by the stx_{1e} phage (Table 2).

To further investigate the stability of the acquired stx_{1e} gene in a new host that already has a stx1a-encoded prophage, the full stx_{1e} operon in T_{20} progenies of the RM8530 lysogen (stx_{1a} positive) was sequenced. Of the 35 randomly picked colonies from 10 cloning plates, all of them were identical to the original sequence of the stx_{1e} gene (Probert, McQuaid and Schrader 2014), and no DNA recombination or mutations were found.

Production of Stx1e and Stx1a in hosts lysogenized by *stx* phages

The production of Stx1e in strains of *Ent. cloacae* 13047, *E. coli* DH5α and *E. coli* 465–97 lysogenized by stx_{1e} phage was evaluated by ELISA (Table 3). Stx1e was detected in all three strains (Table 3), although the percentage of colonies that produced the toxin varied from one host to another (Table S1, Supporting Information). For the *E. coli* DH5α lysogen, Stx1e was only detected

Table 2. Detection of stx_{1e} and stx_{1a} genes in strains immediately (T_0), at 10 (T_{10}) and 20 (T_{20}) subcultivation steps after infection by stx_{1e} and stx_{1a} phages. Ten colonies were randomly picked from all T_0, T_{10} and T_{20} cultures respectively, and colony PCR was performed for the presence of stx_{1e} and/or stx_{1a} genes.

		No. of colonies containing a stx gene/total no. of colonies tested ($n = 10$)		
stx_1 phage	Lysogenized strains	T_0	T_{10}	T_{20}
stx_{1e} phage	*Enterobacter cloacae* 13047	10/10	9/10	9/10
	Escherichia coli DH5α	10/10	10/10	0/10
	Escherichia coli 465–97	10/10	10/10	10/10
	Escherichia coli O157 RM8530	10/10	10/10	10/10
stx_{1a} phage	*Enterobacter cloacae* M12X01451	10/10	0/10	0/10
	Enterobacter cloacae 13047	10/10	10/10	6/10
	Escherichia coli DH5α	10/10	7/10	1/10
	Escherichia coli 465–97	10/10	9/10	0/10
stx_{1e} phage	*Escherichia coli* O157 RM8530[a]	10/10	10/10	8/10
stx_{1e} phage	*Enterobacter cloacae* M12X01451[a]	10/10	10/10	10/10

[a]Denotes the stability of the stx gene carried on the original prophage in *E. coli* O157 RM8530 (stx_{1a}-positive) and *Ent. cloacae* M12X01451 (stx_{1e} positive) strains after being lysogenized with the stx_{1e} and stx_{1a} phage, respectively.

Table 3. Detection of Stx1e toxin in supernatants of host strains lysogenized by stx_{1e} phage at different subcultivation steps by ELISA. Results were considered Stx1e positive when ELISA signal to noise (s/n) ≥ 3.

Lysogenized strains	Subcultivation step	No. of Stx1e-positive colonies/total no. of colonies tested ($n = 10$)
Enterobacter cloacae 13047	T_0	5/10
	T_{10}	7/10
	T_{20}	8/10
Escherichia coli DH5α	T_0	0/10
	T_{10}	9/10
	T_{20}	0/10
Escherichia coli 465–97	T_0	10/10
	T_{10}	8/10
	T_{20}	8/10

in culture supernatants of T_{10} (90%), but not in those of T_0 or T_{20}; however, their s/n values seemed very low compared to those recorded for other Stx1e-positive supernatants of the other lysogenized strains. Stx1e was detected in 50% of cells tested from the T_0 lysogen culture of *Ent. cloacae* 13047, and subsequent subculturing resulted in more Stx1e-positive colonies (70% at T_{10}, and 80% at T_{20}). For lysogen *E. coli* 465–97, Stx1e was detectable in most colonies, including those from T_0 (100%), T_{10} (80%) and T_{20} (80%) cultures. Notably, higher levels of Stx1e, as demonstrated by the ELISA s/n data (Table S2, Supporting Information), were detected in *E. coli* 465–97 cells from T_{10} cultures, compared to those from T_0 and T_{20} cultures.

To avoid the cross-reaction between Stx1a and Stx1e, production of Stx1e in RM8530 lysogenized by stx_{1e} phage was evaluated by western blot analysis, instead of ELISA, using a Stx1e-specific monoclonal antibody. Figure 3 indicates that Stx1e was detected in all 10 colonies picked from the T_0, T_{10} and T_{20} plates; however, levels of Stx1e varied significantly as shown by the intensity of the protein bands.

