Transduction of Enteric *Escherichia coli* Isolates with a Derivative of Shiga Toxin 2-Encoding Bacteriophage ϕ 3538 Isolated from *Escherichia coli* O157:H7

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Received 19 May 1999/Accepted 7 July 1999

We investigated the ability of a detoxified derivative of a Shiga toxin 2 (Stx2)-encoding bacteriophage to infect and lysogenize enteric *Escherichia coli* strains and to develop infectious progeny from such lysogenized strains. The *stx*₂ gene of the patient *E. coli* O157:H7 isolate 3538/95 was replaced by the chloramphenicol acetyltransferase (*cat*) gene from plasmid pACYC184. Phage ϕ 3538(Δ *stx*₂::*cat*) was isolated after induction of *E. coli* O157:H7 strain 3538/95 with mitomycin. A variety of strains of enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), Stx-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), and *E. coli* from the physiological stool microflora were infected with ϕ 3538(Δ *stx*₂::*cat*), and plaque formation and lysogenic conversion of wild-type *E. coli* strains were investigated. With the exception of one EIEC strain, none of the *E. coli* strains supported the formation of plaques when used as indicators for ϕ 3538(Δ *stx*₂::*cat*). However, 2 of 11 EPEC, 11 of 25 STEC, 2 of 7 EAEC, 1 of 3 EIEC, and 1 of 6 *E. coli* isolates from the stool microflora of healthy individuals integrated the phage in their chromosomes and expressed resistance to chloramphenicol. Following induction with mitomycin, these lysogenic strains released infectious particles of ϕ 3538(Δ *stx*₂::*cat*) that formed plaques on a lawn of *E. coli* laboratory strain C600. The results of our study demonstrate that ϕ 3538(Δ *stx*₂::*cat*) was able to infect and lysogenize particular enteric strains of pathogenic and nonpathogenic *E. coli* and that the lysogens produced infectious phage progeny. Stx-encoding bacteriophages are able to spread *stx* genes among enteric *E. coli* strains.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains have emerged as a serious foodborne pathogen that has caused large-scale outbreaks of intestinal diseases in the developed countries (10). Whereas *E. coli* O157:H7 is the predominant STEC serotype isolated from patients in the United States, Canada, and Japan (3, 10), STEC strains belonging to other serotypes (non-O157 STEC) have been responsible for outbreaks and sporadic cases of human disease in continental Europe (6). Pathogenic STEC strains share several determinants implicated in pathogenesis. These include Stx and a pathogenicity island termed LEE, the latter encoding proteins responsible for the intimate adherence of STEC to the intestinal mucosa and for the subsequent characteristic destruction of the microvilli termed “attaching and effacing” lesions (15, 18).

Stx are the major pathogenicity factors of these organisms, and there is evidence that they are involved in the development of extraintestinal complications, such as the hemolytic-uremic syndrome (HUS) (25). Stx of *E. coli* are a family of proteins which are cytotoxic to eucaryotic cells expressing the glycolipid receptor globotriaosylceramide (Gb3) (17). They function as rRNA-N-glycosidases and inhibit protein biosynthesis in target cells (9). Moreover, it has been demonstrated that Stx may induce apoptosis in renal tubular cells (37). The members of the Stx family of *E. coli* share genetic, structural, and functional features and consist of Stx1, Stx2, and variants of Stx2 (18). Stx1 is nearly identical to Stx of *Shigella dysenteriae* type 1, but it shares only 55% amino acid sequence identity with Stx2 (18).

In addition, Stx1 and Stx2 are not neutralized by heterologous antisera (34, 36).

Stx1 and Stx2 are encoded in the genomes of several lambdoid prophages (8, 13, 22, 36). It has been shown that such Stx-encoding phages are heterogeneous in their genetic structure and size (39). The Stx1-converting bacteriophages H19J (24) and H19B (35), originally isolated from Stx-producing O26:H11 strain H19, and the Stx2-converting bacteriophage 933W derived from *E. coli* O157:H7 EDL933 (24) have been characterized intensively (12, 21, 35, 36). It has been demonstrated that the *stx*₁ and *stx*₂ genes in STEC O157:H7, O26, and O111 are closely associated with a *p*-like gene which is similar to the *p* gene of bacteriophage λ (8). This implicated that the *stx* genes of different Stx-converting bacteriophages were located in the same site. This was confirmed in a later study which also demonstrated the presence of an *ileX* tRNA gene upstream of the *stx*₂ gene in STEC O157:H7, O26:H11, O103:H2 and O111:H2 strains (33). In addition, Neely and Friedman (21) characterized a 17-kb DNA segment of the Stx1-encoding phage H19B that covers an analogue of the λ Q transcription activator, the *stx*₁ gene, and an open reading frame homologous to the λ holin lysis gene. They suggested a role for the λ -like Q protein and the holin lysis genes in the regulation of *stx* expression and release of the protein from the bacterial cell. They also showed that the Stx2-encoding phage 933W had a similar structure in this region (21). The complete sequence of phage 933W was recently published and also provides evidence for the linkage of phage induction and *stx* expression (27).

