Immunity Profiles of Wild-Type and Recombinant Shiga-Like Toxin-Encoding Bacteriophages and Characterization of Novel Double Lysogens

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The pathogenicity of Shiga-like toxin (stx)-producing *Escherichia coli* (STEC), notably serotype O157, the causative agent of hemorrhagic colitis, hemolytic-uremic syndrome, and thrombotic thrombocytopenic purpura, is based partly on the presence of genes (stx_1 and/or stx_2) that are known to be carried on temperate lambdoid bacteriophages. Stx phages were isolated from different STEC strains and found to have genome sizes in the range of 48 to 62 kb and to carry either stx_1 or stx_2 genes. Restriction fragment length polymorphism patterns and sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles were relatively uninformative, but the phages could be differentiated according to their immunity profiles. Furthermore, these were sufficiently sensitive to enable the identification and differentiation of two different phages, both carrying the genes for Stx2 and originating from the same STEC host strain. The immunity profiles of the different Stx phages did not conform to the model established for bacteriophage lambda, in that the pattern of individual Stx phage infection of various lysogens was neither expected nor predicted. Unexpected differences were also observed among Stx phages in their relative lytic productivity within a single host. Two antibiotic resistance markers were used to tag a recombinant phage in which the *stx* genes were inactivated, enabling the first reported observation of the simultaneous infection of a single host with two genetically identical Stx phages. The data demonstrate that, although Stx phages are members of the lambdoid family, their replication and infection control strategies are not necessarily identical to the archetypical bacteriophage λ, and this could be responsible for the widespread occurrence of *stx* genes across a diverse range of *E. coli* serotypes.

Strains of Shiga-like toxin (Stx)-producing *Escherichia coli* (STEC), especially serotype O157, are increasingly implicated as causative agents of human disease, including hemorrhagic colitis, hemolytic-uremic syndrome, and thrombotic thrombo-cytopenic purpura (21). There are many virulence factors that contribute to the pathogenic personalities of STEC strains, including the locus of enterocyte effacement (17), which provides genes for promoting both attachment and effacement lesions and a type III secretion system (10), two different hemolysins (29, 35), attachment fimbriae (11), a heat-stable enterotoxin (28), an iron transport system (14, 19), and Stx (22). Genes encoding Stx are most typically associated with *Shigella dystenteriae* and *E. coli* O157:H7, but they have been found in *E. coli* strains encompassing >100 serotypes, as well as other species of gram-negative bacteria, including *Citrobacter freundii* (30, 37), *Vibrio cholerae* (20), *Enterobacter cloacae* (23), and *Aeromonas* spp. (7).

There are two forms of Stx: Stx1 is almost identical to the toxin from *Shigella dysenteriae* type 1; Stx2 shares only 50 to 60% amino acid homology with Stx1, and there is a greater degree of antigenic heterogeneity among members of this toxin class (18). Although both toxins have the same mode of action, their effects in vitro and in vivo differ considerably. Stx2 pro-

duction alone is reported to be associated with more serious disease than strains that produce either Stx1 or a combination of Stx1 and Stx2 (12). Stx1 binds more efficiently to the mammalian globotriaosylceramide receptor and is more potent against Vero cells (36). Stx2, when injected intravenously, is 400 times more lethal for mice (36) and 1,000 times more active on human renal microvascular endothelial cells (16), the putative target for the development of hemolytic-uremic syndrome (15). These observations should, however, be interpreted with caution, since there is also a report that Stx1 is as potent an inhibitor of protein synthesis as Stx2 when challenged against highly purified human glomerular microvascular endothelial cells (39).

Both Stx1 and Stx2 have been shown to be encoded on bacteriophages (34, 38), and such phages have been isolated from a variety of *E. coli* strains of human, bovine, and porcine origin. The phages appear to belong to the lambdoid family of bacteriophages (27). *E. coli* strains that produce both Stx1 and Stx2 have been shown to carry two separate phages, each of which encodes only one of the toxins although possessing similarities in gross morphology and genome size and a high degree of DNA homology (27). The potential phage-mediated transfer of *stx* genes between *E. coli* strains and other bacterial species is cause for concern. Stx expression has been described in species representing several genera of gram-negative bacteria, not all of which are members of the *Enterobacteriaceae*. Thus, the presence of *stx* genes in combination with other virulence factors could lead to the emergence of new patho-

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gens. Phages involved in *stx* dissemination must be relatively nonspecific, belong to a heterogeneous group, and/or interact with a highly conserved ligand or receptor to account for this etiology.

Recent studies characterizing Stx phages have been limited because of the assumption that immunity to subsequent infection was conferred to a lysogen by related or closely related phages (27, 33, 41). The host range of the recombinant Stx phage described in the present study has been reported (9). Here we characterize the group of Stx phages from which this was isolated, with emphasis on the investigation of Stx phage immunity profiles because of the observance of multiple phage infection events in a single host cell. The bacteriophage λ immunity mechanism is well studied, and multiple infections of genetically identical phage are obviated (26). An important objective of the present study reported here was to determine whether Stx phage deviated from this immunity model to account for the aetiology of multiple Stx phage and *stx* genes within a single host cell.

MATERIALS AND METHODS

Bacterial strains and phage isolates. Thirty-one wild-type STEC strains were obtained from the Central Public Health Laboratory (CPHL; Colindale, London, United Kingdom), one strain was from the Department of Medical Microbiology (University of Liverpool, Liverpool, United Kingdom), and six were isolated on sorbitol-MacConkey agar plates from samples of cattle feces. Phages obtained from all of these STEC strains were named according to their bacterial strain origin and the type of toxin produced. The *E. coli* K-12 derivative strain, MC1061 (42), was used as the nontoxigenic host for Stx phage infections and propagation.

Isolation of phage particles from STEC lysogens. Putative STEC lysogens were cultured overnight in 10 ml of nutrient broth (NB) at 37°C, and the cultures were membrane filtered (Millipore; 0.45-μm pore diameter). These potential phage-containing suspensions were used to infect *E. coli* strain MC1061, and individual plaques were picked and suspended in LB medium plus 0.01 M CaCl$_2$ to isolate a single phage type. Where indicated, UV induction of lysis was used to enhance the recovery of phage (20).

Phage infections. Nontoxigenic strains of *E. coli* were grown to an optical density at 600 nm (OD$_{600}$) of 0.5 in either NB or Luria-Bertani (LB) medium containing 0.01 M CaCl$_2$ at 37°C with shaking. Aliquots (200 μl) of these cultures were incubated at 37°C with 200 μl of serially diluted phage suspensions. After 30 min, 6 ml of Stx top agar (1% tryptone, 0.5% yeast extract, 1% NaCl, 0.4% [wt/vol] Difco agar, 0.01 M CaCl$_2$) was added to the infection mix and poured onto the LB agar. The agar plates were vented and incubated overnight at 37°C before the plaques were identified, counted, and picked.

