I. Article 1

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I.1. Objectifs

Ces travaux avaient pour objectifs d'explorer la diversité génétique d'une collection de STEC O26:H11 d'origines humaine, alimentaire (principalement des produits laitiers) et animale (bovins) en étudiant leur principal facteur de virulence. Ils visaient à caractériser les sous-types du gène *stx* et les sites d'insertion chromosomiques des phages Stx. De plus, une étude phylogénétique par la méthode MLST (Multi-Locus Sequence Type) a aussi été menée en parallèle sur un petit nombre de souches.

L'étude des sous-types du gène *stx* a été réalisée par une méthode de PCR conventionnelle développée par Scheutz *et al*., basée sur l'amplification des trois sous-types du gène *stx1* et des sept sous-types du gène *stx2* (Scheutz *et al.*, 2012).

L'identification des sites d'insertion chromosomiques des phages Stx, a été, dans un premier temps, effectuée par PCR conventionnelle à partir de couples d'amorces décrits dans la littérature, ou développés au cours des travaux de thèse. Neuf sites d'insertion ont ainsi été étudiés. Il s'agit des sites *wrbA*, *yehV*, *yecE*, *sbcB*, *Z2577*, *ssrA*, *prfC*, *argW* et de la région intergénique *torS-torT*. Deux études PCR ont été réalisées, la première était basée sur l'amplification du site *attB* et témoigne de l'absence de génome de phage dans un locus donné, tandis que la seconde était basée sur l'amplification du site jonction *attL* et témoigne de la présence du prophage (génome du phage intégré dans le chromosome bactérien).

Dans un deuxième temps, des modèles de PCR en temps réel ont été développés pour quatre des neuf sites d'insertion étudiés (*wrbA*, *yehV*, *yecE* et *sbcB*), afin de réduire le temps des analyses et effectuer des dosages des sites *attB* et *attL*. Le but du dosage des sites *attB* et *attL* était d'évaluer la stabilité des prophages Stx au sein d'une culture pure de STEC, selon les sites d'insertion dans lesquels ils sont intégrés. Cette étude avait pour but de comprendre l'origine des souches *stx*-négatives.

Enfin la caractérisation des sites d'insertion des phages Stx a aussi été effectuée sur une collection de 29 souches *E. coli* O26:H11 *stx*-négatives (AEEC). Cette étude avait pour objectif, de déterminer si ces souches peuvent correspondre à des STEC ayant perdu leurs phages Stx ou seulement leur gène *stx*.

I.2. Résultats et discussion

I.2.1. Détermination des sous-types du gène stx et des sites d'insertion des phages Stx

L'étude des sous-types du gène *stx* a montré que les 74 souches étudiées possèdent essentiellement les sous-types *stx*$_{1a}$ (n = 56) et *stx*$_{2a}$ (n = 20). Un autre sous-type, *stx*$_{2d}$, a été retrouvé dans une seule souche d'origine humaine. De plus, trois souches possèdent simultanément les deux sous-types (*stx*$_{1a}$ et *stx*$_{2a}$) tandis que cinq souches possèdent deux copies du même sous-type. Lorsque l'on prend en compte l'origine des souches, on constate que la majorité des souches laitières (88,2%) et la totalité des souches bovines possèdent le sous-type *stx*$_{1a}$ tandis que les souches humaines possèdent en proportion équivalente les deux sous-types (*stx*$_{1a}$ et *stx*$_{2a}$).

L'étude des sites d'insertion des phages Stx a permis de mettre en évidence l'existence de quatre sites d'insertion préférentiellement occupés par les phages Stx dans les STEC O26:H11. Il s'agit des sites *wrbA* (37 souches), *yehV* (28 souches), et dans une moindre mesure, des sites *yecE* et *sbcB*. Aucun phage Stx n'a été détecté dans les cinq autres sites d'insertion étudiés (*Z2577*, *prfC*, *argW*, *ssrA* et la région intergénique *torS-torT*). De plus, pour les souches possédant deux phages Stx, des méthodes de PCR « *long-template* », visant à amplifier de long fragments d'ADN (>15kb), ont été développées pour déterminer le site d'insertion de chacun des phages. Ainsi, pour la souche 09QMA277.2, le phage Stx$_{1a}$ est intégré dans le site *yehV* et le phage Stx$_{2a}$ est intégré dans *wrbA*. Les souches 3073/00 et 3901/97 ont leur phage Stx$_{2a}$ localisé dans *yecE* et leur phage Stx$_{1a}$ intégré respectivement dans *yehV* et *wrbA*.

Une analyse des combinaisons formées d'un sous-type du gène *stx* et d'un site d'insertion chromosomique du phage Stx en fonction de l'origine des souches a été effectuée. On a constaté que les souches d'origines laitière et bovine ont leurs phages Stx intégrés essentiellement dans *wrbA* et *yehV*, tandis que les souches humaines ont leurs phages Stx intégrés dans *wrbA* et *yehV* mais aussi dans le site *yecE*. En effet, *yecE* sert de site d'insertion dans 28% des souches humaines. Il est important de noter que toutes les souches humaines possédant un phage Stx$_{2a}$ intégré dans *wrbA* et *yecE* ont été à l'origine de SHU, indiquant une

forte virulence de ces souches. En revanche, ces profils sont absents (ou rarement retrouvés) dans les souches d'origines laitière et bovine.

Pour finir, l'état des sites d'insertion a aussi été investigué dans 29 souches *stx*-négatives, pour vérifier l'absence de phages Stx ou bien seulement une délétion du gène *stx* qui se serait produite au cours du temps. Les résultats ont montré que les sites d'insertion sont intacts pour 25 des 29 souches *stx*-négatives, indiquant bien l'absence de phage Stx dans la majorité d'entre elles. Pour les quatre souches restantes, deux observations ont été faites. La première observation concerne les souches 5021/97 et 5080/97 pour lesquelles les sites *yehV* et *yecE* sont occupés par un phage Stx mais dont le gène *stx* est *a priori* délété. L'observation suivante concerne les souches 07QMA144.1 et F61-523 pour lesquelles un autre élément génétique, différent du phage Stx, a été détecté dans les sites *argW* et *Z2577* respectivement.

I.2.2. Caractérisation phylogénétique des STEC O26:H11

Une étude phylogénétique par typage MLST a été réalisée sur 14 souches STEC de la collection. Les données de la littérature ont permis de classer ces souches selon deux séquences types, ST21 et ST29. Les souches *stx*$_{2a}$ qui sont à l'origine de SHU se distribuent entre ST21 et ST29. L'association du gène *stx2* à ST21 ou à ST29 a été démontrée comme un indicateur de SHU. En revanche, aucune combinaison d'un sous-type du gène *stx* et d'un site d'insertion donné du phage Stx n'a révélé de corrélation avec un ST particulier.

I.2.3. Stabilité des prophages Stx

Pour explorer la relation entre les STEC O26:H11 et les souches *stx*-négatives, une étude de la stabilité des prophages Stx a été effectuée au sein des cultures pures de STEC. Les quantités de sites *attB* et *attL*, témoignant respectivement de l'absence et de la présence d'un phage ont été déterminées. On a pu constater, pour chaque culture pure, une amplification simultanée des sites *attB* et *attL*, témoignant de l'excision spontanée du prophage Stx au cours de la croissance cellulaire. Des calculs de ratio *attB/attL* ont été effectués au niveau des quatre sites d'insertion chromosomiques (*wrbA*, *yehV*, *yecE* et *sbcB*). Ces ratios ont montré que ce phénomène d'excision spontanée n'est pas dépendant des sites d'insertion des phages Stx.

I.2.4. Mise en évidence de la présence d'un phage EspK dans le site ssrA

Ces travaux ont aussi permis de mettre en évidence la présence dans les STEC O26:H11 d'un phage différent des phages Stx dans un site nommé *ssrA*. En effet, lors de la détermination des sites d'insertion des phages Stx, on a constaté que le site *ssrA* était souvent occupé chez les STEC O26:H11. L'hypothèse de la présence d'un phage EspK, codant un effecteur de type III, et intégré dans le site *ssrA*, a donc été émise. Les résultats ont montré que le phage EspK intégré dans *ssrA* est présent dans la majorité des STEC O26:H11 (soit 87,8%). En revanche, ce phage n'est présent que dans seulement 17,2% des souches O26:H11 *stx*-négatives.

I.3. Conclusion

En conclusion, ces travaux ont permis de mettre en évidence une variabilité des profils génétiques entre les souches STEC O26:H11 d'origines différentes. Il a été observé une grande diversité des sous-types du gène *stx* et des sites d'insertion des phages Stx issus de STEC O26:H11, avec quelques différences observées entre les souches humaines et les souches provenant des aliments et des bovins. Ces différences confirment d'autres travaux portant sur l'existence de différents clones de STEC O26:H11 possédant des niveaux de pathogénicité variés.

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Diversity of Shiga Toxin-Producing *Escherichia coli* (STEC) O26:H11 Strains Examined via *stx* Subtypes and Insertion Sites of Stx and EspK Bacteriophages

Ludivine Bonanno,[a,b] Estelle Loukiadis,[c,d] Patricia Mariani-Kurkdjian,[e] Eric Oswald,[f] Lucille Garnier,[a] Valérie Michel,[b] Frédéric Auvray[a]

Université Paris-Est, Anses, Laboratory for Food Safety, Department of Microbiology, Maisons-Alfort, France[a]; ACTALIA Produits Laitiers, Laboratoire de Microbiologie d'Intérêt Laitier, La Roche sur Foron, France[b]; Université de Lyon, VetAgro Sup, Laboratoire d'Études des Microorganismes Alimentaires Pathogènes/Laboratoire National de Référence pour les Escherichia coli y Compris les E. coli Producteurs de Shiga-Toxines, Marcy l'Etoile, France[c]; Université de Lyon, UMR 5557 Ecologie Microbienne, Université Lyon 1, CNRS, VetAgro Sup, Equipe Bactéries Pathogènes et Opportunistes, Villeurbanne, France[d]; Associated-National Reference Center for Escherichia coli, AP-HP, Service de Microbiologie, Hôpital Robert-Debré, Paris, France[e]; INSERM UMR1043/CNRS UMR5282/Université Toulouse III, Centre de Physiopathologie de Toulouse Purpan (CPTP), Toulouse, France[f]

Shiga toxin-producing *Escherichia coli* (STEC) is a food-borne pathogen that may be responsible for severe human infections. Only a limited number of serotypes, including O26:H11, are involved in the majority of serious cases and outbreaks. The main virulence factors, Shiga toxins (Stx), are encoded by bacteriophages. Seventy-four STEC O26:H11 strains of various origins (including human, dairy, and cattle) were characterized for their *stx* subtypes and Stx phage chromosomal insertion sites. The majority of food and cattle strains possessed the stx_{1a} subtype, while human strains carried mainly stx_{1a} or stx_{2a}. The *wrbA* and *yehV* genes were the main Stx phage insertion sites in STEC O26:H11, followed distantly by *yecE* and *sbcB*. Interestingly, the occurrence of Stx phages inserted in the *yecE* gene was low in dairy strains. In most of the 29 *stx*-negative *E. coli* O26:H11 strains also studied here, these bacterial insertion sites were vacant. Multilocus sequence typing of 20 *stx*-positive or *stx*-negative *E. coli* O26:H11 strains showed that they were distributed into two phylogenetic groups defined by sequence type 21 (ST21) and ST29. Finally, an EspK-carrying phage was found inserted in the *ssrA* gene in the majority of the STEC O26:H11 strains but in only a minority of the *stx*-negative *E. coli* O26:H11 strains. The differences in the *stx* subtypes and Stx phage insertion sites observed in STEC O26:H11 according to their origin might reflect that strains circulating in cattle and foods are clonally distinct from those isolated from human patients.

Shiga toxin-producing *Escherichia coli* (STEC) strains are a diverse group of food-borne pathogens, including enterohemorrhagic *E. coli* (EHEC), that are responsible for diseases in humans such as diarrhea, hemorrhagic colitis (HC), and hemolytic-uremic syndrome (HUS) (1). The most important natural reservoirs of STEC are cattle (2). Transmission to humans occurs through food, water, and direct contact with animals or their environment. A large number of STEC serotypes are known. Although O157:H7 is the most important, four non-O157 STEC serotypes, O26:H11, O103:H2, O145:H28, and O111:H8, have emerged as leading causes of infection. Serotype O26:H11 was first identified as a cause of HUS in 1983 (3, 4) and is the second most frequently detected serotype in Europe, accounting for 12% of all clinical EHEC isolates in 2012 (5). It has also been isolated in the United States and several countries in Europe (6–8).

Shiga toxins (Stx) are considered the major virulence factor of STEC (9, 10). There are two Stx groups, Stx1 and Stx2, which are divided into three (a, c, and d) and seven (a to g) subtypes, respectively (11, 12). STEC strains carry Stx1, Stx2, or both. However, Stx2 is more often associated with severe disease (12, 13). In the mid-1990s, a new highly virulent stx_{2a}-positive *E. coli* O26:H11 clone of sequence type 29 (ST29) emerged in Europe (6). The genetic information for the production of Stx1 and Stx2 is located in the genome of lambdoid prophages (2, 14–17). During infection of *E. coli* cells, Stx phages can insert their DNA into specific chromosomal sites and remain silent (16, 18), allowing their bacterial hosts to survive as lysogenic strains. In contrast to many genetic elements that are frequently integrated within tRNA genes (19), Stx phages insert their DNA preferably into genes from the basic genetic equipment of the *E. coli* chromosome (20). Nine Stx phage insertion sites have been described, including *wrbA*, which codes for a tryptophan repressor-binding protein (21); *yehV*, which codes for a transcriptional regulator (22, 23); *yecE*, whose function is unknown (24); *sbcB*, which produces an exonuclease (25, 26); *Z2577*, which codes for an oxidoreductase (27); *ssrA*, which encodes a tmRNA (28, 29); *prfC*, which encodes peptide chain release factor 3; *argW*, which codes for tRNA-Arg; and the *torS-torT* intergenic region (30–32). The *ssrA* gene is also known as an insertion site for EspK phages carrying the type III effector EspK-encoding gene (33). By studying the Stx phage insertion sites among 606 EHEC O157 strains of various geographic origins, Mellor et al. showed that the genotype *wrbA yehV* stx_1 stx_2 was more frequent in the United States while the profile *argW sbcB*

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Address correspondence to Frédéric Auvray, frederic.auvray@anses.fr.

