Shiga toxins (Stx) are potent AB$_5$ cytotoxins that inhibit eukaryotic protein synthesis, eventually leading to host cell death (1). Infections with bacteria that produce Stx result in hemorrhagic colitis and can lead to serious complications like hemolytic uremic syndrome (HUS) (2). Although 4 species of Shigella cause bacillary dysentery, historically only Shigella dysenteriae type 1 has been recognized as carrying the genes for Shiga toxin (stx). Likewise, S. dysenteriae 1 is the only Shigella species that causes HUS as a complication of infection (3).

The genes encoding the toxin are found in an operon consisting of stxA and stxB. The stx locus in S. dysenteriae 1 is surrounded by DNA sequence homologous to lambdoid bacteriophage sequence; however, the toxin genes are not associated with a complete prophage genome (4,5). Insertion sequences flanking the stx region suggest that gene rearrangements occurred and resulted in a defective phage. As a consequence, viable phage are not recovered from S. dysenteriae 1 cultures even under conditions that induce phage production (6).

Stx has been extensively studied in Shiga toxin–producing Escherichia coli (STEC), notably E. coli O157:H7. STEC produce 2 variants of Stx: Stx1a (which differs from S. dysenteriae 1 Stx by 1 aa), and Stx2 (which shares 56% identity with Stx1a) (7, 8). In contrast to the toxin genes in S. dysenteriae 1, those in STEC are generally carried by lambdoid prophages, which integrate into the host bacterial chromosome (9). The phage remains in a lysogenic state until environmental conditions induce expression of phage lytic cycle genes, leading to new phage production and lysis of the host bacterium. The stx$^{1a}$ and stx$^{2}$ loci are found within the late gene regions of the phage; therefore, induction of the lytic cycle increases expression of the toxin genes and host cell lysis allows for toxin release (10).

Recently, acquisition of stx genes in clinical isolates of other Shigella species has been reported (11,12). Three cases of infection with S. dysenteriae 4 were described, and all were shown to express stx$^{4}$. No further characterization of the stx$^{4}$-encoding S. dysenteriae 4 strains was reported; however, all 3 infected patients had reported recent travel to Hispaniola (11). An isolate of Shiga toxin–producing S. sonnei from a patient returning from the Ukraine was also characterized; the toxin genes were determined to be
carried by a lambdoid prophage homologous to stx-encoding phages found in STEC (13).

We identified 26 clinical isolates of _S. flexneri_ 2 that encode stx<sub>1a</sub>. DNA sequence and PCR analyses determined that stx<sub>1a</sub> is encoded by a lambdoid prophage. Characterization of the phage indicated that it behaves similarly to stx-encoding phages that infect STEC. Like the patients from whom stx<sub>1a</sub>-encoding S. _dysenteriae_ 4 was isolated, patients from whom stx<sub>1a</sub>-encoding _S. flexneri_ 2 was isolated and who reported foreign travel had also recently visited Hispaniola. The potential consequences of an epidemiological link to this region are discussed.

**Methods**

**Bacterial Strains and Growth Conditions**

_Shigella_ clinical isolates used in this study are listed in Table 1. _S. flexneri_ strains were grown in Tryptic Soy Broth (BD Difco, Franklin Lakes, NJ, USA) at 37°C with aeration or on Tryptic Soy Broth plates containing 1.5% agar and 0.025% Congo red. _E. coli_ K-12 strain MG1655 was grown in Luria-Bertani broth and on Luria-Bertani agar plates. Kanamycin and ampicillin were used at 50 µg/mL, respectively.

**PCR Analysis of stx<sub>1a</sub>-Encoding _S. flexneri_**

DNA lysates were used for PCR for stx<sub>1a</sub> with primers stxl-det-F1 and stxl-seq-R1, and for stx<sub>2</sub> with primers stx_F4/stxR1. All strains were verified to contain the virulence plasmid of _S. flexneri_ by PCR amplification of _virF_ with primers VirF1 and VirF2.