For strains *Ent. cloacae* M12X01451, 13047, *E. coli* DH5α and *E. coli* 465–97 lysogenized by the stx_{1a} phage, Stx1a was not detected either by ELISA or western blot analyses in any representative colonies after mitomycin C induction, despite confirming the presence of the stx_{1a} gene by PCR analysis (Table 2). The RM8530 strain did express detectable levels of Stx1a (s/n ≥ 3, data not shown).

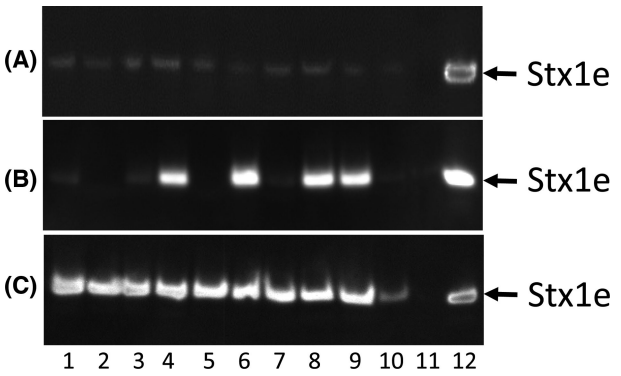

Figure 3. Western blot analysis of Stx1e produced by strain RM8530 infected with the stx_{1e} phage. Lanes 1–10 were loaded with supernatants (7 μL) of bacterial cultures from 10 colonies randomly picked from T_0 (**A**), T_{10} (**B**) and T_{20} (**C**) plates. Lanes 11–12 were loaded with 0.2 μg of purified Stx1a and Stx1e, respectively. Proteins were separated by SDS-PAGE and probed with a Stx1e-specific monoclonal antibody, Stx1e-3. The Stx1e positions on the blots are indicated by arrows.

DISCUSSION

Lysogenic phage conversion constitutes an efficient mechanism for rapid dissemination of phage-encoded virulence genes (Krylov 2003; Feiner *et al.* 2015). While *in vitro* transmission of

Stx1 phages between different bacteria within the same species has been described (Sváb *et al.* 2015), studies that provide information on cross-genera conversions involving toxigenic and non-toxigenic strains are still limited, in particular, those regarding susceptibility of commensal strains to Stx1-converting phages from non-*E. coli* strains. In this study, bacterial strains from two genera, RM8530 (*Escherichia*) and M12X01451 (*Enterobacter*), were analyzed and the presence of Stx1a- and Stx1e-encoding phages, both in their lysogenic and virion forms, in these strains was confirmed by PCR analysis of the stx_1 genes using genomic and phage DNA with or without DNase treatment. Gene products of stx_{1a} in RM8530 and stx_{1e} in M12X01451 were detected in both genomic and phage DNA preparations without antibiotic induction, suggesting the occurrence of spontaneous induction of *stx*-encoding phages (Veses-Garcia *et al.* 2015). No attempts were made to investigate the presence of converting phages other than the stx_{1a} or stx_{1e} phages in the donor strains.

Reproducibility of plaque assays is dependent on both the host strain and the lytic capacity of the phage, and has been shown to be severely limited for Stx-converting phages (Grau-Leal *et al.* 2015). Despite the efforts to select the optimal conditions for high phage titers in this study, the basal titer in stx_{1e}-positive phage stock solutions was $<10^3$ PFU mL^{-1} (Muniesa *et al.* 2004). Plaques formed by both stx_1-encoding prophages (though the stx_{1e} was more readily identifiable than the stx_{1a}) were very small and turbid on agar plates (data not shown), which is consistent to the characteristic feature of Stx-converting phages described by others (Saunders *et al.* 2001; Muniesa *et al.* 2003). The plaque assay may have not been the ideal method for *stx* phages enumeration in this study, therefore culture-independent methods (McDonald *et al.* 2010; Rooks *et al.* 2010) would be considered for improving the quantification of *stx* phages in future investigations.

