2007

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Recommended Citation

Bielaszewska, Martina; Prager, Rita; Kock, Robin; Mellmann, Alexander; Zhang, Wenlan; Tschape, Helmut; Tarr, Phillip I.; and Karch, Helge, "Shiga toxin gene loss and transfer in vitro and in vivo during enterohemorrhagic Escherichia coli O26 infection in humans." Applied and Environmental Microbiology. 73, 10. 3144-3150. (2007).
https://digitalcommons.wustl.edu/open_access_pubs/2029

Applied and Environmental Microbiology

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***Appl. Environ. Microbiol.* 2007, 73(10):3144. DOI: 10.1128/AEM.02937-06.**
Published Ahead of Print 30 March 2007.

Applied and Environmental Microbiology, May 2007, p. 3144–3150 Vol. 73, No. 10
0099-2240/07/$08.00+0 doi:10.1128/AEM.02937-06

Shiga Toxin Gene Loss and Transfer In Vitro and In Vivo during Enterohemorrhagic *Escherichia coli* O26 Infection in Humans▽

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Received 19 December 2006/Accepted 6 March 2007

Escherichia coli **serogroup O26 consists of enterohemorrhagic *E. coli* (EHEC) and atypical enteropathogenic *E. coli* (aEPEC). The former produces Shiga toxins (Stx), major determinants of EHEC pathogenicity, encoded by bacteriophages; the latter is Stx negative. We have isolated EHEC O26 from patient stools early in illness and aEPEC O26 from stools later in illness, and vice versa. Intrapatient EHEC and aEPEC isolates had quite similar pulsed-field gel electrophoresis (PFGE) patterns, suggesting that they might have arisen by conversion between the EHEC and aEPEC pathotypes during infection. To test this hypothesis, we asked whether EHEC O26 can lose *stx* genes and whether aEPEC O26 can be lysogenized with Stx-encoding phages from EHEC O26 in vitro. The stx_2 loss associated with the loss of Stx2-encoding phages occurred in 10% to 14% of colonies tested. Conversely, Stx2- and, to a lesser extent, Stx1-encoding bacteriophages from EHEC O26 lysogenized aEPEC O26 isolates, converting them to EHEC strains. In the lysogens and EHEC O26 donors, Stx2-converting bacteriophages integrated in *yecE* or *wrbA*. The loss and gain of Stx-converting bacteriophages diversifies PFGE patterns; this parallels findings of similar but not identical PFGE patterns in the intrapatient EHEC and aEPEC O26 isolates. EHEC O26 and aEPEC O26 thus exist as a dynamic system whose members undergo ephemeral interconversions via loss and gain of Stx-encoding phages to yield different pathotypes. The suggested occurrence of this process in the human intestine has diagnostic, clinical, epidemiological, and evolutionary implications.**

Escherichia coli serogroup O26 has members classified as enterohemorrhagic *E. coli* (EHEC) or atypical enteropathogenic *E. coli* (aEPEC). EHEC O26 strains constitute the most common non-O157 EHEC group associated with diarrhea and hemolytic uremic syndrome (HUS) in Europe (16, 18, 19, 25, 48, 51). EHEC O26 is also the most common non-O157 EHEC serogroup in the United States, where, between 1983 and 2002, it accounted for 22% of non-O157 EHEC clinical isolates (10). In a recent prospective study from Montana, half of EHEC O26 isolates originated from patients with bloody diarrhea (23). Moreover, EHEC O26 has spread globally (24).

EHEC O26 strains produce Shiga toxin 1 (Stx1) and Stx2, either singly or together (10, 54). Indeed, phage H19B from a clinical EHEC O26 isolate that carries stx_1 was one of the first Stx-converting phages described (45). Moreover, these strains contain the intimin-encoding *eae* gene (6, 54), an important characteristic of EHEC (33). EHEC O26 represents a highly dynamic group of organisms that rapidly engender new pathogenic clones (54). This is exemplified by emergence of a novel EHEC O26:H11 clonal subgroup in Germany in the 1990s that possessed stx_2 as the sole *stx* gene, in contrast to stx_1, exclusively identified in EHEC O26 previously. The pathogenicity of this clone was demonstrated by its strong association with HUS (29, 54) and its ability to spread rapidly (2, 54).

aEPEC O26 strains do not harbor *stx* genes (9, 20, 42) but share with EHEC the *eae* gene (20, 34) and the ability to produce attaching and effacing lesions in intestinal epithelial cells via actin rearrangement (9, 20, 42). Unlike typical EPEC strains (49), aEPEC O26 strains lack the EPEC adherence factor plasmid (6) encoding bundle-forming pili that mediate localized adherence on cultured epithelial cells. The absence of the EPEC adherence factor plasmid is a common feature of aEPEC strains, which cause gastroenteritis in children (12, 20, 49).

