

Genotypic and Phenotypic Diversity among Induced, *stx*₂-Carrying Bacteriophages from Environmental *Escherichia coli* Strains[∇]

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Shiga toxin 2 (*stx*₂) gene-carrying bacteriophages have been shown to convert *Escherichia coli* strains to Shiga toxin-producing *Escherichia coli* (STEC). In this study, 79 *E. coli* strains belonging to 35 serotypes isolated from wastewaters of both human and animal origin were examined for the presence of *stx*₂-carrying bacteriophages in their genomes. The lytic cycle of the bacteriophages was induced by mitomycin, and the bacteriophage fraction was isolated and used for morphological and genetic characterization. The induced bacteriophages showed morphological diversity, as well as restriction fragment length polymorphism variation, in the different strains belonging to different serotypes. The ability to infect new hosts was highly variable, although most of the induced phages infected *Shigella sonnei* host strain 866. In summary, in spite of carrying either the same or different *stx*₂ variants and in spite of the fact that they were isolated from strains belonging to the same or different serotypes, the induced bacteriophages were highly variable. The high level of diversity and the great infectious capacity of these phages could enhance the spread of the *stx*₂ gene and variants of this gene among different bacterial populations in environments to which humans may be exposed.

Horizontal transmission of pathogenic factors through mobile elements, such as bacteriophages, plasmids, or transposable elements, is important in the emergence of new pathogenic bacteria (25). The pathogenic factors that are encoded in bacteriophages include toxins, such as the *Streptococcus pyogenes* toxin (48), the A, D, and E enterotoxins and toxic shock syndrome 1 toxin of *Staphylococcus aureus* (4), the C, D, and E neurotoxins of *Clostridium botulinum* (44), the diphtheria toxin of *Corynebacterium diphtheriae* (15), the enterotoxin of *Vibrio cholerae* (48), and the Shiga toxins (Stxs) of *Shigella dysenteriae* and *Escherichia coli* (5, 24).

Shiga toxin-producing *E. coli* (STEC) emerged in 1982 as a human pathogen that causes hemorrhagic colitis and the hemolytic-uremic syndrome (30, 49). STEC strains have been shown to produce bacteriophage-encoded toxins belonging to two main groups, Stx1 and Stx2, and some variants of these main groups have been described (Stx1, Stx1c, Stx1d, Stx2, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g) (10, 20, 24, 37, 43, 45). Although the presence of *stx* genes is not sufficient to cause infection, the fact that these genes were first detected in association with increased disease in humans suggests that they may be linked to the emergence of STEC. However, little information is available about the epidemiological associations of *stx*₂ genes before the association with STEC was found (23). Thus, establishing a link to evolutionary trends in *E. coli* is difficult without further study of the dispersal of *stx*₂ genes in the environment and in other genetically related bacteria, such as *Shigella*.

*stx*₂ gene-carrying bacteriophages (Stx2 phages) have been shown to occur naturally in the environment (18, 22) and have

been shown to spread *stx*₂ genes among bacterial populations (1, 36, 41). It has been suggested that these phages play a direct role in STEC pathogenesis since Stx production is linked to induction of the lysis cycle of the phages (17, 47). Several mechanisms induce lysis, and mitomycin treatment and UV irradiation are the methods most commonly used for research purposes (26). These treatments induce the SOS system, leading to an increase in phage gene expression and consequently an increase in Stx production (14, 50). The environment is a potential reservoir for the phages since they show high resistance in the environment (19). The ability of these phages to transduce *stx* genes has been demonstrated in vivo and in vitro (2, 35).

The genetic structure of Stx2 phages is similar to that of the lambda bacteriophage, and thus they are frequently referred to as lambdoid bacteriophages (33). Some of these phages have been totally or partially sequenced (13, 20, 28, 34, 46). Rietra et al. concluded that induced bacteriophages from *E. coli* O157:H7 isolates of human origin exhibited similar restriction fragment length polymorphism (RFLP) patterns and high levels of DNA-DNA cross-hybridization, suggesting that these bacteriophages were genetically closely related (29). However, other authors have shown that there is heterogeneity in Stx2 phages isolated from strains with different pulsed-field gel electrophoresis profiles (26, 31). The DNA phage heterogeneity of different isolates derived from the same strain may be due to different rearrangements of the Stx phages with other temperate bacteriophage genes present in the genomes of these strains (11, 27). In fact, in two previously sequenced *E. coli* O157 strains, different prophages with a structure similar to the lambda structure were detected, suggesting that these phages have an important role in the evolution of pathogenic strains since they have not been found in *E. coli* K-12.

The genetic heterogeneity reported for the Stx phages is consistent with some diversity in virion morphology. Thus, some bacteriophages are morphologically similar to the lambda

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bacteriophage and have icosahedral capsids with hexagonal symmetry and long tails, while other bacteriophages have long capsids with hexagonal symmetry and noncontractile tails (21). Moreover, there have been several descriptions of strains carrying two different bacteriophages (1, 3, 21).