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yehV stx_1 stx_{2c} was more prevalent in Australia, suggesting a divergent evolution of EHEC O157 in Australia and the United States (34). Prophages of non-O157 EHEC strains were also shown to be remarkably divergent in their structure and integration sites from those of EHEC O157 (Sakai strain) (30).

Considered highly mobile genetic elements, Stx phages are involved in the horizontal transfer of *stx* genes (20, 35, 36). Loss of Stx phage and hence of the *stx* genes by EHEC O26:H11 was also shown to occur *in vitro* and *in vivo* in humans, leading to the production of *stx*-negative *E. coli* O26:H11 (37, 38). Contamination of raw-milk cheeses with STEC and *stx*-negative *E. coli* O26:H11 was reported previously (39). It was noteworthy that O26:H11 was the *E. coli* serotype most frequently found in the cheeses studied. The presence of STEC and *stx*-negative *E. coli* O26:H11 strains has also been detected in food products during French surveillance plans (40), with samples containing either *stx*-positive or *stx*-negative *E. coli* strains identified in equivalent proportions. In contrast, the average annual incidence of HUS cases in France remains low (<0.8/100,000 children under 15 years old [41]), with a predominance of the O157:H7 serotype, therefore questioning the virulence level of STEC O26:H11 isolates contaminating these foodstuffs. It is not known whether the *stx*-negative *E. coli* O26:H11 detected in foods originated from STEC O26:H11 upon the loss of Stx phages, i.e., in cattle or other animal hosts, within the food matrix, or during isolation in a laboratory. Consequently, assessment of food safety by molecular screening methods such as ISO/TS 13136 can be problematic when food enrichment broths are found *stx* positive by PCR and STEC isolation attempts only lead to the recovery of *stx*-negative *E. coli* O26:H11 strains. Indeed, when such diagnostic results are obtained, the presence of STEC O26:H11 in food and loss of the Stx phage during enrichment and strain isolation steps cannot be excluded.

In this study, 74 STEC O26:H11 strains were selected and analyzed for their *stx* subtypes and Stx phage insertion sites, and the results obtained were compared according to strain origins, i.e., human, dairy, and cattle. An additional group of 29 *stx*-negative *E. coli* O26:H11 strains was also studied to evaluate the state of Stx phage insertion sites.

MATERIALS AND METHODS

Bacterial strains. Seventy-four STEC O26:H11 isolates from humans ($n = 31$), dairy products ($n = 31$), and cattle ($n = 12$) and 29 *stx*-negative *E. coli* O26:H11 isolates from humans ($n = 8$), dairy products ($n = 9$), and cattle feces or ground beef ($n = 12$) were used in this study (see Tables 2 and 3). Bacterial strains of dairy and cattle origins were isolated in Europe (mainly in France) between 2007 and 2012 and those of human origin were isolated between 1994 and 2011 (except for two strains, H19 and H30, that were isolated in 1977 in Canada). *E. coli* strains were cultivated in tryptone soy broth-yeast extract at 37°C overnight. Bacterial DNA was extracted with the InstaGene Matrix 100 as described by the supplier (Bio-Rad Laboratories, Marnes-la-Coquette, France) and stored either at 4°C before PCR analysis or at −20°C for longer storage.

PCR techniques. Subtyping of *stx* genes allowing the identification of three subtypes of the stx_1 gene (stx_{1a}, stx_{1c}, and stx_{1d}), and seven subtypes of the stx_2 gene (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, and stx_{2g}) was performed by conventional PCR as described by Scheutz et al. (11), with a 9700AB thermocycler (Applied Biosystems).

Amplification of the bacterial *attB* site by conventional PCR was performed to determine the absence of inserted Stx phage into *wrbA*, *yehV*, *yecE*, *sbcB*, *Z2577*, *argW*, *prfC*, *ssrA*, and the *torS-torT* intergenic region in each strain. When no *attB* DNA amplification occurred, amplification of the *attL* junction site was performed to demonstrate the presence of inserted Stx phage (Table 1). The amplification reactions were performed in a total volume of 50 μl and contained 0.6 μM primers, 100 μM each deoxynucleoside triphosphate (Roche Diagnostics), 1× PCR buffer with $MgCl_2$, 2.5 U of FastStart-*Taq* polymerase (Roche Diagnostics), and 2 μl of genomic DNA. The reactions were performed in a Veriti thermocycler (Applied Biosystems) with the thermal profiles described in Table 1. The presence of EspK phage inserted in *ssrA* was determined by PCR with primers ssrAF (TGCTGACGAGTGGTTTGTTC) and ssrA-R2 (TGTGATTTCGCTTTTGATGC) for amplification of the 770-bp-long bacterial-EspK phage junction site at the *ssrA* locus. The PCR conditions were as described above, and the thermal profile consisted of an initial denaturation at 94°C for 5 min, followed by 30 s at 94°C, 60 s at 60°C, and 60 s at 72°C for 30 cycles and a final elongation at 72°C for 5 min. PCR products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide.

To allow rapid and high-throughput analysis of the Stx phage insertion site state at four chromosomal loci, i.e., *wrbA*, *yehV*, *yecE*, and *sbcB*, and to avoid postamplification manipulations, eight quantitative PCR (qPCR) assays were also designed with primers and probes specific for the *attB* and *attL* sites from each locus (Table 1) and compared to the conventional PCR assays. The reactions were performed with the LightCycler 480 instrument (Roche Diagnostics) in a total volume of 20 μl with the thermal profile described in Table 1. The optimal amplification reaction mixture contained 1× LightCycler 480 Probes Master mix (Roche Diagnostics), 200 nM each primer, 200 nM each probe (except for yecE-B, yehV-BL, and yehV-L2, 400 nM), and 2 μl of extracted DNA. The cycle threshold (C_T) value was defined as the PCR cycle at which the fluorescent signal exceeded the background level. The C_T was determined automatically by the LightCycler 480 software by the second derivative maximum method.

For strains containing two different *stx* subtypes, long-template PCR was used to determine at which sites the corresponding two phages were inserted. For this analysis, the LongAmp *Taq* PCR kit (BioLabs) was used with primers stx2-rev (42) and EC11 (37) for a ca. 17-kbp-long stx_2-*yecE* target and primers stx1-rev (42) and yehV-B (43) for a ca. 19-kbp-long stx_1-*yehV* target. The optimal amplification reaction mixture contained 1× LongAmp *Taq* Reaction Buffer (BioLabs), 5 U of LongAmp *Taq* DNA polymerase (BioLabs), 300 μM deoxynucleoside triphosphates (BioLabs), 400 nM each primer, and 1 μl of extracted DNA. The reactions were performed in a Veriti thermocycler (Applied Biosystems) with the thermal profile described in Table 1. PCR products were analyzed by electrophoresis in a 0.8% agarose gel stained with ethidium bromide.

qPCR-based quantification of *attB* and *attL* DNA copies in STEC O26:H11 cultures. qPCR assays targeting the *attB* and *attL* sites at the *wrbA*, *yehV*, *yecE*, and *sbcB* chromosomal loci (Table 1) were used as described above in order to examine the level of spontaneous excision of Stx phages during the cultivation of STEC. The numbers of *attB* and *attL* DNA copies quantified by qPCR for each strain and for each insertion locus were used to calculate *attB*/*attL* ratios (i.e., ratios of bacterial cells whose Stx prophage is excised from the chromosome against those with chromosomally integrated Stx prophage, respectively). The linearity and limit of quantification of each qPCR assay were formerly determined by using calibrated suspensions of STEC corresponding to dilutions of pure cultures of *attL*-positive control strains 11368, H19, 245.2, and VTH7 containing an Stx phage inserted in the *wrbA*, *yehV*, *yecE*, and *sbcB* genes, respectively, and to dilutions of a pure culture of *attB*-positive control strain MG1655 (*E. coli* K-12). Amplification efficiency (E) was calculated with the 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 C_T values. The concentrations of DNA samples from the 74 STEC O26:H11 strains of the collection were determined with NanoDrop instruments (Thermo Scientific), and each DNA was then diluted to a fixed concentration of 30 ng/μl prior to qPCR analysis. Student's t test was used to determine whether there were statistically signif-

TABLE 1 Primers and probes for conventional PCR, real-time qPCR, and long-range PCR determinations of Stx phage insertion sites and status of insertion sites as intact (*attB*) or occupied (*attL*)

Target DNA	Primer or probe	Nucleotide sequence (5′→3′)	Amplicon size (bp)	PCR conditions	Reference
Conventional PCR[a]					
wrbA-attB	wrbA1	ATGGCTAAAGTTCTGGTG	600	94°C, 30 s; 59°C, 60 s; 72°C, 60 s	49
	wrbA2	CTCCTGTTGAAGATTAGC			
wrbA-attL	wrbA	CGCCATCCACTTTGCTTG	1,045	94°C, 30 s; 59°C, 60 s; 72°C, 90 s	37
	Int933W	TATGCTACCGAGGCTTGG			
yehV-attB	yehV-A	AAGTGGCGTTGCTTTGTGAT	340	94°C, 30 s; 62°C, 30 s; 72°C, 60 s	43
	yehV-B	AACAGATGTGTGGTGAGTGTCTG			
yehV-attL	yehV-F	CACCGGAAGGACAATTCATC	702	94°C, 30 s; 62°C, 30 s; 72°C, 60 s	43
	yehV-B	AACAGATGTGTGGTGAGTGTCTG			
yecE-attB	EC10	GCCAGCGCCGAGCAGCACAATA	400	94°C, 30 s; 63°C, 60 s; 72°C, 60 s	37
	EC11	GGCAGGCAGTTGCAGCCAGTAT			
yecE-attL	Int258	CATAGCAAACCAAATGGGCCA	425	94°C, 30 s; 57°C, 60 s; 72°C, 60 s	37
	EC11	GGCAGGCAGTTGCAGCCAGTAT			
sbcB-attB	sbcB1	CATGATCTGTTGCCACTCG	1,800	94°C, 30 s; 60°C, 60 s; 72°C, 90 s	49
	sbcB2	AGGTCTGTCCGTTTCCACTC			
sbcB-attL	sbcBF	ATTGTCGCGCTAAAGCTGAT	250	94°C, 30 s; 60°C, 60 s; 72°C, 60 s	25
	stx2cphiB	CAACGATGCTCGTTATGGTG			
Z2577-attB	z2577F	AACCCCATTGATGCTCAGGCTC	909	94°C, 30 s; 59°C, 90 s; 72°C, 60 s	27
	z2576R	TTCCCATTTTACACTTCCTCCG			
argW-attB	argW-A	CCGTAACGACATGAGCAACAAG	216	94°C, 30 s; 58°C, 45s; 72°C, 90 s	32
	argW-D	AATTAGCCCTTAGGAGGGGC			
argW-attL	argW-C	GCATCTCACCGACGATAACA	462	94°C, 30 s; 58°C, 45s; 72°C, 90 s	32
	argW-D	AATTAGCCCTTAGGAGGGGC			
prfC-attB	yiiG1	CCCACCTGGACCGTTTCTTC	348	94°C, 30 s; 55°C, 60 s; 72°C, 60 s	This study
	prfC1	CCCACGCTGCTTTTCCATCT			
prfC-attL	ECO5234	GGAAGAACTGCGGCAGCGAT	914	94°C, 30 s; 56°C, 60 s; 72°C, 90 s	This study
	prfC1	CCCACGCTGCTTTTCCATCT			
torST-attB	torS2	TGCGCGGCGAAAAGTTCCCA	533	94°C, 30 s; 60°C, 60 s; 72°C, 60 s	This study
	torT2	CCGCCTGCCTCCAGCACTTT			
ssrA-attB	ssrA1	GGATTCGACGGGATTTGCGA	838	94°C, 30 s; 55°C, 60 s; 72°C, 90 s	This study
	ypjA-R1	AACGGTATGGAAATTGAGC			
qPCR[b]					
wrbA-attB	wrbA-F1	GCGAATCGCTACGGAATAGA	163	95°C, 10 s (4.4°C/s none)	This study
	wrbA-R1	CGGTACACGCTTAACGACAA		60°C, 30 s (2.2°C/s single)	
	wrbA-B	FAM-CATATTGAAACGATGGCACG-BHQ1		40°C, 30 s (4.4°C/s none)	
wrbA-attL	intW	CCAAAGTGACCAGGAGGATG	200	95°C, 10 s (4.4°C/s none)	This study
	wrbA-R2	GGTGCAGTTTGCGTTTTACC		60°C, 30 s (2.2°C/s single)	
	wrbA-L	FAM-TTAAGCGTGTACCGGAAACC-BHQ1		40°C, 30 s (4.4°C/s none)	
yehV-attB	yehV-F1	AGTGGCGTTGCTTTGTGATA	216	95°C, 10 s (4.4°C/s none)	This study
	yehV-R1	CCGTTCTGCACATCAACATT		60°C, 30 s (2.2°C/s single)	
	yehV-BL	FAM-TTCAACGATGCCGATATTGA-BHQ1		40°C, 30 s (4.4°C/s none)	

(Continued on following page)