It has been hypothesized (15) that aEPEC O26 is ancestral to EHEC O26. According to this hypothesis, the acquisition of stx_1 by aEPEC O26 gave rise to globally distributed toxigenic EHEC O26 (15). Furthermore, replacement of stx_1 with stx_2 has been postulated as the cause of the recent emergence of the new stx_2-harboring EHEC O26 clonal subgroup in Europe (15, 54). A prerequisite for such an evolutionary process is that aEPEC O26 strains undergo lysogeny by Stx-encoding bacteriophages. However, this has not yet been systematically investigated. Moreover, it is not clear if the sequence of events proposed for the evolution of EHEC O26 is unidirectional, where aEPEC O26 strains are always progenitors of EHEC O26 strains, or bidirectional, with EHEC O26 also being converted to aEPEC by loss of an *stx* gene. Therefore, we investigated the role of Stx-encoding bacteriophages in the postulated transition between EHEC and aEPEC O26 to determine if (i) Stx-encoding phages originating from EHEC O26 lysogenize aEPEC O26 under laboratory conditions, (ii) *stx* genes

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▽ Published ahead of print on 30 March 2007.

and their encoding phages are lost by EHEC O26 in vitro, (iii) the loss and gain of Stx-encoding phages influence the genomic architecture, (iv) there is an identifiable site where these bacteriophages integrate into the genomes of EHEC O26 and the lysogens, and (v) there is bidirectional conversion between EHEC O26 and aEPEC O26 during human infection.

MATERIALS AND METHODS

Bacterial strains. Three EHEC (stx_2-positive, *eae*-positive, *bfpA*-negative) and three aEPEC (*stx*-negative, *eae*-positive, *bfpA*-negative) O26:H11 strains were isolated from initial stools (collected 5 days after the onset of diarrhea) and follow-up stools (collected 9 days after the initial samples), respectively, of three children (13, 16, and 17 months old) during an outbreak of HUS in Germany (29). The other EHEC and aEPEC O26:H11 strains used in transduction experiments were isolated from patients between 1971 and 1999; they were epidemiologically unrelated except for EHEC strain 46 and aEPEC strain 47 (Table 1), the latter being a spontaneous stx_2-negative laboratory derivative of the former. The donors and recipients of Stx-encoding phages were selected from our strain collection to contain strains with related as well as dissimilar pulsed-field gel electrophoresis (PFGE) patterns. *E. coli* strain C600(ϕH19B), which contains Stx1-converting phage H19B from a clinical EHEC O26:H11 isolate H19 (44), was described previously (45).

PCR techniques. PCRs were performed in an iCycler (version 1.259; Bio-Rad, München, Germany) or a Biometra TGradient 96 cycler (Biometra GmbH, Göttingen, Germany) (46) using reagents from PEQLAB Biotechnologie (Erlangen, Germany) and primers synthesized by Sigma Genosys (Haverhill, United Kingdom). stx_1, stx_2, *eae*, and *bfpA* (encoding the structural subunit of bundle-forming pili) were detected using published protocols (6, 18). The chromosomal loci that serve as integration sites for Stx-encoding phages in *E. coli* O157 were interrogated using primer pairs A-B (*yehV*) (43), wrbA1-wrbA2 (*wrbA*) (47), EC10-EC11 (*yecE*) (14), and sbcB1-sbcB2 (*sbcB*) (47). The linkage between *yecE* and the integrase gene (*int*) of stx_2-harboring bacteriophage $\phi 258_{320}$, which integrates in *yecE* in *E. coli* O157 (7), was tested using primers Int-258_{320} and EC11 (7). The linkage between *wrbA* and the *int* gene of stx_2-harboring bacteriophage ϕ933W, which integrates in *wrbA* in *E. coli* O157:H7 strain EDL933 (35, 37), was investigated using primers WrbA (5′-CGCCATCCACTTTGCTTG-3′) and Int933W (5′-TATGCTACCGAGGCTTGG-3′); the PCR consisted of 30 cycles of denaturing (94°C, 30 s), annealing (55°C, 1 min), and extension (72°C, 90 s) followed by a final extension (72°C, 5 min). The specificity of PCR products was confirmed by analyzing the sequence of representative amplicons as described below.

PFGE and Southern hybridization. PFGE was performed using the PulseNet protocol (22) and with XbaI-digested DNA of *Salmonella enterica* serovar Braenderup strain H9812 (22) as a standard. Restriction patterns were analyzed with BioNumerics, version 4.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). XbaI-digested, PFGE-separated genomic DNAs were hybridized with a digoxigenin-11-dUTP-labeled (DIG High Prime kit; Roche Molecular Biochemicals, Mannheim, Germany) $stxA_2$ probe (7).