The infectivity of the stx_{1a} and stx_{1e} phages to host strains including *Ent. cloacae* 13047, *Ent. cloacae* M12X01451, *E. coli* DH5α, *E. coli* 465–97, RM8530, *E. coli* K-12 and *E. coli* 25922 was determined using the spot test based on the development of lysis areas and the OD method (except for the two latter strains), which compares the final ODs of infected cultures and the corresponding counts of the surviving populations 5 h after phage infection. All host strains responded differently to the infection by the *stx*-phage stock solutions in terms of formation of clear lysis plaques and the reduction in population counts (Holguín *et al.* 2015). The decrease in the culture OD is a relevant qualitative measurement of prophage induction and Stx-phage production (Tyler, Mills and Friedman 2004), and the higher the level of phage production, the lower was the OD value due to bacterial lysis. In this study, the most susceptible hosts to the stx_{1e} and stx_{1a} phages were *Ent. cloacae* 13047 and *E. coli* 465–97 strains, which was in line with previously published findings suggesting that *Enterobacter* and O157:H7 are the origins of the respective prophages (McDonough and Butterton 1999; Strauch, Lurz and Beutin 2001). To better understand the mechanisms and evolutionary forces that extend the genetic spectrum of phages from different bacterial genera, each donor strain (RM8530 from *E. coli* and M12X01451 from *Enterobacter*) was respectively grown in the presence of its original phage stock solution (prepared from each strain's induced culture) and the phage stock solution of the opposite donor strain. A sharp decrease in culture OD of M12X01451 strain grown in its original phage stock solution was observed; however, this decrease was not found for the STEC (RM8530) strain grown in the stx_{1a} phage, possibly due to a mechanism of superinfection immunity present in the stx_{1a} phage.

Susceptibility of *stx* lysogens to new phages was observed to be greatly dependent on the absence of a highly related bacteriophage within their genome, which confers immunity against the new infecting phage (Bielaszewska *et al.* 2007). This may serve as a plausible explanation to the spot test data and the OD results, where only 1 log reduction in final population counts of M12X01451 culture after infection by stx_{1a} phage was observed. M12X01451 was not easily lysed in presence of stx_{1a} phage, which could be attributed to the spontaneous release of its own prophage (confirmed by PCR) in concentrations that masked the effect of the stx_{1a} phage (Muniesa *et al.* 2003).

In this study, the *E. coli* K-12 and *E. coli* ATCC 25922 strains failed to produce any visible lysis areas on overlays seeded with these strains after being spotted with stx_{1a}- and stx_{1e}-phage stock solutions. Presence of glucose moieties instead of galactose residues in the terminal position of cell wall lipopolysaccharides (Rakhuba *et al.* 2010), lack of receptors for the *stx* phages recognition or presence of cryptic phages that mediate immunity (Shaikh and Tarr 2003; Besser *et al.* 2007) may have contributed to the non-specific phage infection to the cells of these strains.

The ability of Stx1 temperate phages from *E. coli* and non-*E. coli* strains to convert a range of non-pathogenic bacteria into Stx-producing bacteria over a prolonged period of time may have clinical implications, if this process takes place in the human GIT. In this study, we determined the stability of transduced *stx* genes based on the ability of the lysogenized strains to retain the acquired genes up to 20 daily passings in LB broth. The stx_{1e} gene was stably maintained in progenies of most hosts lysogenized by stx_{1e} phage as examined by PCR, and PCR products were detected in at least 90% of colonies picked from T_{10} and T_{20} cultures of *Ent. cloacae* 13047, *E. coli* 465–97 and RM8530. On the other hand, the stx_{1e} gene was completely lost in *E. coli* DH5α cells after 20 subcultivation steps. It is possible that the stx_{1e} phage is more likely maintained within the genome of closely related bacteria (similar serotypes). In contrast, the stx_{1a} gene was less stable in progenies of host strains infected with the stx_{1a} phage and seemed to be carried transiently in subcultured cells (Tozzoli *et al.* 2014). The instability of *stx* genes has been noted among STEC strains (Grotiuz *et al.* 2006; Yang 2014) and appears to be a fairly common characteristic of non-*E. coli* Stx-producing microorganisms. Subtle differences in phage coat proteins may have an impact on phage stability in these host genomes (Lima *et al.* 2004).

The ability of the stx_{1e} phage to infect RM8530 and form stable double lysogens provides an ideal intracellular environment for recombination to expand diversity among Stx phages (Allison *et al.* 2003; Allison 2007). The generation of double lysogens in the GIT may be of clinical significance, as it contributes to the spread of *stx* genes among different serotypes, and fosters the emergence of new STEC pathotypes with elevated virulence. Additionally, the presence of several *stx* gene copies within the same host may lead to the production of more Stx upon lysis, resulting in severe disease pathology. In this particular study, we randomly picked 35 colonies from the T_{20} cultures of the RM8530 double lysogens and sequenced the stx_{1e} gene. No recombination or sequence variation was found in the acquired stx_{1e} gene isolated from these colonies that were originally stx_{1a} positive and repeatedly subcultured over 20 consecutive days.

