Diversity and Host Range of Shiga Toxin-Encoding Phage

Shantini D. Gamage, Angela K. Patton, James F. Hanson and Alison A. Weiss


Updated information and services can be found at: http://iai.asm.org/content/72/12/7131

These include:

This article cites 39 articles, 30 of which can be accessed free at: http://iai.asm.org/content/72/12/7131#ref-list-1

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to: http://journals.asm.org/site/subscriptions/
Diversity and Host Range of Shiga Toxin-Encoding Phage

Shantini D. Gamage, Angela K. Patton, James F. Hanson,† and Alison A. Weiss*

Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, Ohio

Received 18 May 2004/Returned for modification 16 August 2004/Accepted 31 August 2004

Shiga toxin 2 (Stx2) from the foodborne pathogen Escherichia coli O157:H7 is encoded on a temperate bacteriophage. Toxin-encoding phages from C600::933W and from six clinical E. coli O157:H7 isolates were characterized for PCR polymorphisms, phage morphology, toxin production, and lytic and lysogenic infection profiles on O157 and non-O157 serotype E. coli. The phages were found to be highly variable, and even phages isolated from strains with identical pulsed-field gel electrophoresis profiles differed. Examination of cross-plaques and lysogenesis profiles further substantiated that each phage is distinct; reciprocal patterns of susceptibility and resistance were not observed and it was not possible to define immunity groups. The interaction between Shiga toxin-encoding phage and intestinal E. coli was examined. Lytic infection was assessed by examining Shiga toxin production following overnight incubation with phage. While not common, lytic infection was observed, with more than a 1,000-fold increase in Stx2 seen in one case, demonstrating that commensal E. coli cells can amplify Shiga toxin if they are susceptible to infection by the Shiga toxin-encoding phages. Antibiotic-resistant derivatives of the Stx2-encoding phages were used to examine lysogeny. Different phages were found to lysogenize different strains of intestinal E. coli. Lysogeny was found to occur more commonly than lytic infection. The presence of a diverse population of Shiga toxin-encoding phages may increase the pathogenic fitness of E. coli O157:H7.

The foodborne pathogen Escherichia coli O157:H7 is responsible for a reported 73,000 cases of illness per year in the United States (16). Disease can range from watery and bloody diarrhea to the life-threatening hemolytic uremic syndrome. A major virulence factor responsible for disease is the production of Shiga toxin (Stx). In Stx-producing E. coli (STEC) such as E. coli O157:H7, Stx can be present in two forms: Stx1, which is about 55% homologous to Stx1 from Shigella dysenteriae, or Stx2, which is essentially identical to Stx from Shigella dysenteriae, or Stx2, which is about 55% homologous to Stx1 at the amino acid level (reviewed in reference 20). A STEC strain can produce either Stx1 or Stx2 or both; however, severe disease has been epidemiologically linked to the presence of Stx2 (3, 24).

The biology of Stx production is unusual. The genes for Stx are encoded in the late gene region of lysogenic phage (21, 28). Late phage genes are expressed only when the phage are engaged in the replicative or lytic cycle (reviewed in reference 11). For E. coli O157:H7, the toxin genes are silent during lysogeny; however, if the phage are induced to enter the lytic cycle, phage and toxin production will occur (37). Induction to the lytic cycle can occur after exposure of the bacteria to DNA damaging agents, such as UV light or mitomycin C (18), or to antibiotics, such as the quinolones (41), which stall DNA synthesis. In addition, neutrophil activation products such as hydrogen peroxide have been shown to induce Stx production (35).

Previous studies indicated that Stx2-encoding phages are highly variable (10, 17, 19, 34, 36). Wagner et al. (36) demonstrated that Stx2 phages from STEC clinical isolates displayed different host ranges and differed in toxin production, suggesting that phage heterogeneity could play a role in disease outcome. Indeed, Muniesa et al. (19) have shown that E. coli O157:H7 isolates from a single outbreak were lysogenized with different toxin-encoding phages. Furthermore, the severity of disease symptoms observed in different patients correlated with in vitro toxin production by their O157:H7 isolate. These studies suggest that phage variability can influence pathogenic fitness.

Shiga toxin-encoding phage can infect non-O157 E. coli. Phage infection can have two outcomes. In some instances, infection of non-toxin-producing E. coli will result in lysogeny, where the phage genome becomes incorporated into the bacterial chromosome (11). Lysogeny has been demonstrated to occur in vivo in a mouse model of disease (1, 7). Lysogeny with toxin-encoding phage has important implications for the evolution of new pathogenic strains. It has been shown that Shigella sonnei can become lysogenized with Stx-encoding phage, which has the potential to convert them to a more pathogenic form (32).

The other outcome of phage infection is lytic infection. Lytic infection of non-O157 E. coli can lead to production of phage and Stx. Our investigators have shown that lytic infection of non-toxin-producing E. coli with Stx-encoding phage in vitro can increase Stx production by more than 1,000-fold (7). Furthermore, in a mouse model of disease, intestinal Stx2 production was dramatically increased in some cases when the toxin-encoding phage were able to infect non-toxin-producing E. coli (7).