Recently, Stx2-converting bacteriophages originating from *E. coli* O157:H7 outbreak strains isolated in Japan were characterized. The authors described one phage, Stx2 ϕ -I, which resembled phage 933W and another phage, Stx2 ϕ -II, which had properties distinct from those of Stx2 ϕ -I (38). Stx2 ϕ -I was

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demonstrated to use the FadL protein as a receptor and the receptor for Stx2 ϕ -II was the LamB protein (38).

Stx-encoding phages may be the major cause for the spread of *stx* genes among wild-type *E. coli* and for the emergence of new STEC types. Evidence for this has been provided by Acheson et al. (1) who showed that a derivative of bacteriophage H19B was able to transduce an *E. coli* recipient laboratory strain in the murine gastrointestinal tract.

In this study, we constructed a detoxified derivative of a Stx2-converting phage— ϕ 3538(Δ stx₂::cat)—which conferred chloramphenicol resistance to assess the range of infectivity of this bacteriophage and to study its postuptake integration, stability, and inducibility.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. Most of the *E. coli* wild-type strains used in this study were from our strain collection and were isolated during routine diagnostic work in recent years from stools of patients with diarrhea or HUS. Pure cultures of wild-type isolates were stored in stab cultures. All in all, from the initial isolation up to the phage experiments, isolates were passaged four times at most. They included 25 STEC (17 serotype O157:H7 or O157:H[−] and 8 non-O157 serotypes), 11 enteropathogenic *E. coli* (EPEC), 6 enteroaggregative *E. coli* (EAEC), 5 enterotoxigenic *E. coli* (ETEC), and 3 enteroinvasive *E. coli* (EIEC) strains. Six *E. coli* strains were isolated from stools of healthy individuals and do not belong to any of the enteric *E. coli* pathogroups. *E. coli* O157:H7 strain 3538/95 was isolated from a patient with HUS in Würzburg, Germany, in 1995. This strain carried *stx*₂, *eae*, and *E-hly* and was cytotoxic to Vero cells. The EAEC O111 strain DEF 53 was kindly provided by Anna Giammanco, University of Palermo, Italy.

E. coli DH5 α (29) was used as a host strain for plasmids and phages and as an indicator strain. *E. coli* SM10 λ pir (*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r λ pir) served as a donor for conjugation experiments with plasmid pGP704 (11). The *E. coli* K-12 derivative C600 strain was used as a positive control in transduction experiments with phage ϕ 3538(Δ stx₂::cat) and as an indicator strain. Plasmid pACYC184 (7) was used as a source of the chloramphenicol acetyltransferase (*cat*) gene. Plasmids pUC18 (Ap^r) and pGP704 (Ap^r ori R6K *mob* RP4 MCS of M13gt131) were used as cloning vectors; the latter delivered suicide functions. Stx2-converting phage 933W was prepared from *E. coli* C600(933W) (24) and used as a control in the plaque assays with wild-type *E. coli* strains.

Culture techniques. Mutants of *E. coli* O157:H7 strain 3538/95 resistant to nalidixic acid were obtained by sequential incubation of this strain in Luria-Bertani (LB) broth containing increasing concentrations of nalidixic acid (Sigma-Aldrich, Deisenhofen, Germany) ranging from 0.5 to 40 μ g/ml. A single colony resistant to 40 μ g of nalidixic acid per ml was stored and designated 3538/95 (Nal^r).

Filter mating of *E. coli* SM10 λ pir/pHS3 and *E. coli* O157:H7 3538/95 (Nal^r) was performed according to the method of Miller (19) in a ratio of 1:4 with minor modifications. The LB agar plate containing the mating mixture was incubated for 8 h at 37°C. Selection of transconjugants was performed on agar plates containing 30 μ g of chloramphenicol per ml (Sigma-Aldrich) and 50 μ g of nalidixic acid per ml that were incubated for 48 h at 37°C.

