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# Heterogeneity in Induction Level, Infection Ability, and Morphology of Shiga Toxin-Encoding Phages (Stx Phages) from Dairy and Human Shiga Toxin-Producing *Escherichia coli* O26:H11 Isolates

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Shiga toxin (Stx)-producing *Escherichia coli* (STEC) bacteria are foodborne pathogens responsible for diarrhea and hemolytic-uremic syndrome (HUS). Shiga toxin, the main STEC virulence factor, is encoded by the *stx* gene located in the genome of a bacteriophage inserted into the bacterial chromosome. The O26:H11 serotype is considered to be the second-most-significant HUS-causing serotype worldwide after O157:H7. STEC O26:H11 bacteria and their *stx*-negative counterparts have been detected in dairy products. They may convert from the one form to the other by loss or acquisition of Stx phages, potentially confounding food microbiological diagnostic methods based on *stx* gene detection. Here we investigated the diversity and mobility of Stx phages from human and dairy STEC O26:H11 strains. Evaluation of their rate of *in vitro* induction, occurring either spontaneously or in the presence of mitomycin C, showed that the Stx2 phages were more inducible overall than Stx1 phages. However, no correlation was found between the Stx phage levels produced and the origin of the strains tested or the phage insertion sites. Morphological analysis by electron microscopy showed that Stx phages from STEC O26:H11 displayed various shapes that were unrelated to Stx1 or Stx2 types. Finally, the levels of sensitivity of *stx*-negative *E. coli* O26:H11 to six Stx phages differed among the 17 strains tested and our attempts to convert them into STEC were unsuccessful, indicating that their lysogenization was a rare event.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) bacteria of various serotypes, including O157:H7, are foodborne pathogens responsible for human infections ranging from mild watery diarrhea to hemorrhagic colitis, which may be complicated by hemolytic-uremic syndrome (HUS), which is sometimes fatal. Domestic ruminants, especially cattle, are a major reservoir of STEC, whose transmission to humans occurs through the ingestion of food or water and through direct contact with animals or their environment. STEC O26:H11 was first identified as a cause of HUS in 1983 (1, 2). O26:H11 is the most commonly isolated non-O157:H7 serotype in Europe, accounting for 12% of all clinical enterohemorrhagic *E. coli* (EHEC) isolates in 2012 (3, 4). Since the early 2000s, a significantly increasing proportion of HUS cases caused by non-O157 serogroups was identified in France, with serogroup O26 accounting for 11% of cases for the period 1996 to 2013 (5). This serogroup also accounted for 22% of clinical non-O157 EHEC isolates in the United States during the period between 1983 and 2002 (6). STEC O26:H11 has been detected in meat and dairy products (7–9). At the end of 2005, STEC O26:H11 was involved in an outbreak in France that included 16 HUS cases and was linked to consumption of contaminated unpasteurized Camembert cheese (10). Another outbreak of STEC O26:H11 occurred in Denmark in 2007 and was caused by beef sausage. Twenty cases of diarrhea, the majority of which occurred in children (average age, 2 years), were reported (11).

Shiga toxins (Stx) are considered the major virulence factor of STEC, and *stx* genes are located in the genome of temperate bacteriophages (Stx phages) inserted as prophages into the STEC chromosome (12–14). There are two Stx groups, Stx1 and Stx2, divided into 3 (a, c, and d) and 7 (a to g) subtypes, respectively

(15). Stx1 and Stx2 can be produced either singly or together by STEC O26:H11 (6), and STEC strains carrying the *stx*<sub>2</sub> gene only are generally associated with a more severe clinical outcome than STEC possessing the *stx*<sub>1</sub> gene (16). The first Stx1 phage described was phage H19B, which was isolated from a clinical EHEC O26 strain (12). In the 1990s, a shift of the *stx* genotype, from isolates carrying *stx*<sub>1</sub> to isolates possessing the *stx*<sub>2</sub> gene either alone or together with *stx*<sub>1</sub>, was observed in Germany in EHEC O26:H11 (17, 18). More recently, a panel of 74 STEC O26:H11 strains of various origins was characterized and the results showed that the majority of food and cattle strains possessed the *stx*<sub>1a</sub> subtype whereas human strains carried mainly *stx*<sub>1a</sub> or *stx*<sub>2a</sub> (19).

Stx phages insert their genome into specific sites in the bacterial chromosome, where they remain silent (20), allowing their bacterial hosts to survive as lysogenic strains. The main Stx phage insertion sites in STEC O26:H11 were the *wrbA* and *yehV* genes followed distantly by *yecE* and *sbcB* (19). Stx phages are inducible from their host strain by DNA-damaging agents such as antibiot-

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ics (21, 22). DNA damage triggers the SOS response of *E. coli* (23), resulting in the derepression of phage lytic genes, lysis of the bacterial host cells, and release of the phage particles. In addition, other conditions such as UV irradiation (24) or high hydrostatic pressure treatment (25) were also shown to induce Stx phages. Stx phage induction is an important feature of STEC as it is closely linked to Stx production (26).

STEC O26:H11 bacteria have the characteristic of frequent loss and acquisition of Stx phages (27, 28). The acquisition of an Stx phage by *stx*-negative *E. coli* O26:H11 has been demonstrated *in vitro* (13, 27). The Stx1 H-19B phage can also be transferred *in vivo* in mice from donor STEC O26:H11 to an *E. coli* recipient strain (29). Stx phage transfer can result in the emergence of new highly pathogenic strains as illustrated by the recent outbreak in Germany in 2011, with nearly 4,000 humans infected, including 900 HUS cases and 50 deaths (30), which was caused by an enteroaggregative *E. coli* O104:H4 strain lysogenized with an Stx phage (31). Phage-mediated transfer of the *stx*<sub>2</sub> gene to *E. coli* has also been shown to occur in food and water samples (32). In contrast, loss of Stx phage by STEC O26:H11 can generate *stx*-negative *E. coli* derivative strains which might interfere in the detection of STEC O26:H11, especially when they are isolated from food samples initially identified as *stx*-positive by PCR. Except for the absence of the *stx* gene, these strains are similar to STEC O26:H11 and are referred to as “attaching/effacing *E. coli*” (AEEC) O26:H11. Madic et al. have demonstrated the presence of STEC and AEEC O26:H11 in raw milk cheese samples (33). Monitoring plans carried out in France between 2007 and 2009 also showed the presence of STEC and AEEC in raw milk cheeses, including serotype O26:H11 (34). Finally, Trevisani et al. also revealed the presence of both *stx*-positive and *stx*-negative *E. coli* O26 strains in milk samples (0.4% and 2% of the samples, respectively) or filters (0.4% and 0.9% of the filters, respectively) (35). The fact that *stx*-negative *E. coli* (or AEEC) O26:H11 strains could be isolated from *stx*-positive food samples raises some questions about the diagnostic result since the possibility that these strains are derivatives of STEC that have lost their Stx phage and hence their *stx* gene during the enrichment procedure or isolation step cannot be excluded. Moreover, Stx phages have also been detected in beef and salad, which may also confound STEC screening methods based on PCR detection of the *stx* gene in food samples (36).