TABLE 1 (Continued)

Target DNA	Primer or probe	Nucleotide sequence (5′→3′)	Amplicon size (bp)	PCR conditions	Reference
yehV-attL	yehV-F4	TGTTTACGGAGCATGGATGA	239	95°C, 10 s (4.4°C/s none)	This study
	yehV-R3	TCAATATCGGCATCGTTGAA		60°C, 30 s (2.2°C/s single)	
	yehV-L2	FAM-AAAGTGTCCCATGTATGCCC-BHQ1		40°C, 30 s (4.4°C/s none)	
yecE-attB	yecE-F1	GCAATGGTCGCATCCTAAAT	180	95°C, 10 s (4.4°C/s none)	This study
	yecE-R1	GTCGCCGGAAACTTAAAACA		60°C, 30 s (2.2°C/s single)	
	yecE-B	FAM-GAGTATGCCCGCCACTTTAA-BHQ1		40°C, 30 s (4.4°C/s none)	
yecE-attL	yecE-F4	AGCCAGACTCTGAAATAATATCTTTA	154	95°C, 10 s (4.4°C/s none)	This study
	yecE-R4	AAGCGGAAGTCATCTGTG		60°C, 30 s (2.2°C/s single)	
	yecE-L2	FAM-TAGTTGCCGTCACATTAACTGCGT-BHQ1		40°C, 30 s (4.4°C/s none)	
sbcB-attB	sbcB-F3	ACGGTAAGCAACAATCTA	170	95°C, 10 s (4.4°C/s none)	This study
	sbcB-R4	CTGGGGTAAATAGTCATCC		60°C, 30 s (2.2°C/s single)	
	sbcB-TQB1	FAM-TACGAAACCTTTGGCACGCACC-BHQ1		40°C, 30 s (4.4°C/s none)	
sbcB-attL	sbcB-F4	GGACAATGCTAGACAATGA	192	95°C, 10 s (4.4°C/s none)	This study
	sbcB-R4	CTGGGGTAAATAGTCATCC		60°C, 30 s (2.2°C/s single)	
	sbcB-TQL1	FAM-AGACACAGATAAGCAACCTACCTTCCT-BHQ1		40°C, 30 s (4.4°C/s none)	
Long-template PCR[c]					
stx_2-*yecE*	stx2-rev	CTGAACTCCATTAACKCCAGATA	17,000	94°C, 30 s; 60°C, 30 s; 65°C, 19 min	This study
	EC11	GGCAGGCAGTTGCAGCCAGTAT			
stx_1-*yehV*	stx1-rev	CGACATYAAATCCAGATAAGAAGTAGT	19,000	94°C, 30 s; 60°C, 30 s; 65°C, 21 min	This study
	yehV-B	AACAGATGTGTGGTGAGTGTCTG			

[a] All PCRs were run for 30 cycles with an initial denaturation step of 5 min at 94°C and a final extension step of 5 min at 72°C.
[b] All qPCRs were run for 40 cycles with an initial denaturation step of 5 min at 95°C (4.4°C/s none). The efficiencies of PCR amplification of *wrbA*, *yehV*, *yecE*, and *sbcB* were 87.1, 96.5, 95.4, and 99.2% for *attL*, respectively, and 96.5, 92.1, 95.4, and 93.6% for *attB*, respectively. None and single indicate the fluorescence acquisition mode selected.
[c] All long-template PCRs were run for 30 cycles with an initial denaturation step of 5 min at 94°C and a final extension step of 10 min at 65°C.

icant differences in the stability of Stx phages according to the insertion site occupied. A *P* value of ≤0.05 was considered a significant difference.

MLST. Multilocus sequence typing (MLST) of 12 *E. coli* O26:H11 strains was performed with the nucleotide sequences of seven housekeeping genes as described previously (37), and the alleles and STs were assigned in accordance with the *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). The STs of six other strains were retrieved from previous reports (6, 44–46) or from the *E. coli* MLST database.

RESULTS

Identification of *stx* subtypes by PCR. A total of 74 STEC O26:H11 strains were analyzed in this study. They corresponded to 53 stx_1-positive strains (human, $n = 14$; dairy product, $n = 27$; cattle, $n = 12$), 18 stx_2-positive strains (human, $n = 15$; dairy product, $n = 3$), and 3 stx_1- and stx_2-positive strains (human, $n = 2$; dairy product, $n = 1$). Subtyping of their *stx* genes showed that all of the stx_1-positive strains harbored the stx_{1a} subtype, while all of the stx_2-positive strains carried the stx_{2a} variant, except for one strain (EH196), which carried the stx_{2d} gene (Table 2).

Insertion site occupancy by Shiga-toxin bacteriophages in STEC O26:H11 strains. Insertion of Stx phages into nine chromosomal loci, i.e., *wrbA*, *yehV*, *yecE*, *sbcB*, *Z2577*, *argW*, *prfC*, *ssrA*, and *torS-torT*, was determined by conventional PCR tests and by newly developed real-time PCR assays for the first four loci listed above. Identical results were obtained by conventional PCR tests and real-time PCR assays, indicating that the latter can reliably determine Stx phage insertion into the *wrbA*, *yehV*, *yecE*, and *sbcB* genes.

Thirty-seven of the 74 STEC O26:H11 strains studied were found to possess an Stx phage inserted in the *wrbA* gene (Table 2), including 16 human strains, 16 dairy strains, and 5 cattle strains. Twenty-eight strains possessed an Stx phage integrated into the *yehV* gene, including 7 human strains, 15 dairy strains, and 6 cattle strains (Table 2). Ten strains from nine humans and one dairy product and two strains from dairy products possessed an Stx phage integrated into the *yecE* and *sbcB* genes, respectively (Table 2).

In the collection studied here, eight strains (from four humans, three dairy products, and one bovine) possessed two Stx bacteriophages. Of these, three strains carried different subtypes (i.e., stx_{1a} and stx_{2a}) and five strains carried two identical subtypes (i.e., four strains with two copies of stx_{1a} and one strain with two copies of stx_{2a}) (Table 2). By long-template PCR, Stx1a and Stx2a phages were found to be inserted in *yehV* and *wrbA*, respectively, in strain 277.2. In the other two strains, 3073/00 and 3901/97, the Stx2a phage was found to be inserted in *yecE* while the Stx1a phage was located in *yehV* and *wrbA*, respectively. Finally, no Stx phage was found integrated in the *Z2577*, *prfC*, or *argW* gene or in the *torS-torT* intergenic region (data not shown).

Association of *stx* subtypes, Stx phage insertion sites and origins of the STEC strains. Most Stx1a phages were inserted in *wrbA* ($n = 25$) and *yehV* ($n = 27$) genes, with only 4 located in either the *yecE* ($n = 2$) or the *sbcB* ($n = 2$) gene. In contrast, most Stx2a phages were located in the *wrbA* ($n = 12$) and *yecE* ($n = 7$) genes, only one Stx2a phage being inserted in the *yehV* gene. The

TABLE 2 Subtyping of *stx* genes, identification of chromosomal insertion sites for Stx and EspK phages in 74 STEC O26:H11, and determination of *attB*/*attL* ratios

Origin[a] or parameter	Strain	Presence of subtype:			Insertion of Stx phage in[b]:				Insertion of EspK phage in *ssrA*	*attB*/*attL* ratio[c]
		stx_{1a}	stx_{2a}	stx_{2d}	*wrbA*	*yehV*	*yecE*	*sbcB*		
Dairy product	ITFF3406	+			Stx1a				+	1.02×10^{-4}
Dairy product	ITFF3407	+			Stx1a				+	1.06×10^{-4}
Dairy product	ITFF3408	+			Stx1a				+	6.86×10^{-5}
Dairy product	09QMA170.2	+			Stx1a				+	7.65×10^{-5}
Dairy product	09QMA238.2	+			Stx1a				+	3.15×10^{-4}
Dairy product	09QMA277.2[f]	+	+		Stx2a	Stx1a			+	2.34×10^{-3} (*wrbA*); 1.27×10^{-4} (*yehV*)
Dairy product	09QMA283.4	+			Stx1a				+	3.42×10^{-5}
Dairy product	F74-476	+			Stx1a	Stx1a			+	4.28×10^{-5} (*wrbA*); 5.57×10^{-4} (*yehV*)
Dairy product	F46-223[f]		+		Stx2a				+	2.82×10^{-3}
Dairy product	10d[e]	+			Stx1a				+	3.26×10^{-4}
Dairy product	2401-4	+			Stx1a	Stx1a			+	2.29×10^{-5} (*wrbA*); 2.24×10^{-4} (*yehV*)
Dairy product	51.2	+			Stx1a				+	3.44×10^{-4}
Dairy product	F15-313	+			Stx1a				+	2.80×10^{-4}
Dairy product	LA3022401		+		Stx2a				+	2.01×10^{-3}
Dairy product	F43-368		+		Stx2a				+	2.48×10^{-3}
Dairy product	AOC 21.04-4	+			Stx1a				+	2.66×10^{-4}
Dairy product	09QMA245.2	+					Stx1a		+	3.19×10^{-6}
Dairy product	09QMA260.3	+				Stx1a			+	2.86×10^{-7}
Dairy product	2976-1	+				Stx1a			+	1.60×10^{-6}
Dairy product	8102-1	+				Stx1a			+	1.40×10^{-6}
Dairy product	7501 POOLA	+				Stx1a			+	4.85×10^{-4}
Dairy product	158.1	+				Stx1a			+	3.15×10^{-4}
Dairy product	L23A	+				Stx1a			+	2.32×10^{-4}
Dairy product	MAC42.4	+				Stx1a			+	9.07×10^{-8}
Dairy product	175 1A	+				Stx1a			+	1.08×10^{-6}
Dairy product	1028	+				Stx1a			+	2.18×10^{-4}
Dairy product	3591.22	+				Stx1a			+	4.82×10^{-5}
Dairy product	1080.2	+				Stx1a			+	6.33×10^{-4}
Dairy product	979.1	+				Stx1a			+	3.84×10^{-4}
Dairy product	95621-1	+							+	ND[g]
Dairy product	09QMA129.2	+							+	ND
Subtotal no. of strains	31	28	4	0	16	15	1	0	31	
Human (NK)	VTH7	+						Stx1a	+	9.12×10^{-5}
Human (NK)	10003174260	+						Stx1a	+	4.39×10^{-4}
Human (D)	ED21	+			Stx1a				+	1.00×10^{-5}
Human (NK)	96-723	+			Stx1a				+	2.04×10^{-3}
Human (HUS)	31131[f]		+		Stx2a				+	3.65×10^{-3}
Human (HC)	31302	+			Stx1a				+	1.78×10^{-5}
Human (D)	EH284	+			Stx1a				+	3.46×10^{-3}
Human (D)	EH324	+			Stx1a				+	3.34×10^{-3}
Human (D)	H30	+			Stx1a				+	4.35×10^{-6}
Human (HUS)	11368[e]	+			Stx1a				+	4.73×10^{-4}
Human (HUS)	3901/97[e]	+	+		Stx1a		Stx2a		+	9.55×10^{-4} (*wrbA*); 8.78×10^{-5} (*yecE*)
Human (HUS)	5917/97[f]		+		Stx2a				+	2.43×10^{-3}
Human (HUS)	6061/96[f]		+		Stx2a				+	2.92×10^{-3}
Human (HUS)	29348[f]		+		Stx2a				+	2.62×10^{-3}
Human (HUS)	25562		+		Stx2a				+	2.97×10^{-3}
Human (HUS)	30993[e]		+		Stx2a				+	4.99×10^{-3}
Human (HUS)	29246		+		Stx2a				+	2.09×10^{-3}
Human (HUS)	29687		+		Stx2a				+	1.05×10^{-2}
Human (D)	EH196			+			Stx2d		+	1.50×10^{-4}
Human (HUS)	1833/98		+				Stx2a			7.77×10^{-5}

(Continued on following page)

TABLE 2 (Continued)

Origin[a] or parameter	Strain	Presence of subtype:			Insertion of Stx phage in[b]:				Insertion of EspK phage in *ssrA*	*attB*/*attL* ratio[c]
		stx_{1a}	stx_{2a}	stx_{2d}	*wrbA*	*yehV*	*yecE*	*sbcB*		
Human (HUS)	31132[f]		+				Stx2a			1.49×10^{-5}
Human (HUS)	21765(1)[e]		+				Stx2a			3.27×10^{-5}
Human (HUS)	21765(2)		+				Stx2a			7.93×10^{-5}
Human (HC)	31212	+				Stx1a			+	1.07×10^{-5}
Human (HC)	31049	+				Stx1a	Stx1a		+	7.85×10^{-4} (*yehV*); 5.07×10^{-4} (*yecE*)
Human (D)	H19	+				Stx1a				0[d]
Human (HUS)	2245/98[e]	+				Stx1a			+	1.68×10^{-7}
Human (HUS)	3073/00[e]	+	+			Stx1a	Stx2a		+	1.12×10^{-3} (*yehV*); 1.18×10^{-3} (*yecE*)
Human (HUS)	28810	+				Stx1a			+	3.68×10^{-6}
Human (HUS)	7662/96		+			Stx2a	Stx2a			2.52×10^{-3} (*yehV*); 1.01×10^{-4} (*yecE*)
Human (D)	CB6307		+						+	ND
Subtotal no. of strains	31	16	16	1	16	7	9	2	25	
Cattle feces	9	+							+	ND
Cattle feces	130	+								ND
Cattle feces	193	+			Stx1a	Stx1a				1.55×10^{-3} (*wrbA*), 1.16×10^{-3} (*yehV*)
Cattle feces	4	+			Stx1a				+	6.49×10^{-5}
Cattle feces	138	+			Stx1a				+	1.53×10^{-3}
Ground beef	54-126B1	+			Stx1a				+	2.47×10^{-4}
Ground beef	85-08.B	+			Stx1a				+	2.40×10^{-5}
Cattle feces	329S89	+				Stx1a				6.47×10^{-2}
Ground beef	37.40	+				Stx1a			+	3.69×10^{-7}
Ground beef	75136	+				Stx1a			+	3.21×10^{-4}
Cattle feces	19	+				Stx1a			+	4.09×10^{-4}
Cattle feces	113	+				Stx1a			+	3.98×10^{-6}
Subtotal no. of strains	12	12	0	0	5	6	0	0	9	
Total no. of strains	74	56	20	1	37	28	10	2	65	

[a] D, diarrhea; NK, not known.
[b] A total of nine loci were tested for each strain for the presence of an Stx phage by lack of *attB* amplification and positive amplification of *attL*. Only the positive results obtained for four loci are indicated.
[c] The *attB*/*attL* ratio for each Stx phage insertion site was determined by dividing the number of *attB* DNA copies by the number of *attL* DNA copies that were quantified by qPCR.
[d] No *attB* DNA copy was detected.
[e] Strain belongs to ST21.
[f] Strain belongs to ST29.
[g] ND, not done.

sole Stx2d phage identified in STEC O26:H11 was inserted in the *yecE* gene.