MLST. Internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) were analyzed using a published multilocus sequence typing (MLST) scheme for *E. coli* (52), except for a newly designed forward primer for *icd* (5′-CCGATTATCCCTTACATTGAAG-3′), which is 79 bp downstream of the original primer. Because of optimized proximity to the analyzed region of *icd*, the sequence trace quality was substantially higher without any ambiguous base callings. After purifying the PCR products, we sequenced both strands in 10 μl containing 0.5 μl premix (ABI Prism BigDye Terminator v3.1 Ready Reaction cycle sequencing kit; Applied Biosystems, Darmstadt, Germany), 1.8 μl 400 mmol/liter Tris-HCl, 10 mmol/liter $MgCl_2$, 10 pmol sequencing primer, and 2 μl PCR product. Sequencing products were purified (Centri-Sep spin columns; Princeton Separations, Adelphia, NJ) and analyzed with the ABI Prism 3100 Avant genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. The alleles and sequence types (ST) were assigned in accordance with the *E. coli* MLST website (http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli).

Induction of Stx-encoding phages and transduction experiments. Stx-encoding bacteriophages were induced using mitomycin C (Sigma-Aldrich, Deisenhofen, Germany) (41) from six wild-type EHEC O26 isolates that contained stx_1 or stx_2 and from strain C600(ϕH19B) (Table 1). To isolate *stx*-harboring phages, sterile filtrates of induced bacterial cultures were subjected to a plaque assay using *E. coli* C600 as an indicator (41); plaques were PCR screened for stx_1 or stx_2 using primer pair KS7-KS8 or LP43-LP44 (18), respectively. *stx*-harboring phages

TABLE 1. Ability of Stx-encoding phages from EHEC O26:H11 to lysogenize aEPEC O26:H11 and *E. coli* K-12 strain C600

EHEC O26 phage donor[a]			Result (frequency) of transduction of aEPEC O26 phage recipient[b,c,f]					Result (frequency) of transduction of *E. coli* C600 recipient[d]
Strain (phage)[e]	Diagnosis or status[c,d]	*stx* gene	10 (D)	15 (D)	22 (D)	47 (LD)	32 (D)	40 (HUS)	
54(ϕ54)	D	stx_1	—	—	—	—	—	—	C600(ϕ54) (1×10^{-6})
49(ϕ49)	D	stx_1	—	—	—	—	—	—	—
51(ϕ51)	HUS	stx_1	—	—	—	—	—	—	C600(ϕ51) (2×10^{-6})
C600(ϕH19B) (ϕH19B)	LS	stx_1	10(ϕH19B) (1×10^{-7})	—	—	—	—	—	C600(ϕH19B) (8×10^{-5})
46(ϕ46)	HUS	stx_2	—	—	—	47(ϕ46) (1×10^{-6})	32(ϕ46) (6×10^{-6})	—	C600(ϕ46) (3×10^{-5})
61(ϕ61)	HUS	stx_2	10(ϕ61) (3×10^{-7})	—	—	—	32(ϕ61) (1×10^{-7})	—	C600(ϕ61) (7×10^{-6})
50(ϕ50)	HUS	stx_2	—	15(ϕ50) (1×10^{-7})	22(ϕ50) (3×10^{-7})	—	—	40(ϕ50) (6×10^{-7})	C600(ϕ50) (4×10^{-6})

[a] Six phage donors are clinical EHEC O26:H11 isolates from our laboratory; *E. coli* strain C600(ϕH19B) (45) contains stx_1-harboring phage ϕH19B from a clinical EHEC O26 isolate H19 (44).

[b] Five phage recipients (strains 10, 15, 22, 32, and 40) are clinical aEPEC O26 isolates, all *stx* negative; aEPEC strain 40 was isolated from a follow-up stool of a HUS patient (patient A) whose initial stool had EHEC strain 50; strain 47 is an stx_2-negative laboratory derivative of EHEC strain 46 that fits the definition of a EPEC.

[c] Data are results of transduction of the respective recipient with the respective phage; lysogen designations are given as the number of the recipient strain (number of the transducing phage). Data in parentheses are rates of transduction (number of lysogens per recipient cell). —, no lysogens were identified.

[d] *E. coli* K-12 strain C600 was a positive control for phage transduction.

[e] *stx*-harboring bacteriophages were designated by numbers of the donor strains.