It is well known that the capacity of Stx-producing bacteria to cause severe disease in humans is closely associated with the Stx production. We studied the ability of lysogens to produce Stx by ELISA and western blot using culture supernatant. The

production of Stx1e was detected by ELISA in all strains lysogenized by stx_{1e} phage, including *Ent. cloacae* 13047, *E. coli* DH5α, *E. coli* 465–97 and RM8530, although the percentage of cells expressing the Stx1e varied by hosts and subcultivation steps. Stx1e was not detected in some colonies that were tested stx_{1e} positive by PCR, which could be reasoned to differences in host response to mitomycin induction (Vethanayagam and Flower 2005). It was reported that the amount of *stx* mRNAs in O157:H7 strains was significantly higher than in strains belonging to other serotypes (de Sablet *et al.* 2008). This was in agreement with our observation that the amount of Stx1e produced by the stx_{1e}-positive *E. coli* O157:H7 465–97 cells was much higher than that produced by their *E. coli* DH5α counterparts. Choi *et al.* (2010) reported that larger cells have higher proportion of protein synthesis machinery than smaller cells due to the difference in cell volume, which was not in accordance with our results, where the smaller cells of *E. coli* O157:H7 lysogenized strain generally seemed to produce more protein than the relatively larger lysogenized cells of *Ent. cloacae* at the time of infection and throughout the repeated passings.

The lack of Stx1e expression in T_0 colonies of *E. coli* DH5α could be associated with a possible abortive infection in the strain (Emond and Molineau 2007). The lysogenized host cells after the primary infection may have experienced interrupted phage development, resulting in the release of few or no progeny particles and the death of the infected cells prior to mitomycin induction. Babu and Aravind (2006) reported that *E. coli* adapts to different growth conditions by fine-tuning protein levels, and that initial mutations on the path to adaptation may alter the mRNA levels of some genes. This may serve as an interpretation to why Stx1e was not detected or at non-detectable levels in the T_o colonies of *E. coli* DH5α strain, where the cells were not yet adapted to the growth medium; however, after 10 days of adaptable transfers, the Stx1e was detected in the supernatants of the T_{10} cells. It should be mentioned that, overall, DH5α strain was a Stx1e-low expresser, and based on ELISA data (Table S1, Supporting Information), the Stx1e levels were much lower compared to those of other lysogenized strains although 90% of the cells at T_{10} are Stx1e positive. It is also possible that the T_o colonies were expressing the protein as well, but with minor levels that were below the detection limit by ELISA. Optimizing growth conditions for DH5α lysogens may improve the expression levels of the target protein, which warrants future investigations. The lack of Stx1e expression in T20 colonies was simply due to the loss of the stx1e gene discussed above.

Surprisingly, Stx1a was not detected in any colonies from strains lysogenized by the stx_{1a} phage, even in those confirmed stx_{1a} positive by PCR. Factors such as the location of stx_{1a} gene in the prophage genome (Aertsen, Faster and Michiels 2005), promoter (Neely and Friedman 1998), host transcription factors (Wagner *et al.* 2001; Wagner and Waldor 2002; Koitabashi *et al.* 2006) or other unknown defects in the phage may account for the impaired Stx1a production in the generated lysogens. The failure to detect Stx1a may be associated with an iron-regulated phage promotor, and less iron concentration in the growth medium may enhance the Stx1a production (Wagner *et al.* 2002). Further studies to compare the morphology, sequence and genomic structure of the stx_{1a} and stx_{1e} phages are necessary to address the impaired Stx1a production in the stx_{1a} lysogens, which may provide interesting perspectives on whether phage control of virulence gene expression is unique to *stx* or can serve as a paradigm that explains regulation of expression of other phage-encoded virulence factors.

CONCLUSIONS

Enterobacter cloacae M12X01451, a strain recently isolated from a human clinical specimen of a patient with a mild diarrheal illness, produces a new subtype of Stx1, named as Stx1e, of prophage origin. The clinical relevance and the role of this strain in human pathogenicity have not yet been addressed. This is the first report of the lysogenization of non-toxigenic/laboratory strains with a stx_{1e}-encoding phage induced from the M12X01451 strain, in comparison with a stx_{1a}-encoding phage induced from a foodborne pathogenic *E. coli* O157:H7 strain; RM8530. The results obtained are of clinical importance, and demonstrate an enormous possibility of occurrence of HGT between clinical strains carrying the *stx*-encoding prophages and bacteria from different genera resembling those colonizing the human GIT. Consequences of this HGT over extended periods of time (frequency of gene acquisition upon transduction, gene stability and Stx production) may result in the conversion of commensal bacterial hosts to pathogens with multiple *stx*-encoding prophages, which is a matter of great clinical concern.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSPD online.