MICs of chloramphenicol were determined with E-test strips (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions.

Plaque assay and preparation of stock lysates of ϕ 3538(Δ stx₂::cat). A log-phase culture (optical density at 600 nm of 0.5) of strain 3538/95(Δ stx₂::cat) in tryptone soya broth (Oxoid, Basingstoke, Hampshire, United Kingdom) containing 5 mM CaCl₂ was adjusted to a final mitomycin (Sigma-Aldrich) concentration of 0.5 μ g/ml, and the cultivation was continued overnight. Bacterial cells were sedimented at 5,000 $\times$ g for 10 min, and the supernatant was filtered through a 0.2- μ m-pore-size membrane filter (Schleicher & Schuell GmbH, Dassel, Germany). In the plaque assay, 100 μ l of the filtrate, 100 μ l of an overnight culture of *E. coli* C600, and 125 μ l of 0.1 M CaCl₂ solution were mixed with 3 ml of LB soft agar (0.7%) at 48°C and poured onto an LB agar plate containing 2% agar. Plaques were observed after overnight incubation at 37°C. A high-titer stock lysate of ϕ 3538(Δ stx₂::cat) was prepared from a single plaque according to the method described by Sambrook et al. (29) for phage λ . This stock lysate contained 10⁸ PFU/ml.

Infection of enteric *E. coli* strains with ϕ 3538(Δ stx₂::cat). One hundred microliters of log-phase cultures of enteric *E. coli* containing 10⁷ CFU was mixed with 100 μ l of a 1:1,000 dilution of ϕ 3538(Δ stx₂::cat) stock lysate (10⁴ PFU), and the ability of wild-type *E. coli* to form plaques and to develop lysogens was investigated. The plaque assay was performed as described above, with the exception that the LB basal layer contained 0.5 μ g of mitomycin per ml.

For the detection of lysogens, bacterial cultures were initially incubated with ϕ 3538(Δ stx₂::cat) for 4 h at 37°C. Then they were transferred to 4 ml of LB broth containing 30 μ g of chloramphenicol per ml, and selective enrichment of lysogenic (chloramphenicol resistant [Cam^r]) bacteria was performed for 48 h at

FIG. 1. Construction of a vector for gene replacement mutagenesis of the *stx*₂ gene. (A) PCR product of the entire *stx*₂ gene obtained by amplification with primers HSB1 and HSB3. (B) Ligation of the PCR product in vector pUC18. (C) Replacement of an internal EcoRV-HincII fragment of the *stx*₂ gene with an BsaAI-HincII fragment of pACYC184 covering the *cat* gene. (D) Ligation of the Δ *stx*₂::*cat* construct in suicide vector pGP704. Restriction sites are depicted. The numbering in panel A corresponds to the beginning of the PCR product. Hatched bars depict the *stx*₂A and *stx*₂B subunit genes. The fragment derived from pACYC184 is noted by a dark box, and the *cat* gene is noted by a light box.

37°C and 180 rpm. Bacteria were then harvested by centrifugation at 5,000 $\times$ g for 30 min and transferred to LB agar plates containing 30 μ g of chloramphenicol per ml (Cam-agar) by single-colony streaking. Colonies grown on Cam-agar after overnight incubation were confirmed to be lysogens by PCR with *stx*₂-specific primers HSB1 and HSB3. The appearance of a 1.57-kb PCR product proved the presence of the *cat* gene linked to the pieces of *stx*₂ (Fig. 1D) and thus lysogenization of the strains with ϕ 3538(Δ stx₂::cat).

General recombinant DNA techniques. Plasmids were purified with the Nucleobond AX100 Preparation kit (Macherey-Nagel, Düren, Germany). Restriction, ligation, and cloning experiments as well as the preparation of phage DNA were performed according to standard methods (29). Total DNA was prepared as described by Datz et al. (8).

For Southern blot hybridization experiments, either plaques from LB plates or DNA fragments from agarose gels were transferred to nylon membranes (Zeta-probe GT; Bio-Rad, Munich, Germany) by standard procedures (29). DNA was fixed with a cross-linker (Stratalinker UV Crosslinker 1800; Stratagene, Heidelberg, Germany) by using the autocross-link mode (120,000 μ J/cm²). Stringent hybridization was achieved with the DIG (digoxigenin) DNA labelling and detection kit (Boehringer GmbH, Mannheim, Germany) according to the manufacturer's instructions. A 1,343-bp BsaAI-HincII fragment of pACYC184 containing the *cat* gene was randomly labeled with digoxigenin-11-dUTP and used as a probe.