Epidemiological studies of E. coli O157:H7 suggest that following exposure, many individuals develop intestinal symptoms; however, only a small percentage of patients go on to develop severe, life-threatening systemic sequelae, such as hemorrhagic colitis or hemolytic uremic syndrome (16). Cer-
tain combinations of toxin-encoding phage and host intestinal flora could result in highly elevated levels of intestinal Stx production, and these individuals may be at increased risk for developing severe systemic disease. Human intestinal flora is highly variable and, furthermore, the host range of Stx-encoding phages is highly variable (2, 9, 22). It is currently unknown how often individuals possess intestinal flora that is susceptible to the toxin-encoding phages. In this study, we examined phage characteristics and immunity profiles of six Stx2-encoding phages from highly related clinical E. coli isolates from the Cincinnati area, with an emphasis on assessing their ability to undergo lysogenic integration and/or lytic infection in E. coli strains. The Stx2-encoding phages were found to be highly variable, even when the E. coli O157:H7 isolates were identical by pulsed-field gel electrophoresis (PFGE) analysis, consistent with the hypothesis that variations in phage host range may play a role in pathogenic fitness.

**TABLE 1. Bacterial strains and phages used in this study**

<table>
<thead>
<tr>
<th>Bacterial strain or phage</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C600</td>
<td>E. coli K-12</td>
<td>22</td>
</tr>
<tr>
<td>E. coli isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECOR set</td>
<td>72 diverse E. coli isolates of animal and human origin</td>
<td>23</td>
</tr>
<tr>
<td>Fecal isolates</td>
<td>29 E. coli isolates from healthy volunteers in the Cincinnati area</td>
<td>7</td>
</tr>
<tr>
<td>FI set</td>
<td>12 E. coli isolates from patients at a Cincinnati hospital</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O157:H7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT22</td>
<td>Isolated in 2000; stx2+ stx1+</td>
<td>This study</td>
</tr>
<tr>
<td>PT27</td>
<td>Isolated in 2001; stx1+ stx2+</td>
<td>This study</td>
</tr>
<tr>
<td>PT32</td>
<td>Isolated in July 1999; stx2+ stx3+</td>
<td>7</td>
</tr>
<tr>
<td>PT38a</td>
<td>Isolated in September 1999; stx1+ stx2+</td>
<td>This study</td>
</tr>
<tr>
<td>PT38b</td>
<td>Isolated in September 1999; stx1+ stx3+</td>
<td>This study</td>
</tr>
<tr>
<td>PT39a</td>
<td>Isolated in September 1999; stx1+ stx1+</td>
<td>This study</td>
</tr>
<tr>
<td>Phages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>φ933W</td>
<td>Stx2 converting phage</td>
<td>22</td>
</tr>
<tr>
<td>φH19B</td>
<td>Stx1 converting phage</td>
<td>22</td>
</tr>
<tr>
<td>φ933WΔtox</td>
<td>φ933W, Stx2 deleted; Cm’, GFP; previously called Δtox</td>
<td>7</td>
</tr>
<tr>
<td>φPT22Δtox</td>
<td>From PT22, Stx2 deleted; Cm’, GFP</td>
<td>This study</td>
</tr>
<tr>
<td>φPT27Δtox</td>
<td>From PT27, Stx2 deleted; Cm’, GFP</td>
<td>This study</td>
</tr>
<tr>
<td>φPT32Δtox</td>
<td>From PT32, Stx2 deleted; Cm’, GFP</td>
<td>This study</td>
</tr>
<tr>
<td>φPT38aΔtox</td>
<td>From PT38a, Stx2 deleted; Cm’, GFP</td>
<td>This study</td>
</tr>
<tr>
<td>φPT39Δαtox</td>
<td>From PT39a, Stx2 deleted; Cm’, GFP</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Isolated at Cincinnati Children’s Hospital Medical Center.

**MATERIALS AND METHODS**

**Bacterial strains.** Luria-Bertani (LB) agar (Difco, Detroit, Mich.) or LB broth was used for routine bacterial propagation. For phage induction and infection experiments, LB agar and broth were supplemented with 10 mM CaCl2 (LB-modified). When indicated, chloramphenicol was added to the medium at a concentration of 15 μg/ml.

The E. coli O157:H7 and other E. coli strains used in this study are listed in Table 1. The clinical E. coli O157:H7 isolates were obtained from The Cincinnati Children’s Hospital Medical Center culture collection. The strains from the ECOR collection were obtained from the STEC Center at Michigan State University (http://foodsafe.msu.edu/whittam/ecor/index.html).

**Phage induction.** Phage from E. coli O157:H7 or from lysogenized strains of C600 was induced with either ciprofloxacin or mitomycin C. For ciprofloxacin inductions, overnight broth cultures were adjusted to an optical density (OD) of 600 nm of 0.08 in LB-modified broth, ciprofloxacin (30 ng/ml) was added, and the culture was incubated at 37°C with shaking for about 16 h. For mitomycin C inductions, overnight broth cultures were adjusted to an OD of about 0.1 in LB-modified broth, the cultures were incubated at 37°C for 2 h with shaking.
mitomycin C (0.5 μg/ml) was added, and cultures were incubated at 37°C for about 16 h with shaking. The OD was used to monitor lysis. The induced cultures were centrifuged (5,000 × g, 10 min), and the supernatants were filter sterilized.

**Determination of Stx concentrations.** Stx concentrations were determined either by ELISA (Premier EHEC ELISA; Meridian Bioscience, Inc., Cincinnati, Ohio) or by a Vero cell assay, as indicated. For the Vero cell assay, twofold serial dilutions of filter-sterilized culture supernatants were made in 25 μl of phosphate-buffered saline in a 96-well plate. Dilutions were overlaid with 100 μl of 10^5 Vero cells/ml, and plates were incubated at 37°C, 5% CO₂ for 3 days. The cells were stained with Giems, and the reciprocal of the dilution at which 50% of the Vero cells were dead was determined. The amount of Stx in the samples was determined by comparison to a standard curve with purified Stx2 (Toxin Technology, Inc., Sarasota, Fla.).