ACKNOWLEDGMENTS

The first author was a visiting scientist on a sabbatical leave at the USDA, ARS, Western Regional Research Center (Foodborne Toxin Detection and Prevention Research Unit) from Faculty of Science, Alexandria University (Egypt) in the period August-2015 to April-2016.

FUNDING

This study was supported by U.S. Department of Agriculture, Agricultural Research Service CRIS project 2030-42000-048-00D.

Conflict of interest. None declared.

REFERENCES

Aertsen A, Faster D, Michiels CW. Induction of Shiga toxin-converting prophage in *Escherichia coli* by high hydrostatic pressure. *Appl Environ Microb* 2005;**71**:1155–62.

Allison HE. Stx-phages: drivers and mediators of the evolution of STEC and STEC-like pathogens. *Future Microbiol* 2007; **2**:165–74.

Allison HE, Sergeant MJ, James CE *et al.* Immunity profiles of wild-type and recombinant Shiga-like toxin-encoding bacteriophages and characterization of novel double lysogens. *Infect Immun* 2003;**71**:3409–18.

Alperi A, Figuera MJ. Human isolates of *Aeromonas* possess Shiga toxin genes (stx1 and stx2) highly similar to the most virulent gene variants of *Escherichia coli*. *Clin Microbiol Infect* 2010;**16**:1563–7.

Anderson B, Rashid MH, Carter C *et al.* Enumeration of bacteriophage particles: comparative analysis of the traditional plaque assay and real-time QPCR-and nanosight-based assays. *Bacteriophage* 2011;**1**:86–93.

Babu MM, Aravind L. Adaptive evolution by optimizing expression levels in different environments. *Trends Microbiol* 2006;**14**:11–4.

Besser TE, Shaikh N, Holt NJ *et al*. Greater diversity of Shiga toxin-encoding bacteriophage insertion sites among *Escherichia coli* O157:H7 isolates from cattle than in those from humans. *Appl Environ Microb* 2007;**73**:671–9.

Bielaszewska M, Prager R, Köck R *et al*. Shiga toxin gene loss and transfer *in vitro* and *in vivo* during enterohemorrhagic *Escherichia coli* O26 infection in humans. *Appl Environ Microb* 2007;**73**:3144–50.

Bloch S, Nejman-Faleńczyk B, Topka G *et al*. UV-sensitivity of Shiga toxin-converting bacteriophage virions Φ24B, 933W, P22, P27 and P32. *Toxins* 2015;**7**:3727–39.

Brabban AD, Hite E, Callaway TR. Evolution of foodborne pathogens via temperate bacteriophage-mediated gene transfer. *Foodborne Pathog Dis* 2005;**2**:287–303.

Carlson K. Appendix: working with bacteriophages: common techniques and methodological approaches. In: Sulakvelidze A, Kutter E (eds). *Bacteriophages: Biology and Applications*. Boca Raton, FL, USA: CRC Press, 2005, 439–90.

Chibani-Chennoufi S, Bruttin A, Dillmann ML *et al*. Phage-host interaction: an ecological perspective. *J Bacteriol* 2004;**186**:3677–86.

Choi C, Kuatsjah E, Wu E *et al*. The effect of cell size on the burst size of T4 bacteriophage infections of *Escherichia coli* B23. *J Exp Microbiol Immunol* 2010;**14**:85–91.

Cornick NA, Helgerson AF, Mai V *et al*. *In vivo* transduction of an Stx-encoding phage in ruminants. *Appl Environ Microb* 2006;**72**:5086–8.

Davin-Regli A, Jean-Marie P. *Enterobacter aerogenes* and *Enterobacter cloacae*; versatile bacterial pathogens confronting antibiotic treatment. *Front Microbiol* 2015;**6**:392.

de Sablet T, Bertin Y, Vareille M *et al*. Differential expression of stx_2 variants in Shiga toxin-producing *Escherichia coli* belonging to seropathotypes A and C. *Microbiol* 2008;**154**:176–86.

Emond E, Molineau S. Bacteriophages and food fermentations. In: McGrathe S, Van Sinderen D (eds). *Bacteriophages, Genetics and Molecular Biology*. Norfolk, UK: Caiste Academic Press, 2007.

Ethelberg S, Olsen KE, Scheutz F *et al*. Virulence factors for hemolytic uremic syndrome, Denmark. *Emerg Infect Dis* 2004;**10**:842–7.

Feiner R, Argov T, Rabinovich L *et al*. A new perspective on lysogeny: prophages as active regulatory switches of bacteria. *Nat Rev Microbiol* 2015;**13**:641–50.