The main objective of this study was to explore the relationship between STEC O26:H11 strains and their *stx*-negative counterparts by focusing on Stx phages whose gain or loss mediates bidirectional conversion. We first addressed whether dairy and human strains could differ in their Stx prophage induction rates, which could be indicative of differential toxin levels and, potentially, of differential levels of virulence. We also addressed whether Stx phages from STEC O26:H11 differentially infect and convert dairy and human *stx*-negative *E. coli* strains. With this aim, the diversity and mobility of Stx phages previously identified among STEC O26:H11 strains (19) were evaluated. Levels of Stx phage induction were compared between clinical and dairy STEC O26:H11 strains containing *stx* prophages inserted into various locations. This comparison was performed in the presence and absence of mitomycin C (MMC), an antibiotic known to effectively induce Stx phages (37). The sensitivity of *stx*-negative *E. coli* isolates to the Stx phages released from STEC O26:H11 was investigated in addition to their lysogenic conversion. Finally, the morphology of Stx phages was studied.

## MATERIALS AND METHODS

**Bacterial strains.** Fourteen STEC O26:H11 isolates collected from humans ( $n = 9$ ) and dairy products ( $n = 5$ ) and 17 *stx*-negative O26:H11 *E. coli* isolates collected from humans ( $n = 8$ ) and dairy products ( $n = 9$ ) were used in this study (Table 1). *E. coli* K-12 strains DH5 $\alpha$  and MG1655 were also used in this study (Table 1). The human and dairy STEC strains contained *stx*<sub>1</sub> ( $n = 3$  of each) or *stx*<sub>2</sub> ( $n = 4$  and  $n = 1$ , respectively) or both *stx*<sub>1</sub> and *stx*<sub>2</sub> genes ( $n = 2$  and  $n = 1$ , respectively). *E. coli* strains were cultivated in lysogeny broth (LB) at 37°C.

**Bacteriophage induction.** A culture of STEC O26:H11 grown overnight was inoculated at 2% in a fresh LB medium with 5 mM CaCl<sub>2</sub> and incubated at 37°C (38). When the culture reached the exponential-growth phase (optical density at 600 nm [OD<sub>600</sub>], 0.3) (26), it was divided into two subcultures, A and B. In subculture A, mitomycin C (MMC) was added to give a final concentration of 0.5  $\mu$ g/ml (26). Subculture B, without MMC, was used to evaluate the spontaneous induction of Stx phages. Cultures were then further incubated overnight at 37°C with shaking at 240 rpm. After incubation, the rate of phage production was evaluated by measuring with a spectrophotometer the optical density at 600 nm of induced and noninduced cultures. All cultures were centrifuged at 7,200  $\times$  *g* for 10 min, and the supernatants were filtered through low-protein-binding 0.22- $\mu$ m-pore-size membrane filters (Millex-GP PES; Millipore) for phage purification.

**Enumeration and isolation of Stx phages by double-agar overlay plaque assay.** *E. coli* DH5 $\alpha$  and MG1655 were used as host strains to screen for the presence of bacteriophages. The suspensions of phage particles obtained after induction (see above) were diluted 10-fold. Two hundred microliters of an overnight culture of the host strain was mixed with 100  $\mu$ l of each diluted phage suspension and incubated 1 h at 37°C. This mixture was added to molten LB top agarose (LB modified broth with agarose at 2 g/liter, 10 mM CaCl<sub>2</sub>, and 10 mM MgSO<sub>4</sub>), which was immediately poured onto LB-agar plates and allowed to solidify. After incubation for 18 to 24 h at 37°C, the plates were examined for the presence of lysis zones. Plaques were counted to determine the titer of the original phage preparation in PFU counts per milliliter by using the following calculation: number of plaques  $\times$  10  $\times$  the inverse of the dilution factor value (39).

**Quantification of Stx phage by qPCR.** Filtered supernatants obtained after Stx phage induction were treated with DNase using a Turbo DNA-free kit (Ambion; Life Technologies). Removal of any contaminating genomic DNA by DNase was verified by detection of the chromosomal STEC O26:H11 *eae* gene by quantitative PCR (qPCR), as described previously (40). Phage DNA was released by heat treatment for 10 min at 100°C. As Stx phages carry only one *stx* gene copy (GC), phage numbers were determined by qPCR assays targeting *stx*<sub>1</sub> or *stx*<sub>2</sub> genes. These were performed with a LightCycler 480 instrument (Roche Diagnostics) as described by Derzelle et al. (41), with minor modifications as follows. The amplification reaction mixture contained 1 $\times$  LightCycler 480 Probes Master mix (Roche Diagnostics), a 500 nM concentration of each primer (*stx*1B-for, *stx*1-rev, *stx*2-for, and *stx*2-rev), and a 200 nM concentration of each probe (*stx*1 and *stx*2 probes). Three microliters of extracted DNAs was used as the template in qPCR. The linearity and limit of quantification of the qPCR assay had formerly been determined by using calibrated suspensions of STEC corresponding to dilutions of pure cultures of *stx*<sub>1</sub>- and *stx*<sub>2</sub>-positive control strain EDL933 containing both *stx*<sub>1</sub> and *stx*<sub>2</sub> genes. The amplification efficiency (*E*) was calculated using the following equation:  $E = 10^{-1/s} - 1$ , where *s* is the slope of the linear regression curve obtained by plotting the log genomic copy numbers of *E. coli* strains in the PCR against threshold cycle (*C*<sub>T</sub>) values. The *C*<sub>T</sub> value was defined as the PCR cycle at which the fluorescent signal exceeded the background level. The *C*<sub>T</sub> was determined automatically by the use of LightCycler 480 software with the second-derivative-maximum method, and the *stx*<sub>1</sub> and *stx*<sub>2</sub> gene copy (GC) numbers were calculated from the standard curve.