Nucleotide sequences were determined by Taq cycle sequencing with universal and reverse primers for pUC/M13 vectors and HSB1 and HSB3 complementary to the sequences of the *stx*₂ gene as described previously (30). Nucleotide sequence analysis was performed with the Lasergene program package (Dnastar, Madison, Wis.).

PCR. Primers HSB1 (5'-CCC GGT ACC ATG AAG TGT ATA TTA TTT AAA TGG-3') and HSB3 (5'-CCC GCA TGC TCA GTC ATT ATT AAA CTG CAC-3') were designed to amplify the entire *stx*₂ gene, yielding a 1,259-bp PCR product containing KpnI and SphI restriction sites at the respective 5' ends. PCRs were performed with the GeneAmp PCR System 9600 (Perkin-Elmer-Applied Biosystems, Weiterstadt, Germany) in a volume of 50 μ l containing each deoxynucleoside triphosphate at 200 μ M, 30 pmol of each primer, 5 μ l of 10-fold-concentrated polymerase synthesis buffer, 3 μ l of 25 mM MgCl₂ solution,

TABLE 1. Infection of enteric *E. coli* strains with ϕ 3538(Δ stx₂::cat) and production of lysogens

Bacterial strain	Serotype	stx geno-type ^a	MIC (μ g/ml) ^b	<i>E. coli</i> group	Source or reference ^c	Result of infection with ϕ 3538(Δ stx ₂ ::cat) ^d				
						Plaque formation on mitomycin-LB	Growth of infected cells on Cam-LB	PCR for detection of lysogens ^e	Inducibility of lysogens with mitomycin	Plaque hybridization with cat probe (%) ^f
11-1/87	O111:H2	—	3.0	EPEC	SC	—	+	+	+	+ (0.5)
6416/87	O26:H ⁻	—	4.0	EPEC	SC	—	+	+	+	+ (0.1)
EDL933	O157:H7	1, 2	4.0	STEC	23	—	+	+	+	+ (2.0)
778/98	O157:H7	1, 2	4.0	STEC	SC	—	+	+	+	+ (0.2)
3574/92	O157:H7	2	3.0	STEC	28	—	+	+	+	+ (52.0)
2987/98	O157:H7	1, 2, 2c	4.0	STEC	SC	—	+	+	+	+ (0.1)
1249/87	O157:H7	2, 2c	3.0	STEC	28	—	+	+	+	+ (63.0)
1658/91	O157:H7	2, 2c	3.0	STEC	28	—	+	+	+	+ (10.0)
1193/89	O157:H ⁻	1	3.0	STEC	28	—	+	+	+	+ (82.0)
7513/91	O157:H ⁻	2c	3.0	STEC	28	—	+	+	+	+ (60.0)
5291/92	O157:H ⁻	1, 2c	3.0	STEC	28	—	+	+	+	+ (53.0)
427/89	O157:H ⁻	1, 2c	2.0	STEC	SC	—	+	+	—	NP ^g
3901/97	O26:H ⁻	1, 2	3.0	STEC	SC	—	+	+	+	+ (30.0)
DEF 53	O111:HND	—	4.0	EAEC	AG	—	+	+	+	+ (0.2)
4140-86	O44:HND	—	3.0	EAEC	SC	—	+	+	NP	NP
12860	O124:HND	—	1.0	EIEC	SC	+	+	+	+	+ (100.0)
7	ND ⁱ	—	4.0	PS ^j	SC	—	+	+	+	+ (15.0)
C600	ND	—	1.5	LS ^k	SC	+	+	+	+	+ (100.0)

^a stx₁ was detected by amplification with primers KS7 and KS8 (32), and the stx₂ and stx_{2c} genes were both amplified with primers GK3 and GK4 and differentiated from each other by restriction analysis with *Hae*III and *Fok*I (28). —, stx negative.

^b MIC of chloramphenicol for wild-type *E. coli* strains.

^c SC, strain collection; AG, strain from Anna Giammanco, Palermo, Italy.

^d Mitomycin-LB, LB agar plates containing 0.5 μ g of mitomycin per ml; Cam-LB, LB agar plates containing 30 μ g of chloramphenicol per ml.

^e Primers HSB1 and HSB3 were used to amplify the cat gene within the Δ stx₂::cat fusion in the genome of ϕ 3538(Δ stx₂::cat).