Isolation of colicin-resistant MC1061 mutants. Briefly, 200 ml of MC1061 culture at OD$_{600}$ of 0.5 was mixed with 1 ml of culture filtrate from an overnight culture of a putative colicin-producing STEC strain. This mixture was plated on LB agar, and any colonies formed were examined for their ability to inhibit naive cultures of *E. coli* MC1061 in addition to producing phage. Colonies that were resistant to the initial colicin and incapable of producing colicin or phage were designated colicin-resistant mutants.

Production of high-titer phage suspensions. *E. coli* MC1061 was cultured in NZCYM medium (1% [wt/vol] NZ amine (Difco), 0.5% [wt/vol] NaCl, 0.5% [wt/vol] Bacto yeast extract, 0.1% [wt/vol] Casamino Acids, 0.2% [wt/vol] MgSO$_4$ · 7H$_2$O) with shaking (100 rpm) at 37°C to an OD$_{600}$ of 0.5. Bacteriophages were added at a multiplicity of infection of 0.1, and the infected culture was further incubated at 37°C with shaking. Phages were then recovered after the removal of whole cells and cellular debris by centrifugation at 12,000 × *g* for 10 min at 4°C, followed by membrane filtration. All phage stocks were stored at 4°C with a few drops of chloroform. With the exception of phage ϕPS14, which decreased in titer overnight from 10^9 to 10^2 PFU ml^{-1}, phage stocks were stable under these conditions for at least 6 months.

Stx oligonucleotide probes. Oligonucleotides that correspond to the published sequences of the *stx$_1$* and *stx$_2$* structural genes (8) were purchased from Genset SA (Paris, France). The oligonucleotide sequences were 5′-CTG ATG ATT GAT AGT GGC TCA-3′ and 5′-TCT GTT ATT AAC CAC ACC CAC-3′, respectively. These oligonucleotides were labeled by using polynucleotide kinase

according to the manufacturer's instructions (Boehringer Mannheim) with 4,500 Ci of ^{32}P (ICN)/mmol.

Colony hybridization. Stx top agar overlay plates were incubated at 4°C for 1 h, and nylon membrane disks (Hybond N+; Amersham Pharmacia Biotech) were then laid on the top agar overlay and removed after 1 min. The membranes were then placed sequentially on Whatman 3MM paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 7 min, in neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.2], 1 mM EDTA) for 3 min (two times), and finally in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 1 min. The DNA was UV cross-linked to the membrane. Cell debris was removed by a wash in 5× SSC. The membranes were placed in prehybridization solution (50 mM Tris-HCl [pH 7.5], 1% [wt/vol] sodium dodecyl sulfate [SDS], 10% [wt/vol] dextran sulfate, 1 M NaCl, 10% [vol/vol] deionized formamide) for 2 to 3 h at 37°C. Radiolabeled probes were then applied and allowed to hybridize overnight at 37°C. Membranes were rinsed several times in 2× SSC containing 0.1% (wt/vol) SDS for 40 min at 42°C until the background radiation was no longer detected. The positive clones were detected by autoradiography.

Electron microscopy. Phage particles were prepared for electron microscopy by dialysis of 10^{10} PFU ml^{-1} against double-distilled H$_2$O overnight. Samples were then negatively stained with 2% (wt/vol) uranyl acetate and then examined by transmission electron microscopy.

Phage DNA preparation. Phage DNA was isolated from 10 ml of the high-titer phage suspensions by using the Promega Wizard DNA purification system according to the manufacturer's instructions.

Southern hybridization. Restriction enzyme-digested phage genomic DNA samples were subjected to 40 mM Tris-acetate–1 mM EDTA–agarose gel electrophoresis. Upon completion, the DNA was depurinated in 0.25 M HCl, denatured in 0.4 M NaOH for 10 min, and neutralized in 1.5 M NaCl–0.5 M Tris-HCl (pH 7.2)–1 mM EDTA. DNA was transferred to a positively charged nylon membrane (Hybond N+) by capillary transfer and fixed to the membrane by UV cross-linking. The prehybridization and hybridization parameters used were identical to those described above for colony hybridization analysis.

SDS-PAGE analysis. Phage particles were precipitated by using 2/5 volume of PEG solution (33% [wt/vol] polyethylene glycol 8000, 3.3 M NaCl), followed by incubation on ice for 1 h prior to centrifugation at 17,500 × *g* for 20 min at 4°C. The pellet was suspended in 6 ml of SM buffer (0.1 M NaCl, 0.01 M MgSO$_4$ · 7H$_2$O, 0.05 M Tris [pH 7.5], 0.01% [wt/vol] gelatin). PEG and cell debris were removed by mixing the sample with 1 volume of chloroform, and the phages were recovered by centrifugation at 1,000 × *g* for 10 min at 4°C. The phage-containing aqueous layer was harvested, and 0.57 g of CsCl ml^{-1} was added. After ultracentrifugation at 90,000 × *g* for 24 h at 4°C, the phage particles were harvested and dialyzed against SM buffer, and the titer was determined. SDS-polyacrylamide gel electrophoresis (PAGE) gels were prepared by the method of Laemmli (13) with a 12% separating gel and a 5% stacking gel. The proteins were separated overnight at 0.5 V cm^{-1} and then subjected to silver staining (Bio-Rad).

Lysogen production. *E. coli* MC1061 lysogens were created by infecting 0.2 ml of culture in NB (OD$_{600}$ = 0.5) with 5 × 10^9 PFU of Stx phage. This infection mix was added to 6 ml of top agar and poured onto LB agar base plates. After overnight incubation, any colonies were regarded as possible lysogens and subcultured on to NB agar plates. Any colonies that could produce phages and were immune to subsequent phage infection were considered lysogens.

Characterization of phages produced by STEC lysogens. *E. coli* MC1061 lysogens were created by using phage preparations obtained from a single plaque obtained from an STEC culture. The culture filtrates from the original STEC strain were then plated against the confirmed MC1061 lysogen. Where plaques were evident, the presence of more than one phage was considered likely, since bacterial lysogens should be immune to subsequent infection by their parent phage.