**Determination of GFP production.** GFP in supernatants from uninduced and induced cultures of the C600::stx lysogens was determined by sodium dodecyl sulfite-polyacrylamide gel electrophoresis (SDS-PAGE) with 8 to 16% precast Tris-glycine gels (Cambrex Bioscience Rockland Inc., Rockland, Maine). Bands were transferred to polyvinylidene difluoride (PVDF) membranes by wet transfer with the use of a Trans-Blot cell (Bio-Rad Laboratories). GFP was detected with probing membranes with anti-GFP antibody (1:500 dilution; BD Biosciences, Clontech, Palo Alto, Calif.) followed by goat anti-rabbit secondary antibody (1:37,500; Cappel, West Chester, Pa.). Bands were visualized with the Western Lightning chemiluminescence reagent plus kit (Perkin-Elmer Life Sciences, Boston, Mass.), and the relative intensity of the signals was determined with ImageQuant (version 5.1; Molecular Dynamics, Amersham Biosciences, Piscataway, N.J.).

**Phage purification.** Phage were isolated from ciprofloxacin-induced cultures (200 ml) by polyethylene glycol precipitation and purified by cesium chloride centrifugation (15, 39). Morphology was assessed by electron microscopy with 2% phosphotungstic acid staining of phage particles (21, 28). Phage protein profiles were determined by SDS-PAGE with a 10% Tris-glycine precast gel (Cambrex Bioscience Rockland Inc.), and bands were visualized with Coomassie stain.

**Southern analysis.** Phage DNA was isolated from ciprofloxacin-induced cultures of the parent O157 strains and the C600 lysogens by using polyethylene glycol precipitation and phenol-chloroform extraction (29). Chromosomal DNA was isolated with the DNeasy tissue kit (QIAGEN, Valencia, Calif.). Phage and chromosomal DNAs were digested with EcoRI for 3 h at 37°C, and bands were resolved by electrophoresis on a 0.7% agarose gel (SeaKem GTG agarose; Biowhittaker Molecular Applications, Rockland, Maine). The digoxigenin-11-dUTP (DIG)-labeled DNA molecular weight marker VII (Roche Diagnostics Corporation, Indianapolis, Ind.) was run as a standard. DNA bands were transferred by upward capillary transfer (29) to nylon membranes (Immobilon-NY +; Millipore Corporation, Bedford, Mass.).

DIG-labeled probes were prepared using the PCR DIG probe synthesis kit (Roche Diagnostics). The primers used to generate the stx2 probe (19) and the GFP probe (7) were described previously. After hybridization of the probes to the Lambda phages (65°C; DIG Easy Hyb; Roche Diagnostics), bands were detected with the DIG wash and block buffer set (Roche Diagnostics) and the DIG luminescent detection kit (Roche Diagnostics).

**Determination of phage immunity profiles.** C600 lysogens were induced with ciprofloxacin as described above and centrifuged (5,000 × g, 10 min), and the phage-containing supernatants were filter sterilized. Five microliters of supernatant were spotted onto LB-modified agar overlaid with LB-modified soft agar (0.7%) containing the test strain. Following overnight incubation at 37°C, plates were examined for the formation of plaques.

**Susceptibility of E. coli strains to lysogeny.** Phages with stx2 and encoding chloramphenicol resistance were used to examine the ability of the phage to lysogenize different E. coli isolates. Approximately 10^6 to 10^10 phage were mixed with 7 ml of overnight cultures of the E. coli strains (approximately 10^9 CFU/ml). The samples were poured onto LB-modified agar plates and incubated overnight at 37°C as static cultures, and 100 μl was plated onto LB agar supplemented with chloramphenicol. Two or three chloramphenicol-resistant colonies were streaked for isolation, and the presence of the phage genome was confirmed by PCR with the primers to GFP as previously described (7).

**Susceptibility of E. coli to lytic infection.** The stx2 genes are under control of the phage late gene promoter (28) and, therefore, stx2 is produced and released during lytic phage infection. Elevated production of Stx2 was used to assess lytic infection. Phage were isolated from E. coli O157:H7 induced with 30 ng of ciprofloxacin/ml for 16 h at 37°C. The amount of Stx in the supernatants was determined by ELISA. Phage preparations were diluted in phosphate-buffered saline such that the amount of toxin added to the non-O157 E. coli strains was less than 50 ng/ml. Inoculated cultures were overlaid onto LB-modified agar and incubated at 37°C overnight as a static culture. The cultures were centrifuged (5,000 × g, 10 min), and the supernatants were filter sterilized. Stx2 production was assessed in the Vero cell assay.

**RESULTS**

Twenty-three clinical E. coli O157:H7 isolates from the Cincinnati area between 1999 and 2001 were characterized for differences in the Stx-encoding phage. The isolates fell into 11 groups based on different polymorphisms by PCR using previously published primers for stx1 and stx2 and surrounding phage late genes (34). Six of the E. coli O157:H7 isolates, which possessed different polymorphisms, and φ933W were chosen for further examination.

