

## Transduction of Porcine Enteropathogenic *Escherichia coli* with a Derivative of a Shiga Toxin 2-Encoding Bacteriophage in a Porcine Ligated Ileal Loop System

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Received 26 February 2003/Accepted 4 September 2003

**In this study, we have investigated the ability of detoxified Shiga toxin (Stx)-converting bacteriophages  $\Phi$ 3538 ( $\Delta$ stx<sub>2</sub>::cat) (H. Schmidt et al., *Appl. Environ. Microbiol.* 65:3855–3861, 1999) and H-19B::Tn10d-bla (D. W. Acheson et al., *Infect. Immun.* 66:4496–4498, 1998) to lysogenize enteropathogenic *Escherichia coli* (EPEC) strains in vivo. We were able to transduce the porcine EPEC strain 1390 (O45) with  $\Phi$ 3538 ( $\Delta$ stx<sub>2</sub>::cat) in porcine ligated ileal loops but not the human EPEC prototype strain E2348/69 (O127). Neither strain 1390 nor strain E2348/69 was lysogenized under these in vivo conditions when *E. coli* K-12 containing H-19B::Tn10d-bla was used as the stx1 phage donor. The repeated success in the in vivo transduction of an Stx2-encoding phage to a porcine EPEC strain in pig loops was in contrast to failures in the in vitro trials with these and other EPEC strains. These results indicate that in vivo conditions are more effective for transduction of Stx2-encoding phages than in vitro conditions.**

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains have emerged as serious food-borne pathogens worldwide that caused large-scale outbreaks of intestinal diseases in developed countries (13, 19). Enterohemorrhagic *E. coli* (EHEC) O157:H7 is the predominant STEC serotype in the United States (12), United Kingdom (4), and Japan (46), frequently causing a broad spectrum of disease in humans ranging (12) from mild diarrhea to hemorrhagic colitis (36), the latter of which can progress to the hemolytic-uremic syndrome (HUS) (20), whereas other non-O157 serotypes (18) are predominant in continental Europe (4). Systemic disease, such as edema, may also develop as a result of STEC infections in swine (6). Most EHEC O157 strains produce one or more Stx, which are thought to be the major pathogenicity factors of these organisms. Shiga toxins comprise a family composed of Stx1, Stx2, and their variants (10, 11, 16, 23, 31, 34, 40, 48), which can be found in STEC strains isolated from either humans or animals (16, 32, 33).

The stx genes are part of the genome of temperate lambdaoid phages, which are integrated in the chromosome of the bacterial host. At present, the ability to produce Stx has been assigned to more than 200 *E. coli* serotypes, which have been isolated from patients, healthy humans (27), animals, food (7), and water (25). Stx production was observed also in other members of the *Enterobacteriaceae*, including *Citrobacter freundii* (39, 44) *Enterobacter cloacae* (30), *Shigella sonnei* (42), and *Shigella dysenteriae* I (43).

In addition to Stx-converting phages, EHEC strains contain

further mobile genetic elements. These include a chromosomal pathogenicity island termed locus of enterocyte effacement (LEE), which was first described in the enteropathogenic *E. coli* (EPEC) O127 strain E2348/69 (21) and a large virulence plasmid (15, 32). LEE encodes proteins responsible for the intimate adherence of EHEC to the intestinal mucosa and for the subsequent characteristic destruction of the microvilli, termed attaching and effacing lesions (24). Sequencing of the *E. coli* O157:H7 genome revealed the presence of additional pathogenicity islands, the function of which has yet to be elucidated (15, 32).

Similar to EHEC, EPEC strains also possess LEE, but in contrast to EHEC, the EPEC strains do not produce Stx and are not associated with HUS. Nevertheless, they are a major cause of infant diarrhea in nonindustrialized countries and are pathogenic to several animal species (27). Clonal analysis derived from multilocus enzyme electrophoresis suggests that *E. coli* O157:H7 evolved from a progenitor strain with the serotype O55:H7 (8, 47). Furthermore, the nearly identical sequences of the H7 flagellin gene (35) and *eae* alleles (22) demonstrate the close relationship between *E. coli* O157:H7 and *E. coli* O55:H7.