Recombinant mutant phage construction. Plasmid NTP707 (43), which was constructed in pACYC184 and contains a 5-kb *Eco*RI-fragment possessing the Stx operon from ϕ933W, served as a mutagenic template for the construction of a selectable Stx-deficient phage. This plasmid was digested with *Pst*I, which interrupts the *a* subunit gene (*stxA$_2$*) of the *stx$_2$* operon. A kanamycin resistance gene, *aph3*, was obtained from pUC4K (Amersham Pharmacia Biotech) after digestion with *Pst*I. These two DNA fragments were ligated together by using T4 ligase (Boehringer Mannheim). The resultant plasmid, NTP707-Kan, was transformed into *E. coli* strain MC1061, which was subsequently infected with ϕE86654-Stx2. The culture filtrates from this infection were then used to subsequently infect naive MC1061 cultures. The infection mix was plated onto NB agar (with 50 μg of kanamycin ml^{-1}) and then replica plated onto NB agar (with 50 μg of kanamycin and 5 μg of tetracycline ml^{-1}). Kanamycin-resistant, tetra-

TABLE 1. Oligonucleotide probing of plaques on an MC1061 bacterial lawn infected with culture filtrates from various STEC strains[a]

STEC strain	Serotype	Phage type	Phage titer/ml of culture filtrate	No. of plaques probed	No. of plaques hybridizing to:	
					Stx1 probe	Stx2 probe
CF16	ND[c]	ND	8.3×10^2	332	0	0
E45040			1.2×10^6	121	0	121
E45040 (UV)[b]	O111:H−	ND	6.7×10^8	670	0	670
E83819			1.7×10^6	171	171	0
E83819 (UV)	O157:H7	14	1.6×10^8	160	160	0
E85539	O157:H7	4	1.4×10^6	175	0	175
E86654	O157:H7	4	7.1×10^4	126	121	5
PS14			1.5×10^6	510	0	510
PS14 (UV)	O157H7	ND	2.4×10^7	240	0	240

[a] Plaques were probed with oligonucleotides specific to Stx1 and Stx2 toxin genes. Data for serotypes and phage types were obtained from the CPHL.
[b] (UV), culture was subjected to UV irradiation to induce phage production.
[c] ND, not determined.

cycline-sensitive colonies were harvested as putative lysogens of the recombinant phage and further examined for their ability to produce plaques.

A second recombinant phage was created by using a similar strategy. Briefly, the plasmid NTP707x, the mutagenic vector, was created from NTP707 by the removal of a previously added green fluorescent protein (Clontech) reporter gene construct (32) that had been cloned into the region between the *Sma*I and *Pst*I sites within $stxA_2$ of the toxin operon. Digestion of this reporter gene construct with *Pst*I and religation of the plasmid resulted in the removal of 0.9-kb fragment of the $stxA_2$ gene, as well as the conservation of the *Pst*I site. The chloramphenicol resistance gene from pLysS (Novagen) was used as a selectable marker. This *cat* gene was amplified from the vector by using the oligonucleotides 5′ChlorAmp (5′-AACTGCAGAAATGAGACGTTGATCGG-3′) and 3′ChlorAmp (5′-AACTGCAGCCTTAAAAAAATTACGCC-3′ [Sigma Geno-Sys]) by using Platinum *Pfx* (Invitrogen Life Technologies) according to the following amplification parameters: denaturation at 94°C for 2 min and 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 68°C for 1 min. A restriction endonuclease site, *Pst*I, was present at the 5′ end of both oligonucleotides so that it could be cloned into the *Pst*I site of NTP707x to create NTP707x-Cat. The strategy for isolation of the recombinant phage was then performed as indicated above with the exception that chloramphenicol-resistant (50 µg ml⁻¹), tetracycline-sensitive (5 µg ml⁻¹) colonies were picked for further study.

Confirmation of lysogen production by using the recombinant phage. A strategy with *Taq* DNA polymerase (MBI) was used to amplify the stx_2 operon containing either the *aph3* or *cat* genes from suspected lysogens with the oligonucleotide primers Stx2A3′ (5′-TCTGTTCAGAAACGCTGC-3′) and Stx2A5′ (5′-TACTGTGCCTGTTACTGG-3′) that were designed from the published sequence of the 933W Stx2-phage (GenBank accession no. NC_000924) (25) to amplify the $stxA_2$ subunit gene (9). Southern blot analysis was also used to confirm the identity of a double lysogen. Chromosomal DNA was treated as described above for Southern blot analysis. Prehybridization, hybridization, and detection methods for the analysis of the double lysogen were performed according to the manufacturer's guidelines by using the DIG nucleic acid detection kit (Roche). Probes for this Southern blot were labeled by using the PCR DIG probe synthesis kit (Roche) with the primer pairs 5′-AAT GTC GGG CAA TCA GG-3′ and 5′-GAA TCC GGT GAG AAT GG-3′, 5′-AAC TGC AGA AAT GAG ACG TTG ATC GG-3′ and 5′-AAC TGC AGC CTT AAA AAA ATT ACG CC-3′, 5-CAC TGG CGA TAA AGA AGG-3′ and 5′-CAC TGG CGA TAA AGA AGG-3′, and 5′-GCA TAT ACC AAG GCC TCT-3′ and 5′-GCG TCA CTG TAT GTC CC-3′ that amplify the genes for the kanamycin resistance cassette, the chloramphenicol resistance cassette, Q, and $substxB_2$, respectively.

RESULTS

Screening *E. coli* strains for the presence of Stx phages. The presence of Stx phages was detected by infecting strain MC1061 with overnight culture filtrates of 48 STEC strains and examining overlaid plates for plaques. This screening procedure was initially hampered by the apparent production of colicins by 13 STEC strains, which killed the indicator strain MC1061. Consequently, two spontaneous colicin-resistant mu-

tants of *E. coli* (MC1061) were isolated to enable examination of phage susceptibility. One of these strains, MC1061-CR1, was isolated by using the colicin-producing O157 STEC strain, PS14, and the other resistant mutant, MC1061-CR2, was isolated by using the colicin-producing O91 STEC strain, E45037. Neither colicin-resistant mutant produced phages or colicins, but both were still susceptible to phage infection. This suggested that their resistance was not mediated by the acquisition of a colicin-producing plasmid or lysogenization by phage. All three MC1061 hosts were used to unequivocally screen all 48 STEC strains for phage production. Culture filtrates from five serotype O157 strains (E83642, E86654, E85539, E83819, and PS14), one O111:H− strain (E45040), and two of the bovine STEC isolates that were not serotyped (CF16 and D155) gave rise to plaques on the MC1061 indicator strains. Of the 48 STEC strains screened, evidence for phage production was obtained in 8 strains.