Strain PT22 was isolated in 2000, and strain PT27 was isolated in 2001. Cincinnati experienced an outbreak of E. coli O157:H7 in 1999. Strain PT32 was isolated in July 1999, and strains PT38a, PT38b, and PT39a were each isolated in the same week in September 1999. PT38b and PT39a had identical PFGE profiles after restriction of total DNA with XbaI (Fig. 1). PT38a had the same profile as the other isolates from September 1999, with an additional band at about 290 kb (Fig. 1). The XbaI profile of strain PT32 had a two-band difference from PT38b and PT39a (Fig. 1). PT32, PT38a, PT38b, and PT39a would be considered to be closely related (33). However, the XbaI digestion profiles of PT22 and PT27 had mul-

![FIG. 1. PFGE of E. coli O157:H7 isolates. E. coli O157:H7 strains were digested with XbaI overnight and run on a 1% agarose gel for 22 h.](image-url)
isolated Stx2-encoding phage, the phage-encoded lysogenic phage (2). To examine the induction profile of an O157:H7 isolates characterized in this study possess the genes and from C600 lysogens. Toxin production from the lysis and toxin production.

Muniesa et al. (19), who reported a strong correlation between similar for all of the phages. These results differ from those of found to be variable (Table 2), even though Stx production was as phage are produced and bacteria are lysed (7). In contrast to grows to an OD of about 0.6, followed by a decrease in the OD typically results in an initial increase in OD as the culture treatment with ciprofloxacin resulted in about 10-fold mitomycin C-induced, and mitomycin C-induced samples are reported as the percentage of the total volume of the bands for each strain.

To assess bacterial lysis following phage induction, the OD was more toxin production than in uninduced cultures, while mit-
toxomal alterations and induce the phage to enter the lytic cycle. Treatment with ciprofloxacin resulted in about 10-fold more toxin production than in uninduced cultures, while mitomycin C resulted in about 20- to 60-fold more toxin (Table 2). To assess bacterial lysis following phage induction, the OD was determined after 16 h (Table 2). Induction of C600::933W typically results in an initial increase in OD as the culture grows to an OD of about 0.6, followed by a decrease in the OD as phage are produced and bacteria are lysed (7). In contrast to the laboratory strain, lysis of the E. coli O157:H7 strains was found to be variable (Table 2), even though Stx production was similar for all of the phages. These results differ from those of Muniesa et al. (19), who reported a strong correlation between lysis and tox production. The ELISA detects both Stx1 and Stx2, and the E. coli O157:H7 isolates characterized in this study possess the genes for both toxins. Furthermore, strains can harbor more than one lysogenic phage (2). To examine the induction profile of an isolated Stx2-encoding phage, the phage-encoded stx2 genes were replaced with two markers, chloramphenicol resistance and GFP, using allelic exchange as previously described (7). Phage with stx2 deleted were lysogenized into E. coli strain C600 to yield the Δtox phage described in Table 1. In the Δtox constructs, GFP expression is under the control of the late phage gene promoter (7), and the production of GFP with and without induction was used to monitor phage-mediated release of Stx2. GFP in the culture supernatant was determined by quantitative Western analysis (Table 2). Little to no GFP was detected in the uninduced supernatants (Table 2). Increased levels of GFP were detected after induction for all strains but were varied among the lysogens with respect to the inducing agent. As observed with the parental strain, lysis was highly variable and the amount of GFP produced after induction did not correlate well with the amount of lysis.

Southern analysis to detect stx2 and GFP genes. E. coli O157:H7 can harbor multiple genes for Stx2. We used Southern analysis to examine the Stx2-encoding genes in the E. coli O157:H7 isolates. The stx2 probe hybridized to a 6.5-kb fragment present in the chromosomal DNA from all of the E. coli O157:H7 isolates; however, in PT27 the stx2 probe also hybridized to a 7-kb band (Fig. 2A). For the Δtox phage derivatives, a single 3.6-kb band hybridized with the GFP probe for both phage DNA (Fig. 2B) and chromosomal DNA (data not shown) for all of the C600 lysogens. These results suggest that PT27 contains two copies of the stx2 gene and Δtox recombined into the copy of stx2 encoded in the 6.5-kb EcoRI fragment.

Phage production and plaquing profiles. Phage from E. coli O157:H7 often produce very small plaques. In initial studies following induction, only supernatants from the C600::933W control, PT22, PT27, and PT32 were able to form visible plaques on the C600 indicator strain, suggesting that the other strains of E. coli O157:H7 might be defective for phage production. However, our ability to lysogenize C600 with all the Δtox derivatives of E. coli O157:H7 strains suggests these strains produce viable phage. To further examine phage production, phage were purified from the C600 Δtox lysogens and C600::H19B, an Stx1-encoding phage (22). The phages were examined by electron microscopy. All six phages from the clinical isolates and Δ933WΔtox were similar in shape (hexa-

### Table 2. Characteristics of phages from clinical E. coli O157:H7 isolates

<table>
<thead>
<tr>
<th>Strain and phage</th>
<th>Uninduced</th>
<th>Ciprofloxacin induction</th>
<th>Mitomycin C induction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lysis (OD)</td>
<td>Stx or GFP concn</td>
<td>Lysis (OD)</td>
</tr>
<tr>
<td>E. coli O157:H7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT22</td>
<td>27</td>
<td>0.238</td>
<td>347</td>
</tr>
<tr>
<td>PT27</td>
<td>23</td>
<td>0.409</td>
<td>354</td>
</tr>
<tr>
<td>PT32</td>
<td>24</td>
<td>0.430</td>
<td>426</td>
</tr>
<tr>
<td>PT38a</td>
<td>18</td>
<td>0.395</td>
<td>355</td>
</tr>
<tr>
<td>PT38b</td>
<td>19</td>
<td>0.376</td>
<td>352</td>
</tr>
<tr>
<td>PT39a</td>
<td>24</td>
<td>0.469</td>
<td>364</td>
</tr>
</tbody>
</table>