The majority of the studies addressing the diversity among Stx2 phages have focused on phages isolated from *E. coli* O157:H7 strains which have been involved in STEC outbreaks (29). Other studies have examined free Stx2 phages in the environment (22). In this study, the presence of inducible bacteriophages in a collection of 79 STEC strains belonging to 35 serotypes isolated from wastewaters from different animal slaughterhouses and human sewage was evaluated for the first time. This study included detection of Stx2 phages by PCR, localization of the *stx*₂ gene in the genomes of these phages by RFLP analysis and specific hybridization of RFLP products with an *stx*₂ probe, and morphological analysis of the phages. Moreover, *in vitro* host infection assays were performed to determine the abilities of the phages to transduce the *stx*₂ gene in different hosts. Toxin production in new lysogenic strains generated upon infection by some of the Stx2 phages was also assessed. As far as we know, this is the first analysis of the prevalence of these phages in nonclinical strains having different origins in which the real prevalence of the phages in environmental strains was assessed.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and culture media. A total of 79 STEC strains carrying the *stx*₂ gene isolated from municipal human sewage (sewage mostly from humans) and animal sewage were screened for the presence of inducible Stx2 phages. Sixty-five of the strains were isolated by using no specific selection method other than selection for the presence of the *stx*₂ gene in the coliform population; the phenotypic and genotypic characteristics of these strains, as well as their serotypes, have been described previously (8). The 14 remaining strains belonged to the O157:H7 serotype and were isolated by using specific recovery on CT-SMAC medium as previously described, and their phenotypic and genotypic characteristics have been described previously (9). The *E. coli* C600 (933W) strain (17), which carries the 933W bacteriophage integrated as a prophage in the genome, was used as a positive control in the bacteriophage induction experiments, while the *E. coli* C600 laboratory strain, which does not carry any Stx2 phage, was used as a negative control.

Bacteriophage induction. The lytic cycle of temperate bacteriophages was induced by mitomycin C treatment as described previously (17). Briefly, 200-ml cultures of each STEC strain were prepared from single colonies in Luria-Bertani (LB) medium supplemented with 5 mM CaCl₂. The cultures were grown at 37°C to an optical density at 600 nm (OD₆₀₀) of 0.3, and then each culture was divided and placed into two flasks. Mitomycin C was added to one of the flasks at a final concentration of 0.5 mg ml⁻¹, and both cultures were then incubated overnight in the dark at 37°C with agitation. Phage-mediated lysis was monitored by determining the decrease in OD₆₀₀.

After overnight incubation, the culture was centrifuged at 14,300 × g for 30 min, and the supernatant was filtered through 0.22-μm-pore-size, low-protein-binding polyether sulfone membranes (Millex-GP; Millipore, Mosheim, France) to recover the bacteriophage fraction.

Phage DNA isolation. Phage DNA was isolated from the 200-ml mitomycin C-induced cultures after centrifugation and filtration as described above by using polyethylene glycol precipitation, DNase treatment, and phenol-chloroform extraction performed as previously described (22).

PCR. The presence of the *stx*₂ gene in the isolated phage DNA fraction was determined by PCR with the specific primers used to detect the different *stx*₂ variants (*stx*_{2a}, *stx*_{2c}, *stx*_{2d}, *stx*_{2e}, and *stx*_{2g}) in the original STEC strains, as previously described (9).

Bacteriophage RFLP analysis and detection of *stx*₂ gene. In order to obtain insight into the genetic diversity of the lysogenic phages found in the environmental STEC strains, isolated phage DNA was subjected to RFLP analysis by digestion with the EcoRI restriction enzyme, analysis by electrophoresis in a

0.8% Tris-borate-EDTA 1× agarose gel, and subsequent staining with ethidium bromide. Alternatively, in some cases the ClaI enzyme was used when an EcoRI pattern was not clearly visible.

Localization of the *stx*₂ gene in the different RFLP patterns obtained for the different DNA phage fractions was performed by Southern blotting using the *stx*₂ gene probe used previously for isolation of the STEC strains by colony blotting, which codes for a 378-bp region of the A subunit (8).

Evaluation of the infectious capacity of Stx2 phages. The infectious capacity of Stx2 phages was evaluated using the following host strains in the infection assays: *E. coli* O157:H7 strain ATCC 43888, three *E. coli* O157 environmental isolates, the *E. coli* DH5α and C600 laboratory strains, *Enterobacter aerogenes* ATCC 13048, a *Citrobacter freundii* environmental isolate, and several clinical isolates, including *Shigella dysenteriae* strain 500H, *Shigella boydii* strains 316 and 351, *Shigella sonnei* strains 635 and 866, *Shigella flexneri* strains 668 and 805, *E. coli* O26 strains 216 and 224, and *E. coli* O111 strain 209. None of these strains carry the *stx*₂ gene. Briefly, 10 μl of induced and filtered phage supernatant was spotted onto LB agar overlaid with LB soft agar supplemented with 5 mM CaCl₂ containing the corresponding host strain at an OD₆₀₀ of 0.3. After overnight incubation at 37°C, the formation of plaques was monitored. Then the plaques were transferred onto nylon membranes using standard procedures (32) and were hybridized using the *stx*₂ probe, as described above. Only the tests showing lysis and a positive hybridization signal were considered positive.

Construction of lysogens. The induced phages were transduced into *S. sonnei* strain 866 and *E. coli* DH5α by spotting 0.5 ml of the phage lysate as described above. After overnight incubation at 37°C, the LB soft agar overlay was removed and resuspended in 2 ml SM buffer (32). The mixture was incubated for 90 min at 37°C, and then 0.1-ml portions of 10-fold dilutions in phosphate-buffered saline were plated onto LB agar. The plates were incubated overnight at 37°C. Lysogens carrying the *stx*₂ gene were selected by a colony hybridization method as described previously (6). The presence of *stx*₂ genes in the new lysogens was confirmed by PCR, as indicated above.

Phage integration site. PCR was used to assess whether the bacteriophages studied were integrated in the same locus as the 933W phage (the *wrbA* locus) or the VT1-Sakai phage (the *yehV* locus), before and after the transduction of the bacteriophages into the new host strains. Briefly, the bacterial DNA with the inserted prophages was extracted, and a PCR was carried out using the primers described previously (39).

Evaluation of Stx production. The production of Stxs (both Stx1 and Stx2, including Stx2 variants) by the constructed lysogens was tested using a commercial Duopath VT detection kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions.