By taking the origins of the strains into account, it was observed that dairy and cattle strains possessed mainly an Stx1a phage that was located in either *wrbA* (in 42.8 and 45.5% of the dairy and cattle strains, respectively) or *yehV* (in 53.6 and 54.5% of the dairy and cattle strains, respectively). In contrast, human strains contained either an Stx1a phage or an Stx2a phage in equivalent proportions. In those strains, the Stx1a phage was preferentially integrated in *wrbA* (53.3%) and *yehV* (40%), as opposed to the Stx2a phage, which was preferentially integrated in *wrbA* (50%) and *yecE* (43.7%).

qPCR-based quantification of spontaneous excision of Stx phage DNA in STEC O26:H11 cultures. For some strains, both the *attB* and *attL* sites could be amplified simultaneously, as observed after electrophoresis of PCR products corresponding to several insertion chromosomal loci (data not shown). PCR products originating from *attB* amplification were less abundant, however, than those from *attL* amplification, suggesting that spontaneous excision of Stx prophage DNA occurred in a subset of the STEC cell population.

Such a simultaneous amplification of *attB* and *attL* was also observed by real-time PCR. The C_T values obtained for the *attB* target varied between the strains, suggesting that the amount of cells with excised Stx phage DNA differed according to the strain tested. The C_T values obtained varied from 22.75 to 37.61 for

TABLE 3 Occupancy of various insertion chromosomal loci by Stx phages, EspK phages, or other genetic element in 29 *stx*-negative *E. coli* O26:H11 strains

Origin[a]	Strain	Insertion of Stx phage whose *stx* gene was deleted								Insertion of EspK phage in *ssrA*
		wrbA	*yehV*	*yecE*	*sbcB*	*Z2577*	*prfC*	*torS-torT*	*argW*	
Dairy product	09QMA04.2	−	−	−	−	−	−	−	−	−
Diary product	09QMA315.2	−	−	−	−	−	−	−	−	−
Dairy product	09QMA306.D	−	−	−	−	−	−	−	−	−
Dairy product	FR14.18[d]	−	−	−	−	−	−	−	−	−
Cattle feces	FFL1.1	−	−	−	−	−	−	−	−	−
Cattle feces	FFL2.6	−	−	−	−	−	−	−	−	−
Cattle feces	FV5.36	−	−	−	−	−	−	−	−	−
Cattle feces	FV2.33	−	−	−	−	−	−	−	−	−
Cattle feces	FV3.11	−	−	−	−	−	−	−	−	−
Cattle feces	FV4.17	−	−	−	−	−	−	−	−	−
Dairy product	4198.1	−	−	−	−	−	−	−	−	+
Dairy product	191.1	−	−	−	−	−	−	−	−	+
Dairy product	64.36[c]	−	−	−	−	−	−	−	−	+
Human (HUS)	5021/97	−	+	−	−	−	−	−	−	+
Human (HUS)	5080/97[c]	−	−	+	−	−	−	−	−	+
Human (HUS)	318/98	−	−	−	−	−	−	−	−	−
Human (HUS)	21474	−	−	−	−	−	−	−	−	−
Human (HUS)	21766	−	−	−	−	−	−	−	−	−
Ground beef	19-57D7	−	−	−	−	−	−	−	−	−
Ground beef	V76-1326[d]	−	−	−	−	−	−	−	−	−
Ground beef	39.1	−	−	−	−	−	−	−	−	−
Human (NK)	MB04	−	−	−	−	−	−	−	−	−
Human (NK)	MB01	−	−	−	−	−	−	−	−	−
Dairy product	09QMA355.2	−	−	−	−	−	−	−	−	−
Ground beef	07QMA144.1	−	−	−	−	−	−	−	−[b]	−
Ground beef	07QMA167.1	−	−	−	−	−	−	−	−	−
Ground beef	07QMA184.3	−	−	−	−	−	−	−	−	−
Human (HUS)	29690[d]	−	−	−	−	−	−	−	−	−
Dairy product	F61-523[d]	−	−	−	−	−	−[b]	−	−	−

[a] NK, not known.
[b] Genetic element other than Stx phage inserted.
[c] Strain belongs to ST21.
[d] Strain belongs to ST29.

wrbA-attB, from 26.59 to 41.86 for *yehV-attB*, from 27.25 to 37.92 for *yecE-attB*, and from 28.85 to 34.84 for *sbcB-attB* (data not shown). In comparison, the positive-control MG1655 strain DNA containing intact *attB* sites showed C_T values of 15.7, 16.9, and 16.2 for *wrbA*, *yehV*, and *yecE*, respectively, and strain 11368 with an intact *sbcB-attB* site displayed a C_T value of 15.4 (data not shown).

As this phenomenon can lead to the conversion of STEC to *stx*-negative *E. coli* O26:H11, spontaneous excision of Stx phages was further examined by evaluating the *attB*/*attL* copy number ratio of each strain (Table 2). The amplification efficiencies of the different real-time PCR assays used to quantify *attL* and *attB* genetic copies were similar and were between 87.1 and 99.2% (Table 1). Although the mean *attB*/*attL* ratio was higher for *wrbA* (1.56×10^{-3}) and *yehV* (2.75×10^{-3}) than for *yecE* (2.26×10^{-4}) and *sbcB* (2.65×10^{-4}), these ratios were not statistically significantly different ($P > 0.1$).

Insertion site occupancy by Stx phages or other genetic elements in *stx*-negative *E. coli* O26:H11 strains. Analysis of the *attB* sites by both conventional and real-time PCRs for 29 *stx*-negative *E. coli* O26:H11 strains showed that these were intact, except for four strains (i.e., 5021/97, 5080/97, 07QMA144.1, and F61-523), for which the *attB* site at the *yehV*, *yecE*, *argW*, and *prfC* genes, respectively, was occupied (Table 3). For strains 5021/97 and 5080/97, *yehV-attL* and *yecE-attL* could be amplified by PCR, respectively, suggesting the presence of a phage similar to an Stx phage but whose *stx* gene is deleted. For strains 07QMA144.1 and F61-523, *argW-attL* and *prfC-attL* could not be amplified by PCR, respectively, suggesting that a genetic element other than an Stx phage was present and interrupted the corresponding genes.

Presence of other phages in the *ssrA* site of STEC and *stx*-negative *E. coli* O26:H11 strains. The *attB* site at the *ssrA* gene of most strains could not be amplified, and no Stx phage could be detected at this location, suggesting the presence of another genetic element, such as an EspK phage (33). The presence of such a phage inserted in *ssrA* was therefore investigated here. The *ssrA* gene hosted an EspK phage in 65 out of 74 STEC O26:H11 strains. These included 100, 80.6, and 75% of the dairy product, human, and cattle strains, respectively (Table 2). In contrast, the *ssrA* site was occupied by an EspK phage in a limited number of *stx*-negative *E. coli* O26:H11 strains, i.e., 5 out of 29 (Table 3).

MLST. Phylogenetic analysis of 14 STEC O26:H11 strains was performed by MLST. As described previously for *E. coli* O26:H11 strains (6, 47), the stx_{1a}-positive strains belonged to ST21 whereas strains containing stx_{2a}, either alone or in combination with stx_{1a}, were distributed into both ST21 and ST29 (Table 2). The six *stx*-

negative *E. coli* strains tested by MLST were also found to belong to ST21 and ST29 (Table 3). The correlation between phylogenetic groups and characteristics of STEC O26:H11 such as *stx* genotypes and Stx phage locations was then examined. Seven strains showing six profiles, i.e., stx_{1a}-*wrbA*, stx_{1a}-*yehV*, stx_{2a}-*wrbA*, stx_{2a}-*yecE*, stx_{1a}-*wrbA*/stx_{2a}-*yecE*, and stx_{1a}-*yehV*/stx_{2a}-*yecE*, belonged to ST21, whereas seven other strains that showed the three profiles stx_{2a}-*wrbA* (five strains), stx_{2a}-*yecE*, and stx_{1a}-*yehV*/stx_{2a}-*wrbA* belonged to ST29 (Table 2). The stx_{2a}-*wrbA* and stx_{2a}-*yecE* profiles were therefore each allocated to both STs.

DISCUSSION

Subtyping of the *stx* gene showed that stx_{1a} and stx_{2a} were the major subtypes found in STEC O26:H11 strains, with 56 stx_{1a}-positive and 20 stx_{2a}-positive strains. Three strains contained both stx_{1a} and stx_{2a}, and five strains contained two copies of the same subtype. Similar results were also observed by Bielaszewska et al. in another study of 272 STEC O26 isolates (6). It is noteworthy that most of the dairy strains (88.2%) contained the stx_{1a} gene, whereas the stx_{1a} and stx_{2a} genes were distributed almost equally in the human strains. No other *stx* subtype was found here, except for the stx_{2d} subtype in one human strain, which has been reported recently in emerging STEC O26:H11 human strains (45).

A total of four genes (i.e., *wrbA*, *yehV*, *yecE*, and *sbcB*) were used as Stx phage chromosomal insertion loci in most of the STEC O26:H11 strains, with *wrbA* and *yehV* being the major insertion sites. In the five remaining STEC strains, none of the nine insertion sites tested here were occupied by an Stx phage, whose location therefore remains to be determined. Other candidates for insertion sites, which were not tested here, could be the *potC*, *yciD*, *ynfH*, *serU*, and *yjbM* genes (48).

All of the STEC O26:H11 strains from dairy products and cattle possessed Stx phages integrated into *wrbA* or *yehV*, except for one strain that contained an Stx1a phage located in *yecE*. The *wrbA* and *yehV* genes also served as Stx phage insertion sites in the human strains. Compared to dairy and cattle strains, more human strains (i.e., $n = 9$, 28%) carried an Stx phage located in *yecE*. To our knowledge, only the integration of Stx2 phages into *wrbA* and *yecE* was already described elsewhere for STEC O26:H11 (37). In our study, all of the strains that possessed an Stx2a phage integrated in *wrbA* and *yecE* caused HUS, which is indicative of high virulence. Interestingly, such a profile was either absent from or rarely identified in the dairy or cattle strains studied here. In addition, *yecE*-located Stx2a and Stx2d phages and *sbcB*-located Stx1a phage were found only in human strains. However, as the number of strains tested here is limited, it is premature to conclude about the absence of STEC O26:H11 harboring such Stx phages in dairy products.

MLST-based phylogenetic analysis of 20 *E. coli* O26:H11 strains showed that the 14 STEC isolates tested belonged to either ST21 or ST29, as described previously (6). The stx_{2a}-positive *E. coli* O26:H11 strains tested that caused HUS were distributed in the ST21 and ST29 subgroups, in agreement with previous findings showing that stx_{2a} rather than the ST is a predictor of HUS development (6). In addition, combinations of an *stx* genotype with an insertion locus (e.g., stx_{2a}-*wrbA* or stx_{2a}-*yecE*) could be assigned to both phylogenetic subgroups, therefore indicating that they did not necessarily correlate with a particular ST. This is not surprising, however, since Stx phages are mobile genetic elements acquired horizontally. The remaining six *stx*-negative *E. coli* O26:H11 strains typed by MLST also belonged to ST21 or ST29, as previously observed (47), suggesting interconversion between STEC and *stx*-negative *E. coli* O26:H11 by loss or gain of Stx phage.

In investigating the origin of *stx*-negative *E. coli* O26:H11, we found that both *attL* and *attB* could be detected simultaneously in STEC O26:H11 genomic DNA extracts, as has been observed previously for STEC O157:H7 (43). This suggests that spontaneous excision of Stx phage DNA occurred in a subset of STEC cells during growth. However, such instability of Stx prophage DNA was not dependent on the insertion site since no significant difference could be identified between the mean *attB*/*attL* ratios calculated for each chromosomal insertion site. Whether spontaneous excision of Stx prophage DNA contributes to loss of Stx phage and concomitant conversion *in vitro* and *in vivo* to *stx*-negative *E. coli* O26:H11 strains (37, 38) remains to be further elucidated.

In addition, apart from *ssrA*, all of the chromosomal bacterial attachment sites were found to be vacant in all *stx*-negative *E. coli* O26:H11 strains, except for four strains, indicating that absence of the *stx* gene from most *stx*-negative *E. coli* O26:H11 strains was due to the absence of Stx phage and not to a deletion within the Stx prophage, as observed for an O103:H25 strain (38, 46). In the remaining four *stx*-negative *E. coli* O26:H11 strains, one of the *attB* sites was found to be interrupted, most probably by an Stx phage whose *stx* gene was deleted or by another genetic element.