C600 lysogens (Stx2 deleted)

<table>
<thead>
<tr>
<th>Strain and phage</th>
<th>Uninduced</th>
<th>Ciprofloxacin induction</th>
<th>Mitomycin C induction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lysis (OD)</td>
<td>Stx or GFP concn</td>
<td>Lysis (OD)</td>
</tr>
<tr>
<td>fPT22Δtox</td>
<td>0.05</td>
<td>0.226</td>
<td>41</td>
</tr>
<tr>
<td>fPT27Δtox</td>
<td>ND</td>
<td>0.209</td>
<td>51</td>
</tr>
<tr>
<td>fPT32Δtox</td>
<td>ND</td>
<td>0.243</td>
<td>33</td>
</tr>
<tr>
<td>fPT38aΔtox</td>
<td>ND</td>
<td>0.430</td>
<td>49</td>
</tr>
<tr>
<td>fPT38bΔtox</td>
<td>ND</td>
<td>0.825</td>
<td>79</td>
</tr>
<tr>
<td>fPT39aΔtox</td>
<td>ND</td>
<td>0.430</td>
<td>72</td>
</tr>
</tbody>
</table>

a Strains were grown overnight in LB broth at 37°C with shaking.
b Strains were induced with 30 ng of ciprofloxacin/ml for 16 h at 37°C with shaking.
c Strains were induced with 0.5 μg of mitomycin C/ml for 16 h at 37°C with shaking.
d Lysis was assessed by measuring the OD_600 after 16 h induction at 37°C with shaking, in the presence of ciprofloxacin or mitomycin C. Results in this trial are representative of the trends observed in at least one other trial.
e GFP production from the C600 lysogens was determined by Western analysis, and bands within a strain were compared using ImageQuant. Results for uninduced, ciprofloxacin-induced, and mitomycin C-induced samples are reported as the percentage of the total volume of the bands for each strain.
f ND, not detected.
gonal heads with short tails) and head size (55 to 65 nm), consistent with published images of 933W (21, 28), the parental phage for \( \theta933W \). In contrast, the Stx1-encoding phage, H19B, had a larger, elongated head and long tail (data not shown). Proteins from the purified phages were separated by SDS-PAGE (Fig. 3). Each phage displayed a unique protein profile, suggesting each phage was distinct.

**Immunity profiles of phage from C600 lysogens.** Bacteriophage lambda lysogens are resistant to lytic infection following superinfection by phage expressing the same immunity protein (11). To determine if the different Stx2-encoding phages in the present study possessed similar immunity genes, phage from C600 lysogenized with \( \theta933W \), \( \theta9278 \), \( \theta9278 \) PT22, \( \theta9278 \) PT27, and \( \theta9278 \) PT38 produced distinct zones of clearing on the C600 indicator (Table 3), while no plaquing was observed when C600 was infected with \( \theta9278 \) PT38a, \( \theta9278 \) PT38b, or \( \theta9278 \) PT39a. These results are identical to those seen when phage were prepared from the parental O157:H7 strain. Since C600 was successfully lysogenized by these phage, these results suggest that these phages are defective in lytic infection, but not lysogeny. This is further supported by the observation that certain phages were able to form plaques only on other lysogens, suggesting genes from the resident phage could complement the lysis defect in the infecting phage. For example, both \( \theta9278 \) PT22 and \( \theta9278 \) PT27 produced phage capable of lytic infection, as evidenced by clear plaques on the C600 indicator. \( \theta9278 \) PT22 produced zones of clearing on C600: \( \theta9278 \) PT22; however, \( \theta9278 \) PT22 did not produce plaques on C600: \( \theta9278 \) PT27.

Cross-plaquing on lysogenic strains was examined. Consistent with lambda immunity to superinfection, all of the lysogens were resistant to lytic infection with their phage. However, highly variable results were obtained when the phages were incubated with the nonhomologous C600 lysogens. No two phages had the same cross-plaquing profile. Furthermore, no clear evidence of reciprocal cross-susceptibility was apparent. For example, both \( \theta933W \) TOX and \( \theta933W \) TOX produced phage capable of lytic infection, as evidenced by clear plaques on the C600 indicator. \( \theta9278 \) PT27 produced zones of clearing on C600: \( \theta9278 \) PT27; however, \( \theta9278 \) PT22 did not produce plaques on C600: \( \theta9278 \) PT27.

**Susceptibility of E. coli O157:H7 strains to lysogeny.** The ability of the \( \Delta \) TOX phage derivatives to infect or lysogenize E. coli O157:H7 was also examined. None of the phages formed
plagues on the *E. coli* O157:H7 strains. To examine lysogeny, overnight cultures of *E. coli* O157:H7 were incubated with the Δtox phage. Chloramphenicol-resistant colonies were selected, and lysogeny was confirmed by a positive PCR for the GFP tox phage. Chloramphenicol-resistant colonies were selected, and lysogeny was confirmed by a positive PCR for the GFP tox phage. Chloramphenicol-resistant colonies were selected, and lysogeny was confirmed by a positive PCR for the GFP tox phage. Chloramphenicol-resistant colonies were selected, and lysogeny was confirmed by a positive PCR for the GFP tox phage.