Electron microscopy imaging. Twenty-milliliter portions of phage-induced cultures were filtered as described above and were concentrated 1,000-fold using Amicon Ultra-4 filters according to the manufacturer's instructions (Millipore). Phages were adsorbed onto Formvar-coated grids and negatively stained with 2% KOH, phosphotungstic acid (pH 7.2) for 2.5 min. Negatively stained samples were observed with a Hitachi 800 STEM microscope (Portland, OR) at 80 kV.

RESULTS

Induction and detection of Stx2 phages from environmental STEC. The levels of induction of the different prophages present in the STEC strains showed great variability. As shown in Fig. 1, for some of the prophages the induction levels were high, and there was a >1-OD₆₀₀ unit difference compared with their noninduced counterparts after mitomycin treatment, while other prophages did not respond to this treatment and there was no significant difference. In summary, around 60% of the prophages present in STEC strains showed high levels of induction, and the values ranged from 0.1 to 0.6 OD₆₀₀ unit. For the corresponding noninduced cultures the values were 1.6 ± 0.2 OD₆₀₀ units after 24 h of incubation.

The amounts of phage DNA extracted from the mitomycin-induced cultures correlated with the levels of induction of the prophages determined by measuring the absorbance, and phage DNA was more abundant in the cultures exhibiting higher levels of induction. A minimum of 0.5 μg/μl of phage

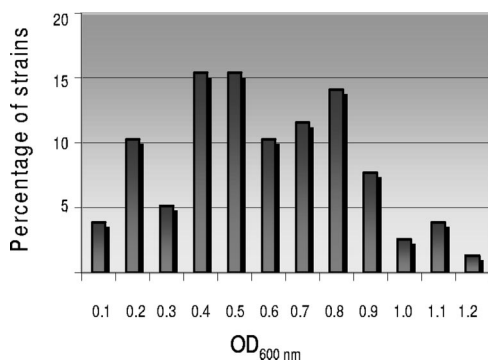


FIG. 1. Levels of induction of STEC integrated prophages, expressed in OD₆₀₀ units, after mitomycin treatment. A low OD₆₀₀ correlates with lysis of bacterial cells and induction. The bars indicate the percentages of the strains having specific OD₆₀₀ values after mitomycin treatment. The average values for noninduced cultures of the same strains were 1.6 ± 0.2 OD₆₀₀ units.

DNA was observed for the strains showing a good level of induction.

Of the 79 STEC strains examined, 70 (89%) contained inducible bacteriophages; 11 of 14 (79%) STEC strains from human sewage contained inducible bacteriophages, 47 of 50 (94%) and 5 of 6 (83%) STEC strains from cattle and pig abattoirs, respectively, contained inducible bacteriophages, and 7 of 9 (78%) STEC strains from mixed sewage contained inducible bacteriophages.

Genetic diversity among Stx2 phages as determined by RFLP analysis. RFLP analysis of phage DNA revealed diversity among induced phages from different STEC strains. However, some prophages isolated from different strains had the same profile, suggesting that the same prophage could be integrated in the different STEC strains. Since any temperate phage present in an STEC strain could have been induced in the assay, detection of the *stx*₂ gene was necessary to confirm the presence of DNA from Stx2 phages. *stx*₂ was detected by Southern blotting and hybridization with the *stx*₂ probe described previously within the RFLP pattern obtained. This probe has neither EcoRI nor ClaI restriction sites, and therefore only one hybridization signal was expected for each *stx*₂ gene copy present in the extracted phage DNA. The *stx*₂ gene was detected by hybridization of the RFLP products with the *stx*₂ probe in 43% of the induced STEC prophages. The RFLP patterns observed were diverse, although some induced prophages from STEC strains having different origins had the same pattern. The molecular weight of the RFLP fragment in which the *stx*₂ gene was located (according to the positive hybridization signal) was highly conserved. The *stx*₂ gene was located in the same RFLP fragment for all phages having the same RFLP pattern. Moreover, for some of the phages with different RFLP patterns, the sizes of the RFLP fragments in which the *stx*₂ gene was located were similar (Fig. 2, lanes 9 and 11). As shown in Table 1, the sizes of the positive RFLP fragments from the induced Stx2 phages from different serotypes ranged from 4.9 to 18 kb. Two bands were detected in the RFLP analysis of the induced prophages from strain O91:H21, which is consistent with previous results showing that this strain carried two *stx*₂ genes (*stx*₂ and the *stx*_{2c} variant). It was not

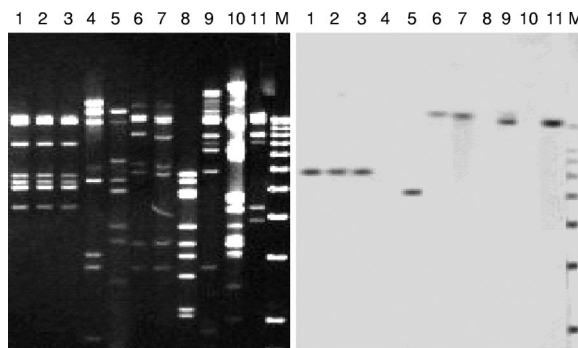


FIG. 2. EcoRI-digested phage DNA from some of the induced phages present in the STEC strains (left panel) and hybridization signals with the *stx*₂ probe after Southern blotting (right panel). Lanes 1 to 11 contained strains belonging to serotypes O157:H7, O157:H7, O157:H7, O157:H7, O146:H⁻, O171:H2, O171:H2, O171:H2, O181:H20, O90:H⁻, and O90:H⁻, respectively. Lane M contained a 1-kb DNA ladder.

possible to detect the *stx*₂ gene after RFLP analysis in some of the induced prophage cultures (Fig. 2, lanes 4, 8, and 10), although it was noticed that a prophage was inside the strain.