To show that stx<sub>1a</sub> was phage encoded, we analyzed lysates by PCR with primer pairs Stx1R2/Phage_stxR2 and Phage_stx1F2/Stx1F2. The insertion site of the phage into _S. flexneri_ locus S1742 was determined by amplifying the upstream region of S1742 and an early phage gene with primers S1742_up/Stx_phage_up and by amplifying a late phage gene and the downstream region of S1742 with primers Stx_phage_dn/S1742_dn. All PCRs were conducted by using PCR Master Mix (2X) (Fermentas, Pittsburgh, PA, USA) according to the manufacturer’s specifications. Primer sequences and expected amplicons are listed in Table 2.

**Construction of recA Mutants**

_recA_ was replaced with a kanamycin-resistance cassette by using λ red recombination (15). Primers RecAko-site1 and RecAko-site2 were used to amplify _kan_ from pKD4 with 5′ and 3′ overhangs homologous to internal regions of _recA_. Kanamycin-resistant colonies of BS766 (15) were double

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**Table 1. Isolation information for clinical strains of _Shigella flexneri_, USA, 2001–2013***

| Strain | Source state (laboratory no.) | Isolation date | Recent travel destination |
|--------|-------------------------------|----------------|---------------------------|
| stx<sub>1a</sub>-positive |
| BS937  | HI (HI_N10–094)               | 2010 Mar       | Haiti                     |
| BS938  | MA (MA_12EN1615)              | 2012 May       | NA                        |
| BS942  | MA (MA_10EN1901)              | 2010 Sep       | NR                        |
| BS943  | MA (MA_11EN1036)              | 2011 Jun       | NA                        |
| BS951  | PA (05E00067)                 | 2005 Jan       | NR                        |
| BS954  | PA (05E02261)                 | 2005 Dec       | Dominican Republic       |
| BS955  | PA (06E00134)                 | 2006 Jan       | NR                        |
| BS957  | PA (06E00281)                 | 2006 Feb       | NR                        |
| BS958  | PA (06E00283)                 | 2006 Jan       | NR                        |
| BS959  | PA (06E00305)                 | 2006 Feb       | NR                        |
| BS960  | PA (06E00941)                 | Unknown        | Haiti                     |
| BS963  | PA (08E01943)                 | 2008 Sep       | NR                        |
| BS965  | PA (M09015890001A)            | 2009 Jul       | NA                        |
| BS968  | PA (M10005231001A)            | 2010 Feb       | Haiti                     |
| BS971  | PA (M11028960001A)            | 2011 Nov       | Haiti                     |
| BS972  | MA (12EN7814)                 | 2012 Nov       | NR‡                       |
| BS974  | IN (01–3105)†                 | 2001 Apr       | Haiti                     |
| BS980  | MA (05–3606)†                 | 2005 Oct       | NR‡                       |
| BS981  | CT (06–3001)†                 | 2005 Dec       | Dominican Republic       |
| BS982  | GA (08–3370)†                 | 2008 Apr       | NR                        |
| BS988  | CT (2012C–3273†               | 2012 Jan       | Haiti                     |
| BS989  | CT (2013C–3310)†              | 2013 (month unknown) | NR‡                   |
| BS998  | PA (M13004940001A)            | 2013 Mar       | Haiti                     |
| BS999  | MA (MA_13EN0428)              | 2013 Jan       | Haiti                     |
| BS1010 | MD (MDA1005139)               | 2009 Dec       | Dominican Republic       |
| BS1011 | MD (MDA12018728)              | 2012 Jan       | NA                        |
| stx<sub>1a</sub>-negative |
| BS952  | PA (05E00414)                 | 2005 Apr       | Peru                      |
| BS959  | PA (M11015188001A)            | 2011 Jun       | India                     |
| BS967  | PA (M11015261001A)            | 2011 Jun       | NR                        |