[f] D, diarrhea; HUS, hemolytic uremic syndrome; LD, laboratory derivative; LS, laboratory strain.

were propagated from single PCR-positive plaques (40). The resulting lysates contained the phages at titers between 2×10^7 and 3.1×10^8 PFU/ml, as determined by plaque assay (41). In transduction experiments, 10^4 PFU of each phage was mixed with 100 μl of log-phase culture (10^7 CFU) of each aEPEC O26 recipient or *E. coli* C600 and 125 μl of 0.1 M $CaCl_2$ solution and incubated for 2 h at 37°C without shaking. The mixtures were then transferred into 4 ml of Luria-Bertani (LB) broth and incubated at 37°C and 180 rpm for 24 h. The cultures (100 μl) were then streaked on LB agar, and overnight bacterial growths that had been harvested into 1 ml of saline were PCR screened for stx_1 or stx_2. Tenfold dilutions of PCR-positive cultures were tested for lysogens using an Stx immunoblot assay (Shiga toxin [verocytotoxin] immunoblot; Sifin, Berlin, Germany). To identify stable lysogens, Stx-producing colonies were subcultured three times on LB agar, and the presence of *stx* genes was confirmed by PCR after the third passage.

Loss of stx in vitro. A single colony of an stx_2-positive EHEC O26 strain was suspended in 50 μl of sterile saline, and 2.5 μl was used to confirm the presence of stx_2 by PCR. Another 5 μl was inoculated into 5 ml of Trypticase soy broth and incubated overnight at 37°C. Tenfold dilutions of the liquid culture were then inoculated onto sorbitol MacConkey agar, and after overnight incubation, 30 to 60 colonies from plates with 150 to 200 well-separated colonies were PCR screened for stx_2. The frequency of stx_2 loss was expressed as the percent stx_2-negative colonies among the total number of colonies tested.

Stx production. Stx1 and Stx2 production was determined using a commercial latex agglutination assay (verotoxin-producing *E. coli* reverse passive latex agglutination; Denka Seiken Co., Tokyo, Japan). Stx cytotoxicity titers were assessed by the Vero cell assay (26).

RESULTS

EHEC and aEPEC O26:H11 strains in consecutive stools collected from patients. During an outbreak of HUS in Germany in 1999, stools from three infected children contained EHEC O26:H11 (stx_2 positive, *eae* positive, *bfpA* negative) in their initial samples and aEPEC O26:H11 (*stx* negative, *eae* positive, *bfpA* negative) in follow-up samples. All six isolates belonged to ST 29 and had similar but not identical PFGE patterns (Fig. 1, lanes 1 to 6). Specifically, EHEC and aEPEC isolates from consecutive stools of individual patients differed by two to five bands; one of these variant bands was always a 550-kb XbaI fragment that contains stx_2 in all EHEC isolates (Fig. 1, lanes 1, 3, and 5) but which is absent from all aEPEC isolates (Fig. 1, lanes 2, 4, and 6). The similarities in the PFGE patterns of the consecutive EHEC and aEPEC isolates from each patient, and the fact that one of the differences was the presence or absence of the genomic fragment containing stx_2, suggested that aEPEC strains were derived from the EHEC strains by the loss of stx_2 in these patients.

Loss of stx_2 in vitro. To test this hypothesis, two EHEC O26 outbreak isolates (strain 50 from patient A and strain 140 from patient B) were tested for the stability of stx_2 in vitro. Both lost stx_2 in each of three independent experiments, with the frequency of the loss ranging from 10 to 14% of colonies tested on first subculture. The ease with which these EHEC strains lost stx_2 in vitro makes it plausible that stx_2 loss might have also occurred during infection in the human host, giving rise to the aEPEC O26 strains isolated from the follow-up stools.

Transduction of aEPEC O26 with Stx1- and Stx2-encoding phages from EHEC O26. To determine if conversion between EHEC and aEPEC O26 is bidirectional, we investigated the ability of Stx-encoding phages from EHEC O26 to lysogenize aEPEC O26. High-titer phage lysates from three EHEC O26:H11 strains harboring stx_2 only, three EHEC O26:H11 strains harboring stx_1 only, and stx_1-harboring *E. coli* strain C600(ϕH19B) were used to infect six aEPEC O26:H11 strains and *E. coli* C600. Stable lysogens were identified based on their ability to retain *stx* genes after three passages on LB agar (Table 1). Three of the four Stx1-encoding phages lysogenized *E. coli* C600, but only phage ϕH19B formed lysogens with one of the aEPEC O26 strains. In contrast, each of the three Stx2-encoding phages from EHEC O26 lysogenized, in addition to *E. coli* C600, at least two of the six aEPEC O26 recipients. Each of the aEPEC O26 recipients could be lysogenized with at least one of the Stx2-encoding phages (Table 1). These phages lysogenized aEPEC recipients with PFGE patterns related to those of the phage donors as well as aEPEC recipients with distant PFGE patterns. Notably, aEPEC strain 40, from the follow-up stool specimen of patient A, was lysogenized with an Stx2-encoding phage from EHEC strain 50, which was isolated from the initial stool of this patient [lysogen 40(ϕ50)] (Table 1). Similarly, aEPEC strain 47, an *stx*-negative laboratory derivative of EHEC strain 46, could be lysogenized with the Stx2-encoding phage originating in the parental EHEC O26 strain 46 [lysogen 47(ϕ46)] (Table 1). The rates of transduction of aEPEC strains with the three different stx_2-harboring phages ranged from 1×10^{-7} to 6×10^{-6} per recipient cell; *E. coli* C600 was transduced with each respective phage at a rate that was approximately 10-fold greater (Table 1). Phage ϕH19B transduced aEPEC strain 10 and *E. coli* C600 at a rate similar to that of stx_2-harboring phages (Table 1).