The size of the total phage DNA was estimated by adding the sizes of the different RFLP fragments, and in most cases it was around 50 kb (data not shown), which was consistent with the assumption that a single phage had been induced.

It should be noted that this study could be performed only with prophages that exhibited sufficient levels of induction, which was possible for only 65 of the 79 STEC strains, even though larger culture volumes were prepared for the strains in which no RFLP of phage DNA was detected.

Evaluation of the infection capacity of the induced Stx2 phages. The ability of induced Stx2 phages to infect different hosts was analyzed both by a plaque-forming assay and by hybridization of the generated plaques with the 378-bp *stx*₂ gene probe described previously. Infection was induced in 50 of the 79 STEC strains (63%) (Table 2). The Stx2 phages infected mainly *S. sonnei* strain 866 (51%), *E. coli* DH5α (46%), *S. sonnei* strain 635 (44%), and *E. coli* C600 (40%). Some STEC strains yielded lytic phages (probably non-Stx2

TABLE 1. Serotypes carrying inducible phages showing positive hybridization signals with the *stx*₂ gene probe after RFLP analysis and Southern blotting

Serotype	No. of phages/ no. of strains	Size(s) of EcoRI fragment(s) (kb)
O146:H ⁻	1/1	4.9
O91:H21	1/1	6, 4.9
O113:H21	1/1	6
O157:H7	14/14	6
O157:H ⁻	1/1	6
O100:H ⁻	1/1	11
O2:H25	3/5	12
O26:H ⁻	1/2	12
O90:H ⁻	1/4	12
O171:H2	6/15	12 (5), 15 (1) ^a
O181:H20	2/2	12
O98:HNT	1/1	15
O1:H20	2/2	18

^a The numbers in parentheses are numbers of phages.

TABLE 2. Detection of bacteriophage infection by plaque blotting and hybridization with the *stx*₂ probe in different host strains analyzed

Serotype ^a	No. of phages	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>	<i>S. sonnei</i>	<i>S. sonnei</i>	<i>S. dysenteriae</i>	<i>S. boydii</i>	<i>S. boydii</i>	<i>S. flexneri</i>	<i>S. flexneri</i>	<i>S. flexneri</i>	<i>E. aerogenes</i>	<i>C. freundii</i>
		C600	DH5a	O111	O26 strain 216	O26 strain 224	O157:H7 strain ATCC 43888	O157 strain 11	O157 strain 44	O157 strain 151	866	635	500	316	351	805	668	
O1:H20	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O2:H2	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O2:H21	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O2:H25	5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O8:H ⁻	1																	
O8:H31	1																	
O8:H9	3																	
O22:H8	2																	
O26:H ⁻	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O54:H21	1																	
O70:H ⁻	1																	
O76:H2	1																	
O89:HI9	1																	
O96:H ⁻	4																	
O91:H21	1																	
O98:HNT	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O100:H ⁻	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O113:H21	1																	
O127:H ⁻	1																	
O136:HI	1																	
O146:H ⁻	1																	
O146:H21	1																	
O157:H ⁻	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O157:H7	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O159:H ⁻	1																	
O162:H7	1																	
O166:H21	1																	
O171:H2	15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O175:HI6	1																	
O177:H ⁻	1																	
O181:H20	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O181:H49	1																	
ONT:H ⁻	4																	
ONT:H21	1																	
ONT:H9	1																	
ONT:HNT	1																	

^a *E. coli* serotypes from which the bacteriophages were induced.^b The numbers in parentheses are the numbers of induced prophages from all of the strains belonging to different *E. coli* serotypes showing a positive signal.

TABLE 3. Genetic and morphological characteristics of the transduced bacteriophages

Transduced bacteriophage ^a	Host	<i>stx</i> ₂ gene	Size of EcoRI fragment (kb)	Morphology
S8	<i>E. coli</i> DH5 α	<i>stx</i> ₂	ND ^b	ND
S48	<i>E. coli</i> DH5 α	<i>stx</i> _{2c}	ND	ND
S62	<i>S. sonnei</i> 866	<i>stx</i> _{2c}	12	H19-like ^c
S63	<i>S. sonnei</i> 866	<i>stx</i> _{2c}	12	H19-like ^c
S67	<i>E. coli</i> DH5 α	<i>stx</i> _{2c}	12	H19-like ^c
S68	<i>E. coli</i> DH5 α	<i>stx</i> _{2c}	ND	H19-like ^c
S86	<i>S. sonnei</i> 866	<i>stx</i> _{2g}	12	933W-like ^d
S134	<i>S. sonnei</i> 866	<i>stx</i> _{2g}	12	933W-like ^d
S150	<i>S. sonnei</i> 866	<i>stx</i> _{2c}	ND	933W-like ^d

^a Induced prophages from the original STEC strains.

^b ND, not detected.

^c Bacteriophage morphology similar to that of phage H19.

^d Bacteriophage morphology similar to that of phage 933W.

phages), which may have accounted for the lack of detection of the *stx*₂ gene in the phage plaques generated. Six induced Stx2 phages from non-O157 STEC strains were able to infect the *E. coli* O157:H7 host strain used in this study. It is noteworthy that all induced Stx2 phages from O157 strains were able to infect the O157 host strains, as well as the most infected host strains described above, including *C. freundii* and *E. aerogenes*.

The presence of infectious Stx2 phages varied according to the isolation source, and such phages were detected mainly in STEC obtained from animal wastewaters. Thus, 80% of the STEC strains isolated from cattle abattoirs and 50% of the STEC strains isolated from pig abattoirs, but only 28 and 33% of the STEC strains isolated from human and mixed sewage, respectively, contained detectable Stx2 infectious prophages.