The dissemination of Stx-encoding phages is the most likely mechanism for the emergence of new STEC serotypes and the intergeneric spread of stx genes. Evidence for this has been provided by Schmidt et al. (38), who were able to lysogenize in vitro a variety of enteric *E. coli*, including two EPEC strains with a chloramphenicol acetyltransferase (*cat*) gene-labeled detoxified derivative of an Stx2-encoding phage isolated from *E. coli* O157. James et al. (17) were also able to lysogenize different wild-type *E. coli* and *Shigella* strains in vitro by using a kanamycin resistance (*aph3*) gene-labeled Stx2-encoding

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TABLE 1. Description of *E. coli* strains used in this study

Strain	Description	Reference
3538 ( $\Delta$ stx <sub>2</sub> ::cat)	Human <i>E. coli</i> O157:H7 strain harboring the detoxified phage $\phi$ 3538 (stx <sub>2</sub> ::cat)	38
MC4100 H19B-Ap-1	<i>E. coli</i> K-12 strain harboring the detoxified phage H-19B::Tn10d-bla	2
1390	Porcine EPEC strain of serogroup O45	49
E2348/69	Human EPEC strain of serotype O127:H6:K1	21
355	Rabbit EPEC strain of serogroup O15	Present study
C600	Laboratory strain	37

phage derivative originated from an *E. coli* O157 strain. Acheson et al. (2) were able to transduce a laboratory strain in the murine gastrointestinal tract with a derivative of phage H19B encoding Stx1. However, there have been so far no data about in vivo transduction of stx<sub>2</sub> genes into wild-type *E. coli* strains.

The aim of this study was to establish an in vivo transduction model for wild-type *E. coli* strains and to examine the putative role of EPEC for the evolution of EHEC by acquisition of stx<sub>2</sub> genes by utilizing a pig ligated ileal loop system.

#### MATERIALS AND METHODS

**Bacterial strains and bacteriophages.** The *E. coli* strains used in this study are listed in Table 1. Three EPEC strains were chosen with different O antigens which were isolated from different sources. Porcine EPEC strain 1390 belonged to serogroup O45, rabbit *E. coli* strain 355 belongs to serogroup O15, and the human EPEC reference strain E2348/69 belonged to serotype O127:H6:K1 (21). None of these wild-type strains carried the stx<sub>1</sub> or stx<sub>2</sub> gene. Strain E2348/69 carried EPEC adherence factor (EAF) and was bfp-positive (see Table 4). *E. coli* strain MC4100 harboring detoxified Stx1 phage H-19B::Tn10d-bla was kindly provided by David Acheson, Boston, Mass. (2). Porcine EPEC O45 strain 1390 was kindly provided by John Fairbrother, Montreal, Canada (49), and was recognized as a colicin producer. The *E. coli* K-12 derivative C600 was used as a host strain for the propagation of phages. Phages  $\Phi$ 3538( $\Delta$ stx<sub>2</sub>::cat) and H-19B::Tn10d-bla were induced by mitomycin C and purified as previously described (38). High-titer stock lysates of  $\Phi$ 3538( $\Delta$ stx<sub>2</sub>::cat) and H-19B::Tn10d-bla phage were propagated from single plaques according to the method described by Sambrook et al. (37) for phage  $\lambda$ .

**Selection of rifampin-resistant *E. coli* mutants.** Selection of spontaneous rifampin-resistant mutants was performed on Luria-Bertani (LB) agar plates containing 100  $\mu$ g of rifampin (Pharmachim)/ml, and 100  $\mu$ l of a culture of a rifampin-sensitive strain was spread onto the surface of a rifampin-containing LB agar plate. After overnight incubation at 37°C Rif<sup>r</sup> colonies were isolated and

purified with a second selection on rifampin agar. One of these Rif<sup>r</sup> colonies was stored for further experiments.