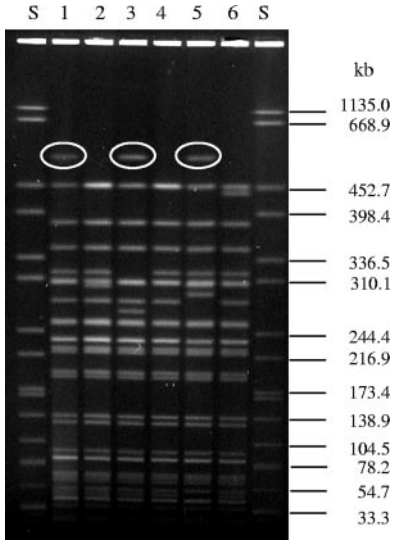

FIG. 1. XbaI-digested genomic DNA from EHEC and aEPEC O26:H11 strains isolated from initial and follow-up stools, respectively, of three patients during a HUS outbreak. Lane 1, EHEC O26 (strain 50), patient A; lane 2, aEPEC O26 (strain 40), patient A; lane 3, EHEC O26 (strain 140), patient B; lane 4, aEPEC O26 (strain 41), patient B; lane 5, EHEC O26 (strain 141), patient C; lane 6, aEPEC O26 (strain 42), patient C; lanes S, molecular size standards (*S. enterica* serovar Braenderup strain H9812; Centers for Disease Control and Prevention, Atlanta, GA). XbaI fragments containing stx_2 as demonstrated by hybridization with an $stxA_2$ probe are circled.

Stx production by the lysogens. All lysogens from aEPEC O26 produced Stx1 or Stx2, depending on the donor. Moreover, supernatants of all lysogens were toxic to Vero cells at dilutions between 1:256 and 1:2,048; the lysogen Stx titers were comparable to those of the phage donors (1:512 to 1:2,048). Thus, aEPEC O26:H11 strains can be converted to EHEC

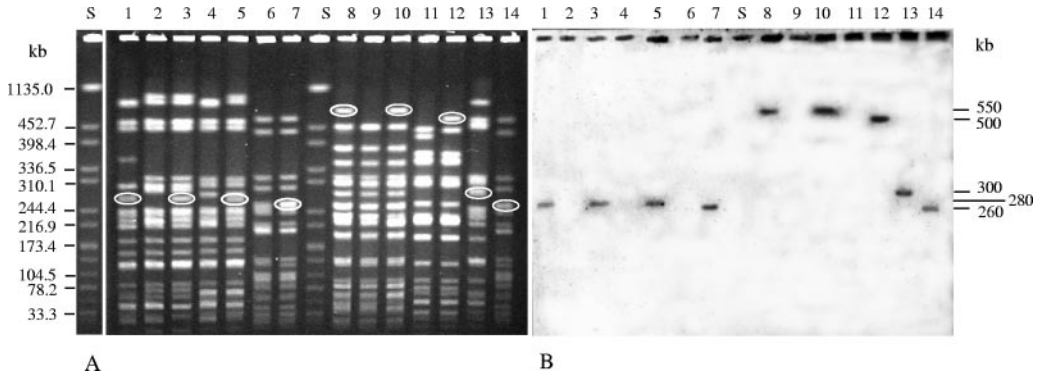

FIG. 2. PFGE (A) and $stxA_2$ hybridization (B) of XbaI-digested genomic DNA from EHEC O26 phage donors, aEPEC O26 recipients, and lysogens transduced with stx_2-harboring bacteriophages from EHEC O26. Lanes 1, EHEC O26 strain 46 (donor of phage ϕ46); lanes 2, aEPEC O26 strain 47; lanes 3, lysogen 47(ϕ46); lanes 4, aEPEC O26 strain 32; lanes 5, lysogen 32(ϕ46); lanes 6, *E. coli* strain C600; lanes 7, lysogen C600(ϕ46); lanes 8, EHEC O26 strain 50 (donor of phage ϕ50); lanes 9, aEPEC O26 strain 40; lanes 10, lysogen 40(ϕ50); lanes 11, aEPEC O26 strain 22; lanes 12, lysogen 22(ϕ50); lanes 13, EHEC O26 strain 61 (donor of phage ϕ61); lanes 14, lysogen C600(ϕ61); lanes S, molecular size standards (*S. enterica* serovar Braenderup strain H9812; Centers for Disease Control and Prevention, Atlanta, GA). The XbaI fragments that hybridized with the $stxA_2$ probe are circled in panel A, and their sizes are given in panel B.