^f Percentage of the total number of plaques present on matrix LB agar plates.

^g NP, not performed.

^h PCR was positive with a pool of colonies; a single PCR-positive colony was not found.

ⁱ ND, not determined.

^j PS, strain of physiological intestinal microflora.

^k LS, laboratory strain.

and 2.0 U of AmpliTaq DNA polymerase (Perkin-Elmer, Applied Biosystems). The DNA was denatured at 94°C for 30 s, annealed at 53°C for 60 s, and then extended for 60 s at 72°C. After 30 cycles were completed, a final extension step of 5 min at 72°C was conducted.

Construction of an stx₂-negative derivative of ϕ 3538 which encodes chloramphenicol resistance. From sequence studies, we did not expect alteration of essential phage functions by mutagenesis in the stx region. Therefore, the stx gene was chosen as a target for mutagenesis of the phage. The entire stx₂ gene of strain 3538/95 was amplified with primers HSB1 and HSB3. The resulting 1,259-bp PCR product (Fig. 1A) was separated on a 0.6% agarose gel, excised from the gel, and then purified with the Prep-a-Gene kit (Bio-Rad, Munich, Germany). After restriction with *Kpn*I and *Sph*I, the fragment was again purified and ligated in plasmid pUC18, which was digested with the same enzymes (Fig. 1B). This plasmid was designated pHS1. Plasmid pHS1 was then digested with *Eco*RV and *Hinc*II, which cleaved at positions 146 and 1168 in the stx₂ gene (Fig. 1A), and electrophoresed on a 0.8% agarose gel. One of the resulting DNA fragments with a size of 2,875 bp was excised from the gel. It contained plasmid vector pUC18 with the remaining short fragments of 138 bp (A subunit gene) and 87 bp (B subunit gene) of the stx₂ gene (Fig. 1C). A 1,343-bp *Bsa*AI-*Hinc*II fragment prepared from pACYC184 was ligated with the 2,875-bp fragment resulting in plasmid pHS2 (Fig. 1C). This plasmid now conferred resistance to chloramphenicol and carried short stx₂ sequences (Fig. 1C) on both sides of the cat gene. The whole Δ stx₂::cat construct was then excised from pHS2 with *Kpn*I and *Sph*I and ligated in suicide vector pGP704, digested with the same enzymes. This yielded pHS3 (Fig. 1D). The ligation samples were transformed in *E. coli* SM10 λ pir. Transformants were checked to carry the Δ stx₂::cat construct by PCR with primers HSB1 and HSB3. A 1,567-bp product was detected, proving the presence of the cat gene. Nucleotide sequence analysis with primers HSB1 and HSB3 also confirmed that the Δ stx₂::cat cassette was inserted correctly.

Plasmid pHS3 was then transferred into *E. coli* O157:H7 strain 3538/95 (Nal^r) by filter mating. Transconjugants were selected on agar plates containing nalidixic acid and chloramphenicol. Only recipient strains containing the recombinant suicide plasmid pHS3 could grow on these plates. Since the recipients did not express the π protein, pHS3 got lost in subsequent culture passages; only those recipients which had integrated the cat gene in their chromosome by homologous recombination could grow. One such isolate was characterized. PCR with primers HSB1 and HSB3 showed that the Δ stx₂::cat fusion was present in the *E. coli* O157:H7 recipient. In addition, plasmids the size of pHS3 could not be detected by preparation methods. The lack of tetracycline resistance also gave evidence that the suicide plasmid was absent. The transconjugant was then

confirmed to be a derivative of its parental strain by serotyping, PCR, and sequencing. However, it was not cytotoxic for Vero cells. The strain was designated *E. coli* 3538/95(Δ stx₂::cat).

Induction of *E. coli* 3538/95(Δ stx₂::cat) with mitomycin and subsequent plaque assay with DH5 α as an indicator confirmed the presence of infectious particles of ϕ 3538(Δ stx₂::cat). In addition, ϕ 3538(Δ stx₂::cat) converted its laboratory host strain DH5 α to express resistance to chloramphenicol.