**In vitro transduction experiments.** One hundred microliters of log-phase cultures of EPEC or *E. coli* K-12 recipient strains containing 10<sup>7</sup> CFU were mixed with 100  $\mu$ l of a phage stock solution containing 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> PFU of phage. After 20 min of incubation at room temperature, 1 ml of LB broth was added to the cultures and incubated without shaking for 4 h at 37°C. Then they were transferred to 4 ml of fresh LB broth containing 30  $\mu$ g of chloramphenicol/ml or 100  $\mu$ g of ampicillin/ml, and selective enrichment of lysogenic bacteria was performed for 16 h at 37°C with shaking at 180 rpm. The bacteria were then harvested by centrifugation, transferred, and spread onto LB agar plates containing selective agents. To isolate  $\Phi$ 3538( $\Delta$ stx<sub>2</sub>::cat) transductants, 30  $\mu$ g of chloramphenicol/ml was mixed with either 50  $\mu$ g of nalidixic acid/ml, when EPEC strain 1390 or E2348/69 was used as a recipient, or 100  $\mu$ g of rifampin/ml. To isolate H-19B::Tn10d-bla transductants, 100  $\mu$ g of ampicillin/ml was combined either with rifampin or nalidixic acid as described above. For selection of *E. coli* C600, 30  $\mu$ g of streptomycin/ml was used. Colonies grown on selective agar after overnight incubation were investigated by PCR with stx<sub>2</sub>-specific primers HSB1 and HSB3 (38) and stx<sub>1</sub>-specific primers B54 and B55 (5).

**Transduction in pig ligated ileal loops.** Phages  $\Phi$ 3538( $\Delta$ stx<sub>2</sub>::cat) and H-19B::Tn10d-bla were induced with mitomycin C from the donor strains as described above and diluted to 3 × 10<sup>7</sup> CFU/ml. These diluted cultures were mixed to an equal amount of the potential recipient bacteria. To ensure phage absorption, these mixtures were incubated at room temperature for 20 min and kept on ice before injecting the culture to a 10-cm ligated ileal loop of 6-week-old weaned piglets. Loops were created by laparotomy in deep anesthesia and kept in postsurgical comfort as described before (26). Piglets were sacrificed 14 to 18 h after surgery, and the loop contents were spread onto LB agar plates containing different combinations of antibiotics. Tetracycline (40  $\mu$ g/ml) and nalidixic acid (50  $\mu$ g/ml) were used as selecting markers for strains 1390 and E2348/69, respectively, with combinations of 30  $\mu$ g of chloramphenicol/ml ( $\Delta$ stx<sub>2</sub>::cat) and 100  $\mu$ g of ampicillin/ml (stx<sub>1</sub>::bla). Twenty-four hours after incubation, the surviving colonies were tested for lactose fermentation and colicin production. Colonies with proper phenotypic markers were tested by PCR as described above. Altogether, 4 loops in 4 pigs were used in these experiments.

**Phenotypic methods.** Colicin production was tested as described previously (1) by using an *E. coli* K-12 strain sensitive against a wide range of colicins.

O-antigen was confirmed with specific hyperimmune sera by slide agglutination.

Antibiotic resistance was examined by the disk diffusion method with the following antibiotics: streptomycin, chloramphenicol, tetracycline, ampicillin, kanamycin, gentamicin, nalidixic acid, and rifampin (Oxoid).

MICs were determined on LB agar plates containing serial dilutions of the following antibiotics: nalidixic acid (6.25 to 100  $\mu$ g/ml), chloramphenicol (3.75 to 30  $\mu$ g/ml), tetracycline (2.5 to 40  $\mu$ g/ml), ampicillin (6.25 to 100  $\mu$ g/ml), and streptomycin (7.5 to 60  $\mu$ g/ml). Bacterial cultures (10<sup>4</sup> CFU) were spread onto the surface of the agar plates, and after overnight incubation, the concentration which caused reduced growth was considered the MIC.

Biochemical profiles of the donor and transductants were determined with the API identification (BioMerieux) program (rapid ID32E).

**DNA methods.** (i) **PCR.** Potential phage recipient strains were characterized by using published PCR primer pairs B54-B55 for stx<sub>1</sub>, B56-B57 for stx<sub>2</sub>, and B52-B53 for eae (5), EAF1-EAF2 for EAF (9), and BFP1-BFP2 for bfp (14) with the respective protocols. Potential transductants were screened with primer pairs B54-B55 for stx1 (5) and HSB1-HSB3 (38) for stx2::cat.