Susceptibility of human intestinal *E. coli* to lysogeny. In initial studies, the ability of φ933W Δtox, a derivative of φ933W (7), to lysogenize C600 and C600::933W was examined. Following overnight incubation, about 10⁶ φ933W Δtox chloramphenicol-resistant lysogens were obtained for C600 and 10⁴ chloramphenicol-resistant lysogens were obtained for C600::933W. While previous studies have shown that C600::933W is resistant to lytic infection by φ933W Δtox, these results suggest integration of the phage does not appear to be blocked by the resident φ933W genome, a result that was not observed for the *E. coli* O157:H7 isolates (Table 4). The C600::933W lysogens were characterized for the presence of Stx2 to determine if chloramphenicol resistance was due to insertion of a second phage genome or if allelic exchange replaced the stx2 genes with the GFP or chloramphenicol cassette. Six of 12 chloramphenicol-resistant C600::933W isolates lacked the stx2 genes, suggesting allelic exchange had occurred. The other six chloramphenicol-resistant lysogens of C600::933W possessed the stx2 genes in addition to the chloramphenicol and GFP genes, suggesting they represented double lysogens.

This same procedure was used to determine the ability of φ933W Δtox to establish lysogeny in natural isolates of *E. coli*. In preliminary studies we used the ECOR strain collection (23). The ECOR collection consists of 72 isolates of *E. coli* from human and animal sources that are thought to be representative of the genetic diversity of the species. These strains have been extensively characterized for many traits. Lysogeny with φ933W Δtox was detected for 31 of the 72 isolates (43%). Forty of the ECOR strains were isolated from humans and 35%, or 14 of 40, of the human isolates were sensitive to lysogeny. Phage susceptibility did not correlate with lipopolysaccharide (LPS) core or O-antigen type. Results are reported only for the positive strains of the 40 ECOR isolates of human origin (Table 5).

Studies examining the lysogeny of the human ECOR isolates were repeated with φPT32 Δtox. Only 10% (4 of 40) of the human ECOR strains were lysogenized by φPT32 Δtox, compared to 35% of the strains by φ933W Δtox, and these four strains were lysogenized by both φ933W Δtox and φPT32 Δtox.

In addition to testing the ECOR set of *E. coli* for lysogeny by φ933W Δtox and φPT32 Δtox, a number of human *E. coli* isolates from the Cincinnati area were also examined. Twenty-nine *E. coli* isolates were obtained from healthy volunteers, and 12 *E. coli* isolates were obtained from stool cultures from patients treated with antibiotics but not infected with *E. coli* O157:H7. Four of 29 (14%) fecal *E. coli* isolates from healthy volunteers and 6 of 12 (50%) fecal *E. coli* isolates from patients were sensitive to lysogeny by φ933W Δtox (Table 5). Only 1 out of 29 *E. coli* isolates (3%) from healthy volunteers and 2 out of 12 isolates (17%) from patients were susceptible to lysogeny by φPT32 Δtox (Table 5). These studies indicate that φ933W Δtox and φPT32 Δtox have different host ranges with respect to lysogeny.

Phylogenetic studies have divided *E. coli* into four main groups: A, B1, B2, and D (31). Virulent strains primarily belong to groups B2 and D, characterized by a large genome size (4). Human commensal intestinal isolates have been reported to primarily belong to groups A and B1, which are characterized by a small genome size (4); however, recent studies suggest this may not be true for all populations (40). For the fecal isolates examined in this study, most (25 of 29, or 79%) of the *E. coli* isolates from the healthy individuals belonged to the larger genome-size groups, group B2 and group D. However, distribution of the 12 *E. coli* isolates from patients was more even, with 5 of 12 (42%) in group A or B1 and 7 of 12 (58%) in groups B2 and D.

Susceptibility to lysogeny by phage φ933W Δtox appeared to

---

**TABLE 3. Phage immunity profiles determined by cross-plaquing**

<table>
<thead>
<tr>
<th>Phage</th>
<th>Result with C600 lysogen indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C600</td>
</tr>
<tr>
<td>φ933W Δtox</td>
<td>+++*</td>
</tr>
<tr>
<td>φPT22 Δtox</td>
<td>++</td>
</tr>
<tr>
<td>φPT27 Δtox</td>
<td>+++</td>
</tr>
<tr>
<td>φPT32 Δtox</td>
<td>++</td>
</tr>
<tr>
<td>φPT38a Δtox</td>
<td>–</td>
</tr>
<tr>
<td>φPT38b Δtox</td>
<td>–</td>
</tr>
<tr>
<td>φPT39a Δtox</td>
<td>–</td>
</tr>
</tbody>
</table>

* – no plaques observed; +, single plaques; ++, hazy zone; +++, distinct clearing.

---

**TABLE 4. Susceptibility of *E. coli* O157:H7 isolates to lysogeny by phages with the toxin deleted**

<table>
<thead>
<tr>
<th>Phage</th>
<th>Result with clinical <em>E. coli</em> O157:H7 recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PT22</td>
</tr>
<tr>
<td>φ933W Δtox</td>
<td>–</td>
</tr>
<tr>
<td>φPT22 Δtox</td>
<td>–</td>
</tr>
<tr>
<td>φPT27 Δtox</td>
<td>–</td>
</tr>
<tr>
<td>φPT32 Δtox</td>
<td>–</td>
</tr>
<tr>
<td>φPT38a Δtox</td>
<td>–</td>
</tr>
<tr>
<td>φPT38b Δtox</td>
<td>–</td>
</tr>
<tr>
<td>φPT39a Δtox</td>
<td>–</td>
</tr>
</tbody>
</table>
be somewhat dependent on phylogenetic group. When the results for all the human *E. coli* isolates were compiled, lysogeny by phage 933W was more prevalent in strains belonging to the phylogenetic groups with the smaller genome size. For example, 59% (19 of 32) of the group A isolates and 48% (10 of 21) of the group B1 isolates were susceptible to lysogeny by phage 933WΔtox. Lysogeny was observed less often in the larger genome-size groups; only 17% (7 of 40) of the group B2 isolates and 19% (3 of 16) of the group D isolates were susceptible to lysogeny by phage 933WΔtox. The relationship between phylogenetic group and susceptibility to lysogeny is less apparent for φPT32Δtox, primarily due to the low number of lysogens. However, four of the seven strains capable of lysogeny were in phylogenetic group A.