**Evaluation of the infectious capacity of Stx phages.** To evaluate the ability of the Stx1 and Stx2 phages to infect *E. coli*, *E. coli* K-12 strain DH5 $\alpha$

TABLE 1 Panel of *stx*-positive and *stx*-negative *Escherichia coli* strains

Origin <sup>a</sup>	Strain	Stx phage type(s)	Insertion site(s) of Stx phage(s) <sup>b</sup>
STEC O26:H11			
Dairy product	2976-1	Stx1a	<i>yehV</i>
Dairy product	10d	Stx1a	<i>wrbA</i>
Dairy product	09QMA277.2	Stx1a, Stx2a	<i>yehV</i> (Stx1), <i>wrbA</i> (Stx2)
Dairy product	09QMA245.2	Stx1a	<i>yecE</i>
Dairy product	F46-223	Stx2a	<i>wrbA</i>
Human (NK)	VTH7	Stx1a	<i>sbcB</i>
Human (D)	H19	Stx1a	<i>yehV</i>
Human (HUS)	3901/97	Stx1a, Stx2a	<i>wrbA</i> (Stx1), <i>yecE</i> (Stx2)
Human (HUS)	11368	Stx1a	<i>wrbA</i>
Human (HUS)	3073/00	Stx1a, Stx2a	<i>yehV</i> (Stx1), <i>yecE</i> (Stx2)
Human (HUS)	5917/97	Stx2a	<i>wrbA</i>
Human (HUS)	29348	Stx2a	<i>wrA</i>
Human (HUS)	31132	Stx2a	<i>yecE</i>
Human (HUS)	21765(1)	Stx2a	<i>yecE</i>
<i>stx</i> -negative <i>E. coli</i> O26:H11			
Dairy product	09QMA04.2		
Dairy product	09QMA315.2		
Dairy product	09QMA306.D		
Dairy product	FR14.18		
Dairy product	4198.1		
Dairy product	191.1		
Dairy product	64.36		
Dairy product	09QMA355.2		
Dairy product	F61-523		
Human (HUS)	5021/97		
Human (HUS)	5080/97		
Human (HUS)	318/98		
Human (HUS)	21474		
Human (HUS)	21766		
Human (NK)	MB04		
Human (NK)	MB01		
Human (HUS)	29690		
Other <i>E. coli</i>			
K-12	DH5 $\alpha$		
K-12	MG1655		

<sup>a</sup> D, diarrhea; HUS, hemolytic-uremic syndrome; NK, not known.

<sup>b</sup> Previously described (19).

and 17 *stx*-negative *E. coli* O26:H11 strains were used as host strains. Each host strain was grown in LB at 37°C overnight with shaking. Two hundred microliters of each culture was added to 5 ml of molten LB top agarose and immediately poured onto LB-agar plates. Ten microliters of filtered supernatants containing Stx phages obtained after induction of six strains (H19, 5917/97, 3901/97, F46-223, 09QMA277.2, and 21765) was spotted onto plates containing the LB top agarose overlay and incubated overnight at 37°C.

**Construction of lysogens.** *E. coli* K-12 strains (DH5 $\alpha$  and MG1655) and *stx*-negative *E. coli* O26:H11 strains were grown overnight in LB broth at 37°C with shaking. For practical reasons, only a subset of five *stx*-negative *E. coli* O26:H11 strains (191.1, 5080/97, 318/98, 21474, and 21766) were used. One milliliter of host culture was mixed with 100, 250, or 500  $\mu$ l (multiplicity of infection [MOI] between 0.1 and 0.5) of the different Stx phages such as Stx1 or Stx2 or a mix of Stx1 and Stx2 phage suspensions resulting from the mitomycin C induction of the H19 strain (phage  $\Phi$ H19s), 5917/97 strain (phage  $\Phi$ 5917s), or 3901/97 strain (phage  $\Phi$ 3901m), respectively, and incubated 1 h at 37°C without shaking. The mixtures were then diluted 10-fold, plated onto LB-agar plates, and incubated for 24 h at 37°C. After incubation, colonies were enumerated and values were compared to the enumeration of the uninfected strains used

as a control, to check whether a majority of the host cells had been lysed and thus infected upon contact with the phage. Finally, 5 to 10 colonies were purified and tested for lysogeny by PCR amplification of *stx*<sub>1</sub> and *stx*<sub>2</sub> genes (as described above) and of host-phage *attL* junction sites (19).

**Propagation and purification of Stx phages and electron microscopy.** *E. coli* laboratory strain MG1655 was used as the host for purification and propagation of Stx1 and Stx2 phages in equal proportions obtained from strains H19, 2976-1, and 11368 and from strains 5917/97, 3901/97, and 21765(1), respectively (Table 1). Stx phage particles, obtained after induction, were amplified and purified in solid medium, as follows. Stx phages were isolated by the double-agar overlay plaque assay, as described above. One lysis plaque was removed with a sterile toothpick and resuspended in 50  $\mu$ l of 10 mM MgSO<sub>4</sub> buffer. Two hundred microliters of an overnight culture of the host strain was mixed with 5 ml of molten LB top agarose, immediately poured onto LB-agar plates, and allowed to solidify. Fifty microliters of phage suspension was spotted onto these plates and incubated 8 h at 37°C. The spots were collected and resuspended in 100  $\mu$ l of 10 mM MgSO<sub>4</sub> buffer. Serial dilution of the lysates was then performed, and the diluted lysates were used in a new round of plaque assays and incubated 8 h at 37°C to produce confluent lysis of the host strain. Finally, 5 ml of 10 mM MgSO<sub>4</sub> buffer was placed

TABLE 2 Level of induction of Stx phage expressed in OD<sub>600</sub> units

Strain	OD <sub>600</sub> <sup>a</sup>	
	–MMC	+MMC
2976-1	3.50 ± 0.10	0.31 ± 0.09
10d	3.33 ± 0.22	0.71 ± 0.17
09QMA277.2	3.10 ± 0.14	0.37 ± 0.08
09QMA245.2	3.20 ± 0.36	0.73 ± 0.11
F46-223	3.25 ± 0.26	0.34 ± 0.14
VTH7	2.73 ± 0.09	0.88 ± 0.04
H19	2.80 ± 0.29	0.78 ± 0.04
3901/97	2.67 ± 0.33	0.68 ± 0.07
11368	2.75 ± 0.06	0.60 ± 0.11
3073/00	3.03 ± 0.38	0.45 ± 0.41
5917/97	2.86 ± 0.33	0.22 ± 0.05
29348	3.28 ± 0.22	0.25 ± 0.06
31132	2.98 ± 0.26	0.37 ± 0.09
21765(1)	2.75 ± 0.17	1.23 ± 0.13

<sup>a</sup> OD<sub>600</sub> of untreated (–MMC) and MMC-treated (+MMC) STEC O26:H11 culture after 24 h of incubation at 37°C. The values are the means of the results of three independent experiments.

onto the top agar and incubated 8 h at 4°C and then recovered and filtered through low-protein-binding 0.22-μm-pore-size membrane filters (Millex-GP PES; Millipore).