TABLE 2. PCR primers and conditions used in this study

Primer	Sequence (5'-3')	PCR conditions (temp [°C], time [s]) for <sup>a</sup> :			PCR product size (kb)	Reference
		Denaturing	Annealing	Extension		
wrbA1	ATG GCT AAA GTT CTG GTG	94, 30	47, 60	72, 60	600	This study
wrbA2	CTC CTG TTG AAG ATT AGC					
sbcB1	CAT GAT CTG TTG CCA CTC G	94, 30	47, 60	72, 60	1,800	29
sbcB2	AGG TCT GTC CGT TTC CAC TC					
yehV-for	AAG TGG CGT TGC TTT GTG AT	94, 30	54, 60	72, 60	340	41
yehV-rev	AAC AGA TGT GTG GTG AGT GTC TG					
yecD-fwd	CGA AGA CGC CTG TAG TGC C	94, 30	47, 60	72, 60	1,371	This study
yecN-rev	CGC AGG GAG AAA ACC AAC TC					

<sup>a</sup> Each PCR was preceded by an initial denaturing step for 5 min at 94°C and followed by a final extension step after 30 cycles for 5 min at 72°C.

TABLE 3. Phenotypic and genotypic characteristics of *E. coli* strain used in this study

Strain	Presence of genotypic characteristic:					Presence of phenotypic characteristic <sup>a</sup> :			
	<i>stx</i> <sub>1</sub>	<i>stx</i> <sub>2</sub>	<i>eae</i>	<i>EAF</i>	<i>bfp</i>	Lac	Col	lys	Resistance
3538 ( $\Delta$ <i>stx</i> <sub>2</sub> :: <i>cat</i> )	-	+	-	-	-	+	-	+	Nal, Cam
MC4100 H19B-Ap1	+	-	-	-	-	-	-	+	Sm, Amp
1390	-	-	+	-	-	+	+	+	Nal, Tc, Sm
E2348/69	-	-	+	+	+	+	-	+	Nal, Sm,
355	-	-	+	-	-	+	-	+	Sensitive
C600	-	-	-	-	-	+	-	-	Sm

<sup>a</sup> Abbreviations: Lac, lactose; Col, colicin producer; lys, lysogenic; Nal, nalidixic acid; Cam, chloramphenicol; Tc, tetracycline; Sm, streptomycin; Amp, ampicillin.

Screening for the occupation of potential phage integration sites *wrbA* (32), *yehV* (32, 41), *yecE* (32), and *sbcB* (29) was performed by PCR under conditions described in Table 2. PCR was performed in a total volume of 50  $\mu$ l containing 5  $\mu$ l of template DNA, 5  $\mu$ l of 10-fold-concentrated polymerase-synthesis buffer (Applied Biosystems Applera, Weiterstadt, Germany), 30 pmol of each primer, 200  $\mu$ mol of each deoxynucleoside triphosphate, and 2.5 U of *Taq* DNA polymerase (Applied Biosystems).

(ii) **DNA sequencing.** The *stx*<sub>2</sub>::*cat* PCR-amplified fragments were purified by a PCR purification kit (Qiagen), and the nucleotide sequence of the amplification products was determined by using HSB1 and HSB3 PCR primers with the Dye Deoxy Terminator cycle sequencing kit with an ABI Prism 377 DNA automatic sequencer (Applied Biosystems Applera).

(iii) **RFLP.** Restriction fragment length polymorphism (RFLP) of PCR-amplified fragments was performed with restriction endonuclease *Pvu*II (Promega) after purification of the PCR product with a QIAquick PCR purification kit (Qiagen) as described by the producer.

(iv) **Plaque hybridization.** Phage lysates prepared from the strain 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*) donor, recipient, and transductant strains after mitomycin C induction were mixed with *E. coli* C600 indicator bacteria in soft agar and poured onto LB agar. Plaques were transferred to nylon membranes and hybridized with a digoxigenin-labeled  $\Delta$ *stx*<sub>2</sub>::*cat* gene probe as described previously (38).