Finally, a prophage encoding the type III effector EspK was located in the *ssrA* gene in the majority (87.8%) of STEC O26:H11 strains. In contrast, this EspK prophage was observed in only 17.2% of the *stx*-negative *E. coli* O26:H11 strains studied. These observations are in agreement with those of Bugarel et al. showing the presence of the *espK* gene in EHEC O26 strains and their derivatives but not in *stx*-negative *E. coli* O26:H11 strains (33). Most of the *stx*-negative *E. coli* O26:H11 strains studied here thus differed from STEC O26:H11 by the absence of two genetic elements, i.e., an Stx prophage and an EspK prophage. Whether these *stx*-negative *E. coli* O26:H11 strains stem directly from STEC O26:H11 by spontaneous loss of these two phages is unknown. Alternatively, these *stx*-negative *E. coli* O26:H11 strains might not be STEC O26:H11 derivatives.

Conclusion. In conclusion, a diverse range of genetic patterns was observed among STEC O26:H11 strains isolated from dairy products, cattle, and human patients. Various *stx* subtypes and insertion sites were identified among the Stx phages that lysogenized STEC O26:H11, with some differences observed between human strains and strains from food and cattle. These results confirm previous reports showing the existence of different clones (or clades) of STEC O26:H11 with various levels of pathogenicity.

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II. Travaux complémentaires de l'article 1

Des travaux complémentaires ont été réalisés en lien avec l'article 1. Ces travaux concernent plus particulièrement l'étude de la stabilité des phages Stx.

II.1. Mise en évidence de deux populations bactériennes au sein d'une culture pure de STEC

Tout d'abord, le phénomène d'excision spontanée des phages Stx au sein d'une culture pure de STEC, a été préalablement mis en évidence lors de l'amplification par PCR des sites d'insertion des phages Stx. L'amplification d'un site jonction *attL* devrait corréler avec l'absence d'amplification du site *attB*. Or, ce n'était pas systématiquement le cas puisque des amplicons des sites *attL* et *attB* ont été observés sur gel d'électrophorèse pour une même souche. Cette co-amplification des sites *attB* et *attL* a ensuite été observée par PCR en temps réel.

La Figure 19 représente les courbes d'amplification par PCR en temps réel du site *attB* au niveau du gène *wrbA* de chaque souche STEC O26:H11 permettant de révéler l'absence de phage Stx. Les deux populations bactériennes mises en évidence sont représentés par un cercle noir ou vert. Les courbes entourées d'un cercle noir possèdent un cycle seuil (Ct, ou « *Cycle Threshold* ») précoce (environ 15-16) et correspondent aux souches d'*E. coli stx*-négatives pour lesquelles le site *wrbA* est inoccupé. En effet, l'absence de phage Stx a été vérifiée par l'absence d'amplification du site *attL*. Ces souches possèdent un phénotype *attB*+++ et *attL*-. En revanche, l'ADN des souches possédant un phage Stx intégré dans *wrbA* ne devrait pas émettre de signal positif pour l'amplification du site *attB*. Pourtant, l'ADN du site *attB* de ces souches est bien amplifié comme l'attestent les courbes d'amplification entourées d'un cercle vert (Figure 19). De plus, les Ct obtenus sont assez tardifs (26 à 35) montrant que la quantité de cibles amplifiées est relativement faible. Ces résultats montrent qu'au sein d'une culture pure d'une souche de STEC, une petite partie de la population a perdu le phage Stx tandis que la partie majoritaire de la population le possède toujours. Ces souches possèdent donc un phénotype *attB*+ et *attL*+++.

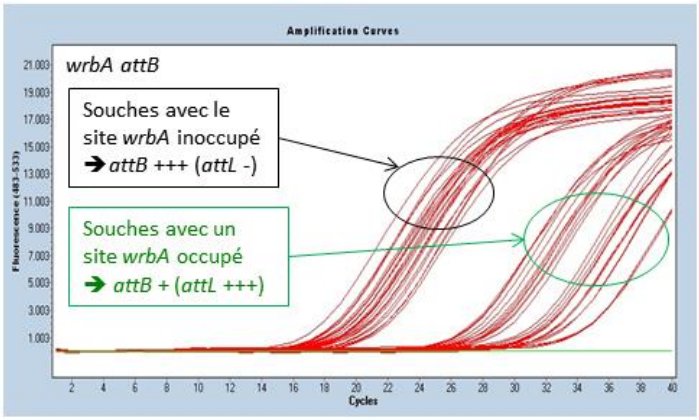

Figure 19. Courbes d'amplification du site *attB* (gène *wrbA*) par PCR en temps réel
Les souches d'*E. coli stx*-négatives sont cerclées en noir et les souches STEC O26:H11 sont cerclées en vert.

II.2. Fréquence d'excision spontanée au cours du réisolement des STEC

La PCR en temps réel permettant de quantifier les cibles d'ADN présentes au départ, les copies de sites *attB* et *attL* ont été quantifiées et des ratios ont été déterminés (présentés dans l'article).

Une seconde étude a été effectuée pour évaluer la fréquence d'excision spontanée au cours du réisolement des STEC. Les 74 souches STEC de la collection ont été réisolées sur des géloses de TSA-YE (« *Tryptone Soy Agar Yeast Extract* ») incubées à 37°C durant 24h en vue d'identifier d'éventuelles colonies dépourvues de phages Stx (et donc de gènes *stx*). Pour chaque réisolement de STEC, 10 à 50 colonies (50 colonies en particulier pour quelques souches *attL*+ ayant une quantité de site *attB* élevé) ont été récupérées puis remises en suspension dans du milieu LB (Lysogeny broth) et les ADN ont été testés pour la présence des gènes *stx1* et *stx2* par PCR en temps réel. Les résultats ont montré que pour 73 des 74 souches STEC, toutes les colonies récupérées étaient positives pour le gène *stx*, excepté pour une souche d'origine laitière, ITFF3408, où 60 colonies ont été testées et pour lesquelles aucune ne possède le gène *stx*. Il est probable que lors de la mise en conservation de cette souche, celle-ci avait perdu son phage Stx. Cette souche témoigne de l'instabilité des phages Stx au cours de la conservation des souches à -20°C.

Chapitre 2. Etude de l'induction des phages Stx : spontanée et en présence de mitomycine C

I. Article 2

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I.1. Objectifs

La première partie de cette étude visait à évaluer l'induction, spontanée et en présence d'un agent inducteur, la mitomycine C (MMC), de différents phages Stx provenant de 14 souches de STEC O26:H11 d'origine laitière (n = 5) et humaine (n = 9).
Les niveaux d'induction ont été comparés en fonction de l'origine des souches STEC, du type de phage Stx (Stx1 et Stx2) et du site d'insertion dans lequel le phage est intégré (*wrbA*, *yehV*, *yecE* et *sbcB*). Parmi cette collection, trois souches humaines et trois laitières possèdent le gène *stx1*. Le gène *stx2* est porté par quatre souches humaines et une laitière. Enfin, deux souches humaines et une laitière contiennent les deux gènes (*stx1* et *stx2*).

Des protocoles d'induction des phages ont tout d'abord été mis au point. La quantité de phage Stx produit a ensuite été déterminée selon deux méthodes, par PCR en temps réel, d'une part, et par dénombrement des plages de lyse, d'autre part.

La seconde partie consistait à évaluer la capacité de quelques phages Stx à infecter des souches d'*E. coli stx*-négatives (AEEC) par un test de lyse des bactéries (dépôt d'une goutte de phage sur un tapis bactérien). L'obtention de lysogènes a ensuite été recherchée.
Enfin, une analyse morphologique de quelques phages Stx a été effectuée, par l'intermédiaire d'un microscope électronique à transmission (MET).

I.2. Résultats et discussion

I.2.1. Induction des phages Stx

Les résultats ont montré que toutes les souches étaient inductibles, et capables de produire des phages Stx. En revanche, le niveau d'induction était variable entre les phages. Cette capacité d'induction a été mise en évidence en présence de l'agent inducteur, la MMC, mais aussi de façon spontanée. L'induction a tout d'abord été observée grâce à la mesure de la densité optique (DO_{600nm}) de la culture bactérienne à 600nm. En présence de MMC, il a été observé une diminution drastique de la DO_{600nm} au bout de 24h.

La PCR en temps réel a ensuite permis de doser les particules de phages Stx issues de l'induction spontanée et en présence de MMC. Les résultats ont montré qu'en présence de MMC, le niveau moyen d'induction est plus important de $2\log_{10}$ par rapport au niveau d'induction spontanée. De plus, l'induction est plus importante pour les souches possédant un phage Stx2 par rapport à celles ayant un phage Stx1. En revanche, aucune différence de niveaux d'induction n'a pu être mise en évidence selon l'origine des souches et selon le site d'insertion du phage Stx. Une exception concerne l'induction spontanée des phages Stx2 intégrés dans *yecE* qui était significativement plus élevée que pour les phages intégrés dans *wrbA* et *yehV*. On pourrait se demander si cette caractéristique implique une virulence accrue des souches possédant un phage Stx2 intégré dans *yecE*. Cette hypothèse a déjà été entrevue lors des travaux décrits précédemment (article 1).

Pour finir sur la quantification des phages Stx, le dénombrement des plages de lyse a permis de démontrer que la quasi-totalité des souches étaient capables de produire des particules phagiques infectieuses. En revanche, quelques phages Stx étaient incapables de générer des plages de lyse isolées. De plus, les quantités observées étaient légèrement inférieures à celles identifiées par la méthode PCRq qui dose tous les phages (y compris les non infectieux ou défectifs).

I.2.2. Pouvoir infectieux des phages Stx

Des tests de lyse en goutte ont été effectués afin d'évaluer la capacité infectieuse des phages Stx sur 17 souches d'*E. coli stx*-négatives (AEEC) et sur une souche d'*E. coli* de laboratoire de type K12 (DH5α). Des différences de sensibilité des souches aux phages Stx ont été observées. La souche DH5α est très sensible aux phages Stx, tandis que seulement 54% (55/102) des interactions AEEC/phage Stx testées, étaient positives pour l'infection. Sur les 17 souches AEEC, une souche était même totalement insensible aux six phages testés. De

plus, les souches humaines semblent plus sensibles aux phages Stx par rapport aux souches laitières.

I.2.3. Obtention d'E. coli lysogènes

Des expériences de conversion lysogénique ont été menées sur des souches AEEC et deux souches d'*E. coli* K12 (DH5α et MG1655). Les résultats ont montré que des bactéries lysogènes issues des souches K12 contenant un prophage Stx2 intégré dans le site *yecE* ont pu être isolées. A la différence, les tentatives de lysogénisation des souches AEEC n'ont pas été concluantes. En revanche, une étude préliminaire de lysogénisation a été effectuée sur une souche AEEC (21474) infectée par un phage Stx2. La particularité est que le phage Stx2 utilisé, est issu d'une purification sur cette même souche. Les résultats ont montré que dans le mélange phage/bactérie, il y avait potentiellement des bactéries lysogènes car le gène *stx2* et le site jonction *yecE-attL* ont été amplifiés par PCR. En revanche, aucune colonie lysogène n'a pu être isolée. D'après la littérature, la lysogénisation des souches d'*E. coli stx*-négatives de terrain est assez rare comparé aux souches K12.

I.2.4. Morphologie des phages

Six phages Stx ont pu être caractérisés morphologiquement, grâce à une observation par microscopie électronique à transmission effectuée au laboratoire de l'INRA de Jouy en Josas (unité Micalis). La morphologie de ces six phages est observable dans la Figure 20. D'après cette étude, trois types de morphologie ont pu être mis en évidence.

Le premier type de morphologie concernait deux phages, Φ3901 (phage Stx2) et ΦH19 (phage Stx1), qui présentent une tête allongée et une longue queue flexible (Figure 20A et 20B).

Le second type de morphologie concernait trois phages, Φ11368 (Stx1), Φ5917/97 (Stx2) et Φ21765 (Stx2) et correspond à celle des phages de type *Siphoviridae* (Figure 20C, 20D et 20E).

Enfin, le phage Φ2976-1 est aussi un phage de type *Siphoviridae*, mais il possède une queue très longue (environ 411nm), comparée à la taille moyenne des queues des autres phages de type *Siphoviridae* (environ 150 nm) (Figure 20F).

Ces résultats montrent que la morphologie des phages Stx des souches STEC O26:H11, est très diverse et n'est pas corrélée avec le type du gène *stx* (*stx1* ou *stx2*).