The lysates (5 to 10 ml) were concentrated by ultracentrifugation at 20,000 × *g* for 2 h in a swinging rotor (SW32Ti), and the pellets were resuspended in 100 μl of 10 mM MgSO<sub>4</sub> buffer. They were tested for the presence of the *stx* gene by qPCR as described above. Five microliters of these suspensions was placed onto copper grids with carbon-coated Formvar films and subjected to negative-contrast analysis using 2% uranyl acetate dehydrate. Samples were examined using a transmission electron microscope (Hitachi HT7700) (Elexience, France) at 80 kV. Microphotographs were acquired with an AMT charge-coupled-device camera.

## RESULTS

**Evaluation of Stx phage induction from clinical and dairy STEC O26:H11 isolates.** Fourteen STEC O26:H11 strains were used to measure the level of induction of their Stx phages occurring either spontaneously (i.e., in the absence of MMC) or with the addition of MMC. For all the strains tested, the OD<sub>600</sub> was lower after 24 h of incubation with MMC (OD<sub>600</sub> < 1.25) than in the absence of MMC (OD<sub>600</sub> > 2) (Table 2). Such a low OD<sub>600</sub> was an indicator of bacterial cell lysis and phage induction. qPCR tests and plaque assays were then performed to assess the amount of Stx phages produced by each strain in the presence and absence of the inducing agent.

Stx phages could be detected by qPCR in all the STEC O26:H11 culture supernatants tested, indicating that all the strains were capable of producing Stx phages (Fig. 1). Levels of Stx phage production were highly variable between the strains (Fig. 1), and in most cases, the presence of MMC increased the phage particle yield. There was no significant difference in the basal induction level of Stx phages between the human and dairy strains, and the same was true in the presence of MMC (Fig. 1). Similarly, there was no significant difference in the induction rates of Stx phages according to their insertion sites, except for the spontaneous induction of Stx phages inserted into *yecE*, the rate of which was significantly higher than that of Stx phages integrated into *wrbA* ( $P < 0.05$ ) or *yehV* ( $P < 0.01$ ) as determined with a Fisher test (Fig. 1).

Production of Stx phages was observed under both conditions,

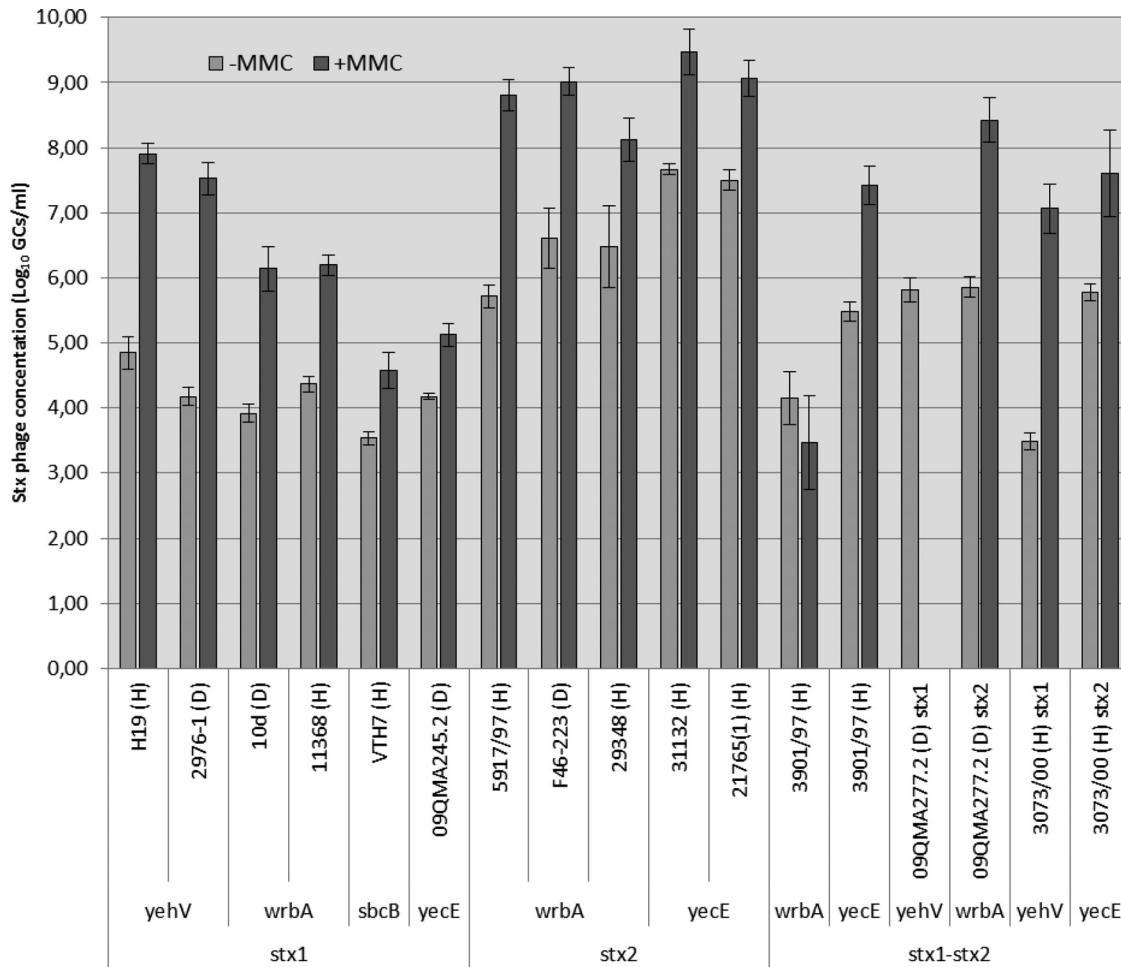
i.e., in the presence and absence of MMC. In the absence of MMC, the level of Stx phage particles spontaneously produced ranged between 3.49 log<sub>10</sub> and 7.67 log<sub>10</sub> GC/ml (numbers of *stx* gene copies/ml) whereas when MMC was added, the Stx phage levels were between 3.47 log<sub>10</sub> and 9.46 log<sub>10</sub> GC/ml. Overall, the addition of MMC resulted in an average increase of 2 log<sub>10</sub> GC relative to the levels seen with spontaneous induction ( $P < 0.05$  [Fisher test]). Two exceptions were observed. For strain 3901/97, the levels of the Stx1 phage were not significantly different with and without MMC, suggesting that the Stx1 phage is a noninducible phage or that its induction pathway is distinct from the SOS response. For strain 09QMA277.2, which contains both Stx1 and Stx2 phages, production of the Stx1 phage became undetectable when MMC was added, suggesting that the production of Stx2 phage upon induction outcompetes that of the Stx1 phage. Additional work is required to support these hypotheses.