## RESULTS

**In vitro transduction of Stx-encoding phages to selected EPEC strains.** (i) **Characterization of recipient strains.** To find selection markers for transduction experiments, we have characterized potential recipient strains for Stx phage transduction. Rabbit *E. coli* strain 355 was sensitive to the tested antibiotics. To promote the selection in the transduction experiments with strain 355, we isolated a spontaneous Rif<sup>r</sup> mutant derivative (355-Rif<sup>r</sup>) from this strain as described in Ma-

terials and Methods. All of these phenotypic and the main genotypic characteristics are summarized in Table 3.

Four independent in vitro transduction experiments with different multiplicities of infection were carried out with the Stx mutant phages. In these experiments, 10<sup>7</sup> bacteria were infected with different amounts of phage, giving multiplicities of infection of 1, 10<sup>-1</sup>, 10<sup>-2</sup>, and 10<sup>-3</sup>. In these experiments, we observed variable amounts of surviving colonies on the selective LB agar plates ranging from none to about 500. Most of these colonies were tested by PCR with *stx*<sub>1</sub>-specific primers B54 and B55 and *stx*<sub>2</sub>::*cat*-specific primers HSB1 and HSB3. These investigations showed that none of the recipients became lysogenic for any of the Stx mutant phages. All surviving colonies were probably not transductants but spontaneous ampicillin-resistant mutants of the recipients.

(ii) **Transduction of Stx mutant phages in the porcine ligated ileal loop system.** To investigate the ability of Stx2-converting bacteriophage  $\Phi$ 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*) to lysogenize EPEC strains in vivo, we conducted transduction experiments in ligated ileal loops of 6-week-old weaned piglets as described in Materials and Methods. By using porcine EPEC O45 strain 1390 (Tc<sup>r</sup> Col<sup>+</sup>) as a recipient, we isolated many Tc<sup>r</sup> and Cam<sup>r</sup> lactose-fermenting (Lac<sup>+</sup>) colonies. As an additional recipient-specific marker, the colicin-production of the Tc<sup>r</sup> Cam<sup>r</sup> Lac<sup>+</sup> colonies was investigated. In two independent experiments, we tested 4 (Tc<sup>r</sup> Cam<sup>r</sup> Lac<sup>+</sup> Col<sup>+</sup>) and 25 (Tc<sup>r</sup> Cam<sup>r</sup> Lac<sup>+</sup> Col<sup>+</sup>) colonies with primers HSB1 and HSB3. Three PCR-positive transductants lysogenized with  $\Phi$ 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*) were identified. These transductant strains were derived from two different loops representing two pigs and were designated E-12, DJ-04, and ME-08. The *eae*-specific PCR revealed that the transductant strains all harbor the *eae* gene (Fig. 1). Transductants E-12, DJ-04, and ME-08 proved to be of serogroup O45. The biochemical profiles of the transductants and parental strains were identical as analyzed by the API identification program. Restriction of the HSB1-HSB3 PCR products of the transductants and wild-type strains with *Pvu*II revealed the same pattern (Fig. 2). Nucleotide sequence analysis also confirmed the presence of *stx*<sub>2</sub>::*cat* in the transductants. Furthermore, investigation of MICs gave further evidence for the functional integration of  $\Phi$ 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*) into the host strain 1390 (Table 4).