Gamage SD, McGannon CM, Weiss AA. *Escherichia coli* serogroup O107/O117 lipopolysaccharide binds and neutralizes Shiga toxin 2. *J Bacteriol* 2004;**186**:5506–12

Gamage SD, Strasser JE, Chalk CL *et al*. Nonpathogenic *Escherichia coli* can contribute to the production of Shiga toxin. *Infect Immun* 2003;**71**:3107–15.

Grau-Leal F, Quirós P, Martínez-Castillo A *et al*. Free Shiga toxin 1-encoding bacteriophages are less prevalent than Shiga toxin 2 phages in extraintestinal environments. *Environ Microbiol* 2015;**17**:4790–801.

Grotiuz G, Sirok A, Gadea P *et al*. Shiga toxin 2-producing *Acinetobacter haemolyticus* associated with a case of bloody diarrhea. *J Clin Microbiol* 2006;**44**:3838–41.

Gyles C, Boerlin P. Horizontally transferred genetic elements and their role in pathogenesis of bacterial disease. *Vet Pathol* 2013;**51**(2):328–340.

He X, Kong Q, Patfield S *et al*. A New Immunoassay for detecting all subtypes of Shiga toxins produced by Shiga toxin-producing E. coli in ground beef. *PLoS One* 2016;**11**:e0148092.

Holguín AV, Rangel G, Clavijo V *et al*. Phage ΦPan70, a putative temperate phage, controls *Pseudomonas aeruginosa* in planktonic, biofilm and burn mouse model assays. *Viruses* 2015;**7**:4602–23.

Imamovic L, Muniesa M. Characterizing RecA-independent induction of Shiga toxin 2-encoding phages by EDTA treatment. *PLoS One* 2012;**7**:e32393.

Kawano K, Okada M, Haga T *et al*. Relationship between pathogenicity for humans and *stx* genotype in Shiga toxin-producing *Escherichia coli* serotype O157. *Eur J Clin Microbiol* 2008;**27**:227–32.

Kelly BG, Vespermann A, Bolton DJ. Horizontal gene transfer of virulence determinants in selected bacterial foodborne pathogens. *Food Chem Toxicol* 2009;**47**:969–77.

Koitabashi T, Vuddhakul V, Radu S *et al*. Genetic characterization of *Escherichia coli* O157: H7– strains carrying the stx_2 gene but not producing Shiga toxin 2. *Microbiol Immunol* 2006;**50**:135–48.

Krylov VN. Role of horizontal gene transfer by bacteriophages in the origin of pathogenic bacteria. *Genetika* 2003;**39**:595–620.

Krzymińska S, Mokracka J, Koczura R *et al*. Cytotoxic activity of *Enterobacter cloacae* human isolates. *FEMS Immunol Med Micr* 2009;**56**:248–52.

Kyle JL, Cummings CA, Parker CT *et al*. *Escherichia coli* serotype O55: H7 diversity supports parallel acquisition of bacteriophage at Shiga toxin phage insertion sites during evolution of the O157: H7 lineage. *J Bacteriol* 2012;**194**:1885–96.

Lima SM, Peabody DS, Silva JL *et al*. Mutations in the hydrophobic core and in the protein-RNA interface affect the packing and stability of icosahedral viruses. *Eur J Biochem* 2004;**271**:135–45.

Łoś JM, Łoś M, Węgrzyn G. Bacteriophages carrying Shiga toxin genes: genomic variations, detection and potential treatment of pathogenic bacteria. *Future Microbiol* 2011;**6**:909–24.

Lunder M, Bratkovic T, Urleb U *et al*. Ultrasound in phage display: a new approach to nonspecific elution. *Biotechniques* 2008;**44**:893–900.

Maaß A, Heiseler T, Maaß F *et al*. A general strategy for antibody library screening via conversion of transient target binding into permanent reporter deposition. *Protein Eng Des Sel* 2014;**27**:41–47.

McAllister LJ, Bent SJ, Petty NK *et al*. Genomic comparison of Two O111:H– enterohemorrhagic *Escherichia coli* isolates from a historic hemolytic-uremic syndrome Outbreak in Australia. *Infect Immun* 2016;**84** :775–81.

McDonald JE, Smith DL, Fogg PC *et al*. High-throughput method for rapid induction of prophages from lysogens and its application in the study of Shiga toxin-encoding *Escherichia coli* strains. *Appl Environ Microb* 2010;**76**:2360–5.