Several non-*Escherichia* fecal isolates were also characterized for susceptibility to lysogeny with φ933WΔtox. These include five isolates of *C. freundii*, two isolates of *K. pneumoniae*, and a single isolate of *K. oxytoca*, *K. ozaenae*, *E. amnigenes*, *E. aerogenes*, *S. marcescens*, *A. lwolfii*, and *Edwardsiella* spp. and a strain that was not identifiable by the Enterotube system. No lysogens were detected with these isolates.

### Table 5. Human *E. coli* isolates susceptible to lysogenic and/or lytic infection by Stx phages

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phylogenetic group</th>
<th>φ933WΔtox or φ933W</th>
<th>φPT32Δtox or φPT32</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lysogeny</td>
<td>Stx amplification</td>
<td>Lysogeny</td>
</tr>
<tr>
<td>Human ECOR† (O:H/core)</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ECOR-1 (O144:H4/R2)</td>
<td>A</td>
<td>+</td>
<td>256×</td>
</tr>
<tr>
<td>ECOR-4 (OR:H7/R4)</td>
<td>A</td>
<td>+</td>
<td>8×</td>
</tr>
<tr>
<td>ECOR-6 (O173:H7/R3)</td>
<td>A</td>
<td>+</td>
<td>512×</td>
</tr>
<tr>
<td>ECOR-9 (O167:H7/R3)</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ECOR-10 (O6:H10/R2)</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ECOR-12 (O7:H32/R2)</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ECOR-13 (OR:H25/K-12)</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ECOR-15 (O25:H30/R1)</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ECOR-71 (OR:H19/R1)</td>
<td>B1</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ECOR-72 (O8:H30/R1)</td>
<td>B1</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ECOR-61 (O2:H4/R1)</td>
<td>B2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ECOR-51 (O25:H1/R1)</td>
<td>B2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ECOR-39 (O7:H/R1)</td>
<td>D</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ECOR-42 (O87:H26/R1)</td>
<td>E</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>ECOR-43 (O7:H18/R4)</td>
<td>E</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Fecal isolates*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FI-13</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>FI-8</td>
<td>B2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>FI-9</td>
<td>B2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>FI-15</td>
<td>B2</td>
<td>+</td>
<td>8×</td>
</tr>
<tr>
<td>FI-16</td>
<td>B2</td>
<td>+</td>
<td>10×</td>
</tr>
<tr>
<td>FI-31</td>
<td>B2</td>
<td>+</td>
<td>9×</td>
</tr>
<tr>
<td>FI-37</td>
<td>B2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>PT-3</td>
<td>A</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>PT-1</td>
<td>B1</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>PT-8</td>
<td>B1</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>PT-2</td>
<td>B2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>PT-10a</td>
<td>B2</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>PT-6-12</td>
<td>D</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

* Lysogeny was assessed by acquisition of GFP DNA from colonies selected for chloramphenicol resistance following infection with φ933WΔtox or φPT32Δtox. +, lysogens detected; –, no lysogens detected.
* Stx, a late gene product, was monitored as evidence of lytic infection following incubation with phage φ933W or φPT32. Fold increase over Stx present in initial phage inoculum is reported when significantly elevated over control values (P < 0.05). –, toxin amplification not detected.
* Serotyping and determination of LPS core type as reported by Amor et al. (2a). OR, rough LPS; ?, unidentifiable.
* Seven of 29 FI isolates and 7 of 12 PT isolates tested positive for lysogeny or lytic infection.

### Shiga toxin production by fecal *E. coli* following lytic infection

We have previously shown that lytic infection of *E. coli* by φ933W can result in highly elevated levels of Stx2 (7). The ability of phage to promote lytic infection was monitored by assessing the ability of bacteria incubated with about 10^2 phage to produce elevated levels of Shiga toxin. Amplified Stx2 following infection with φ933W was detected for three strains, ECOR-4, ECOR-6, and ECOR-13, all of which were capable of being lysogenized with the Δtox derivative of φ933W (Table 5).

The ability of phages from the six Cincinnati *E. coli* O157:H7 isolates to promote lytic infection and amplify Shiga toxin production was also monitored (Table 5). Only the phage from clinical isolate PT32 amplified toxin in any of the non-toxin-producing *E. coli* strains tested. Susceptibility profiles to φ933W and φPT32 were different. ECOR-6 and ECOR-13 were susceptible to lysogenic and lytic infection by φPT32 as well as φ933W (Table 5). ECOR-51 was susceptible to lytic infection by φPT32 but not φ933W; however, lysogeny of this strain with either phage was not detected. Overall, the non-O157 isolates were highly variable in their susceptibility to lytic
or lysogenic infection with the different phages. Interestingly, while φ933W was much more likely to lysogenize the non-
O157 isolates than φPT32, infection with φPT32 was much more likely to result in amplified Shiga toxin expression.