Construction of new lysogens. The abilities of the Stx2 phages to infect and to establish lysogeny in two previously described hosts (*E. coli* DH5 α and *S. sonnei* strain 866) were assessed. Nine bacteriophages carrying different *stx*₂ genes (*stx*₂, *stx*_{2c}, *stx*_{2g}, and *stx*_{2e}) were successfully transduced (Table 3). The infection and subsequent stabilization of the *stx*₂ gene within the different hosts generated new lysogenic strains, which in some cases changed some phenotypic characteristics. The new lysogens showed levels of induction different than those obtained with the original strains. In general, the ability to produce Stxs was maintained, but there were some exceptions. Thus, in the case of strain S8 (serotype O146:H⁻, carrying an Stx2 prophage), after transduction of the Stx2 phage

to *E. coli* DH5 α , the prophage was poorly induced and no Stx2 production was detected. The opposite was found to be the case for strain S150 (serotype O26:H⁻, carrying an Stx2e prophage), in which a higher level of prophage induction was observed and the Stx2 protein was detected after transduction to *S. sonnei*. The seven other transduced prophages retained the same phenotypic characteristics in the new lysogenic strains. The RFLP prophage patterns obtained were the same as the patterns obtained for the induced prophages from the original strains.

Phage integration site. One strain belonging to serotype O146:H⁻ was shown to amplify the *wrbA* phage-bacterium attachment site, suggesting that the phage was integrated in this locus. Unexpectedly, 3 strains amplified only one of the *wrbA* locus junctions (1 serotype O146:H21 strain, 1 serotype O162:H7 strain, and 1 serotype O90:H⁻ strain), while 22 strains amplified only one of the *yehV* locus junctions (3 serotype O171:H2 strains, 1 serotype O113:H21 strain, 15 serotype O157:H7 strains, 1 serotype O91:H21 strain, and 2 serotype O22:H8 strains). Additionally, two other strains belonging to serotypes O127:H⁻ and O90:H⁻ amplified one junction from both the *wrbA* and *yehV* loci. It should be noted that in the newly constructed lysogens most of the prophages showed the same integration profile; the only exception was a prophage that became integrated in the *yehV* locus after transduction.

Morphological diversity of STEC prophages. In most cases the morphology of the induced bacteriophages resembled that of the previously described Stx2 phages 933W and H19, which have icosahedral capsids and a short tail and long capsids and a long tail, respectively. In a few cases other phages were detected (Fig. 3), but they could not be transduced, and therefore the presence of the *stx*₂ gene could not be determined. No relationship between the presence of a particular *stx* variant and the morphology of the observed phage was found.

DISCUSSION

Phages can be considered important reservoirs of genes that can be mobilized in the bacterial population, which is especially relevant for the emergence of new pathogenic strains. Bacteriophage-mediated transmission of the *stx*₂ gene has been examined in various studies since the emergence of STEC strains. However, most authors have focused on Stx2 phages isolated from clinical STEC strains or on the O157:H7 serotype. In this study, the presence of inducible bacteriophages in a collection of 79 STEC strains belonging to 35 serotypes

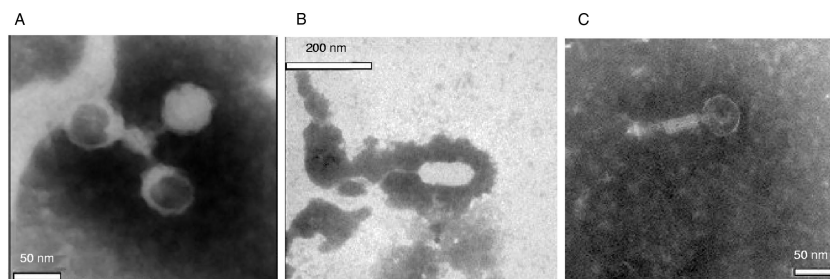


FIG. 3. Electron micrographs showing the Stx2 phages isolated most frequently from the newly constructed 933W-like and H19-like lysogens (A and B, respectively) and one non-Stx2 phage isolated from the original STEC (C), which may have hindered the detection of Stx2 phages.

isolated from different animal wastewaters and human sewage was evaluated in order to assess the real prevalence of these phages in environmental strains.

Eighty-nine percent of the strains contained mitomycin-inducible bacteriophages, as measured by *stx*₂ gene PCR amplification of phage DNA, and a high proportion (63%) of the bacteriophages were capable of infecting the laboratory strains used in this study. However, it should be noted that only 28% of the strains isolated from human sewage carried infectious Stx₂ prophages. It could be hypothesized that loss of functional *stx*₂ genes occurs as STEC emerges in human populations. The lack of infectious and inducible Stx₂ phages in human sewage isolates was supported by the absence of Stx production, a feature that is highly related to the pathogenicity of the STEC strains, although this was not the subject of this study. This finding suggests that inactivation of the phages may have occurred after the phage integration process. Moreover, only a small proportion of the phages (9 of 79) were maintained after several passages in the host strain to generate new lysogens, which implies that although the phages are infectious in different hosts, their insertion and persistence as prophages in the host genome are dependent on the genetic background of the strains (38), including the presence of other bacteriophages. This effect was also obvious after transduction of some of the phages whose lysogenic strains lost or gained the ability to produce Stx.