McDonough MA, Butterton JR. Spontaneous tandem amplification and deletion of the Shiga toxin operon in *Shigella dysenteriae* 1. *Mol Microbiol* 1999;**34**:1058–69.

Mauro SA, Koudelka GB. Shiga toxin: expression, distribution, and its role in the environment. *Toxins* 2011;**3**:608–25.

Mezzatesta ML, Gona F, Stefani S. *Enterobacter cloacae* complex: clinical impact and emerging antibiotic resistance. *Future Microbiol* 2012;**7**:887–902.

Muniesa M, Blanco JE, de Simón M *et al*. Diversity of stx_2 converting bacteriophages induced from Shiga toxin-producing *Escherichia coli* strains isolated from cattle. *Microbiol* 2004;**150**:2959–71.

Muniesa M, De Simon M, Prats G *et al*. Shiga toxin 2-converting bacteriophages associated with clonal variability in *Escherichia coli* O157:H7 strains of human origin isolated from a single outbreak. *Infect Immun* 2003;**71**:4554–62.

Muniesa M, Serra-Moreno R, Jofre J. Free Shiga toxin bacteriophages isolated from sewage showed diversity although

the *stx* genes appeared conserved. *Environ Microbiol* 2004; **6**:716–25.

Neely MN, Friedman DT. Arrangement and functional identification of genes in the regulatory region of lambdoid phage H-19B, a carrier of a Shiga-like toxin. *Gene* 1998;**223**:105–13.

Ohnishi M, Terajima J, Kurokawa K *et al.* Genomic diversity of enterohemorrhagic *Escherichia coli* O157 revealed by whole genome PCR scanning. *P Natl Acad Sci USA* 2002;**99**:17043–8.

Ooka T, Seto K, Kawano K *et al.* Clinical significance of *Escherichia albertii*. *Emerg Infect Dis* 2012;**18**:488–92.

Orth D, Grif K, Khan AB *et al.* The Shiga toxin genotype rather than the amount of Shiga toxin or the cytotoxicity of Shiga toxin *in vitro* correlates with the appearance of the hemolytic uremic syndrome. *Diagn Micr Infec Dis* 2007;**59**:235–42.

Pacheco AR, Sperandio V. Shiga toxin in enterohemorrhagic *E. coli*: regulation and novel anti-virulence strategies. *Front Cell Infect Microbiol* 2012;**2**:1–2.

Paul JH, Weinbauer M. Detection of lysogeny in marine environments. In: Wilhelm SW, Weinbauer MG, Suttle CA (eds). *Manual Aquatic Viral Ecology*. ASLO: Waco, TX, USA, 2010, pp 30–33.

Probert WS, McQuaid C, Schrader K. Isolation and identification of an *Enterobacter cloacae* strain producing a novel subtype of Shiga Toxin type 1. *J Clin Microbiol* 2014;**52**:2346–51.

Rakhuba DV, Kolomiets EI, Dey ES *et al.* Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. *Pol J Microbiol* 2010;**59**:145–55.

Rooks DJ, Yan Y, McDonald JE *et al.* Development and validation of a qPCR-based method for quantifying Shiga toxin-encoding and other lambdoid bacteriophages. *Environ Microbiol* 2010;**12**:1194–204.

Sambrook J, Russell DW. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2001.

Saunders JR, Allison H, James CE *et al.* Phage-mediated transfer of virulence genes. *J Chem Technol Biot* 2001;**76**:662–6.

Schmidt H, Montag M, Bockemuhl J *et al.* Shiga-like toxin II-related cytotoxins in *Citrobacter freundii* strains from humans and beef samples. *Infect Immun* 1993;**61**:534–43.

Shaikh N, Tarr PI. *Escherichia coli* O157:H7 Shiga toxin encoding bacteriophages: integrations, excisions, truncations, and evolutionary implications. *J Bacteriol* 2003;**185**:3596–605.

Scheutz F, Teel LD, Beutin L *et al.* Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. *J Clin Microbiol* 2012; **1**:2951–63.

Skinner C, Patfield S, Khalil R *et al.* New monoclonal antibodies against a novel subtype of Shiga toxin 1 produced by *Enterobacter cloacae* and their use in analysis of human serum. *mSphere* 2016;**1**:e00099–15.

Skinner C, Patfield S, Stanker L *et al.* Development of monoclonal antibodies and immunoassays for sensitive and specific detection of Shiga toxin Stx2f. *PLoS One* 2013;**8**:e76563.

Smith DL, Rooks DJ, Fogg PC *et al.* Comparative genomics of Shiga toxin encoding bacteriophages. *BMC Genomics* 2012;**13**:311.