O26:H11 strains that produce active Stx via transduction with Stx-encoding bacteriophages from EHEC O26:H11.

Genomic positions of stx_2 genes in EHEC O26 donors and lysogens. To compare genomic positions of stx_2 genes in the EHEC donors and lysogens, XbaI-digested, PFGE-separated DNA was hybridized with an $stxA_2$ probe (Fig. 2). In lysogens derived from aEPEC strains that had PFGE patterns related to that of the respective EHEC phage donor (Fig. 2A, lanes 1 to 5 and lanes 8 to 10), stx_2 was located on the same XbaI fragment as in EHEC (Fig. 2B, lanes 1, 3, and 5 and lanes 8 and 10). In a lysogen (Fig. 2A, lane 12) derived from an aEPEC strain (Fig. 2A, lane 11) that differed in PFGE pattern from the EHEC donor (Fig. 2A, lane 8), the stx_2 genomic position (Fig. 2B, lane 12) differed from the one in the donor (Fig. 2B, lane 8). In *E. coli* C600 transduced with phage ϕ46 [lysogen C600(ϕ46)] (Fig. 2B, lane 7) or with phage ϕ61 [lysogen C600(ϕ61)] (Fig. 2B, lane 14), stx_2 was on a 260-kb XbaI fragment. In contrast, *E. coli* C600 transduced with phage ϕ50 contained stx_2 on a 440-kb XbaI fragment (data not shown). Thus, stx_2-harboring phages excised from their integration sites in the genomes of EHEC O26 donors integrate into the same locus in the aEPEC O26 transductants, and there are at least two different integration sites for stx_2-harboring phages in the genomes of EHEC O26 and corresponding lysogens.

Integration sites of Stx2-encoding phages in EHEC O26: H11. In EHEC O26 strains 46 and 61, *yecE* was occupied by foreign DNA (Fig. 3, lanes 4 and 5), but this locus was intact in an stx_2-negative laboratory derivative of strain 46, strain 47 (Fig. 3, lane 8). Moreover, strains 46 and 61 produced an amplicon of 425 bp in PCR linking *yecE* with the *int* gene of

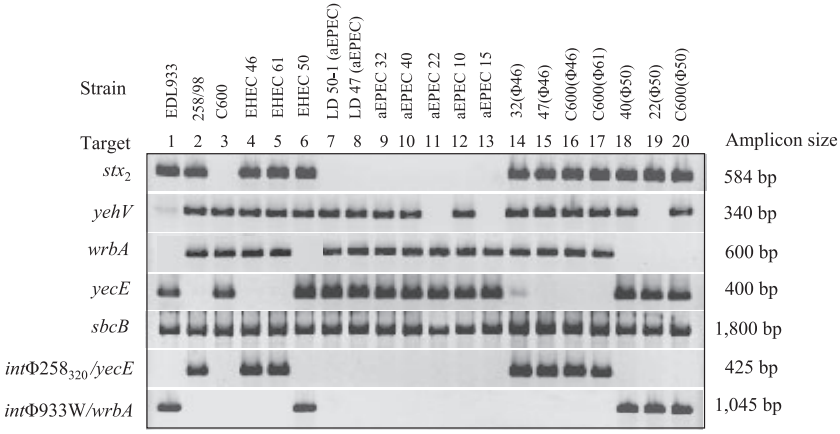

FIG. 3. PCR analyses of phage integration sites in EHEC O26, aEPEC O26, and lysogens. Strains tested, loci examined, and lengths of resulting amplicons are listed across the top and to the left and right of the rows of amplicons, respectively. stx_2-negative laboratory derivatives (LD) 47 and 50-1 fit the definition of aEPEC. Strains EDL933 (stx_1- and stx_2-harboring phages integrated in *yehV* and *wrbA*, respectively) (35, 37), 258/98 (stx_2-harboring phage $\phi 258_{320}$ integrated in *yecE*) (7), and *E. coli* K-12 C600 (all the genes investigated as putative phage integration sites intact) (8) were used as controls. In PCRs targeting *yehV*, *wrbA*, *yecE*, and *sbcB*, the presence of an amplicon indicates that the locus is intact, whereas the absence of an amplicon (or a very weak amplicon) indicates that the locus is occupied by foreign DNA. In PCRs connecting *yecE* with the *int* gene of phage $\phi 258_{320}$ and *wrbA* with the *int* gene of phage ϕ933W (rows 6 and 7, respectively), the presence of an amplicon indicates that a phage with a homologous *int* gene is integrated in the respective locus; the absence of an amplicon indicates the absence of such a phage.