*NA, no information available; NR, none reported; stx<sub>1a</sub>, Shiga toxin 1a gene.
†Obtained by the Centers for Disease Control and Prevention.
‡No foreign travel reported, but patient had contact with a person who recently returned from Haiti.
that was required to kill 50% of the Vero cells. By determining the inverse dilution of the bacterial sample (cytotoxic dose that kills 50% of the cells) was calculated in 5% CO₂ samples were incubated with antibody for 2 hours at 37°C clonal antiserum against Shiga Toxin 1a (Stx1a), or a 1:25 dilution of rabbit polyclonal antiserum against Shiga Toxin 2a (Stx2a). In brief, 100 μL medium, a 1:25 dilution of F45 polyclonal anti-Stx1/2 antibody for 2 hours at 37°C in 5% CO₂. We then applied 100 μL of the toxin–antibody mixture to Vero cells and incubated as above.

Cytotoxicity Assay
The cytotoxicity of bacterial samples for Vero cells was determined as previously described (16,17). In brief, 100 μL of diluted samples was overlain in 96-well plates containing confluent monolayers of Vero cells and incubated for 48 hours at 37°C in 5% CO₂. Viable cells were fixed with 10% formalin and stained with 0.13% crystal violet. The optical density (OD) of the stained wells was measured at 630 nm by using a BioTek (Winooski, VT, USA) EL800 spectrophotometric plate reader. The CD₅₀ (cytotoxic dose that kills 50% of the cells) was calculated by determining the inverse dilution of the bacterial sample that was required to kill 50% of the Vero cells.

In Vitro Neutralization of Stx1a
Overnight supernatants were serially diluted 10-fold in medium. We mixed 100 μL of diluted samples with 100 μL medium, a 1:25 dilution of F45 polyclonal anti-Stx1/2 antiserum (17,18), or a 1:25 dilution of rabbit polyclonal antiserum against Shiga flexneri whole cell lysate. Toxin samples were incubated with antibody for 2 hours at 37°C in 5% CO₂. We then applied 100 μL of the toxin–antibody mixture to Vero cells and incubated as above.

Mitomycin C Induction of Bacterial Lysis, Shiga Toxin 1a Production, and Prophage Induction
Overnight cultures of bacteria were inoculated 1:100 into Tryptic Soy Broth, and 2 hours after inoculation a final volume of 0.5 μg/mL mitomycin C (Sigma, St. Louis, MO, USA) was added to the cultures. To monitor the induction of bacterial lysis, we read the OD₆₀₀ hourly over a period of 8 hours. Induction of bacterial lysis was noted as a 3–4-fold decrease in OD₆₀₀ compared with the stx₁α-negative control strains.

To determine the effect of mitomycin C on production of Stx1a and prophage induction, we collected whole cell lysates and supernatants 3 hours after addition of mitomycin C. Samples were then analyzed for cytotoxicity on Vero cells. For isolation of phage particles, supernatants were prepared similarly, except that a final concentration of 10 mmol/L MgSO₄ and a drop of chloroform were added after centrifugation. As described previously, 100 μL of phage lysate was absorbed onto 100 μL of Stx1a-negative control strains.

Isolation of Lysozymes
Supernatants containing phage were prepared from mitomycin C–induced culture of BS937. Phage lysate was spotted onto an L-soft agar overlay containing either E. coli MG1655 or Shiga flexneri 2457T and incubated overnight at 37°C. A loop from the zone of clearing was streaked into Tryptic Soy Broth, and 2 hours after inoculation a final volume of 0.5 μg/mL mitomycin C (Sigma, St. Louis, MO, USA) was added to the cultures. To monitor the induction of bacterial lysis, we read the OD₆₀₀ hourly over a period of 8 hours. Induction of bacterial lysis was noted as a 3–4-fold decrease in OD₆₀₀ compared with the stx₁α-negative control strains.