Strauch E, Hammerl JA, Konietzny A *et al.* Bacteriophage 2851 is a prototype phage for dissemination of the Shiga toxin variant gene 2c in *Escherichia coli* O157:H7. *Infect Immun* 2008;**76**: 5466–77.

Strauch E, Lurz R, Beutin L. Characterization of a Shiga Toxin-encoding temperate bacteriophage of *Shigella sonnei*. *Infect Immun* 2001;**69**:7588–95.

Strauch E, Schaudinn C, Beutin L. First-time isolation and characterization of a bacteriophage encoding the Shiga toxin 2c variant, which is globally spread in strains of *Escherichia coli* O157. *Infect Immun* 2004;**72**:7030–9.

Sváb D, Bálint, Maróti G *et al.* A novel transducible chimeric phage from *Escherichia coli* O157: H7 Sakai strain encoding Stx1 production. *Infect Genet Evol* 2015;**29**:42–47.

Tóth I, Schmidt H, Dow M *et al.* Transduction of porcine enteropathogenic *Escherichia coli* with a derivative of a Shiga toxin 2-encoding bacteriophage in a porcine ligated ileal loop system. *Appl Environ Microb* 2003;**69**:7242–7.

Tóth I, Sváb D, Bálint B *et al.* Comparative analysis of the Shiga toxin converting bacteriophage first detected in *Shigella sonnei*. *Infect Genet Evol* 2016;**37**:150–7.

Tozzoli R, Grande L, Michelacci V *et al.* Shiga toxin-converting phages and the emergence of new pathogenic *Escherichia coli*: a world in motion. *Front Cell Infect Microbiol* 2014;**4**:80.

Tyler JS, Mills MJ, Friedman DI. The operator an early promoter region of the Shiga toxin type 2-encoding bacteriophage 933W and control of toxin expression. *J Bacteriol* 2004;**186**:7670–9.

USDA, FSIS. Detection, isolation and identification of *Escherichia coli* O157:H7 from meat products. Microbiology Laboratory Guidebook (MLG) 5.06, 2012.

Velandia CVG, Krüger A, Parma YR *et al.* Differences in Shiga toxin and phage production among stx2g-positive STEC strains. *Front Cell Infect Microbiol* 2012;**2**:82.

Veses-Garcia M, Liu X, Rigden DJ *et al.* Transcriptomic analysis of Shiga-toxigenic bacteriophage carriage reveals a profound regulatory effect on acid resistance in *Escherichia coli*. *Appl Environ Microb* 2015;**81**:8118–25.

Vethanayagam JG, Flower AM. Decreased gene expression from T7 promoters may be due to impaired production of active T7 RNA polymerase. *Microb Cell Fact* 2005;**4**:3.

Wagner PL, Acheson DW, Waldor MK. Isogenic lysogens of diverse Shiga toxin 2-encoding bacteriophages produce markedly different amount of Shiga toxin. *Infect Immun* 1999;**67**:6710–4.

Wagner PL, Neely MN, Zhang X *et al.* Role for a phage promoter in Shiga Toxin 2 expression from a pathogenic *Escherichia coli* strain. *J Bacteriol* 2001;**183**:2081–5.

Wagner PL, Livny J, Neely MN *et al.* Bacteriophage control of Shiga toxin 1 production and release by *Escherichia coli*. *Mol Microbiol* 2002;**44**:957–70.

Wagner PL, Waldor MK. Bacteriophage control of bacterial virulence. *Infect Immun* 2002;**70**:3985–93.

Węgrzyn G, Licznerska K, Węgrzyn A. Phage λ-new insights into regulatory circuits. *Adv Virus Res* 2012;**82**:155–78.

Węgrzyn G, Węgrzyn A. Genetic switches during bacteriophage lambda development. *Prog Nucleic Acid Re* 2005; **79**:1–48.

Wick LM, Qi W, Lacher DW *et al.* Evolution of genomic content in the stepwise emergence of *Escherichia coli* O157:H7. *J Bacteriol* 2005;**187**:1783–91.

Yang YA. Genetic diversity of Shiga toxin-producing prophages among *Escherichia coli* isolated from cattle in multiple herds. *Doctoral Dissertation*, Michigan State University, 2014.

Zhang W, Bielaszewska M, Kuczius T *et al.* Identification characterization and distribution of a Shiga toxin 1 gene variant (*stx*1c) in *Escherichia coli* strains isolated from humans. *J Clin Microbiol* 2002;**40**:1441–6.

Zhang X, McDaniel AD, Wolf LE *et al.* Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. *J Infect Dis* 2000;**181**:664–70.