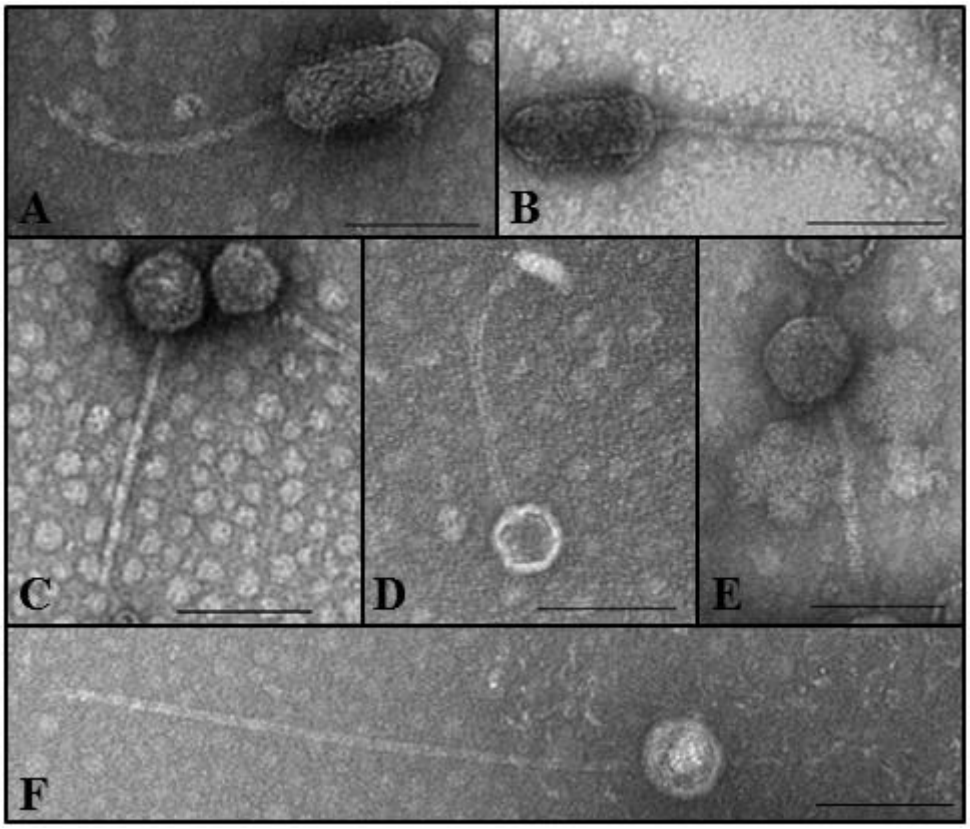

Figure 20. Observation au microscope électronique à transmission (MET) de particules de phages Stx issues de STEC O26:H11

(A) phage Φ3901; (B) phage ΦH19; (C) phage Φ11368; (D) phage Φ5917; (E) phage Φ21765; (F) phage Φ2976-1. (A) et (B) représentent des phages Stx avec une tête allongée et une longue queue flexible. (C) à (E) représentent des phages Stx de type *Siphoviridae* avec une longue queue. (F) est un phage Stx de type *Siphoviridae* avec une très longue queue. L'échelle est matérialisée par une barre qui équivaut à 100 nm.

I.3. Conclusion

En conclusion, les phages Stx1 et Stx2 des STEC O26:H11, sont très diversifiés y compris au sein de chaque type. Cette variabilité a été observées, à la fois au niveau de l'induction, morphologiquement et dans la capacité des phages à infecter les souches d'*E. coli*.

Induction of Stx phages from STEC O26:H11 isolated from humans and dairy products and infection of *stx*-negative *E. coli* O26:H11

BONANNO Ludivine [1,2], PETIT Marie-Agnès [3], LOUKIADIS Estelle [4,5], MICHEL Valérie [2], AUVRAY Frédéric [1*]

[1] Université Paris-Est, Anses, Laboratory for Food Safety, Department of Microbiology, Maisons-Alfort, France

[2]Actalia Produits Laitiers, Laboratoire de microbiologie d'intérêt laitier, La Roche sur Foron, France

[3] INRA, UMR1319, Micalis, domaine de Vilvert, Jouy en Josas, France

[4] Université de Lyon, VetAgro Sup, Laboratoire d'études des microorganismes alimentaires pathogènes/Laboratoire national de référence pour les *Escherichia coli* y compris les *E. coli* producteurs de shiga-toxines, Marcy l'Etoile, France

[5] Université de Lyon, UMR 5557 Ecologie Microbienne, Université Lyon 1, CNRS, VetAgro Sup, Equipe « Bactéries pathogènes et opportunistes », Villeurbanne, France

[*]Corresponding author: Frédéric Auvray, Anses, Laboratoire de Sécurité des Aliments, 14 rue Pierre et Marie Curie, 94706 Maisons-Alfort cedex, France. Tel: +33 1 49 77 28 36; Fax: +33 1 49 77 46 66. E-mail: frederic.auvray@anses.fr

Running title: Stx phage induction and infection of *E. coli*

ABSTRACT

The Shiga toxin producing *Escherichia coli* (STEC) are food-borne pathogens responsible for human infections ranging from mild watery diarrhea to hemorrhagic colitis (CH), which may be complicated by hemolytic uremic syndrome (HUS), sometimes fatal. 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 serotype O26:H11 is considered as the second HUS-causing serotype worldwide after O157:H7, and the first detected in dairy products. The consumption of raw milk cheeses contaminated with STEC O26:H11 could therefore contribute to HUS cases although only a small number of HUS cases caused by serotype O26:H11 are identified each year in France.

Here we evaluated the induction rate *in vitro* of different Stx phages from human and dairy strains of STEC O26:H11, either spontaneously or in the presence of mitomycin C. Overall the Stx2 phages were more inducible 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 displayed various shapes that were unrelated to Stx1 or Stx2 types. In addition, sensitivity of *stx*-negative *E. coli* O26:H11 to six Stx phages varied among the 17 strains tested and our attempts to convert them into STEC were unsuccessful indicating that their lysogenization was a rare event.

Keywords: STEC, O26:H11, Stx phages, induction, dairy product

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) O26:H11 were first identified as causes of hemolytic uremic syndrome (HUS) in 1983 (1, 2). O26:H11 is the most commonly isolated non-O157:H7 serotype in Europe, accounting for 12% of all clinical enterohaemorrhagic *E. coli* (EHEC) isolates in 2012 (3, 4). Since the early 2000s, InVS has observed a significant increasing proportion of HUS cases identified in France due to non-O157 serogroups including O26 which accounted for 11% of cases for the period 1996-2013 (5). This serogroup also accounted for 22% of clinical non-O157 EHEC isolates in the United States, between 1983 and 2002 (6).

Transmission of STEC to humans occurs through food, water and direct contact with animals and their environment. 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 (7). Another outbreak of STEC O26:H11 occurred in Denmark in 2007 and was caused by beef sausage. Twenty cases of diarrhea were reported, the majority of which occurred in children (average age of two years) (8).

Shiga toxins (Stx) are considered as 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 (9-11). There are two Stx groups, Stx1 and Stx2, each divided into 3 (a, c and d) and 7 subtypes (a-g), respectively (12). Recently, a panel of 74 STEC O26:H11 strain was characterized, showing that the majority of food and cattle strains possessed the stx_{1a} subtype, while human strains carried mainly stx_{1a} or stx_{2a} (13). Stx1 and Stx2 can be produced either singly or together by STEC O26:H11 (6) and STEC carrying the *stx2* gene only are generally associated with more severe clinical case compared to STEC possessing the *stx1* gene (14). The first Stx_1 phage described was phage H19B and was isolated from a clinical EHEC O26 strain (9). In the 1990s, a shift of the *stx* genotype was observed in

Germany in EHEC O26:H11, from isolates carrying *stx1* to isolates possessing the stx_2 gene either alone or together with stx_1 (15).

Stx phages insert their genome into specific sites in the bacterial chromosome where they remain silent (16), allowing their bacterial hosts to survive as lysogenic strains. The main Stx phage insertion sites in STEC O26:H11 were *wrbA* and *yehV* genes followed distantly by *yecE* and *sbcB* (13). Stx phages are inducible from their host strain by DNA-damaging agents such as antibiotics (17, 18). These observations led to controversy regarding treatment of STEC infection with antibiotics. Wong et al. showed that the risk of developing HUS was significantly increased in children receiving antibiotics (19). DNA damages trigger the SOS response of *E. coli* (20), 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 ultraviolet irradiation (21) or high hydrostatic pressure treatment (22) were also shown to induce Stx phages.

STEC O26:H11 have the particularity to frequently lose and acquire Stx phages (23, 24). The acquisition of an Stx phage by *stx*-negative *E. coli* O26:H11 was demonstrated *in vitro* (10, 23). The Stx1 phage H-19B can also be transferred *in vivo* in mice from STEC O26:H11 to an *E.coli* recipient strain (25). Moreover, the recent outbreak which occurred in Germany in 2011 with 4000 infected humans including 900 HUS cases and 50 deaths (26), was caused by an enteroaggregative *E. coli* O104:H4 strain that had acquired an Stx phage (27).

Loss of Stx phage can generate *E. coli stx*-negative O26:H11 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 *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 (28). Monitoring plans carried out in France between

2007 and 2009 also showed the presence of STEC and AEEC in raw milk cheeses, including the serotype O26:H11 (29). Finally, Trevisani *et al.* also revealed the presence of both *E. coli* O26 *stx*-positive and *stx*-negative strains in samples of milk (0.4% and 2%, respectively) or in milk filters (0.4% and 0.9%, respectively) (30). The fact that *E. coli* stx-negative or AEEC O26:H11 strains were 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 cannot be excluded.

In this study, the induction of Stx phage from different STEC O26:H11 strains was evaluated in the presence and absence of mitomycin C (MMC), an antibiotic known to effectively induce Stx phages (31). The objective was to compare the induction level of Stx phages according to their Stx type (Stx1 or Stx2) and insertion site in the bacterial chromosome, and to the origin of the STEC strains. The sensitivity of *stx*-negative *E. coli* to Stx phages was also investigated in addition to their lysogenic conversion. Finally the morphology of Stx phages was studied to evaluate the diversity of Stx phages circulating in STEC O26:H11.

MATERIALS AND METHODS

Bacterial strains.

Fourteen STEC O26:H11 isolated from humans (n = 9) and dairy products (n = 5), 17 *stx*-negative O26:H11 *E. coli* isolated from humans (n =8) and dairy products (n = 9), and the *E. coli* K12 strains DH5α and MG1655 were used in this study (Table 1). The human and dairy STEC strains contained *stx1* (n = 3, each), *stx2* (n = 4 and n = 1, respectively) and both *stx1* and *stx2* genes (n = 2 and n = 1, respectively). *E. coli* strains were cultivated in Luria broth (LB) at 37°C.

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

Origin [a]	Strain	Stx phage Type	Insertion site of Stx phage [b]
STEC O26:H11			
Dairy product	2976-1	Stx1a	*yehV*
Dairy product	10d	Stx1a	*wrbA*
Dairy product	09QMA277.2	Stx1a – Stx2a	*yehV* (Stx1), *wrbA* (Stx2)
Dairy product	09QMA245.2	Stx1a	*yecE*
Dairy product	F46-223	Stx2a	*wrbA*
Human (NK)	VTH7	Stx1a	*sbcB*
Human (D)	H19	Stx1a	*yehV*
Human (HUS)	3901/97	Stx1a – Stx2a	*wrbA* (Stx1), *yecE* (Stx2)
Human (HUS)	11368	Stx1a	*wrbA*
Human (HUS)	3073/00	Stx1a – Stx2a	*yehV* (Stx1), *yecE* (Stx2)
Human (HUS)	5917/97	Stx2a	*wrbA*
Human (HUS)	29348	Stx2a	*wrA*
Human (HUS)	31132	Stx2a	*yecE*
Human (HUS)	21765(1)	Stx2a	*yecE*
***stx*-negative 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 *E. coli*			
K12	DH5α	-	-
K12	MG1655	-	-

[a] *D, Diarrhea; HUS, Hemolytic Uremic Syndrome; NK, Not known.*
[b] *Previously described (13)*

Bacteriophage induction.

An overnight culture of STEC O26:H11 was inoculated at 2% in a fresh LB medium with 5mM of $CaCl_2$ and incubated at 37°C. At the exponential growth phase (OD_{600} 0.3), the culture was divided into two subcultures, A and B. In subculture A, MMC was added to a final concentration of 0.5 µg/ml. The 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 at 600nm the optical density of induced and non-induced cultures. All cultures were centrifuged at 7,200 x *g* for 10 min, and the supernatants were filtered through low-protein-binding 0.22 µm-pore-size membrane filters (Millex-GP PES; Millipore) for phage purification.

Enumeration and isolation of Stx phages by double-agar overlay plaque assay.

The *E. coli* DH5α was used as the host strain to screen for the presence of bacteriophages. The suspensions of phage particles obtained after induction (see above) were diluted tenfold. Two hundred microliters of an overnight culture of the host strain was mixed with 100 µl 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 2g/L, 10 mM $CaCl_2$ and 10 mM $MgSO_4$) immediately poured on LB-agar plates and allowed to solidify. After incubation for 18-24 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 plaque-forming units per millilitre (pfu/ml) by using the following calculation: number of plaques x 10 x inverse of the dilution factor (32).

Quantification of Stx phage by quantitative PCR.

Filtered supernatants obtained after Stx phage induction were treated with DNase using the Turbo DNA-free™ kit (Ambion®, life technologies). Removal of any contaminating genomic DNA by DNase was verified by detection of chromosomal STEC O26:H11 *eae* gene by quantitative PCR (qPCR), as described previously (33). 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 *stx1* or *stx2* genes. These were performed with the LightCycler® 480 instrument (Roche Diagnostics) as described by Derzelle *et al.*

(34), with minor modifications as follows. The amplification reaction mixture contained 1X LightCycler® 480 Probes Master mix (Roche Diagnostics), 500 nM of each primer (stx1B-for, stx1-rev, stx2-for, stx2-rev), and 200 nM of each probe (stx1 and stx2 probes). Three microliters of extracted DNAs were used as templates in qPCR. Linearity and limit of quantification of the qPCR assay was formerly determined by using calibrated suspensions of STEC corresponding to dilutions of pure cultures of *stx1* and *stx2*-positive control EDL933 strain containing both *stx1* and *stx2* 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 reaction against Ct values. The cycle threshold value (Ct) was defined as the PCR cycle at which the fluorescent signal exceeded the background level. The Ct was determined automatically by the Lightcycler 480 software with the second derivative maximum method and the *stx1* and *stx2* 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*, the *E. coli* K12 strain DH5α 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 were added to 5 ml of molten LB top agarose and immediately poured on 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), were spotted onto plates containing the LB top agarose overlay and incubated overnight at 37°C.

Construction of lysogens.