Under the same experimental conditions, we were not able

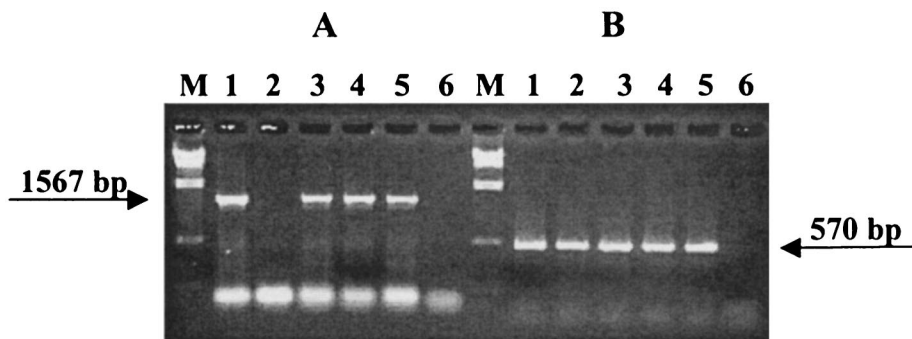


FIG. 1. Agarose gel electrophoresis of *stx*<sub>2</sub>::*cat*-specific (A) and *eae*-specific (B) PCR products. Samples: M,  $\lambda$  phage DNA digested with *Hind*III; 1, *stx*<sub>2</sub>::*cat* donor 3538, *eae*<sup>+</sup>; 2, EPEC recipient strain 1390, *eae*<sup>+</sup>; 3, transductant E-12 (*stx*<sub>2</sub>::*cat*), *eae*<sup>+</sup>; 4, transductant DJ-04 (*stx*<sub>2</sub>::*cat*), *eae*<sup>+</sup>; 5, transductant ME-08 (*stx*<sub>2</sub>::*cat*), *eae*<sup>+</sup>; 6, *E. coli* C600 (negative control).

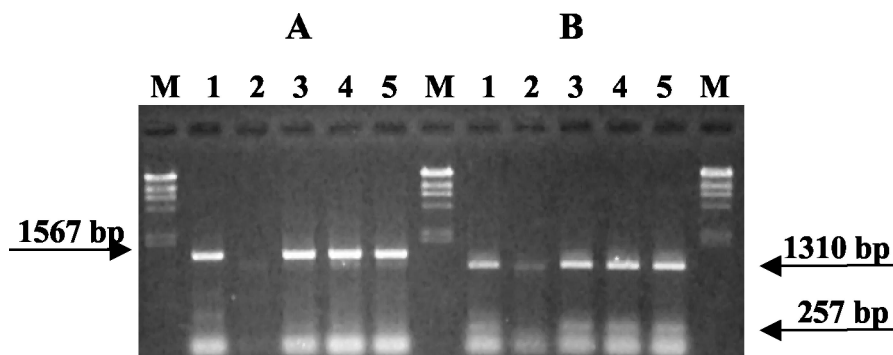


FIG. 2. RFLP of *stx*<sub>2</sub>::*cat*-specific PCR products. The undigested (A) and *Pvu*II-digested (B) *stx*<sub>2</sub>::*cat* amplicons were electrophoresed in a 1% agarose gel. Samples: M,  $\lambda$  phage DNA digested with *Hind*III; lane 1, strain 3538; 2, strain 1390; 3, strain E12; 4, strain DJ-04; 5, strain ME-08. The *Pvu*II-digested *stx*<sub>2</sub>::*cat*-specific PCR uniformly yielded two fragments of 257 and 1,310 bp, respectively.

to transduce the EPEC reference strain E2348/69 with phage  $\Phi$ 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*).

By repeating these loop experiments with *E. coli* H-19B::Tn10d-*bla* as the phage donor and with the same recipient strains as mentioned above, we could not isolate any transductant of strains 1390 and E2348/69.

(iii) **Induction of  $\Phi$ 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*) from the lysogens.** Transductant strains E-12, DJ-04, and ME-08 and, as a control, bacterial host strain 1390 were investigated for the ability to produce infectious  $\Phi$ 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*) particles after induction with mitomycin C by the plaque method. Plaques were then hybridized with *stx*-specific probes. In these experiments, large amounts of phage were induced from the recipient and transductant strains, but none of the plaques hybridized with the *stx*<sub>2</sub>::*cat*-specific probe, suggesting that the plaques consist of phage other than  $\Phi$ 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*).