RESULTS

Transduction of various enteric *E. coli* strains with ϕ 3538(Δ stx₂::cat). In order to investigate the ability of ϕ 3538(Δ stx₂::cat) to spread among enteric *E. coli* strains, we performed transduction experiments which exploited the fact that the phage could convert its host to express chloramphenicol resistance. Before starting these experiments, we determined the MIC of chloramphenicol for all 57 wild-type strains. The MICs ranged from 0.5 to 4.0 μ g/ml and were mostly $\leq$ 3.0 μ g/ml. The MICs for *E. coli* C600 and DH5 α were 1.5 and 4.0 μ g/ml, respectively, and that for strain 3538/95(Δ stx₂::cat) was 128 μ g/ml. Since the breakpoint for sensitivity of *E. coli* to chloramphenicol is $\leq$ 8 μ g/ml (20), all strains used in this study except for 3538/95(Δ stx₂::cat) were sensitive to chloramphenicol. The concentration of 30 μ g/ml that represented a 10-fold MIC for more than half of the wild-type strains and inhibited growth of all of them was chosen for selection of lysogens. The MICs for the relevant strains are shown in Table 1.

In the experiments, 10⁷ CFU of log-phase cultures of the test strains were exposed to 10⁴ PFU of phage ϕ 3538(Δ stx₂::cat), and development of plaques and production of lysogens were determined. Only 1 of 57 wild-type strains, EIEC 12860, formed plaques upon infection with ϕ 3538(Δ stx₂::cat) (Table 1). None of the other wild-type strains used as indicator strains

FIG. 2. Plaque hybridization. Phage lysates prepared from the lysogens *E. coli* O157:H⁻ strain 1193/89(Δ *stx*₂::*cat*) (A) and *E. coli* O157:H7 strain 3574/92(Δ *stx*₂::*cat*) (B) after mitomycin 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 *cat* gene probe. Plasmid pACYC184 DNA was used as a positive control and is seen in the upper part of the membranes (large dots). *stx*₇*B* and *stx*₂*B* PCR products, respectively, were used as negative controls on the membranes.

supported the formation of plaques. The same results were obtained when the *Stx*2-converting phage 933W and the original (unlabeled) phage ϕ 3538/95 were used as positive controls to infect these strains.

Since the phage-associated *cat* gene confers resistance to chloramphenicol, the ability to grow on Cam-agar was used as a marker to screen for lysogens. The 57 wild-type strains were subjected to the selective chloramphenicol enrichment procedure after having been exposed to ϕ 3538(Δ *stx*₂::*cat*). Cam^r colonies developed in 26 of 57 strains. PCR with primers HSB1 and HSB3 performed with 15 pooled colonies of each of these Cam^r strains confirmed that 17 of the 26 strains were lysogenized with ϕ 3538(Δ *stx*₂::*cat*). From the remaining nine PCR-negative strains, up to 100 colonies were subsequently investigated, but the presence of the phage could not be demonstrated.

Control experiments were conducted under the same conditions with wild-type strains that had not been exposed to ϕ 3538(Δ *stx*₂::*cat*). Only two strains developed Cam^r colonies, with a frequency of 4×10^{-8} during the selective enrichment procedure.

In total, lysogenization with ϕ 3538(Δ *stx*₂::*cat*) occurred in 2 of 11 EPEC strains, 2 of 7 EAEC strains, 1 of 3 EIEC strains, and 1 of 6 physiological *E. coli* strains. None of five ETEC strains was lysogenized. Among the STEC strains tested, 1 of 8 non-O157 strains and 10 of 17 O157 strains were lysogenized by ϕ 3538(Δ *stx*₂::*cat*). The 17 strains that produced lysogens are characterized in Table 1.

The laboratory strain *E. coli* C600 was used as a positive control in ϕ 3538(Δ *stx*₂::*cat*) transduction experiments. As demonstrated in Table 1, the strain formed plaques upon infection with the phage and produced lysogens from which infectious phage particles were induced by treatment with mitomycin.

Location of the *cat* gene in the lysogens. In order to determine whether the lysogens had inserted ϕ 3538(Δ *stx*₂::*cat*) into the chromosome or into a plasmid, plasmid and total DNA were prepared from five selected lysogens. Among these there were *stx*-negative as well as *stx*-carrying strains. The DNA preparations were restricted with *EcoRV* and *HincII* and sep-

arated in parallel on agarose gels. Hybridization with the *cat* probe demonstrated a signal with a size of approximately 2,800 bp in the total DNA preparations of all strains tested. However, no hybridization signals were present in the respective plasmid preparations (data not shown). These experiments clearly proved the association of the *cat* gene with the bacterial chromosome.