Phages carrying different *stx*₂ gene variants, including the recently reported *stx*_{2g} variant, were detected in this study (16). The differences between *stx* genes from *S. dysenteriae*, which traditionally appear to be chromosomally encoded, and *stx* genes from *E. coli* strains, which are bacteriophage encoded, are unknown. However, an *stx* gene-carrying bacteriophage was isolated from the related species *S. sonnei* (5). This bacteriophage was adapted to different *Shigella* species (42). Adaptation of the induced Stx₂ phages from STEC strains to *S. sonnei* could be inferred from the results obtained in this study since the majority of the prophages were able to infect *S. sonnei* 866. This is not surprising since similar results were obtained with free Stx₂ phages isolated from sewage (22). These observations support the hypothesis that *Shigella* could act as a reservoir of the *stx*₂ gene. To our knowledge, no studies have examined the prevalence of Stx₂ phages in the phages in nonclinical isolates of *Shigella* species, which could provide new insight into the horizontal transmission of *stx*₂ genes in the environment. In any case, it is important to point out that the Stx₂ phages that are present mostly in potentially nonpathogenic STEC strains are similar to the phages that have previously been linked to human outbreaks (8, 9).

Ten STEC strains contained two *stx*₂ genes, although only one phage was induced from the majority of these strains. Similar results have been obtained in other studies, showing that simultaneous induction of two prophages results in an induction rate for one of the prophages that is lower than the induction rate when a single prophage is present in the strain (21).

A low proportion of the induced Stx₂ phages from non-O157 STEC strains were capable of infecting the *E. coli* O157 host strains used in this study. It has been reported previously that short-tailed Stx phages share a common receptor, YaeT, which is an outer membrane protein commonly found in members of

the *Enterobacteriaceae* (40). Although the possibility of the presence of another receptor in O157 that is preferentially used by induced phages from O157 strains cannot be ruled out, this information suggests that some other mechanisms in the genetic background of the O157 strains can also hinder infection with Stx phages isolated from non-O157 strains. Accordingly, 14 of 15 induced phages from O157 STEC were able to infect the O157 serotype host strains, as well as the majority of the host strains tested.

The RFLP studies revealed a high degree of heterogeneity among the different induced STEC prophages, which could be attributed to the presence of more than one phage (probably non-Stx₂) in the genomes of the strains. However, the degree of heterogeneity of Stx₂ phages was supported by the finding that the *stx*₂ gene was located in fragments with different molecular weights in the RFLP patterns. The differences were attributed to variations in the genetic sequences of the phages. Recombination of Stx₂ phages with other prophages or phage genes in intergenic regions that do not disrupt functional modules is a plausible explanation for reorganization events leading to an increase in such genetic heterogeneity (7). Genes that are present in some phage genomes may also increase the fitness of the phage (12). It is noteworthy that some of the induced Stx₂ phages from different STEC strains having different origins had similar RFLP patterns, suggesting that the horizontal transfer of these phages could have been a recent event or that the phages are subjected to high selective pressure. In addition, two induced phages were from a strain belonging to *E. coli* serotype O91:H21, which is consistent with a previous study that found two *stx*₂ genes in a genome (8). The heterogeneity of the Stx₂ prophages present in the environmental STEC strains is consistent with the heterogeneity reported in previous studies performed with free environmental Stx₂ phages (22).

Numerous Stx phages have been shown to be integrated in the *wrbA* and *yehV* loci (39). In this study only 1 strain showed PCR amplification of both *wrbA* bacteriophage-bacterium junctions, 2 strains showed simultaneous PCR amplification of a single *wrbA-yehV* junction, while 3 and 22 strains showed amplification of the *wrbA* and *yehV* junctions, respectively. The two strains that showed PCR amplification of both loci had previously been shown to carry two *stx*₂ gene variants (8). The change of integration site that occurred in one of the transduced bacteriophages is not surprising since it has been reported that an Stx phage has a preferred integration site when it is available but is also able to integrate at different sites when this site is occupied (38). The high amplification rate for only one of the locus junctions may be related to slight variability in one of the junctions, although this has not been reported to date.

Some instability of free Stx₂ phages after propagation has been reported previously (22), which suggests that these phages could have evolved so that they were integrated in an STEC bacterial host as a prophage. A prophage can be induced when the environmental conditions activate its lytic cycle. Accordingly, in this study all the strains studied allowed induction of their Stx phages at moderate or high levels, ensuring that free viral particles remained free in the environment, which ensured the survival necessary to allow new infection of a suitable host once environmental conditions were

optimal again. The cycle is thus closed and allows the *stx* genes to be prevalent in the environment, which may be their real reservoir.