(iv) **Chromosomal integration site of  $\Phi$ 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*).** Bacteriophage  $\Phi$ 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*) was originally isolated from *E. coli* O157:H7 strain 3538/96. The *wrbA* and *sbcB* genes have been described as integration sites for Stx2-encoding phages in *E. coli* O157:H7 (28, 31). This prompted us to screen these sites for integration of  $\Phi$ 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*) in parental strains and transductants. However, the generation of 600- and 1,800-bp PCR products in strains 1390, E-12, DJ-04, and ME-08 for *wrbA* and *sbcB*, respectively, demonstrated that these genes are intact and therefore not occupied by phage sequences. With the same approach, we investigated the *yecE* and *yehV* genes, but these genes are also not used as integration sites in any of the strains described above. Therefore,

$\Phi$ 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*) obviously uses an integration site that is yet not described for Stx phage.

## DISCUSSION

STEC strains demonstrate a broad range of phenotypic and genotypic heterogeneity (27). STEC strains belong to more than 200 *E. coli* serotypes, and Stx production was described also for several other bacterial species (30, 39, 42, 43, 44). Bacteriophages have played an important role in the evolution of many bacterial pathogens by promoting the horizontal transfer of genes between strains of the same species as well as between distantly related bacteria. Phage can alter host bacterial properties relevant to all stages of the infectious process including bacterial adherence, colonization, invasion, exotoxin production, resistance to immune defenses and antibiotics (reviewed by Wagner and Waldor [45]). Transduction of Stx-encoding phages to nontoxigenic strains could be involved in the emergence of novel STEC serotypes (17, 38).

The genome sequences of EHEC O157 strains Sakai and EDL933 revealed the presence of 18 prophages (15, 32). Among the 18 prophages on the O157 Sakai chromosome, 13 are lambda-like phages, and in addition to Shiga toxins, they encode further virulence-related proteins, such as zinc/copper-type superoxide dismutases, Bor proteins, and Lom homologues (15). On the basis of these results, *E. coli* O157 bacteria might be considered phage factories, as described by Ohnishi et al. (28), releasing chimeric or mosaic phages into the environment that could lead to new pathogenic characters, first of all new STEC clones. However, the conditions, which favor lysogenic conversion in vivo, are not well established.

Recently, Strauch et al. (42) isolated and characterized an Stx-encoding temperate phage in an *S. sonnei* strain, which could be transduced to a nontoxigenic *S. sonnei* strain and to an *E. coli* laboratory K-12 strain. Schmidt et al. (38) lysogenized a broad range of enteric *E. coli* strains with a derivative of a Shiga toxin 2-encoding phage designated  $\Phi$ 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*). They were able to transduce different pathotypes, including EPEC, STEC, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli*, and *E. coli* isolated from stool microflora of a healthy individual. In the EPEC group, interestingly, only 2 of 11 strains were successfully lysogenized.

TABLE 4. MIC values of EPEC recipient 1390, phage donor 3538 ( $\Delta$ *stx*<sub>2</sub>::*cat*), and transductant E-12, DJ-04, and ME-08 *E. coli* strains

Strain	MIC ( $\mu$ g/ml) of <sup>a</sup> :				
	Nal	Cam	Tc	Amp	Sm
1390	>100	$\leq$ 4	>40	$\leq$ 6	>60
3538 ( $\Delta$ <i>stx</i> <sub>2</sub> :: <i>cat</i> )	$\leq$ 50	>30	$\leq$ 3	$\leq$ 6	$\leq$ 8
E-12	>100	>30	>40	$\leq$ 6	>60
DJ-04	>100	>30	>40	$\leq$ 6	>60
ME-08	>100	>30	>40	$\leq$ 6	>60

<sup>a</sup> Abbreviations: Nal, nalidixic acid; Cam, chloramphenicol; Tc, tetracycline; Sm, streptomycin; Amp, ampicillin.

The lysogenized EPEC strains belonged to classical EPEC serotypes O111:H2 and O26:H, which are also known to frequently produce Stx and cause HUS (18).

We first carried out in vitro transduction experiments. In these experiments we were not able to lysogenize several selected recipient strains with the Stx mutant phage. James et al. (17) lysogenized several *E. coli* and *Shigella* strains in vitro by using an *aph3* gene-labeled Stx2-encoding phage originating from an *E. coli* O157 strain, but neither of their 2 EPEC strains were successfully lysogenized. One reason for the failure with *E. coli* strains E2348/69 and 355 could be that their cryptic prophages might have mediated superimmunity (3). Another reason for the failure could be that the prophages of these EPEC strains have already occupied the potential integration sites for labeled Stx-encoding phages (38). Strain 1390 was also lysogenic, but its prophage did not prevent the integration of  $\Phi$ 3538 ( $\Delta$ stx<sub>2</sub>::cat) in the porcine loop.