Comparing the production levels of Stx1 phages to those of Stx2 phages, a difference was observed ( $P < 0.001$  [Fisher test]). This was true both in the presence and in the absence of MMC, since the amounts of Stx2 phages were 2.11 and 3.16 log<sub>10</sub> greater, respectively, than those of Stx1 phages.

The Stx phage titers were then determined by enumeration of infectious Stx phages using the double-agar layer method and compared to the concentrations of Stx phage DNA determined by qPCR. Except for one strain (11368), all the strains generated infectious particles capable of producing plaques with the *E. coli* DH5α recipient strain. Four strains (2976-1, 09QMA245.2, VTH7, and 3073/00) also produced infectious Stx phages capable of promoting confluent lysis on plates, but these failed to generate visible and identifiable isolated plaques (Fig. 2). As observed with qPCR, the mean titers for Stx2 phages were higher by 2 log<sub>10</sub> than those for Stx1 phages. However, the Stx phage titers determined by plaque enumeration were lower by an average of 1.98 log<sub>10</sub> ± 1.01 (decreases of a minimum of 0.79 for strain 3901/97 and of a maximum of 4.28 log<sub>10</sub> for strain H19) than the concentrations of Stx phage genomes determined by qPCR. Using *E. coli* MG1655 instead of DH5α as a recipient strain, the Stx phage titers obtained were similar or even lower (data not shown).

**Susceptibility of *stx*-negative *E. coli* O26:H11 to Stx phages and lysogenization.** Seventeen *stx*-negative *E. coli* O26:H11 strains and the *E. coli* K-12 DH5α strain were evaluated using spot agar tests for their sensitivity to six filtered supernatants containing Stx phages released after induction with MMC from strains H19, 5917/97, 3901/97, F46-223, 09QMA277.2, and 21765(1). The results obtained from the 102 different *E. coli*/Stx phage interactions tested are reported in Table 3. The 17 strains were not infected equally by Stx phages. One strain (64.36) was not sensitive to any of the six Stx phages, and two strains (09QMA04.2 and 09QMA355.2) were infected by only one Stx phage. In contrast, other strains such as 191.1, 5080/97, and 5021/97 were sensitive to all six of the Stx phages. Overall, 55 (54%) of the 102 *E. coli*/Stx phage interactions tested were positive for infection. A total of 68.7% of the human strains were sensitive to Stx phages, whereas only 40.7% of dairy strains were infected, a difference which was statistically significant ( $P = 0.008$  [chi-square test]). In addition, the levels of turbidity of the lysis area differed among the strains tested (Table 3). Clear lysis was obtained with DH5α, in contrast to most of the *stx*-negative *E. coli* O26:H11 strains, which generated lysis areas that were more opaque.

Phages Φ5917 and Φ3901m could infect 76.4% and 70.6% of



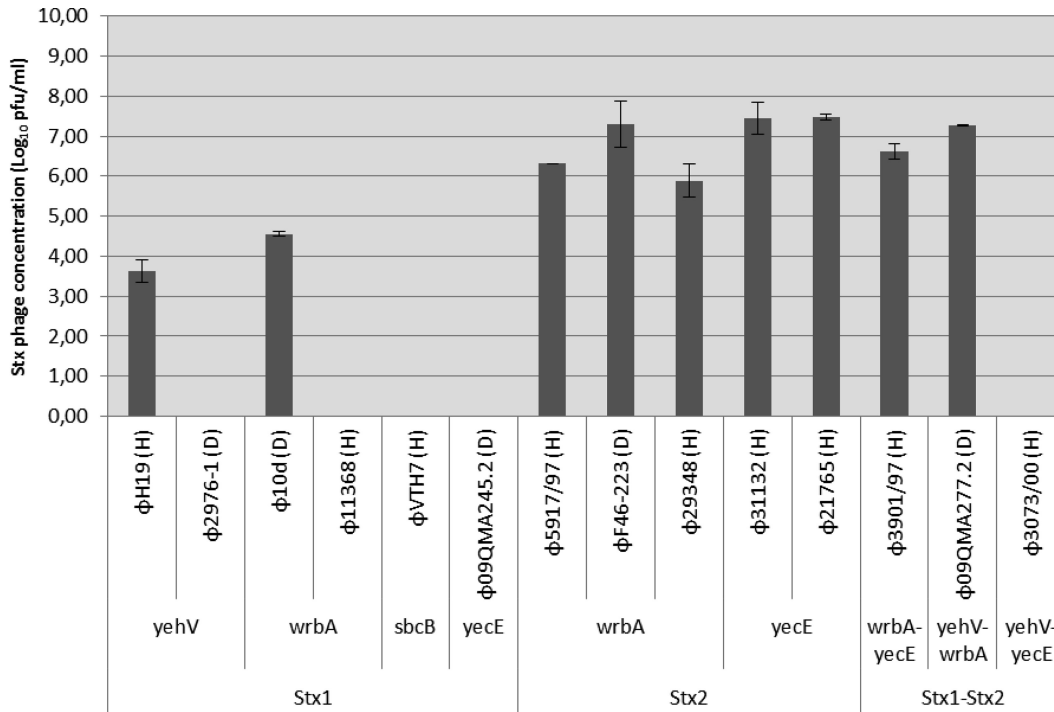
**FIG 1** Quantification of DNA from Stx1 and Stx2 phages. Quantification by qPCR of DNA of Stx1 and Stx2 phages extracted from supernatants obtained from untreated (–MMC; gray bars) or MMC-treated (+MMC; black bars) STEC O26:H11 cultures was performed. The STEC strains were from dairy (D) and human (H) origins, and their Stx phages were integrated into *wrbA*, *yehV*, *yecE*, or *sbcB* sites. The concentration of Stx phages was expressed in log<sub>10</sub> values of *stx* gene copies per milliliter (log<sub>10</sub> GC/ml). The data were obtained from three independent analyses, and the average copy numbers for each phage DNA are shown. Bars indicate standard deviations.