E. coli K12 strains (DH5α and MG1655) and *stx*-negative *E. coli* O26:H11 strains were grown overnight in LB broth at 37°C with shaking. One milliliter of host culture was

mixed with 100, 250 or 500 µl (MOI between 0.1 and 0.5) of the different Stx phages such as Stx1, Stx2 or a mix of Stx1 and Stx2 phage suspensions resulting from the mitomycin C induction of the H19 strain (phage ΦH19s), 5917/97 strain (phage Φ5917s) and 3901/97 strain (phage Φ3901m), respectively, and incubated 1 h at 37°C without shaking. The mixtures were then diluted tenfold, plated onto LB-Agar and incubated 24 h at 37°C. After incubation, colonies were enumerated and compared to the enumeration of the uninfected strains used as a control, to check that a majority of the host cells had been lysed and thus infected upon contact with the phage. Finally, 5-10 colonies were purified and tested for lysogeny by PCR amplification of *stx1* and *stx2* genes (as described above) and of host-phage *attL* junction sites (13).

Propagation and purification of Stx phages and transmission electron microscopy.

The *E. coli* laboratory strain MG1655 was used as the host for purification and propagation of Stx phages from strains H19, 5917/97, 3901/97, 21765(1), 2976-1 and 11368 (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 µl of 10 mM $MgSO_4$ buffer. Two hundred microliters of an overnight culture of the host strain was mixed with 5ml of molten LB top agarose, immediately poured onto LB-agar plates and allowed to solidify. Fifty microliters of phage suspension were spotted onto these plates and incubated 8 hours at 37°C. The spots were collected and resuspended in 100 µl of 10 mM $MgSO_4$ buffer. Serial dilution of the lysates were then performed and used in a new round of plaque assay and incubated 8 hours at 37°C to produce confluent lysis of the host strain. Finally, 5 ml of 10 mM MgSO4 buffer were placed onto the top agar and incubated 8 hours 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 ultra-centrifugation at 20,000x*g* for 2 h in a swinging rotor SW32Ti and the pellets were resuspended in 100µL of 10 mM $MgSO_4$ buffer. Five microliters of these suspensions were placed onto copper grids with carbon-coated Formvar films and negatively contrasted with 2% uranyl acetate dehydrate. Samples were examined using a transmission electron microscope (HITACHI HT 7700) (Elexience – France) at 80kV. Microphotographs were acquired with a charge-coupled device camera AMT.

RESULTS

Evaluation of Stx phage induction from STEC O26:H11.

Fourteen STEC O26:H11 strains were used to measure the level of induction of their Stx phages, either spontaneously (i.e. in the absence of MMC) or with the addition of MMC. For all the strains tested, the OD_{600} was lower after 24h of incubation with MMC (OD_{600} < 1.25) than that obtained in the absence of MMC (OD_{600} > 2) (Table 2). This low OD_{600} was an indicator of bacterial cell lysis and 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.

Table 2. Level of induction of Stx phage expressed in OD_{600} units.

	OD_{600} [a]	
Strain	- 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

[a] *OD_{600} of untreated (-MMC) and MMC-treated (+MMC) STEC O26:H11 culture after 24h of incubation at 37°C. The values are the mean of three independent experiments.*

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. Stx phage production was highly variable between the strains (Fig. 1), and in most cases, 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 site, except for the spontaneous induction of Stx phages inserted into *yecE* which was significantly higher than that of Stx phages integrated into *wrbA* ($P < 0.05$) or *yehV* ($P < 0.01$) (Fig. 1).

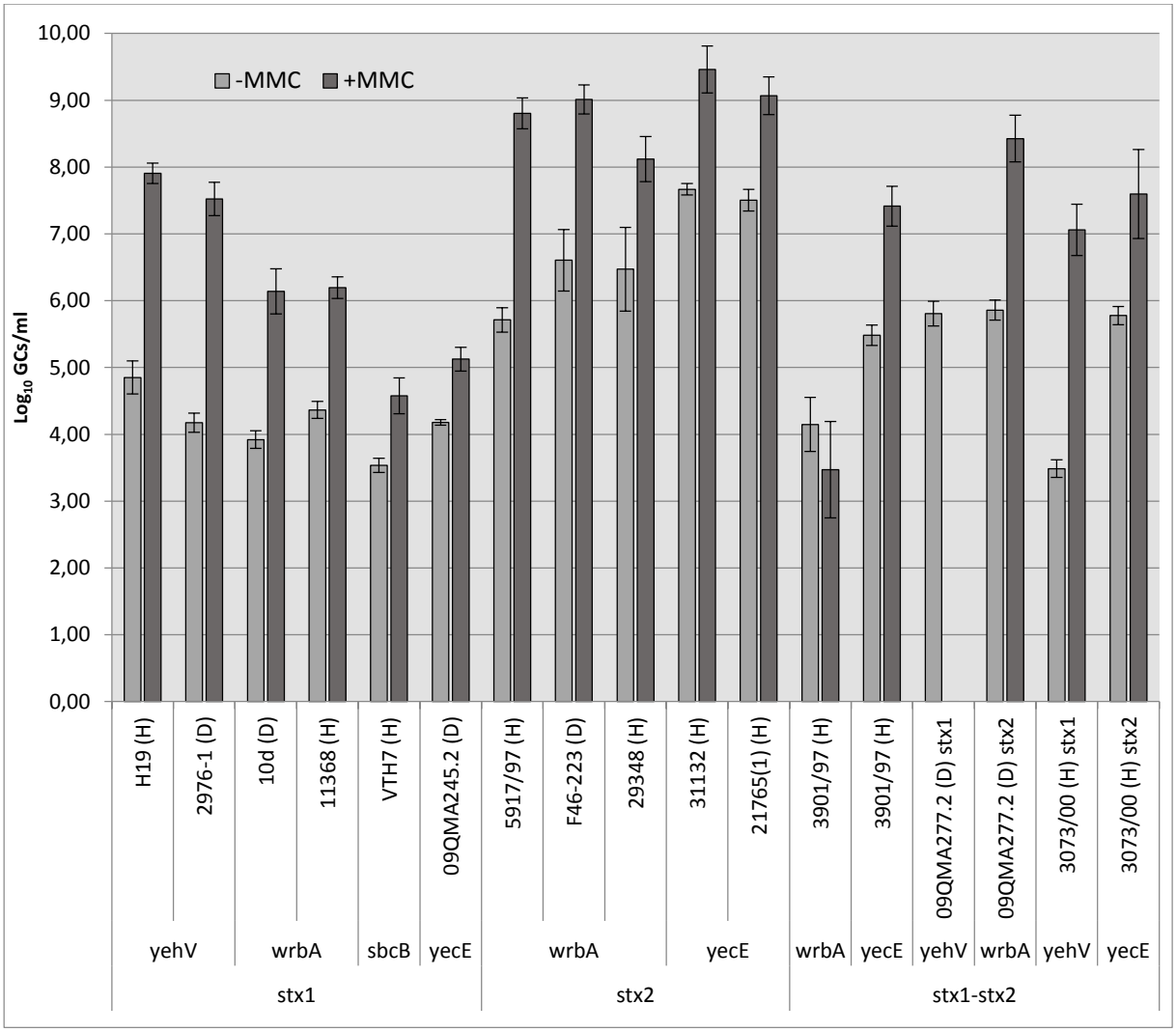

Figure 1. Quantification of Stx1 and Stx2 phages DNA. *Quantification by qPCR of Stx1 and Stx2 phages DNA extracted from culture supernatants obtained from untreated (-MMC, grey bars) or MMC-treated (+MMC, black bars) STEC O26:H11 cultures. The STEC strains were from dairy (D) and human (H) origins and their Stx phages were integrated into wrbA, yehV, yecE or sbcB sites. The concentration of Stx phages was expressed in log_{10} of stx gene copies per milliliter (log_{10} 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.*

Production of Stx phages was observed in both conditions, i.e. in the presence and absence of MMC. In the absence of MMC, the level of Stx phage particles spontaneously produced

varied between 3.49 $\log_{10}$ and 7.67 $\log_{10}$ GC/ml (no. of *stx* gene copies/ml) whereas when MMC was added, the Stx phage levels were between 3.47 $\log_{10}$ and 9.46 $\log_{10}$ GC/ml. Overall, the addition of MMC resulted in an average increase of 2 $\log_{10}$ relative to the spontaneous induction ($P < 0.05$). Two exceptions were observed. For strain 3901/97, the levels of the Stx1 phage were not significantly different with and without MMC, and for strain 09QMA277.2 which contains both Stx1 and Stx2 phages, production of the Stx1 phage became undetectable when MMC was added.

When the production levels of Stx1 phages were compared to those of Stx2 phages, a difference was observed ($P < 0.001$). This was true both in the presence and in the absence of MMC, since the amount of Stx2 phages was higher of 2.11 and 3.16 $\log_{10}$, respectively, compared to that 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* recipient strain DH5α. Four strains (2976-1, 09QMA245.2, VTH7 and 3073/00) also produced infectious Stx phages but these failed to generate isolated plaques (Fig. 2). As observed with qPCR, the mean titers for Stx2 phages were higher of 2 $\log_{10}$ than that for Stx1 phages. However, the Stx phage titers determined by plaque enumeration were lower on average of 1.98 $\log_{10} \pm 1.01$ (decrease of min. 0.79 for 3901/97 strain and of max. 4.28 $\log_{10}$ for H19 strain) than the concentrations of Stx phage genomes determined by qPCR.

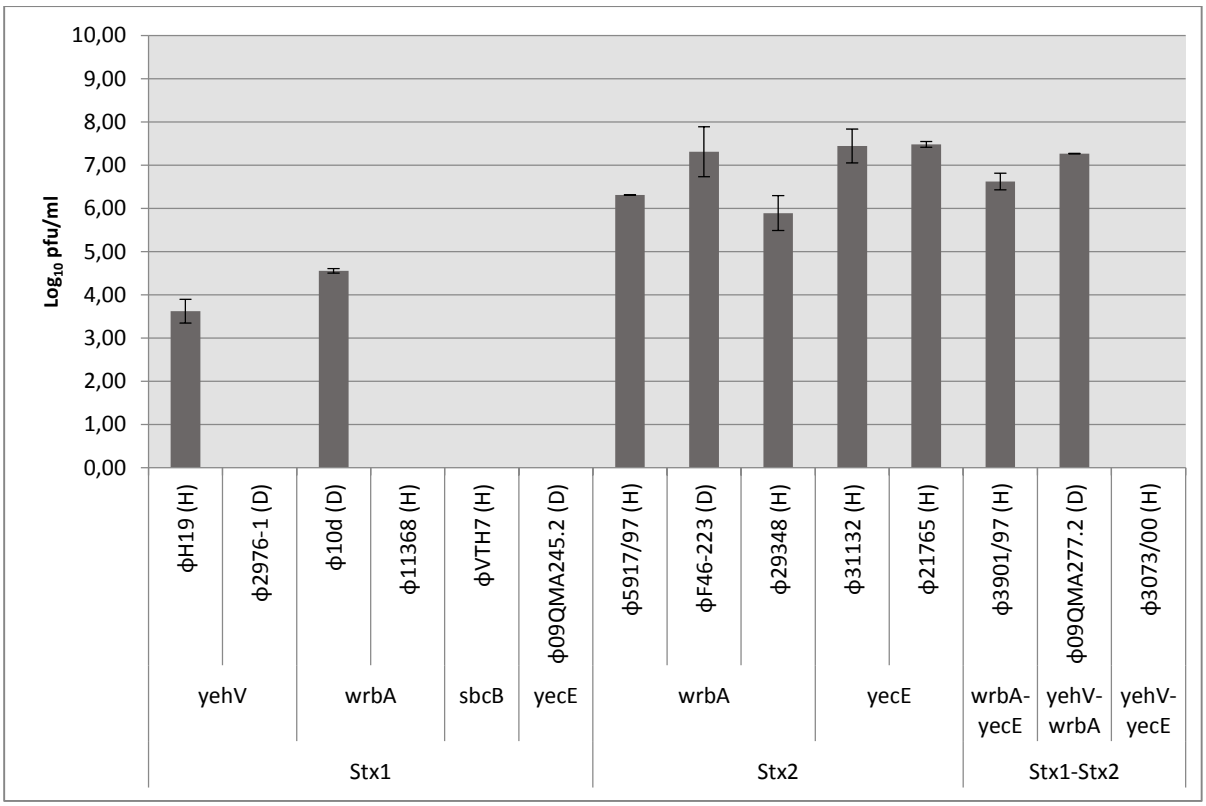

Figure 2. Quantification of Stx1 and Stx2 phage particles. *Quantification by enumeration of lysis-plaques from Stx1 and Stx2 phages particles obtained from culture supernatants derived from MMC-treated STEC O26:H11 cultures. The STEC strains were from dairy (D) and human (H) origins and their Stx phages were integrated into wrbA, yehV, yecE or sbcB sites. The titer of the original phage preparation was expressed in plaque-forming units 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.*

Host infectivity of Stx phages and construction of lysogens.

Seventeen *stx*-negative *E. coli* O26:H11 strains and the *E. coli* K12 strain DH5α were evaluated using spot agar tests for their sensitivity to six filtered supernatants containing Stx phages (obtained after induction of strains H19, 5917/97, 3901/97, F46-223, 09QMA277.2, and 21765). The results obtained from the 102 different *E. coli* / Stx phage interactions tested are reported in Table 3.

The 17 strains were not equally infected 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. By contrast, other strains such as 191.1, 5080/97 or 5021/97 were sensitive to all of the 6 Stx phages. Overall, 55 (54%) out the 102 *E. coli* / Stx phage interactions tested were positive for infection. Among the human strains, 68.7% were sensitive to Stx phages while only 40.7% of dairy strains were infected, a difference which was statistically significant (P = 0.008 with a chi2 test). In addition, the turbidity of the lysis area varied among the strains tested (Table 3). Clear lysis was obtained with DH5α in contrast to most stx-negative *E.coli* O26:H11 strains which generated more opaque lysis area.