In the present study, we established a suitable model for studying the in vivo spread of Stx-encoding phages. We were able to lysogenize the porcine EPEC O45 strain 1390 with phage  $\Phi$ 3538 ( $\Delta$ stx<sub>2</sub>::cat) in porcine ligated ileal loops but not the human EPEC prototype strain E2348/69. Under the same conditions, neither strain 1390 nor strain E2348/69 was lysogenized when *E. coli* K-12 containing H-19B::Tn10d-bla was used as the phage donor. However, two commensal non-EPEC strains of the loop flora might have been lysogenized with H-19B::Tn10d-bla, but the phage was unstable in these strains and after the first passage these phages were eliminated (data not shown). Acheson et al. (2) also lysogenized laboratory *E. coli* strains with phage H-19B::Tn10d-bla in the murine gastrointestinal tract in vivo. They have not detected any transduction of commensal strains, perhaps because they eliminated most of the normal intestinal flora by streptomycin application before the injection of donor and recipient *E. coli* K-12 strains.

The success of the in vivo transduction of Stx2 to the target EPEC strain and Stx1 to *E. coli* strains of the porcine intestinal flora indicates that there are important differences in the porcine intestinal environment acting as inducers of phage transduction in vivo. The exact nature of some of these in vivo effectors will be determined in future studies. Among these factors, redox potential, pH value, oxygen, and perhaps a concerted action of several factors may play a role in making in vivo conditions so much different from in vitro conditions.

The bacteriophages encoding Stx2 have been shown to integrate into *wrbA* or *sbcB* in the genomes of O157:H7 strains (15, 32). Recktenwald and Schmidt (34) reported that an Stx2e-encoding bacteriophage is integrated into *yecE* in STEC O157:H7. Recently Shaikh and Tarr (41) analyzed the integration of Stx2- and Stx1-encoding phages into the chromosome of *E. coli* O157 and non-O157 STEC strains by PCR with *wrbA*- and *yehV*-specific primers, respectively. Their study revealed that stx<sub>2</sub> bacteriophage usually occupies *wrbA* in stx<sub>1</sub><sup>+</sup> and stx<sub>2</sub><sup>+</sup> *E. coli* O157:H7 strains, but instead of *wrbA*, truncated bacteriophages occupy *yehV* in most stx<sub>1</sub>-negative and stx<sub>2</sub>-positive *E. coli* O157:H7 strains (41). In the present study, we have examined the integrity of *wrbA*, *sbcB*, *yehV*, and *yecE* genes by PCR in *E. coli* O157:H7 strain 3538 and transductant strains. Our investigations revealed that all of the previously identified *stx1* and *stx2* phage integration sites were intact in the donor and transductant strains, indicating that stx<sub>2</sub> occu-

pies a new as yet undescribed site in the chromosome of *E. coli* O157:H7 strain 3538 and transductants.

In conclusion, we have demonstrated that Stx-encoding phages are able to spread in vivo among wild-type enteric *E. coli* strains, and the failures in the in vitro trials with the same strains suggest that in vivo conditions are more effective than in vitro conditions. Results suggest that EHEC may emerge from EPEC by acquisition of stx genes, but further studies are necessary to define the bacterial features promoting the Stx-encoding phage transduction and to determine the phage integration site in the porcine EPEC strain.

#### ACKNOWLEDGMENTS

This work was supported by grants from the European Union (E.U. project number QLK2-2000-0060) and by the Hungarian Basic Scientific Fund (OTKA) (no. T34970 and T37890).

We are solely responsible for the work described in this paper, and the opinions expressed are not necessarily those of the E.U.

We thank Marta Puruczki (Budapest) and Stefanie Müksch (Dresden) for skillful technical assistance.

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