*stx*-negative *E. coli* O26:H11 bacteria, respectively, while phages ΦF46-223, Φ277.2, and Φ21765 could infect between 47% and 59% of *stx*-negative *E. coli* O26:H11 bacteria. In contrast, phage ΦH19 infected only 23.5% of the *stx*-negative *E. coli* O26:H11 strains. These differences between the phages were not significant, however, except for phages Φ5917 and Φ3901m, whose infectivity was significantly higher than that of the phage ΦH19 ( $P = 0.006$  and  $P = 0.016$ , respectively [chi-square test]). Finally, the *E. coli* K-12 DH5α strain used as a control was susceptible to infection with all the phages tested, showing marked lytic areas.

Attempts to lysogenize *E. coli* K-12 strains DH5α and MG1655 with filtered supernatants containing Stx phages were then performed. Suspensions of Stx1 phage (ΦH19) or of Stx2 phage (Φ5917) obtained from strain H19 or strain 5917/97, respectively, as well as a mixed suspension of both Stx1 and Stx2 phages (Φ3901m) obtained from 3901/97 strain were tested. A high decrease in DH5α bacterial viability, i.e., from  $-5.32$  to  $-6.19$  log<sub>10</sub>, respectively, was observed in the presence of phages Φ5917 and Φ3901m compared to the control conditions in the absence of phages, and similar results were observed with MG1655 (data not

shown). In contrast, only a small decrease ( $<1.01$  log<sub>10</sub>) in DH5α bacterial viability was observed using phage ΦH19. Lysogens could be obtained only with phage Φ3901m, with both strain DH5α and strain MG1655, and these acquired the Stx2 phage but not the Stx1 phage (data not shown). For these two *E. coli* lysogens, the left attachment (*attL*) bacterium/phage junction site could be amplified by PCR at the *yecE* site (data not shown), suggesting that the Stx2 phage integrated its genome into the *yecE* bacterial chromosomal gene.

When the same lysogenization assays were conducted with four *stx*-negative *E. coli* O26:H11 strains (318/98, 191.1, 5080/97, and 21766), the presence of Stx phages did not decrease viability of the recipient strains and no lysogens could be recovered, in contrast to DH5α used as a positive control (data not shown). This suggested to us some level of resistance to infection in liquid, although growth occurred in top agar, as turbid plaques were obtained (see above). To test whether phage resistance was due to restriction or modification, Stx2 phage Φ3901 was propagated onto a fifth recipient *stx*-negative *E. coli* O26:H11 strain (21474). Using this new stock for a lysogenization assay performed on



**FIG 2** Quantification of Stx1 and Stx2 phage particles. Quantification by enumeration of lysis plaques from particles of Stx1 and Stx2 phages obtained from supernatants derived from MMC-treated STEC O26:H11 cultures was performed. The STEC strains were from dairy (D) and human (H) origins, and their Stx phages were integrated into *wrbA*, *yehV*, *yecE*, or *sbcB* sites. The titer of the original phage preparation was expressed in PFU per milliliter (PFU/ml). The data were obtained from three independent analyses, and the average titers for each phage are shown. Bars indicate standard deviations.

strain 21474, no lysogens could be isolated either. However, after the step of adsorption of phage Φ3901 onto the 21474 strain, the mixture was positive for PCR amplification of the *attL* junction at the *yecE* site, suggesting that lysogenic bacterial cells were present (data not shown).

**Electron microscopy.** Six Stx phages, including three Stx1 phages (ΦH19, Φ2976-1, and Φ11368) and three Stx2 phages (Φ5917, Φ3901, and Φ21765), were subjected to plaque purification from STEC O26:H11 and amplified on the MG1655 K-12 strain, which is devoid of functional prophages (see Materials and Methods). Using observation by transmission electron microscopy, three different kinds of phages were identified (Fig. 3). Two phages (Φ3901 and ΦH19) presented an elongated (prolate) capsid measuring 42 to 52 nm in width and 106 to 121 nm in length, with a long flexible tail of ca. 190 to 209 nm, and were members of the *Siphoviridae* family (Fig. 3A and B). Three phages (Φ11368, Φ5917, and Φ21765) showed also a morphology of *Siphoviridae*, with an isometric head of ca. 44-nm to 64-nm diameter and a long tail ca. 150 to 195 nm in length (Fig. 3C, D, and E). Finally, phage Φ2976-1 was also a member of the *Siphoviridae* family, with an isometric head of ca. 56-nm diameter and a very long tail ca. 411 nm in length (Fig. 3F).

## DISCUSSION

All of the 14 (100%) STEC O26:H11 strains examined here contained inducible Stx phages and were capable of producing Stx phages both spontaneously and in the presence of MMC. Other studies showed that 18% to 89% of STEC bacteria isolated from cattle or wastewaters and belonging to a wide variety of serotypes contained inducible Stx2 phages (26, 38).

According to previous work, the concentrations of phage DNA obtained after induction are inversely proportional to the optical densities of the culture (26). This was also the case here, although several exceptions were observed. For example, strains 31132 and 21765(1) showed the same Stx2 phage concentrations of ca.  $9 \log_{10}$  GC/ml but distinct  $OD_{600}$  values of 0.37 and 1.23, respectively. Conversely, strains VTH7 and H19 displayed similar  $OD_{600}$  values (i.e., 0.88 and 0.78, respectively), but H19 produced a hundred times more Stx1 phage than VTH7. Although  $OD_{600}$  values still represent a good indicator of qualitative Stx phage induction, the use of alternative assays such as qPCR seems therefore preferable for a more accurate and quantitative assessment of Stx phage induction.