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REFERENCES

- Acheson, D. W. K., and G. T. Keusch. 1996. Which Shiga toxin-producing types of *Escherichia coli* are important? *ASM News* **6**:302–306.
- Acheson, D. W. K., J. Reidl, X. Zhang, G. T. Keusch, J. J. Mekalanos, and M. K. Waldor. 1998. In vivo transduction with Shiga toxin 1-encoding phage. *Infect. Immun.* **66**:4496–4498.
- Allison, H. E., M. J. Sergeant, C. E. James, J. R. Saunders, D. L. Smith, R. J. Sharp, T. S. Marks, and A. J. McCarthy. 2003. Immunity profiles of wild-type and recombinant Shiga-like toxin-encoding bacteriophages and characterization of novel double lysogens. *Infect. Immun.* **71**:3409–3418.
- Betley, M., and J. Mekalanos. 1985. Staphylococcal enterotoxin A is encoded by a phage. *Science* **229**:185–187.
- Beutin, L., E. Strauch, and I. Fischer. 1999. Isolation of *Shigella sonnei* lysogenic for a bacteriophage encoding gene for production of Shiga toxin. *Lancet* **353**:1498.
- Blanch, A. R., C. García-Aljaro, M. Muniesa, and J. Jofre. 2003. Detection, enumeration and isolation of strains carrying the *stx*₂ gene from urban sewage. *Water Sci. Technol.* **47**:109–116.
- Brüssow, H., C. Canchaya, and W. D. Hardt. 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol. Mol. Biol. Rev.* **68**:560–602.
- García-Aljaro, C., M. Muniesa, J. E. Blanco, M. Blanco, J. Blanco, J. Jofre, and A. R. Blanch. 2005. Characterization of Shiga toxin producing *Escherichia coli* isolated from aquatic environments. *FEMS Microbiol. Lett.* **246**:55–65.
- García-Aljaro, C., X. Bonjoch, and A. R. Blanch. 2005. Combined use of an immunomagnetic separation method and immunoblotting for the enumeration and isolation of *E. coli* O157 in wastewaters. *J. Appl. Microbiol.* **98**:589–597.
- García-Aljaro, C., M. Muniesa, J. Jofre, and A. R. Blanch. 2006. Newly identified bacteriophages carrying the *stx*_{2g} gene isolated from *Escherichia coli* strains in polluted waters. *FEMS Microbiol. Lett.* **258**:127–135.
- Hayashi, T., K. Makino, M. Ohnishi, K. Kurokawa, K. Ishii, K. Yokoyama, C. G. Han, E. Ohtsubo, K. Nakayama, T. Murata, M. Tanaka, T. Tohe, T. Iida, H. Takami, T. Honda, C. Sasakawa, N. Ogasawara, T. Yasunaga, S. Kuhara, T. Shiba, M. Hattori, and H. Shinagawa. 2001. Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Res.* **8**:11–22.
- Hendrix, R. W., J. G. Lawrence, G. F. Hatfull, and S. Casjens. 2000. The origins and ongoing evolution of viruses. *Trends Microbiol.* **8**:504–508.
- Johansen, B. K., Y. Wasteson, P. E. Granum, and S. Brynstad. 2001. Mosaic structure of Shiga-toxin-2-encoding phages isolated from *Escherichia coli* O157:H7 indicates frequent gene exchange between lambdoid phage genomes. *Microbiology* **147**:1929–1936.
- Kimmitt, P. T., C. R. Harwood, and M. R. Barer. 2000. Toxin gene expression by Shiga toxin-producing *Escherichia coli*: the role of antibiotics and the bacterial SOS response. *Emerg. Infect. Dis.* **6**:458–465.
- Laird, W., and N. Groman. 1976. Orientation of the *tox* gene in the prophage of corynebacteriophage beta. *J. Virol.* **19**:228–231.
- Leung, P. H., J. S. Peiris, W. W. Ng, R. M. Robins-Browne, K. A. Bettelheim, and W. C. Yam. 2003. A newly discovered verotoxin variant, VT_{2g}, produced by bovine verocytotoxigenic *Escherichia coli*. *Appl. Environ. Microbiol.* **69**:7549–7553.
- Mühdorfer, I., J. Hacker, G. T. Keusch, D. W. Acheson, H. Tschäpe, A. V. Kane, A. Ritter, T. Otschlager, and A. Donohue-Rolfe. 1996. Regulation of the Shiga-like toxin II operon in *Escherichia coli*. *Infect. Immun.* **64**:495–502.
- Muniesa, M., and J. Jofre. 1998. Abundance in sewage of bacteriophages that infect *Escherichia coli* O157:H7 and that carry the Shiga toxin 2 gene. *Appl. Environ. Microbiol.* **64**:2443–2448.
- Muniesa, M., F. Lucena, and J. Jofre. 1999. Comparative survival of free Shiga toxin 2-encoding phages and *Escherichia coli* strains outside the gut. *Appl. Environ. Microbiol.* **65**:5615–5618.
- Muniesa, M., J. Recktenwald, M. Bielaszewska, H. Karch, and H. Schmidt. 2000. Characterization of a Shiga toxin 2e-converting bacteriophage from an *Escherichia coli* strain of human origin. *Infect. Immun.* **68**:4850–4855.
- Muniesa, M., M. de Simon, G. Prats, D. Ferrer, H. Panella, and J. Jofre. 2003. 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.* **71**:4554–4562.
- Muniesa, M., R. Serra-Moreno, and J. Jofre. 2004. Free Shiga toxin bacteriophages isolated from sewage showed diversity although the *stx* genes appeared conserved. *Environ. Microbiol.* **6**:716–725.
- O'Brien, A. D., M. K. Gentry, M. R. Thompson, B. P. Doctor, P. Gemski, and S. B. Formal. 1979. Shigellosis and *Escherichia coli* diarrhea: relative importance of invasive and toxigenic mechanisms. *Am. J. Clin. Nutr.* **32**:229–233.
- O'Brien, A. D., J. W. Newland, S. F. Miller, R. K. Holmes, H. W. Smith, and S. B. Formal. 1984. Shiga-like toxin-converting phages from *Escherichia coli* strains that cause hemorrhagic colitis or infantile diarrhea. *Science* **226**:694–696.
- Ochman, H., J. G. Lawrence, and E. A. Groisman. 2000. Lateral gene transfer and the nature of bacterial innovation. *Nature* **405**:299–304.
- Osawa, R., S. Iyoda, S. I. Nakayama, A. Wada, S. Yamai, and H. Watanabe. 2000. Genotypic variations of Shiga toxin-converting phages from enterohaemorrhagic *Escherichia coli* O157:H7 isolates. *J. Med. Microbiol.* **49**:565–574.
- Perna, N. T., G. Plunkett III, V. Burland, B. Mau, J. D. Glasner, D. J. Rose, G. F. Mayhew, P. S. Evans, J. Gregor, H. A. Kirkpatrick, G. Posfai, J. Hackett, S. Klink, A. Boutin, Y. Shao, L. Miller, E. J. Grothbeck, N. W. Davis, A. Lim, E. T. Dimalanta, K. D. Potamouisis, J. Apodaca, T. S. Anantharaman, J. Lin, G. Yen, D. C. Schwartz, R. A. Welch, and F. R. Blattner. 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* **409**:529–533.
- Plunkett, G., III, D. J. Rose, T. J. Durfee, and F. R. Blattner. 1999. Sequence of Shiga toxin 2 phage 933W from *Escherichia coli* O157:H7: Shiga toxin as a phage late-gene product. *J. Bacteriol.* **181**:1767–1778.
- Rietra, P. J. G. M., G. A. Willshaw, H. R. Smith, A. M. Field, S. M. Scotland, and B. Rowe. 1989. Comparison of vero-cytotoxin-encoding phages from *Escherichia coli* of human and bovine origin. *J. Gen. Microbiol.* **135**:2307–2318.
- Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N. Engl. J. Med.* **308**:681–685.
- Rüssmann, H., H. Schmidt, J. Heesemann, A. Caprioli, and H. Karch. 1994. Variants of Shiga-like toxin II constitute a major toxin component in *Escherichia coli* O157 strains from patients with haemolytic uraemic syndrome. *J. Med. Microbiol.* **40**:338–343.
- Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sanger, F., A. R. Coulson, G. F. Hong, D. F. Hill, and G. B. Petersen. 1982. Nucleotide sequence of bacteriophage λ DNA. *J. Mol. Biol.* **162**:729–773.
- Sato, T., T. Shimizu, M. Watarai, M. Kobayashi, S. Kano, T. Hamabata, Y. Takeda, and S. Yamasaki. 2003. Distinctiveness of the genomic sequence of Shiga toxin 2-converting phage isolated from *Escherichia coli* O157:H7 Okayama strain compared to other Shiga toxin 2-converting phages. *Gene* **309**:35–48.
- Schmidt, H., M. Bielaszewska, and H. Karch. 1999. Transduction of enteric *Escherichia coli* isolates with a derivative of Shiga toxin 2-encoding bacteriophage φ3538 isolated from *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* **65**:3855–3861.
- Schmidt, H., J. Scheef, H. I. Huppertz, M. Frosch, and H. Karch. 1999. *Escherichia coli* O157:H7 and O157:H⁻ strains that do not produce Shiga toxin: phenotypic and genetic characterization of isolates associated with diarrhea and hemolytic-uremic syndrome. *J. Clin. Microbiol.* **37**:3491–3496.
- Scotland, S. M., H. R. Smith, G. A. Willshaw, and B. Rowe. 1983. Vero cytotoxin production in a strain of *Escherichia coli* determined by genes carried on bacteriophage. *Lancet* **ii**:216.
- Serra-Moreno, R., J. Jofre, and M. Muniesa. 2007. Insertion site occupancy by *stx*₂ bacteriophages depends on the locus availability of the host strain chromosome. *J. Bacteriol.* **189**:6645–6654.
- Shaikh, N., and P. I. Tarr. 2003. *Escherichia coli* O157:H7 Shiga toxin-encoding bacteriophages: integrations, excisions, truncations, and evolutionary implications. *J. Bacteriol.* **185**:3596–3605.
- Smith, D. L., C. E. James, M. J. Sergeant, Y. Yaxian, J. R. Saunders, A. J. McCarthy, and H. E. Allison. 2007. Short-tailed *stx* phages exploit the conserved YacT protein to disseminate Shiga toxin genes among enterobacteria. *J. Bacteriol.* **189**:7223–7233.
- Smith, H. W., P. Green, and Z. Parsell. 1983. Vero cell toxins in *Escherichia coli* and related bacteria: transfer by phage and conjugation and toxic action in laboratory animals, chickens, and pigs. *J. Gen. Microbiol.* **129**:3121–3137.
- Strauch, E., R. Lurz, and L. Beutin. 2001. Characterization of a Shiga toxin-encoding temperate bacteriophage of *Shigella sonnei*. *Infect. Immun.* **69**:7588–7595.
- Strockbine, N. A., L. R. M. Marques, J. W. Newland, H. W. Smith, R. K. Holmes, and A. D. O'Brien. 1986. Two toxin-converting phages from *Escherichia coli* O157:H7 strain 933 encode antigenically distinct toxins with similar biological activities. *Infect. Immun.* **53**:135–140.