| Primer pair              | Sequence  | Ampli size, bp | Source |
|-------------------------|-----------|----------------|--------|
| stx1-det-F1             | GTACGGGGATGCAGATAATCCTGC | 698 (14) |        |
| stx1-seq-R1             | GAAAGAAGACGTGAGATTTGCCATCTG | 627 (14) |        |
| stx2_F4                 | GCCATGTGCTAAAGCTGCTTGT | 907 | This study |
| stx2R1                  | ATTAACCTGACATCCTACAGAA | 897 | This study |
| VirF1                   | GCAAATCATGCTTGGTGGACAGAG | 628 | This study |
| VirF2                   | GGCGCTGATATCGGATAAGTC | 628 | This study |
| VirB01                  | TCTTACCATCATAATCTCCCTCC | 1,059 |        |
| IpaAFwd                 | GTACTGACGCCCCTCAGCAAG | 1,155 |        |
| IpaHF                   | GCTTACCGTGGACCGCTTTCGATAACCG | 1,155 |        |
| IpaHR                   | CTTTCAAGCAGGTACAGCCTCCTGAGAG | 1,059 |        |
| Stx1R2                  | AGCGGAATGACATCTCGGAACTCTA | 1,155 |        |
| Phage_stxR2             | GAGCGCAATCAAGAAGTC | 1,155 |        |
| Stx9F2                  | GACGCTGATTGTAACCTGAAAA | 1,224 |        |
| Phage_stx1F2            | CACTCGCGTACGTGATG | 1,155 |        |
| Stx_phage_up            | GACGCGACATCTGCTAC | 1,224 |        |
| Stx_phage_dn            | CCCTGCGGTTATTTAAAATAGGG | 1,059 |        |
| S1742 up                | AGTCAACCGGCCTATTGG | 1,155 |        |
| S1742 dn                | TGCATGACGAGGCAATAAACCAGG | 1,224 |        |
| RecAko-site1            | GCTATCGACGAAAACAAACAGAAAGCTTGGGGCGACGACTGGCAGA | 1,155 |        |
| RecAko-site2            | TGTGTAAGCTTGAGCTGCTTC | 1,059 |        |
| RecAKo-site3            | AAGAATATCCCTTTTA | 1,148, 1,701* |        |
| RecA-1                  | AAATCTTTCTTGTGAAGTTTGGTCACTCCTCCGGTGATCATCTAGAGAATCC | 1,148, 1,701* |        |
| RecA-3                  | ATGAAATATCCCTCTTA | 1,148, 1,701* |        |
| Phage_stxR2             | GACGCGCAATCAAGAAGTC | 1,059 |        |
| Stx9F2                  | GACGCTGATTGTAACCTGAAAA | 1,155 |        |
| Phage_stx1F2            | CACTCGCGTACGTGATG | 1,155 |        |
| Stx_phage_up            | GACGCGACATCTGCTAC | 1,224 |        |
| Stx_phage_dn            | CCCTGCGGTTATTTAAAATAGGG | 1,059 |        |
| S1742 up                | AGTCAACCGGCCTATTGG | 1,155 |        |
| S1742 dn                | TGCATGACGAGGCAATAAACCAGG | 1,224 |        |

*Ampli sizes for wild-type recA or insertion of kan cassette into recA, respectively.
for isolation of single colonies, which were subsequently screened for stx<sub>1a</sub> genes by PCR. Positive colonies from the initial screening were double colony purified, and PCR was repeated to ensure that the colonies were positive for stx<sub>1a</sub>. MG1655 lysogens were confirmed to not be contaminants of the donor strain, BS937, by testing for S. flexneri chromosomal and virulence plasmid genes by use of PCR primer pairs IpaHF/IpaHR and VirB01/IpaFWD (Table 2), respectively. Similarly, 2457T lysogens were analyzed by PCR as described above. Two independently isolated lysogens of MG1655 and 2457T were used for further analysis.

**Virulence Assays**

Virulence-associated phenotypes were determined by a gentamicin protection invasion assay in HeLa cells and by plaque formation in L2 monolayers, as previously described (20,21). Both assays were conducted 3 independent times and included technical duplicates or triplicates in each individual experiment.