The addition of MMC resulted in Stx phage induction levels 2  $\log_{10}$  higher than those obtained under spontaneous conditions, and a higher level of induction was observed for Stx2 phages than for Stx1 phages. This less-pronounced effect of MMC on Stx1 production was also observed by Ritchie et al. (42). Moreover, it had been shown previously that spontaneous induction of Stx1 phages could be increased significantly when STEC were grown in a low-iron medium (43), although no correlation between induction levels obtained in low-iron medium and in the presence of MMC was observed for various Stx1 phages (42). Finally, although the levels of Stx phages evaluated here were highly variable, there was no significant difference between the human and the dairy strains in the Stx phage induction levels in the presence and absence of MMC. As there is a direct relationship between Stx phage induction and toxin production (26), this result would therefore indicate that isolates from dairy strains are capable of producing Stx at levels comparable to those seen with human

TABLE 3 Susceptibility of *E. coli* O26:H11 host strains to Stx phages obtained from STEC O26:H11

Origin <sup>a</sup>	Strain	Stx phage to which strain is susceptible <sup>f</sup>						Total no. of Stx phages
		ΦH19	Φ5917	Φ3901	ΦF46-223	Φ277.2	Φ21765	
Dairy product	09QMA04.2	–	–	+	–	–	–	1
Dairy product	09QMA315.2	+	++	+	–	+	–	4
Dairy product	09QMA306.D	–	+	–	–	+	–	2
Dairy product	FR14.18	–	+	+	–	–	–	2
Dairy product	4198.1	–	–	+	++	++	+	4
Dairy product	191.1	+	+	+	+	+	+	6
Dairy product	64.36	–	–	–	–	–	–	0
Dairy product	09QMA355.2	–	+	–	–	–	–	1
Dairy product	F61-523	–	+	–	–	++	–	2
Human (HUS)	5021/97	+	+	+	+	+	+	6
Human (HUS)	5080/97	+	+	+ <sup>b</sup>	+	+	++	6
Human (HUS)	318/98	–	–	+	+	++	–	3
Human (HUS)	21474	–	+ <sup>c</sup>	++++ <sup>d</sup>	+++	++	+++	5
Human (HUS)	21766	–	+	+	–	–	+	3
Human (NK)	MB04	–	+	+	+	–	+	4
Human (NK)	MB01	–	+	–	+	+	+	4
Human (HUS)	29690	–	+	++	–	–	–	2
K-12	DH5α <sup>e</sup>	++++ <sup>c</sup>	++++ <sup>c</sup>	++++	++++ <sup>c</sup>	++++ <sup>c</sup>	+++	6
Total	18	5	14	13	9	11	9	

<sup>a</sup> HUS, hemolytic-uremic syndrome; NK, not known.

<sup>b</sup> Presence of small lysis plaques instead of a confluent lysis area was detected.

<sup>c</sup> Presence of a blurred halo around the lysis area was detected.

<sup>d</sup> Clear lysis with colonies in the lysis plaque was seen.

<sup>e</sup> The *E. coli* K-12 strain was used as a control strain.

<sup>f</sup> –, nondetectable lysis in the spot area; +++++, clear lysis in the spot area; +++, minimally opaque lysis in the spot area; ++, moderately opaque lysis in the spot area; +, maximally opaque lysis in the spot area.

isolates. Similarly, no differences were observed according to the Stx phage insertion sites, except for spontaneous induction, which was higher for Stx phages inserted into *yecE* than for those inserted into *wrbA* and *yehV*. Interestingly, strains with Stx2 phage integrated into *yecE* were described previously as highly virulent (19).

Most of the STEC O26:H11 strains generated infectious Stx phage particles capable of producing plaques with the *E. coli* DH5α recipient strain. Nevertheless, as a few Stx phages were unable to generate isolated plaques, improvements of the plaque assay used here could be considered in the future, as described by Islam et al. (44). In addition, the phage titers determined by plaque enumeration were lower than the concentrations of phage genomes determined by qPCR, as reported previously (45). Several factors have been proposed to influence plaque enumeration, including the susceptibility of the host strain and the presence of noninfectious particles in the phage suspension tested (45, 46). Nevertheless, despite this difference, the relative levels of Stx1 and Stx2 phages determined by the two methods among the panel of STEC O26:H11 strains were in agreement.

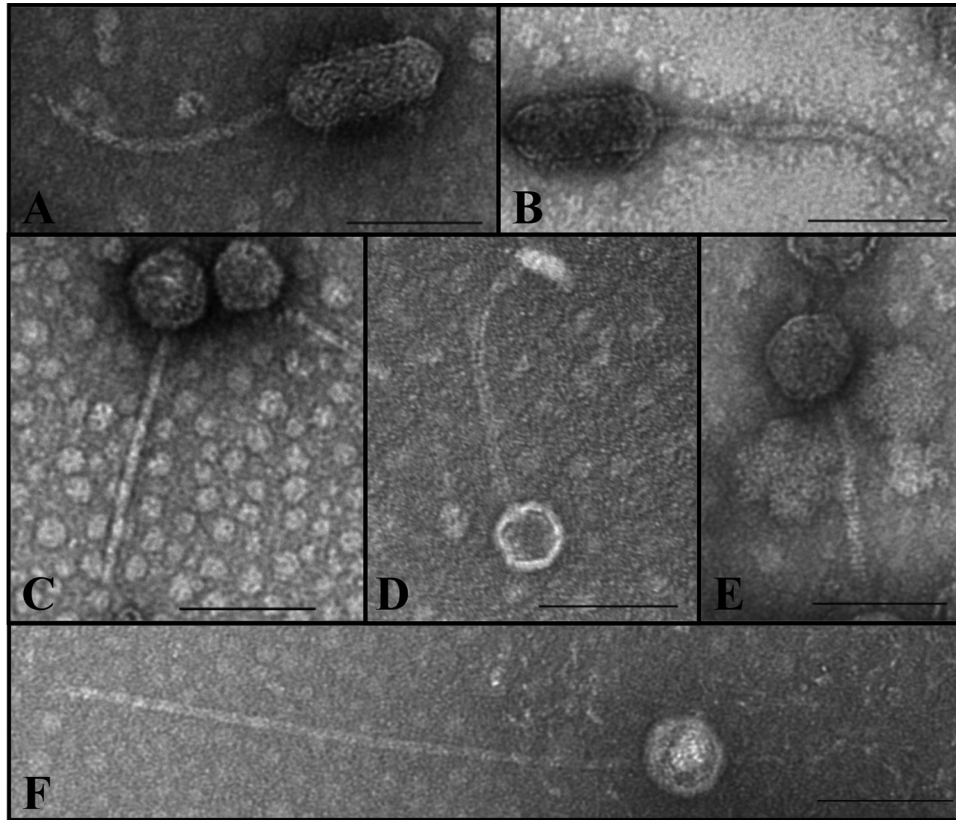
Considering the susceptibility of *stx*-negative *E. coli* O26:H11 strains to a subset of six Stx phages from STEC O26:H11, a large variability in sensitivity was observed, as one strain (64.36) was not infected by any of the six Stx phages tested whereas, in contrast, three strains (191.1, 5080/97, and 5021/97) were sensitive to all of the Stx phages. This phenomenon was previously observed by Muniesa et al. when *E. coli* strains of various serotypes were tested as recipients (47). Indeed, *E. coli* O26 strains 216 and 224 were infected by 1 and 7 of 11 Stx phages tested, respectively (47). Interestingly, the sensitivity of the human strains to Stx phages here was higher than that observed with the dairy strains. It is