Confirmation of the presence of multiple phages within a single host strain. STEC strains produce one or more types of Stx. To determine whether these different toxins are encoded by more than one phage, experiments were designed to differentiate the phages. Culture filtrates from strains that produced more than one Stx were used to infect MC1061. The resultant plaques were transferred to nylon membranes and sequentially probed with stx_1 and then stx_2 gene-specific oligonucleotide probes (Table 1). Only plaques produced by CF16 culture filtrates failed to hybridize with either probe, possibly indicating that the plaques formed on the indicator strain by CF16 culture filtrates were not due to Stx phages. Although the CPHL had reported that this strain produced Stx1, we were unable to confirm this through either Southern blot analysis or hybridization to plaques. The phage titer produced by CF16 (ca. 10^3 PFU ml⁻¹) was many orders of magnitude lower than all of those produced by the other STEC strains (ca. 10^5 to 10^9 PFU ml⁻¹) (Table 1). Except for strain E86654, phages encoding only one toxin type were detected.

In order to isolate as many different phages as possible from the STEC strains, an alternative procedure was used. It was based on infecting an MC1061 lysogen with phages such that any virion in the filtrate able to plaque on that lysogen was assumed to be different from the original bacteriophage. Again, the presence of both Stx1 and Stx2 phages in strain E86654 was revealed, but two other strains (E83819 and

TABLE 2. Isolation and differentiation of phages from STEC strains after MC1061 lysogen infection

Strain	Culture filtrate PFU[b]	Primary phage isolated	PFU of secondary phage[c]	Secondary phage isolated	Ratio of primary to secondary phage isolate
E45040	1.23×10^5	φE45040-Stx2	0	None	NA[d]
E45040 (UV)[a]	8.34×10^8	φE45040-Stx2	0	None	NA
E83819	2.90×10^5	φE83819-Stx1	5.60×10^3	φE83819-Stx2	50:1
E85539	1.41×10^6	φE85539-Stx2[1]	1.01×10^6	φE85539-Stx2[2]	2:5
E86654	6.90×10^4	φE86654-Stx1	6.60×10^3	φE86654-Stx2	10:1
PS14	1.47×10^6	φPS14-Stx2	0	None	NA
PS14 (UV)	1.90×10^9	φPS14-Stx2	0	None	NA

[a] (UV), culture was subjected to UV irradiation to induce phage production.
[b] That is the total amount of PFU found in the culture filtrates.
[c] Calculated as the titer against MC1061 − the titer against the primary phage lysogen.
[d] NA, not applicable.

E85539) were now shown to produce more than one Stx phage (Table 2). This approach also allowed the identification and isolation of two different Stx2 phages from the same strain (E85539), which would not have been possible by using the simple plaque hybridization assay (Table 1). Although strains E45040 and PS14 (both from the CPHL) were originally characterized as possessing stx_1 and stx_2 genes, we could only isolate a single Stx2 phage from each, even when UV induction was also applied.

Characterization of Stx phages. Two of the Stx phages were examined by transmission electron microscopy. Both φE86654-Stx2 and φE86654-Stx1 particles had regular hexagonal heads and short stubby tails (Fig. 1) that were similar in morphology to Stx phages described previously (27).

Restriction patterns of phage genomic DNA digested with *Eco*RI and *Hin*cII (Fig. 2) indicated that the Stx2 phages had broadly similar restriction fragment length polymorphism (RFLP) patterns (Fig. 2, lanes 1, 3, 4, 7, and 8 to 10); the Stx1 phages were more heterogeneous (Fig. 2, lanes 2, 5, and 9).

After TAE-gel electrophoresis, the restriction endonuclease-digested phage genomic DNA samples were transferred to a nylon membrane and probed with oligonucleotides specific for either stx_1 or stx_2 (8). The stx_2 oligonucleotide probe usually identified a 5.0-kb *Eco*RI or 2.2-kb *Hin*cII fragment (Fig. 2, column III). However, the stx_2 gene was present on a 5.9-kb *Eco*RI fragment and 1.5 *Hin*cII fragment in phage φE83819. The size of the *Eco*RI and *Hin*cII fragments harboring the stx_1 gene varied in all three Stx1-phages. The phage genome sizes were estimated from the molecular weights of DNA fragments produced by the RFLP analysis (Fig. 2). Four of the five Stx2-phages possessed 61.7-kb genomes, the remaining Stx2-phage had a 49.2-kb genome, and the three Stx1-phages had genomes ranging from 48.2 to 59.7 kb.

Total phage proteins were analyzed by SDS-PAGE for some of the Stx phages, and most had very similar gross protein profiles (Fig. 3). In some cases, Stx phages that could be readily distinguished by the RFLP analysis described above exhibited identical gross protein profiles. However, φE85539-Stx2[2] had
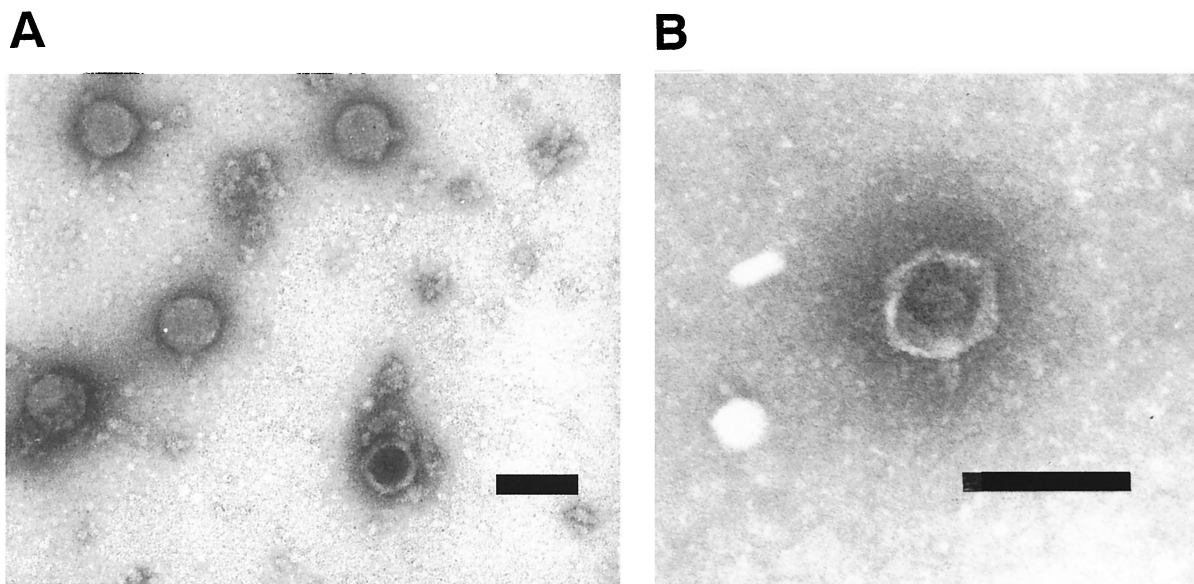