Phages Φ5917 and Φ3901m could infect 76.4 and 70.6% of *stx*-negative *E. coli* O26:H11, respectively, while phages ΦF46-223, Φ277.2 and Φ21765 could infect between 47 and 59% of *stx*-negative *E. coli* O26:H11. By 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 with a chi2 test). Finally, the *E. coli* K12 strain DH5α used as a control was susceptible to infection with all the phages tested, showing marked lytic areas.

Table 3. Stx phage infectivity of *E. coli* hosts.

		Phages						
Origin[a]	Strain	ΦH19	Φ5917	Φ3901m	ΦF46-223	Φ277.2	Φ21765	Total
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	+	+	+[b]	+	+	++	6
Human (HUS)	318/98	-	-	+	+	++	-	3
Human (HUS)	21474	-	+[c]	++++[d]	+++	++	+++	5
Human (HUS)	21766	-	+	+	-	-	+	3
Human (NK)	MB04	-	+	+	+	-	+	4
Human (NK)	MB01	-	+	-	+	+	+	4
Human (HUS)	29690	-	+	++	-	-	-	2
K12	DH5α[e]	++++[c]	++++[c]	++++	++++[c]	++++[c]	+++	6
Total	18	5	14	13	9	11	9	

[a] *HUS, Hemolytic Uremic Syndrome; NK, Not known*

[b] *Presence of small lysis plaques instead of a confluent lysis area*

[c] *Presence of a blurred halo around the lysis area*

[d] *Net lysis with colonies in the lysis plaque*

[e] *E. coli K12 strain was used as a control strain*

-, Non-detectable lysis in the spot area; ++++, clear lysis in the spot area; +++ to +, lysis increasingly opaque in the spot area

Attempts to lyzogenize *E. coli* K12 strains DH5α and MG1655 with filtered supernatants containing Stx phages were then performed. Supernatants containing only an Stx1 phage (ΦH19) or only an Stx2 phage (Φ5917), obtained from the induction with MMC of H19 and 5917/97 strains, respectively, were tested, as well as a mix of both Stx1 and Stx2 phages (Φ3901m) obtained from the induction with MMC of 3901/97 strain. A maximum decrease in DH5α bacterial viability was observed from -5.32 to -6.19 $\log_{10}$ in the presence of phages Φ5917 and Φ3901m, respectively, compared to the same conditions in the absence of phages, and similar results were observed with MG1655 (data not shown). By contrast only a

small decrease (< 1.01 log_{10}) in DH5α bacterial viability was observed using phage ΦH19. Lysogens could only be obtained with Φ3901m, with both DH5α and MG1655 (data not shown), and these acquired the Stx2 phage but not the Stx1 phage (data not shown). For these two lysogens, PCR amplification of *attL* junction tested from their DNA was positive at the *yecE* site (data not shown), suggesting that the Stx2 phage integrated its genome into the bacterial chromosomal gene *yecE*.

When the same lysogenization assays were conducted with four *stx*-negative *E. coli* O26:H11 strains (318/98, 191.1, 5080/97 and 21766), there was no difference in bacterial concentrations in the presence or absence of phages, and no lysogens could be recovered (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/modification, the Stx2 phage Φ3901 was propagated on the *stx*-negative *E. coli* O26:H11 strain 21474 and this new stock was used for a lysogenization assay on strain 21474.No lysogens could be isolated either. However, after the adsorption step of phage Φ3901 on 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 an Stx1 phage (ΦH19), and five Stx2 phages (Φ5917, Φ3901, ΦF46-223, Φ277.2 and Φ21765) were plaque-purified and amplified on the MG1655 K12 strain that is devoid of functional prophages (see Methods). The six purified Stx phages from STEC O26:H11 were observed by Transmission electron microscopy. Three different kinds of phages were identified (Fig. 3). Two phages (Φ3901 and ΦH19) presented an elongated (prolate) capsid measuring 42-52 nm wide and 106-121 nm long, with a long flexible tail of *ca* 190-209 nm, and were member of the *Siphoviridae* family (Fig. 3A and 3B).

Three phages (Φ11368, Φ5917 and Φ21765) showed a morphology of *Siphoviridae*, with a head of *ca* 44-64 nm diameter and a long tail of *ca* 150-195 nm (Fig. 3C, 3D and 3E). 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 of *ca* 411 nm (Fig. 3F).

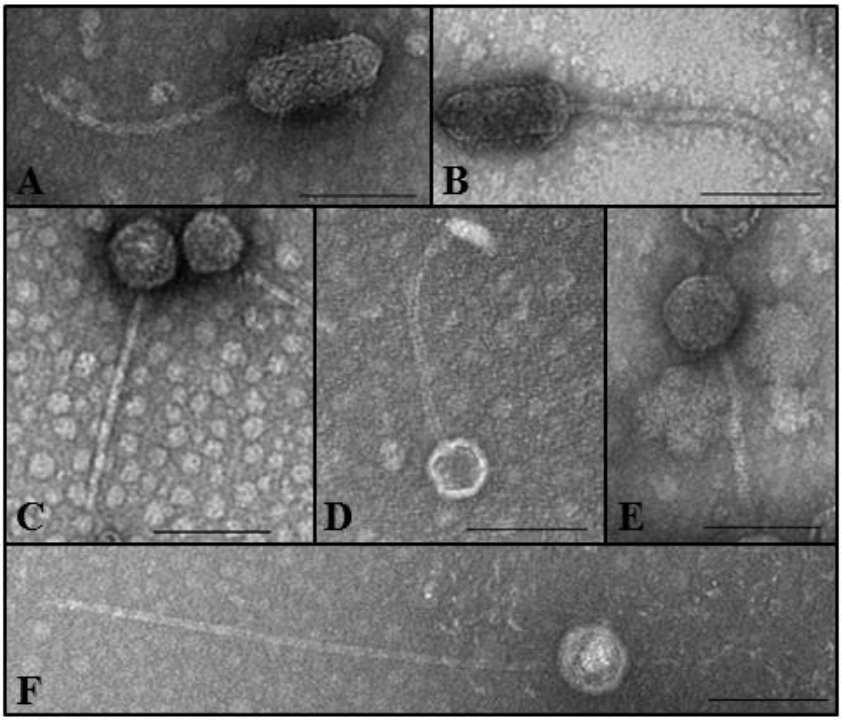

Figure 3. Electron micrographs of Stx phage particles obtained from STEC O26:H11. *Electron micrographs of six phages. (A) phage Φ3901; (B) phage ΦH19; (C) phage Φ11368; (D) phage Φ5917; (E) phage Φ21765; (F) phage Φ2976-1. (A) and (B), Stx phage particles with an elongated head and a long flexible tail. (C) to (E), Siphoviridae phages with a long tail. (F), Siphoviridae phage with a very long tail. Bars, 100 nm.*

DISCUSSION

In this study the level of Stx phages induction from 14 STEC O26:H11 was analyzed. Various strains were selected according to their origin (i.e. human and dairy) and Stx phages (i.e. Stx types 1 and 2, and insertion sites *wrbA*, *yehV*, *yecE* and *sbcB*), in order to evaluate the variability of Stx phages inducibility depending on these parameters. Induction of Stx phages was evaluated in spontaneous conditions as well as in the presence of an inducing agent, mitomycin C.

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 from 18% to 89% of STEC isolated from cattle or wastewaters and belonging to a wide variety of serotypes contained inducible Stx2 phages (35, 36).

According to previous work, the concentrations of phage DNA obtained after induction are inversely proportional to the optical densities of the culture (35). This was also the case here although several exceptions were observed. For example, for strains 31132 and 21765(1) which showed the same Stx2 phage concentration of *ca* 9 $\log_{10}$ GC/ml, the OD_{600} values were 0.37 and 1.23, respectively. Conversely, for strains VTH7 and H19 whose OD_{600} values were similar (i.e. 0.88 and 0.78 respectively), H19 produced hundred times more Stx1 phage than VTH7. Although OD_{600} values might represent a good indicator of qualitative Stx phage induction, quantitative assessment using other assays seems therefore preferable.

In this study, the induction levels of Stx phages in the presence of MMC were significantly 2 $\log_{10}$ higher than those obtained from spontaneous induction, and a higher induction was observed for Stx2 phages compared to Stx1 phages. This less pronounced effect of MMC on Stx1 production was also observed by Ritchie *et al.* (37). Moreover, it has been shown previously that spontaneous induction of Stx1 phages could be increased

significantly when STEC were grown in a low-iron medium (38) although no correlation was observed between Stx1 phage induction levels obtained in low-iron medium and in the presence of MMC (37). Finally, although the levels of Stx phages evaluated here showed a high variability, there was no significant difference in the Stx phage induction levels depending on the origin of the strains (human *versus* dairy), whatever the presence or absence of MMC. No difference were observed either according to the Stx phage insertion sites, except for the spontaneous induction which was higher for Stx phages inserted into *yecE versus wrbA* and *yehV*. Interestingly, strains with Stx2 phage integrated into *yecE* were described previously as highly virulent (13).

Enumeration of Stx phages demonstrated that 13 out of 14 strains generated infectious phage particles capable of producing plaques on the *E. coli* DH5α recipient strain. As some Stx phages were also unable to generate isolated plaques, improvements of our protocol could be considered, as described by Islam *et al.* (39). In addition, the phage titers determined by plaque enumeration were lower than the concentrations of phage genomes determined by qPCR suggesting that only a fraction of phage particles were infectious, the remaining ones corresponding to defective particles which could not generate plaques. Nevertheless, despite this difference, the relative levels of Stx phages produced by the STEC O26:H11 strains and determined by both methods were in agreement.

When considering the infectivity of different *stx*-negative *E. coli* O26:H11 strains towards a group of six Stx phages from STEC O26:H11, variability in sensitivity was observed as one *stx*-negative *E. coli* strain (64.36) was not infected by any six Stx phages while, at the opposite, three strains (191.1, 5080/97 and 5021/97) were sensitive to all Stx phages. This phenomenon was previously observed by Muniesa *et al*. with different serotypes of *E. coli* including the *E. coli* serogroup O26 strains for which 1 and 7 out 11 phages tested on strain 216 and 224, respectively, resulted in positive infection (40). Interestingly, a higher sensitivity of the human strains was observed here compared to the dairy strains.

When the lysogenic conversion was investigated, lysogenic *E. coli* DH5α and MG1655 isolates could be obtained, with Stx2 phages integrated into *yecE*. However, all our attempts to lysogenize *stx*-negative *E. coli* O26:H11 strains failed. This could be due to a low frequency of lysogenization preventing the isolation of lysogenic strains on agar plates. Indeed, Bielaszewska *et al.* showed that three Stx2 phages lysogenized only two or three of six clinical *stx*-negative *E. coli* strains, with rates of lysogenization ranging from 1 x 10^{-7} to 6 x 10^{-6} per recipient cell, i.e. 10x lower than that obtained with laboratory strain *E. coli* C600. More importantly, in the same study, only one out of four Stx1 phages was successful in lysogenization, and this event occurred with only one out of six clinical *stx*-negative *E. coli* strains, at a rate similar to that of Stx2 phages (23).

The *stx*-negative *E. coli* strains tested in our study contained vacant Stx phage integration sites, indicating that availability of free insertion site was not the reason for the low lysogenization rates observed here. Interestingly, insertion of Stx phage genomes into the chromosome of an *stx*-negative *E. coli* O26:H11 strain (21474) could be demonstrated here by PCR amplification of *attL* junction at the *yecE* site within suspensions corresponding to a mixture of phage and recipient strain. This result suggested that Stx phage 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, it is also tempting to speculate that a certain degree of instability of Stx prophage might have prevented the isolation of lysogens. Indeed, although lysogenization of *stx*-negative *E. coli* such as Enteropathogenic *E. coli* (EPEC) and Enteroaggregative *E. coli* (EAEC or EAggEC) with Stx phages was shown previously (23, 41, 42), the stable Stx phage acquisition was also observed to rarely occur (23, 42).

Finally 3 types of morphology were observed by electron microscopy among the six Stx phages analyzed. Two phages (Φ3901 and ΦH19) presented an elongated (prolate) capsid and a long flexible tail and were considered as *Siphoviridae*. The Stx1 phage ΦH19 studied

here was identical to Stx1 phage H-19B isolated from the strain (H19) and described elsewhere (43). The fact that Stx2 phage Φ3901 was also similar to the Stx1 phage H-19B indicated that phages harboring either an *stx*$_2$ or an *stx*$_1$ gene in their genomes can share a similar shape, as previously observed by Muniesa *et al.* (2003) (44). The second phage morphology observed here corresponded to the family of *Siphoviridae* and was shared by an Stx1 phage (Φ11368) and two Stx2 phages (Φ5917/97 and Φ21765). This morphology was similar to that of Stx2 phages previously observed (35, 40). Finally, the third type of phage morphology was found for phage Φ2976-1 and corresponded to a *Siphoviridae* with a very long tail of about 411 nm. This type of tail of a particularly large size was observed by Hoyles *et al.* for virus-like particles corresponding to bacteriophages and associated with human faecal or caecal samples (45). Altogether 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 (36). This also confirmed the diversity of phage morphologies circulating in STEC population, and in STEC O26:H11 strains in particular.

In conclusion, Stx1 and Stx2 phages of STEC O26:H11 are characterized by a high diversity with variations observed in their induction levels, morphologies and ability to infect *E. coli* strains. Interestingly, we noted that the lysogenization of *stx*-negative *E. coli* with Stx phages was a rare event requiring more appropriate conditions to successfully isolate stable lysogens. Molecular methods such as PCRq could represent alternative assays to identify and quantify lysogens within an *E. coli* population infected by Stx phages.

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