tempting to speculate that the clinical strains were modified by passage through humans and became more susceptible to Stx phages. However, a more extensive comparison with a higher number of human and dairy strains is required to confirm these findings.

Investigating lysogenic conversion by Stx phages using *E. coli* DH5α and MG1655 as recipient strains, lysogenic *stx*-positive isolates could be obtained, with Stx2 prophages inserted into *yecE*. However, all our attempts to lysogenize *stx*-negative *E. coli* O26:H11 strains failed. Difficulties in the production of lysogens and variability in transduction effectiveness have already been reported for Stx phages. Muniesa et al. obtained stable lysogens with only 6 of 30 Stx phages derived from cattle STEC strains (26). Bielaszewska et al. also reported that lysogenization of clinical *E. coli* O26:H11 was not systematic, since only two or three of six *stx*-negative *E. coli* O26:H11 strains could be lysogenized by Stx2 phages, with rates of lysogenization ranging from  $1 \times 10^{-7}$  to  $6 \times 10^{-6}$  per recipient cell, i.e.,  $10 \times$  lower than that obtained with *E. coli* laboratory strain C600. More importantly, transfer of Stx1 phages was even less successful since, in the same study, only one of four Stx1 phages lysogenized *E. coli* O26:H11, and this was true for only one of the six *stx*-negative *E. coli* O26:H11 strains tested (27).

The *stx*-negative *E. coli* strains used as recipients in our study contained vacant Stx phage insertion sites (19), indicating that the lack of a free insertion site was not the reason for the failure to obtain lysogens with *E. coli* O26:H11. Interestingly, chromosomal insertion of Stx phage genome into a fraction of bacterial cells from an *stx*-negative *E. coli* O26:H11 strain (21474) suspension could be demonstrated here by PCR amplification of an *attL* junc-





**FIG 3** Electron micrographs of Stx phage particles obtained from STEC O26:H11. Electron micrographs depict the following six phages: (A) phage  $\Phi$ 3901; (B) phage  $\Phi$ H19; (C) phage  $\Phi$ 11368; (D) phage  $\Phi$ 5917; (E) phage  $\Phi$ 21765; (F) phage  $\Phi$ 2976-1. (A and B) Stx phage particles with an elongated head and a long flexible tail. (C to E) *Siphoviridae* phages with a long tail. (F) *Siphoviridae* phage with a very long tail. Bars, 100 nm.

tion at the *yecE* site, suggesting that Stx phages have the ability to lysogenize *stx*-negative *E. coli* O26:H11, presumably with a low frequency that did not allow the isolation of lysogens in the absence of selective pressure. Alternatively, Stx prophage instability might also prevent the isolation of lysogens. Indeed, although lysogenization of *stx*-negative *E. coli* strains such as enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC or EAaggEC) strains with Stx phages was shown previously (27, 48, 49), stable Stx phage acquisition was also observed to rarely occur (27, 49). Despite the fact that our results are in agreement with the difficulties of lysogenization observed by others, it should be noted that an MOI higher than those used here might have increased the rate of lysogen production.

Finally, Stx phages from STEC O26:H11 showed some variability in their morphology since three distinct shapes were observed by electron microscopy among six Stx phages analyzed. The two phages  $\Phi$ 3901 (Stx1) and  $\Phi$ H19 (Stx2) displayed an elongated (prolate) capsid and a long flexible tail, as described previously in the case of phage  $\Phi$ H19 (50). This result indicates that phages with genomes harboring either an *stx*<sub>2</sub> or an *stx*<sub>1</sub> gene can share similar shapes, as previously observed by Muniesa et al. (51). The second phage morphology observed here was characterized by isometric head and was shared by an Stx1 phage ( $\Phi$ 11368) and two Stx2 phages ( $\Phi$ 5917/97 and  $\Phi$ 21765). Such a morphology has been previously observed for two Stx2 phages,  $\Phi$ SW13 and  $\Phi$ SW16 (26, 47). Finally, the third type of phage morphology was found for phage  $\Phi$ 2976-1, which possessed a very long tail of about 411 nm.

Such a long tail has already been observed by Hoyles et al. for virus-like particles that corresponded to bacteriophages and were associated with human fecal or cecal samples (52). Taken together, these results are consistent with previous reports showing that there was no relationship between the presence of a particular *stx* variant and the morphology of the corresponding phage (38). This also confirmed the diversity of phage morphologies circulating in the STEC population, and in STEC O26:H11 strains in particular.

In conclusion, Stx1 and Stx2 phages of STEC O26:H11 are characterized by high diversity, with variations observed in their induction levels, morphologies, and abilities to infect *E. coli* strains. Interestingly, we noted that the lysogenization of *stx*-negative *E. coli* with Stx phages was a rare event and that more appropriate conditions were required for successful isolation of stable lysogens. Nevertheless, lysogenization of AEEC O26:H11, although at a low frequency, seems possible, and such an event would convert them into STEC strains potentially harmful for humans. Molecular methods such as qPCR could represent alternative assays to identify and quantify lysogens within an *E. coli* population infected by Stx phages.

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