Induction of ϕ 3538(Δ *stx*₂::*cat*) from the lysogens. Sixteen of 17 lysogens were further investigated for their ability to produce infectious particles of phage ϕ 3538(Δ *stx*₂::*cat*) after induction with mitomycin. The EAEC strain 4140-86 was excluded from this study because we were not able to isolate lysogens from the PCR-positive colony pool (Table 1). As demonstrated in Table 1, 15 of 16 lysogens could be induced with mitomycin and produced phage progeny that formed plaques on a lawn of the indicator strain *E. coli* C600.

We hybridized these plaques with a probe specific for the *cat* gene. Signals, examples of which are shown in Fig. 2, were obtained from plaques of all of the lysogens. However, in 14 of 15 lysogens, only some of the plaques hybridized with the *cat* probe; the proportion of the ϕ 3538(Δ *stx*₂::*cat*)-associated plaques ranged from 0.1% to 82% (Table 1). To ensure that the hybridization signals were specific, control hybridization experiments were performed. All 15 parental *E. coli* strains (not transduced with ϕ 3538(Δ *stx*₂::*cat*)) were also induced, and the supernatants were investigated for plaque formation. Plaques were observed with supernatants of 12 of the 15 strains. Plaque hybridization with the *cat* probe was performed and was negative in all cases, demonstrating that no *cat*-like sequences were present in the parental *E. coli* strains. Moreover, hybridization of chromosomal DNA of all 57 wild-type strains with the same probe was also negative.

In addition, phages isolated from five selected lysogens were tested for the ability to transduce chloramphenicol resistance into the laboratory strain DH5 α . After mitomycin induction, we were able to isolate phages containing the *cat* gene from all lysogens. Laboratory strain DH5 α was transduced with these phages, and the *cat*-harboring phages could be reisolated from such transductants. Control experiments were performed with mitomycin-induced supernatants of the parental strains not

FIG. 3. Agarose gel electrophoresis (A) and Southern blot hybridization (B) of phage DNA restricted with *Eco*RI. DNA of the original phage $\phi 3538(\Delta stx_2::cat)$ (lanes 1) was compared with DNA from phage progeny that has been isolated from the lysogens 1193/89($\Delta stx_2::cat$) (lanes 2), 3574/92($\Delta stx_2::cat$) (lanes 3), 12860($\Delta stx_2::cat$) (lanes 4), 7513/91($\Delta stx_2::cat$) (lanes 5), and 1249/87($\Delta stx_2::cat$) (lanes 6) and processed in *E. coli* laboratory strain DH5 α as described in the text. M, molecular weight standard (1-kb DNA ladder; Gibco BRL, Eggenstein, Germany).

harboring $\phi 3538(\Delta stx_2::cat)$. However, DH5 α could not be converted to chloramphenicol resistance by any of the supernatants.

In order to study putative recombination events that could have occurred in the phage during the alternating lysogenic and lytic cycles, we compared restriction fragment profiles of the original phage $\phi 3538(\Delta stx_2::cat)$ and the phages that we have isolated from the DH5 α transductants as described above. It could be demonstrated (Fig. 3) that the phages had identical restriction patterns. Hybridization experiments demonstrated that the same restriction fragment of the phage DNAs hybridized with the *cat* probe.

DISCUSSION

STEC strains demonstrate a broad range of genotypic and phenotypic heterogeneity. Until now, the mechanisms by which this heterogeneity has evolved have been poorly understood. Besides recombination events and spontaneous mutations, mobile genetic elements could play a role. Although Stx are known to be encoded by temperate, lambdoid prophages, the role of these phages in the spread of *stx* genes has not been well elucidated.

STEC strains belong to more than 100 serotypes (14), and transduction of *stx* genes by phages to other enteric *E. coli* strains may contribute to the observed heterogeneity in STEC serotypes. Acheson et al. (1) demonstrated that it was possible to infect and lysogenize an *E. coli* laboratory strain with an Stx1-converting phage in the murine gastrointestinal tract. In addition, transductants which produced infectious phage particles were isolated. Although these experiments were the first evidence for in vivo transduction activity of Stx-encoding phages, wild-type isolates were not used as recipients.

Similar experiments were performed with CTX ϕ , a filamentous *Vibrio cholerae* bacteriophage which encodes cholera toxin (16). Using a model with suckling CD1 mice, Lazar and Waldor showed transduction of CTX ϕ from a lysogen to a recipient strain in the small intestine (16). It was also reported that

lysogenic conversion from a nonpathogenic *Corynebacterium diphtheriae* strain to a toxinogenic strain could occur in the upper respiratory tract (26).