FIG. 1. Transmission electron micrographs of φE86654-Stx1 and φE86654-Stx2. (A) φE86654-Stx1; (B) φE86654-Stx2. Both phages appear to be lambda-like in that they possess icosahedral heads and short stubby tails. Bar, 100 nm.
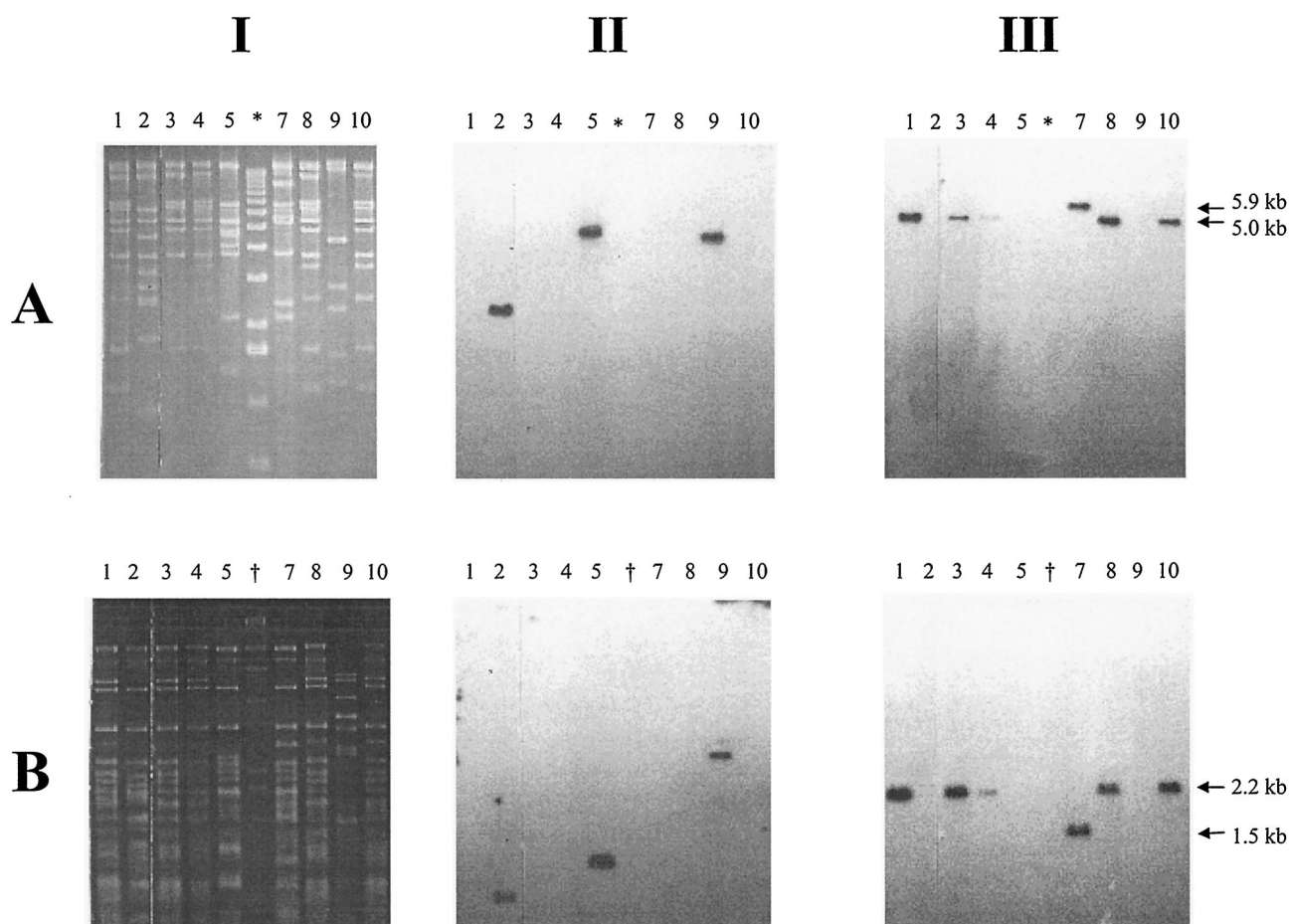
FIG. 2. RFLP and Southern blot analysis of phage genomic DNA. (A) *Eco*RI-digested DNA; (B) *Hin*cII-digested DNA. Columns: I, RFLP patterns on TAE-agarose gel; II, Southern blot analysis of I with a Stx1-specific probe; III, Southern blot analysis of I with an Stx2-specific probe. Phage DNA were from the following sources: lane 1, φE86654-Stx2; lane 2, φE86654-Stx1; lane 3, φE85539-Stx2; lane 4, φE85539-Stx2′; lane 5, φE83819-Stx1; lane 7, φE83819-Stx2; lane 8, φE45040-Stx2; lane 9, φD155-Stx1; lane 10, φPS14-Stx2; lane ✻, molecular mass marker X (Roche); lane †, molecular weight marker II (Roche). Sizes: ✻, 12,216, 1,1198, 10,180, 9,162, 8,144, 7,126, 6,108, 5,090, 4,072, 3,054, 2,036, 1,636, 1,018, 517, 506, 396, 433, 298, 220, 201, 154, 134, and 75 bp; †, 23,130, 9,416, 6,557, 4,361, 2,322, 2,027, 564, and 125 bp.

an unusually distinct protein profile but had an RFLP pattern identical to that for φE85539-Stx2[1].

Immunity profiles of Stx phages and their lysogens. Phage immunity patterns were derived by plating each phage against every lysogen, and all phages failed to infect their own lysogens. The immunity profiles varied considerably; some phages infected all lysogens, while others, e.g., φPS14-Stx2, infected few lysogens (Table 3). Three of the STEC strains each yielded two different Stx phages. Their immunity patterns were not uniform in that one phage could infect the MC1061 lysogen of the other, but the reciprocal infection was either not obtained or was at a much reduced level (Table 3). Both phages obtained from STEC strain E86654 were unusual because they were able to infect many lysogens but often with much reduced efficiency. There are a number of examples of an absence of infection reciprocity between two phages and their lysogens (i.e., φE83819 Stx1 and φE45040 Stx2 [see Table 3]), and this finding was unexpected because it does not fit the established lambda bacteriophage immunity model (4), wherein the order of infection would have no effect on the immunity of the lysogen to the second phage.