$\phi 258_{320}$ (Fig. 3, lanes 4 and 5), which integrates in *yecE* (7), demonstrating that the Stx2-encoding phages in these strains are integrated in *yecE*. In contrast, in EHEC strain 50, the *wrbA* insertion site was occupied (Fig. 3, lane 6), but *wrbA* was intact in its stx_2-negative laboratory derivative, strain 50-1 (Fig. 3, lane 7). Strain 50 (Fig. 3, lane 6), but not strain 50-1 (Fig. 3, lane 7), produced an amplicon of 1,045 bp in PCR linking *wrbA* with the *int* gene of ϕ933W, which integrates in *wrbA* (35, 37). The Stx2-encoding phage in EHEC strain 50 is thus integrated in *wrbA*.

Integration sites of Stx2-converting phages in lysogens. *yecE* and *wrbA* were intact in all aEPEC strains (Fig. 3, lanes 8 to 13) and a control *E. coli* C600 strain (Fig. 3, lane 3). The acquisition of phage ϕ46 or ϕ61 resulted in occupation of *yecE* and a positive signal in PCR linking *yecE* with *int* of $\phi 258_{320}$ in lysogens arising from aEPEC (Fig. 3, lanes 14 and 15) and from *E. coli* C600 (Fig. 3, lanes 16 and 17). In contrast, phage ϕ50 integrated in *wrbA* in the aEPEC and *E. coli* C600 lysogens (Fig. 3, lanes 18 to 20). Thus, Stx2-encoding phages integrate in EHEC O26 in more than one locus, including *yecE* and *wrbA*. These loci are intact in aEPEC O26 and represent hot spots for the integration of such phages into the genomes and the conversion of aEPEC O26 to EHEC O26.

Impact of loss and gain of Stx-converting bacteriophages on PFGE patterns. As demonstrated in Fig. 2A, loss and gain of Stx2-converting phages diversifies PFGE patterns, even in organisms that are epidemiologically related or are derivatives of the same isolate.

DISCUSSION

Several non-O157 EHEC serogroups are important causes of human disease, and the most common of these is O26 (10, 24, 25). We demonstrate that stx_2- and stx_1-harboring phages from EHEC O26 can transduce aEPEC O26 strains, converting them to stable lysogens that produce Stx in amounts comparable to that produced by wild-type EHEC O26. Conversely, EHEC O26 can lose *stx* genes at appreciable frequencies, thereby reverting to being aEPEC. EHEC O26 and aEPEC O26 represent a dynamic system in which bidirectional conversion yields different pathotypes; Stx-encoding bacteriophages are the major elements facilitating this conversion. Data from in vitro experiments correlate with observations obtained with patients infected with *E. coli* O26, in whom we observed the frequent loss (reference 28 and this study) as well as gain (though at considerably lower frequencies) (H. Karch, unpublished data) of stx_2 genes.

EHEC O26:H11 and aEPEC O26:H11 belong to a common phylogenetic group, EHEC 2 (15). It has been hypothesized that aEPEC O26:H11 is ancestral to the EHEC 2 group (15). According to this hypothesis, the acquisition by the ancestral cell of *stx* genes, the EHEC plasmid, and a high-pathogenicity island that encodes an iron uptake system gave rise to EHEC O26:H11 (15). However, aEPEC O26:H11 already possesses a high-pathogenicity island and the EHEC plasmid (6) and differs from EHEC O26 most notably by the absence of *stx* genes (6). This observation and our data support the concept of interconversions between aEPEC O26 and EHEC O26 involving loss as well as gain of Stx-encoding bacteriophages. Such bidirectional conversion in nature is also evidenced by the fact that EHEC O26 and aEPEC O26 from different sources have closely related core genomes (3) and highly conserved housekeeping genes (39, 52) and share multiple non-Stx virulence and fitness genes (6). A proposed progenitor of these pathogenic *E. coli* O26 strains has yet to be identified.