Recently, Beutin et al. (4) demonstrated that Stx produced by a *Shigella sonnei* isolate was encoded by a bacteriophage which could be transduced to a nontoxigenic *S. sonnei* reference strain and *E. coli* laboratory strains.

In the present study, we demonstrated that the detoxified phage $\phi 3538(\Delta stx_2::cat)$, which was isolated from a pathogenic STEC O157:H7 strain, was able to infect and lysogenize certain wild-type strains of *E. coli*. Moreover, lysogenic transductants produced infectious virus particles upon induction with mitomycin.

In total, we were able to detect 17 of 57 wild-type *E. coli* strains transduced with the phage, and these belonged to a broad spectrum of various enteric pathogroups as well as to the physiological intestinal microflora.

There are some limitations to the techniques used for the detection of lysogens described in this paper. First, the selection of lysogens depended on both the initial MIC of chloramphenicol for the host strains and the ability to develop spontaneous chloramphenicol resistance during the selective enrichment procedure.

To address this, we determined the MICs for all strains used in this study and found them to be low. All wild-type strains were sensitive to chloramphenicol. In control experiments, two of the wild-type strains which were not exposed to the phage $\phi 3538(\Delta stx_2::cat)$ developed chloramphenicol resistance during the selective enrichment procedure. In these strains, the frequency of the appearance of Cam^r colonies was 4×10^{-8} . This means that spontaneous mutation to Cam^r is a relatively rare process.

In contrast, after exposure to the phage, we found 26 Cam^r strains. From these, 17 strains contained phage $\phi 3538(\Delta stx_2::cat)$ and 9 did not. The nature of the Cam^r in the latter strains is not known.

Second, we were able to isolate lysogens from strains which did not support the formation of plaques. This may reflect the methodology used, lysogen isolation being more sensitive because of the selective chloramphenicol enrichment step. Without this step, we would not have been able to isolate any lysogen from wild-type isolates. In contrast, selective enrichment was not necessary for the isolation of lysogens from laboratory *E. coli* strains (31).

The lysogenized *E. coli* O157:H7/H⁻ strains contained *stx*₁, *stx*₂, and *stx*_{2c} genes alone or in combination. Since it was shown in earlier studies that Stx1 and Stx2 are generally phage encoded (8, 21, 36), this was assumed for the *E. coli* O157 strains tested here. The fact that it was possible to lysogenize such strains with $\phi 3538(\Delta stx_2::cat)$ indicates that the Stx-encoding prophages which were likely to be present in these strains were different from $\phi 3538(\Delta stx_2::cat)$. Otherwise superinfection immunity would have prevented infection.

The presence of temperate Stx-converting phages that were not related to $\phi 3538(\Delta stx_2::cat)$ could explain why in most of the lysogens, only a part of plaques hybridized with the *cat* probe (Table 1). Natural *E. coli* strains frequently contain sequences which are related to phage λ and presumed to be cryptic prophages (2). The most intensively studied were those present in *E. coli* K-12, whose chromosome contains, besides phage λ itself, four phage-related elements, including DLP12, Rac, Qin, and e14 (5). Cryptic prophages can alter the host phenotype and directly or indirectly interact with active phages. They can mediate superinfection immunity, recombine with active phage DNA, or modify the host surface properties (5). Moreover, natural *E. coli* strains can harbor active lamb-

doid prophages, examples of which are HK022, P2, and 434 (5). In order to successfully infect a host cell, Stx-converting phages probably have to compete with naturally occurring phages or phage-related elements. This could lead to the failure of establishment of the superinfecting phage.

The obvious presence of several prophages in *E. coli* isolates could also contribute to the failure of the labeled phage to establish in the chromosome in a way that the insertion sites could be occupied, and therefore the phage could not replicate.

Another interesting aspect of our study concerns the genetic stability of ϕ 3538(Δ stx₂::cat). We could not detect any recombination of this phage with extant phages of *E. coli* O157 strains, even under chloramphenicol selection.

In conclusion, using a model of a detoxified derivative of an Stx2-converting bacteriophage, we have demonstrated that Stx2-converting bacteriophages are able to spread among enteric *E. coli* strains and may thus contribute to the genotypic and phenotypic heterogeneity observed in the STEC family. The findings described here suggest a role for Stx-encoding bacteriophages in the spread of stx genes among enteric *E. coli* strains.

ACKNOWLEDGMENTS

We thank Beatrix Henkel for excellent technical assistance and Phil I. Tarr for helpful discussions.

This work was supported by grant Ka 717/3-1 from the Deutsche Forschungsgemeinschaft.

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