Construction of an Stx phage for lysogen selection. A recombinant phage, φ24$_B$::Kan, was created from phage φE86654-Stx2. The laboratory *E. coli* strain MC1061 was very susceptible to lytic infection by this wild-type phage, so a derived recombinant phage could provide an experimental system that does not require high-level laboratory containment. Plasmid NTP707, which contains the *stx*$_2$ operon from φ933W and a tetracycline resistance gene in its vector backbone (43), was used to create a mutagenic template. The *stxA*$_2$ gene from the toxin operon was interrupted with a kanamycin resistance cassette (see Materials and Methods). This recombinant plasmid was introduced into *E. coli* MC1061, and the resulting transformant was infected with phage φE86654-Stx2. Double recombinational events at both the 5′ and the 3′ ends of the Stx operon were selected for on LB agar containing 50 µg of kanamycin ml^{-1}, and loss of the plasmid backbone was confirmed by screening for loss of the tetracycline resistance phenotype. Kanamycin-resistant, tetracycline-sensitive clones were further examined for the production of phage particles with or without UV induction. Phages obtained in this manner were also shown to produce kanamycin-resistant lysogens in strain
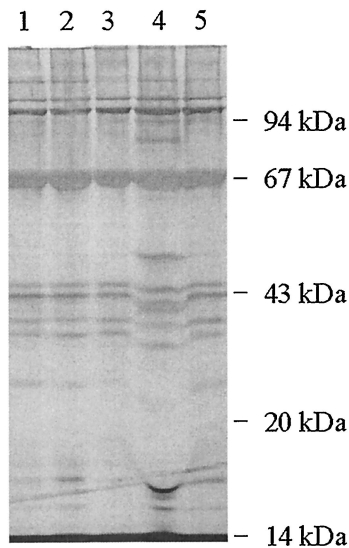
FIG. 3. Silver-stained SDS-PAGE analysis of protein profiles of phages isolated from various STEC strains. The locations of the molecular mass markers are indicated. Lane numbers represent phage proteins from ϕE86654-Stx1 (lane 1), ϕE86654-Stx2 (lane 2), ϕE85539-Stx2 (lane 3), ϕE85539-Stx2$'$ (lane 4), and ϕE45040-Stx2 (lane 5).

MC1061. Plaques obtained with the recombinant phage were phenotypically identical to those produced by the wild-type phage ϕE86654-Stx2 (data not shown). No differences in phage morphology could be detected by electron microscopy. The MC1061 lysogens produced with this recombinant phage could easily be detected and enumerated by culturing infection mixtures on LB agar containing 50 μg kanamycin ml^{-1}.

An alternative selective marker, the gene for chloramphenicol resistance, was introduced into the $stxA_2$ gene of the Stx operon in ϕE86654-Stx2 to create ϕ24$_B$::Cat. This enabled experiments in which both ϕ24$_B$::Kan and ϕ24$_B$::Cat could be used to doubly infect *E. coli*, and the markers allowed for strong selection to aid in the identification of lysogens and/or double lysogens. The latter would be unexpected if the underlying mechanism for immunity in these lambdoid Stx phages conformed to the lambda bacteriophage model (4). Regardless of the order of infection, both phages were able to produce

lytic infection on the lysogen of the other, and double lysogens were detected at a very low frequency (data not shown). A single colony of a PCR-confirmed double lysogen was subjected to Southern analysis. The chromosomal DNAs of naive strain MC1061, a PCR-confirmed ϕ24$_B$::Kan lysogen, a PCR-confirmed ϕ24$_B$::Cat lysogen, and the double lysogen were digested with either *Eco*RI or *Afl*III (Fig. 4). Labeled probes specific to *aph3*, *cat*, and *Q* and to a region, *substxB$_2$*, located downstream of the *stxB$_2$* genes were synthesized. After Southern hybridization analysis it was definitively demonstrated that two intact toxin operons existed within the chromosome of the double lysogen. As shown in Fig. 4, the *aph3* gene probe hybridized to the predicted fragments in the ϕ24$_B$::Kan lysogen and in the ϕ24$_B$::Kan ϕ24$_B$::Cat double lysogen, only (Fig. 4IIIA, lanes 1, 2, 5, and 6). When the *cat* gene probe was used, it hybridized to DNA only from the lysogens containing ϕ24$_B$::Cat (Fig. 4IIIB, lanes 3, 4, 5, and 6). The *Q*- and *substxB$_2$*-specific probes hybridized to DNA fragments of the predicted sizes in both single lysogens (Fig. 4IIIC and D, lanes 1, 2, 3, and 4), whereas the double lysogens possessed all DNA fragments that were unique to each single lysogen (Fig. 4IIIC and D, lanes 5 and 6). Since all of the DNA fragments detected were of the expected sizes and both *Afl*III and *Eco*RI sites were found outside the *stx$_2$* toxin operon, it is clear that both toxin operons must be intact. No probes recognized DNA fragments in uninfected *E. coli* MC1061 (data not shown). It was also demonstrated that both ϕ24$_B$::Cat and ϕ24$_B$::Kan were released as infective phage particles from the double-lysogen host (Table 4). The lysogens that were formed after infection of naive host with phage released from the double lysogens were characterized by their growth on selective media containing kanamycin and/or chloramphenicol and by PCR as described previously (9). Recombinant phages containing both selectable markers were never produced by a double lysogen.

DISCUSSION

There are many reports of bacteria that can produce Stx, although not all species are normally associated with toxin production (7, 20, 23, 30, 37). The production of Stx-like toxins in strains of *C. freundii* and *Aeromonas* spp., for example, may be due to bacteriophage-mediated transfer of *vt* genes from the STEC reservoir. This could have serious implications for the

TABLE 3. Immunity patterns of MC1061 lysogens and phages isolated from STEC strains

Lysogen	Immunity pattern[a] with phage:								
	ϕD155-Stx1	ϕE45040-Stx2	ϕE83819-Stx1	ϕE83819-Stx2	ϕE85539-Stx2[1]	ϕE85539-Stx2[2]	ϕE86654-Stx1	ϕE86654-Stx2	ϕPS14-Stx2
MC1061(ϕD155-Stx1)	(−)	++	++	++	++	++	++	++	++
MC1061(ϕE45040-Stx2)	++	(−)	++	++	++	++	++	++	−
MC1061(ϕE83819-Stx1)	++	−	(−)	−	++	++	++	+	−
MC1061(ϕE83819-Stx2)	++	++	++	(−)	++	++	++	++	++
MC1061(ϕE85539-Stx2[1])	++	−	++	++	(−)	++	++	++	−
MC1061(ϕE85539-Stx2[2])	++	−	++	++	−	(−)	+	−	−
MC1061(ϕE86654-Stx1)	++	++	++	++	++	++	(−)	++	++
MC1061(ϕE86654-Stx2)	++	++	++	++	++	++	+	(−)	−
MC1061(ϕPS14-Stx2)	++	++	++	++	++	++	++	++	(−)