44. Sunagawa, H., and K. Inoue. 1992. Biological and biophysical characteristics of phages isolated from *Clostridium botulinum* type C and D strains, and physicochemical properties of the phage DNAs. *J. Vet. Med. Sci.* **54**:675–684.
45. Teel, L. D., A. R. Melton-Celsa, C. K. Schmitt, and A. D. O'Brien. 2002. One of two copies of the gene for the activatable Shiga toxin type 2d in *Escherichia coli* O91:H21 strain B2F1 is associated with an inducible bacteriophage. *Infect. Immun.* **70**:4282–4291.
46. Unkmeir, A., and H. Schmidt. 2000. Structural analysis of phage-borne *stx* genes and their flanking sequences in Shiga toxin-producing *Escherichia coli* and *Shigella dysenteriae* type 1 strains. *Infect. Immun.* **68**:4856–4864.
47. Wagner, P. L., D. W. Acheson, and M. K. Waldor. 1999. Isogenic lysogens of diverse Shiga toxin 2-encoding bacteriophages produce markedly different amounts of Shiga toxin. *Infect. Immun.* **67**:6710–6714.
48. Waldor, M. K., and J. J. Mekalanos. 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**:1910–1914.
49. Wells, J. G., B. R. Davis, I. K. Wachsmuth, L. W. Riley, R. S. Remis, R. Sokolow, and G. K. Morris. 1983. Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. *J. Clin. Microbiol.* **18**:512–520.
50. Yoh, M., and T. Honda. 1997. The stimulating effect of fosfomycin, an antibiotic in common use in Japan, on the production/release of verotoxin-1 from enterohaemorrhagic *Escherichia coli* O157:H7 in vitro. *Epidemiol. Infect.* **119**:101–103.