**Whole-Genome Sequencing**

DNA was isolated from overnight cultures by using a QIAGEN DNEasy Kit (Valencia, CA, USA). Samples were prepared for sequencing by using a Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequenced on an Illumina MiSeq sequencing system. The phage sequence was assembled by mapping the reads to the reference phage NC_004913.2 by using Bowtie 2 version 2.1.0 (http://sourceforge.net/projects/bowtie-bio/). The complete phage sequence of φPOC-J13 from strain BS937 was submitted to GenBank. The sequence is available under accession no. KJ603229.

**Nucleotide Sequence Accession Number**

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**Results**

**Identification and Epidemiology of stx<sub>1a</sub>-positive S. flexneri 2 Strains**

BS937 and BS938 (Table 1) were acquired from the Hawaii and Massachusetts state laboratories, which had determined the isolates to be positive for stx<sub>1a</sub> by PCR. Both strains shared the same pulsed-field gel electrophoresis (PFGE) XbaI pattern, JZXX01.0357, as indicated in the database (http://www.cdc.gov/pulsenet). To identify other clinical isolates of S. flexneri 2 that might be stx<sub>1a</sub>-positive, we searched the PulseNet database for strains that matched this PFGE pattern. From state public health laboratories, we obtained 18 additional strains of S. flexneri that matched this PFGE pattern. We also received time-matched, but not PFGE-matched, strains of S. flexneri as negative controls. Six additional strains that had been confirmed to be stx<sub>1a</sub>-positive S. flexneri were acquired from the Centers for Disease Control and Prevention. Clinical strains included in this study and their sources are listed in Table 1.

**Verification of Stx1a in S. flexneri Isolates**

The presence of stx<sub>1a</sub> in the S. flexneri strains was confirmed by PCR (data not shown). All isolates that matched PFGE pattern JZXX01.0357 or had previously been shown to encode stx<sub>1a</sub> yielded a PCR product of the correct size for the toxin gene. PCR analysis for stx<sub>2</sub> did not produce a product. The 3 negative controls did not generate a PCR product for either stx<sub>1a</sub> or stx<sub>2</sub>.

To determine if Stx1a was released from the bacteria, we tested supernatants from overnight cultures in a Vero cell cytotoxicity assay. All stx<sub>1a</sub>-positive isolates released a toxin into the supernatant, which killed Vero cells. To confirm that the toxin responsible for killing the cells was Stx1a, we tested overnight supernatants from 3 representative isolates for Vero cell cytotoxicity after neutralization by anti-Stx/Stx1a antiserum. After neutralization, supernatants were no longer cytotoxic to Vero cells (Figure 1). These findings demonstrate that the extracellular product responsible for cytotoxicity to Vero cells is indeed Stx1a and not a different protein being released by the stx<sub>1a</sub>-expressing S. flexneri.

**Effects of Mitomycin C on Stx1a and Prophage Production**

Stx is generally found encoded by functional prophages (22). The prophage lytic cycle can be induced with DNA damaging agents (23); therefore, to address whether the toxin in carried in stx<sub>1a</sub>-encoding S. flexneri was associated with a prophage, we tested sensitivity to lysis when grown in the presence of mitomycin C. All Stx1a-producing S. flexneri isolates showed a sharp decrease in OD<sub>600</sub> within 3–4 hours after addition of mitomycin C, whereas the stx<sub>1a</sub>-negative strains showed no decrease in OD<sub>600</sub> (data not shown).
Because all stx₁a-encoding S. flexneri isolates behaved similarly in the assays, we selected 3 isolates (BS937, BS938, and BS974) to characterize more thoroughly. To further investigate the response to mitomycin C, we grew log-phase cultures and collected samples to measure cell-associated and released toxin. Supernatants and whole cell lysates from bacteria treated with mitomycin C exhibited elevated cytotoxicity to Vero cells (Figure 2). To determine if stx₁a-producing S. flexneri generated infectious phage, we analyzed supernatants from untreated and mitomycin C–treated cultures in a PFU assay. After treatment with mitomycin C, PFUs in supernatants of BS937, BS938, and BS974 increased ≈1,000-fold (Figure 3).