[a] Pattern: (−), phage plated against its own lysogen; ++, lytic infection (PFU count was identical to that obtained from infection of naive MC1061); +, lytic infection (PFU count was reduced several orders of magnitude compared to infection of naive MC1061); −, no plaques found.
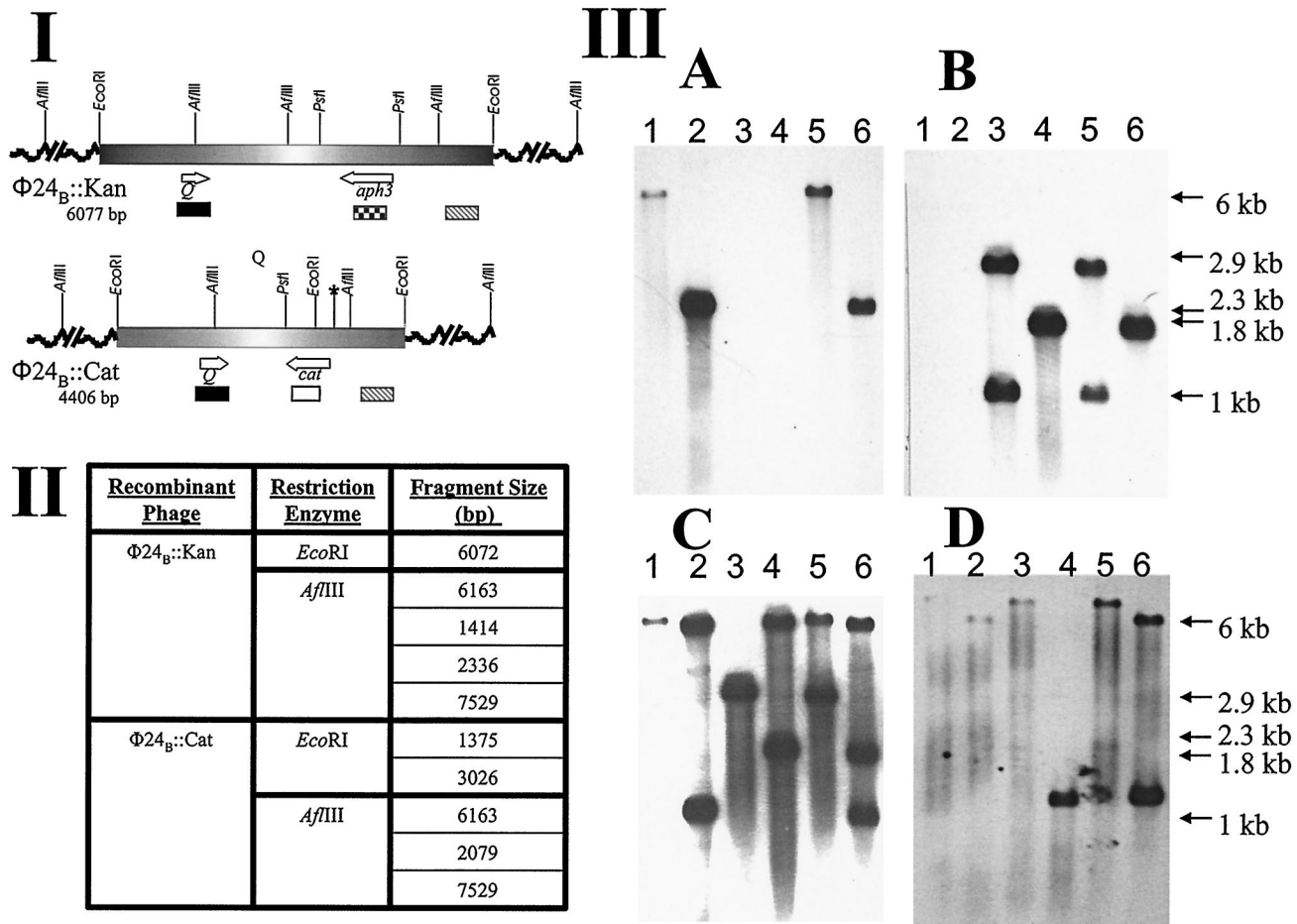
FIG. 4. Confirmation of the residence of two complete toxin operons within the double lysogen genome. (I) Genetic maps showing the location of relevant restriction endonuclease sites and areas recognized by the relevant probes: *aph3* (▨), *cat* (□), *Q* (■), and *substxB₂* (▧). The asterisk (∗) indicates the position of a *Pst*I site that was lost after a single nucleotide substitution, as confirmed by DNA sequencing. (II) Tabulated data of the RFLP lengths of the relevant *Afl*III and *Eco*RI fragments from each isogenic recombinant phage. Fragment sizes are listed in consecutive order beginning at the 5′ end of the maps in part I. (III) Southern blots with probes specific to *aph3* (A), *cat* (B), *Q* (C), and *substxB₂* (D). Lanes 1, 3, and 5 on blots A to D contain *Eco*RI-digested DNA, and lanes 2, 4, and 6 contain *Afl*III-digested DNA. The sources of the DNA are *E. coli* MC1061 lysogens containing the following prophages: lanes 1 and 2, φ24$_B$::Kan; lanes 3 and 4, φ24$_B$::Cat; lanes 5 and 6, φ24$_B$::Kan and φ24$_B$::Cat.

increase and spread of STEC-like diseases, and the presence of *stx* genes in "unusual" hosts may further complicate the detection of new Stx-producing pathogens in outbreaks. Consequently, characterization of Stx phages, and particularly their

infection and immunity profiles, is important but rarely reported.

Here, Stx phages were isolated from a variety of STEC serotypes that had been isolated previously from various ani-

TABLE 4. Characterization of phage released from the double lysogens

Double lysogen[a]	Total PFU released (SEM)	LFU (SEM)[b]		
		φ24$_B$::Kan	φ24$_B$::Cat	φ24$_B$::Kan φ24$_B$::Cat
φ24$_B$::Kan φ24$_B$::Cat				
Uninduced	8.0×10^5 (5.3×10^4)	355 (45)	0 (0)	0 (0)
Induced	2.1×10^8 (1.3×10^7)	1,030 (141)	673 (64)	0 (0)
φ24$_B$::Cat φ24$_B$::Kan				
Uninduced	7.6×10^5 (1.6×10^4)	3 (3)	0 (0)	0 (0)
Induced	2.6×10^8 (4.0×10^6)	1,430 (57)	837 (22)	0 (0)

[a] Uninduced, phage spontaneously released after overnight culture of the double lysogen in LB medium plus Ca; induced phage released from the double lysogen after norfloxacin treatment (1 µg/ml, 1 h).
[b] LFU, lysogen-forming units. Lysogens were detected after infection of naive MC1061 cells with culture filtrate from double-lysogen culture.

mal or human hosts. Stx phages were isolated by infecting laboratory strains of *E. coli* with culture filtrates from each of the STEC cultures. In a few cases, where inhibitory products (probably colicins) were produced by STEC strains, resistant host strains were generated to enable phage detection. Only 3 of 17 non-O157 STEC strains and 5 of 31 O157 STEC strains spontaneously released phages that produced plaques on *E. coli* MC1061. All of these plaques hybridized to either the stx_1 or the stx_2 gene probe. Approximately 10% of these STEC strains spontaneously released Stx phages, a frequency similar to those reported previously (27, 33). If phages are present in the other strains, they may be defective, unable to be induced, not induced into the lytic cycle within the parameters of the present study, or incapable of infecting strain MC1061.