Loss and acquisition of stx_2-harboring bacteriophages in vitro alter the genomic architecture of *E. coli* O26, as reflected by changed PFGE patterns. This observation agrees with previous findings that the loss of *stx* genes is associated with variant PFGE patterns in EHEC O157:H7/NM (7, 17, 30). These data are also in keeping with the concept that bacteriophages are major drivers of genome diversity in *E. coli* O157:H7 (31). Therefore, we hypothesize that a diversification of the genome of the infecting EHEC O26 HUS outbreak strain via multiple losses and gains of Stx2-converting phages during human infection accounts for EHEC and aEPEC O26 strains with related, but not identical, PFGE patterns in the same individual and within the outbreak (Fig. 1). Such a possibility should be considered when PFGE is applied to epidemiological investigations of outbreaks caused by *E. coli* O26. It is noteworthy that variations in PFGE patterns caused by insertions or deletions of phage-associated DNA fragments have also been reported for *Campylobacter jejuni* (4).

The ability of Stx-encoding phages from clinical EHEC O26 and EHEC O157 isolates to transduce *E. coli* laboratory strain K-12, commensal *E. coli*, and EPEC of various origins within the gastrointestinal tract has been demonstrated in mice (1, 55), sheep (13), and house flies (*Musca domestica*) (36) in vivo and in porcine ligated ileal loops (47). These findings support the hypothesis that interconversion between aEPEC O26 and EHEC O26 can occur in the human intestine during an infection. However, conditions favoring lysogenic conversion or loss in vivo are poorly understood (47, 50, 55) and require further studies.

The ability of EHEC O26 strains to cause HUS indicates that such organisms are more virulent than aEPEC strains lacking the *stx* gene. If this is true, a change in the pathotype of the infecting *E. coli* O26 strain during an infection by the loss or acquisition of an Stx-encoding bacteriophage might have clinical implications. For example, using the paradigm that Stx causes HUS, the loss of an *stx* gene early in infection, before Stx is produced, might prevent the development of HUS in the infected patient. Furthermore, the *stx* loss by the infecting EHEC at a later stage in the infection, after Stx has already bound to the target organs and has injured the microvasculature, could confound diagnostic schemes dependent on toxin detection. Acquisition of an *stx* gene by an aEPEC O26 strain during an infection appears to be possible (Karch, unpublished) but is extremely rare. Therefore, the question of whether such an event could trigger HUS cannot be answered at present.

Three integration sites for Stx2-encoding bacteriophages have been identified in EHEC O157:H7, including *wrbA* (5, 21, 27, 35, 37, 43), *sbcB* (32), and *yecE* (14). This last locus is also a common integration site for Stx2-encoding phages in sorbitol-fermenting EHEC O157:NM strains (7), as well as an integration site for phage ϕP27, which encodes Stx2e in a non-O157 EHEC strain (38). In this study, we demonstrate for the first time that *yecE* is also an integration site for Stx2-encoding phages in EHEC O26:H11; the other is *wrbA*. Taken together,

these data indicate that *yecE* and *wrbA* may be common integration sites for Stx2-encoding bacteriophages in EHEC strains. These loci were intact in all aEPEC O26:H11 strains we investigated and thus represent hot spots where stx_2-harboring phages can enter the genomes of aEPEC O26, converting them to EHEC.

A remaining question is why all three Stx2-encoding phages, but only one of four Stx1-encoding phages (phage ϕH19B), lysogenized aEPEC O26. In general, the susceptibility of a wild-type strain to lysogenization by a phage depends on the presence of the phage receptor, the absence of a highly related bacteriophage within the host that confers immunity against the infecting phage, and the availability of a free integration site for the infecting phage in the genome of the recipient. One reason why wild-type aEPEC strains were not so easily lysogenized by Stx1-encoding phages might be their lack of receptors for such phages or the presence of cryptic prophages that mediate immunity (11). It is possible that such cryptic or truncated bacteriophages occupy a genomic integration site(s) for stx_1-harboring phages in aEPEC O26, as has been recently shown in EHEC O157:H7 (5, 43). Although the integration site for stx_1-harboring phages in EHEC O26 is unknown, it is noteworthy that in two of the six aEPEC recipients we examined, *yehV*, an integration site for stx_1-harboring phages in EHEC O157:H7 (5, 35, 53), was already occupied by DNA of unknown origin. Future studies should examine the factors promoting transduction of aEPEC O26 by Stx1-converting phages from EHEC O26 and determine the nature of the phage integration site(s).

ACKNOWLEDGMENTS

This work was supported by a grant from the EU Network of Excellence EuroPathoGenomics (number LSHB-CT-2005-512061), by a grant from the EU Network ERA-NET PathoGenoMics (project number 0313937C), and by NIH grant AI47499.

The skillful technical assistance of Dagmar Mense, Nadine Brandt (Münster), and Gerlinde Bartel and Evelyn Skiebe (Wernigerode) is greatly appreciated.

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