**DISCUSSION**

A number of phage-related factors have been described that influence the severity of disease caused by Shiga toxin-encoding
*E. coli*. Toxin production is regulated by phage late gene expression. Antibiotic treatment has been shown to induce late gene expression and increase toxin expression in vitro (12, 13, 41). Furthermore, epidemiological studies have suggested that the use of antibiotics may exacerbate the disease process in humans (12, 38). This aspect of phage biology has seriously limited the treatment options for *E. coli* O157:H7.

In addition, Muniesa et al. (19) showed that *E. coli* O157:H7 isolates from different patients in a single outbreak were highly variable with respect to their ability to produce phage and Shiga toxin in vitro. Toxin production was genetically linked to the phage type expressed by the strain, and isolates capable of producing high levels of Shiga toxin were more likely to be isolated from patients with severe systemic disease. The inherent ability of the phage to produce high levels of Stx is likely to be a significant factor in influencing whether a patient develops self-limiting disease or life-threatening complications, such as hemolytic uremic syndrome.

In this study we examined the diversity of Stx2-encoding phage isolated from highly related strains of *E. coli* O157:H7. In contrast to the study by Muniesa et al. (19), all of the *E. coli* O157:H7 isolates characterized in this study produced similar, high levels of toxin in the presence or absence of treatments which induce the phage lytic cycle. This difference could be due to the fact that the *E. coli* O157:H7 strains characterized in this study were all obtained from Cincinnati Children’s Hospital and may represent isolates likely to be highly virulent. Livny and Friedman (14) demonstrated that Stx-encoding phages were more readily induced than non-Stx-encoding phages and suggested that there is selective pressure on Stx-encoding phages to enter the lytic cycle and release Stx as a competitive advantage for pathogenic bacteria in the intestine. The isolates used in our studies may have demonstrated particular fitness in patients due, in part, to the high levels of Stx released.

Electron microscopy revealed that all of the phages had morphological characteristics similar to those reported for other Stx2-encoding phages (2, 28) but different from Stx1-encoding phages from *E. coli* O26 strain H19 (22). In contrast to published reports (2) the protein profiles for the phages used in this study differed. The Stx2 phages were also found to be remarkably different with regard to host range for both lysogenic and lytic infection, despite being isolated, in some cases, from otherwise indistinguishable *E. coli* O157:H7 strains. The sequence of *E. coli* O157:H7 strain EDL933 revealed that this strain possessed only a single Stx2-encoding phage, but multiple defective phage genomes were present (26). Recombination between phage genomes could generate diversity, even between highly related strains, as observed in this study. Phage diversity and changes in either lysogenic or lytic host range within an otherwise clonal population of *E. coli* O157:H7 have implications for disease. Future studies that include epidemiological data of the isolates will add valuable information to the role of phage variability and host range in an outbreak.

Lysogeny with Stx2-encoding phage is thought to be a driving force for evolution of new pathogens. For example, lysogeny of *S. sonnei* with Stx-encoding phage (32) is a very disturbing development. We examined a small sample of non-*E. coli* commensal *Enterobacteriaceae* and did not detect lysogeny of φ933WΔtox. Stx-related disease has been documented that was reportedly due to strains of *C. freundii* (30) and *Enterobacter* spp. (25), and it is possible that a larger sampling of *Enterobacteriaceae* or a broader range of Stx-encoding phages would yield lysogens.

While lysogeny may influence evolution of pathogens, lytic infection may directly influence disease outcome. Two Stx2-encoding phages were able to infect normal *E. coli* cells and amplify Shiga toxin production, and lytic infection increased toxin production by more than 1,000-fold in one case (Table 5). The susceptible *E. coli* isolates varied with respect to O-antigen and LPS core types, suggesting that factors other than LPS influence susceptibility. *E. coli* isolates from all phylogenetic groups were susceptible to lysogeny by Shiga toxin-encoding phage; however, *E. coli* isolates belonging to the phylogenetic groups with smaller genome sizes, groups A and B1, were more likely to be susceptible to phage. Initial reports on subjects from around the world have suggested that *E. coli* strains in groups A, B1, and D are the predominant colonizers of the intestine (6, 27, 40). A recent study from the United States (40) and our examination of fecal isolates from the Cincinnati area indicate that in some populations the group B2 *E. coli*, usually associated with extraintestinal disease, predominate. The composition of the intestinal flora could influence the susceptibility of individuals and populations to lytic infection by Shiga toxin-encoding phages. Having a diverse host range may confer a selective advantage for toxin-encoding phages by allowing them to infect intestinal *E. coli*.

Similar to previous studies, the phages examined in this study were found to be highly variable, despite the relatedness of the *E. coli* O157:H7 strains from which they originated. Phage diversity with regard to toxin production can directly influence the ability to cause disease. Variations in phage host range with regard to lysogeny can influence the evolution of new pathogenic strains. We suggest that variation in phage host range may also confer a selective advantage for toxin-encoding phage by increasing the probability that infected intestinal *E. coli* will produce toxin. The ability of toxin-encoding phage to influence disease outcome underscores the importance for understanding the diversity of these phages.

**ACKNOWLEDGMENTS**

We thank Melanie Cushion and Sandy Rebholz for assistance with the PFGE analysis, Joel Mortensen and the Cincinnati Children’s Hospital Medical Center culture collection for the *E. coli* O157:H7 strains, and Judith Rhodes for patient isolates.

This work was supported by grant R21-AI-02-008 to A.A.W. S.D.G. was supported by T32-AI055406.

**REFERENCES**