The phages that were isolated in the present study displayed similar, though not identical, genomic DNA restriction patterns. Two phages were examined by electron microscopy and exhibited typical lambdoid phage morphology (26). The calculated genome sizes for nine of the Stx phages were in the range of 48 to 62 kb, with a pronounced cluster of phages with genomes of 61.7 kb in size. These general features are of limited significance, but they did reveal that Stx1- and Stx2-phages from the same strain (φE83819-Stx1 and φE83819-Stx2) are more similar to one another than to Stx1- and Stx2-phages from different *E. coli* strains. This finding supports previous work in which stx_1 and stx_2 genes were always located on separate phages within a single bacterial host, and the restriction patterns of both phage genomes displayed strong similarity to one another (27). Strains E83819, E85694, and E86654 possessed more than one Stx phage and produced both types of phage progeny simultaneously. Interestingly, the ratio of the yield of the two phages was highly variable, and in strain E83819, the Stx1/Stx2 ratio was 50:1. It is not known whether these variations are due to differences in the controls of the lytic cycles of the relevant bacteriophages, to the assembly of virion particles, to the location and hence the activity in the host genome, or to the overall fitness of the phages. It has been reported that genotypic variation in Stx phages does affect the expression of late genes (40). This indicates that it is possible for infectious particles to remain undetected, especially if the host is multiply infected, and one phage is a more efficient pathogen of the host. If a strain was infected with a phage that was biased toward the lysogenic cycle due to stringent regulatory controls, it would be difficult, if not impossible, to detect a lytic cycle and subsequent phage release. The full genome sequence analysis of a strain of O157:H7 has confirmed the presence of at least one complete Stx phage and multiple areas in the genome that appear to represent sequences of otherwise-uncharacterized cryptic, or presumed cryptic, Stx-prophages (24). The results presented here indicate that at least some of these cryptic phages could be competent viruses whose activity has yet to be detected.

Most of the properties of the Stx phages isolated here are similar to those described previously (27, 33). However, one of the interesting and distinguishing features of the Stx phages studied here was their immunity profile pattern. Unexpectedly, two phages with identical RFLP patterns, φE85539-Stx2[1] and φE85539-Stx2[2], that would normally have been indistinguishable were isolated from the same strain. Identification of different phages by superinfection of lysogens had the limitation of only separating phages with different immunity profiles. Rietra et al. (27) showed that Stx1- and Stx2 phages from the same strain had identical immunity patterns. The results reported here indicate that this is not always the case, although the RFLP patterns of Stx1- and Stx2 phages isolated from the same host exhibited a level of similarity that was not evident in phages isolated from different hosts. Recombination between prophages within a single host could result in the minimization of heterogeneity. The implication of the data presented here is that the immunity conferred on an *E. coli* Stx phage lysogen is not absolute. Multiple, complete Stx phages can be carried by a single *E. coli* host strain, and this has clear implications for the pathogenicity and epidemiology of STEC.

All of the genes necessary to propagate phage lambda under laboratory conditions are present on 30 kb of DNA (1). The remainder of the genome, sometimes greater than 30 kb as found in the Stx phages reported here, presumably comprises genes that are not essential for propagation, at least in *E. coli* K-12. However, these genes are clearly maintained, suggesting that they offer some selective advantage, either to the Stx phage itself or to a lysogen once the phage genome has become integrated. This is the case with lambda, which encodes additional virulence determinants *lom* and *bor* that are expressed by lysogens, and may aid fitness in its natural environment and increase host growth rate in laboratory conditions (1). Sequences homologous to these two genes have been found on 933W and other stx_2-carrying Stx phages (25). Thus, in nature, the carrier phages and possibly the Stx genes might confer some selective advantage assisting in their maintenance within bacterial populations. From an evolutionary standpoint, a phage with a different immunity grouping will have some selective advantage within a population simply because it is able to escape immunity control and grow on lysogens of otherwise closely related phages. This could enable phage infection of gut commensals or other bacteria not typically associated with Stx production, and result in the creation of new pathogens or add to the refractory nature of the disease process.

The mechanisms underlying the immunity patterns observed here need to be examined further because lysogens of Stx phages are not necessarily immune to superinfection. The experiment in which we created a double lysogen by using the same phage with different selectable markers, i.e., φ24$_B$::Kan and φ24$_B$::Cat, is unique. The data in Fig. 4 provide unequivocal evidence that genetically identical phages can coexist in the same *E. coli* host despite similar genetic arrangement of the Stx phage loci homologous to the lambda phage immunity region (5). In previous studies on lambda phage, double lysogens resulted from the integration of a second phage into the first prophage genome by homologous recombination (2, 3), which resulted in a population of mosaic phage particles upon entry into the lytic cycle. In later unrelated studies, double lysogens were obtained when immunity mutants were used (6, 31). We used powerful antibiotic resistance selection criteria and never detected phage particles that possessed both resistance markers. These selection criteria would assure the detection of every instance of phage incorporation into the host cell chromosome, regardless of the mechanism. We are therefore confident that the double lysogens of this Stx phage have been formed by single phage-directed integration into the host chromosome, in contrast to the observations of Calef et al. (2,

3) in bacteriophage lambda. We also used a phage background that was only recently acquired from a pathogenic *E. coli* strain and, other than the replacement of the stx_2 operon, is unlikely to have undergone mutations that make it markedly different from Stx phages found in the environment. It is therefore unlikely that we are studying a laboratory-induced immunity mutant, but even if the double lysogen is derived from a naturally occurring immunity mutant of the Stx phage, then the data presented here are still very relevant to phage-mediated dissemination of *vt* genes. The numbers of double lysogens obtained by using the isogenic recombinant phage were found at a frequency sufficiently high to reinforce the notion that this mechanism could play a significant role in *stx* gene dissemination. Double lysogens could also have a role in creating a genetic pool within a host cell for the production of novel mosaic phages after recombination events in situ. Future studies with our recombinant Stx phage may help explain the unusual immunity data reported here, as well as enable general Stx phage carriage investigations, both in vitro and in vivo.

ACKNOWLEDGMENTS

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