Induction of the prophage lytic cycle by mitomycin C is caused by the SOS response (24). During the SOS response, the bacterial protease RecA becomes active and cleaves the phage repressor cI, which maintains the phage in a quiescent state under non-SOS conditions. Cleavage of cI enables transcription of the phage antiterminator Q, which activates the late phage genes, including stx (25). STEC recA mutants no longer release toxin or respond to agents that trigger the SOS response (24,26,27).

To investigate whether similar regulation occurs in Stx₁a-producing S. flexneri, we constructed recA deletions in BS937, BS938, and BS974. recA mutants were cultured with mitomycin C as above, and samples were collected to measure the presence of toxin and phage. In the absence of mitomycin C, the recA mutants produced and released toxin in amounts comparable to those of the parental strains; however, when cultured with mitomycin C, the recA mutants did not exhibit increased cytotoxicity to Vero cells (Figure 2). Additionally, in the absence or presence of mitomycin C, no PFUs were enumerated from the recA mutants. Collectively, these data suggest that stx₁a in S. flexneri is carried by a lambdoid prophage.

**stx₁a Carriage by a Lambda Prophage in S. flexneri**

To identify the location of stx₁a, we sequenced BS937, BS938, and BS974. Whole-genome sequencing confirmed that stx₁a was encoded within a 62-kb lambdoid prophage. To extend the analysis to all the clinical isolates in this study, we used the PCR strategy and primer design shown in Figure 4. To ensure that stx₁a in all isolates was phage encoded, we designed primers to amplify from stxA₁a and stxB₁a (encoding subunits A and B of Stx₁a) and 1 kb either upstream or downstream of the stx₁a operon. All stx₁a-positive strains yielded a PCR product consistent with the toxin being phage encoded. No DNA was amplified from the stx₁a-negative isolates.

Whole-genome sequencing revealed that the phage was inserted into locus S1742 (which encodes a putative oxidoreductase) of the S. flexneri chromosome. Primers were designed (Figure 4) to determine if the stx₁a-encoding phage inserted into S1742 for all isolates. All stx₁a-positive strains generated the expected PCR product when amplified with primers specific for the early phage sequence and upstream of S1742 and with primers directed to the late phage sequence and downstream of S1742. None of the stx₁a-negative strains yielded an amplified product with the primer pairs. A representative gel of the 4 amplifications for 1 stx₁a-negative and 6 stx₁a-positive isolates is shown in Figure 5. These data suggest that a phage has integrated into all stx₁a-positive isolates. We named this stx₁a-encoding phage φPOC-J13.

**Lysogeny of Laboratory Strains of E. coli and S. flexneri with φPOC-J13**

*E. coli* MG1655 and *S. flexneri* 2457T were lysogenized as described earlier. To test for production and release of Stx₁a, we examined whole cell lysates and supernatants from overnight cultures of MG1655 and 2457T lysogens in Vero cell cytotoxicity assays. The average CD₅₀/mL of supernatants from MG1655 and 2457T lysogens was 1 × 10⁵, similar to that of the stx₁a-positive *S. flexneri* clinical isolates. Lysogens were also tested for the presence of phage in supernatants of overnight cultures by determining PFUs. The 2457T lysogens released ≈10⁶ PFUs/mL; however, no viable phage could be recovered from MG1655 lysogens.

To confirm that stx₁a in the lysogens was phage associated, we conducted PCR amplification as described above (Figure 4). The MG1655 and 2457T lysogens yielded a

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**Figure 1.** Shiga toxin 1a (Stx₁a) is produced and released from stx₁a-encoding Shigella flexneri isolates. Overnight supernatants from BS937, BS938, and BS974 were serially diluted 10-fold in medium alone, medium containing anti-Stx/Stx₁a antiserum, or antiserum against whole cell lysates of S. flexneri. After 2 hours of incubation, samples were analyzed in a Vero cell cytotoxicity assay. Stx from S. dysenteriae 1 was included as a positive control. CD₅₀/mL is defined as the reciprocal of the dilution of Stx1 that kills 50% of Vero cells. *CD₅₀/mL was below the level of detection. Data are averages of 3 independent experiments. Error bars indicate standard error.
PCR product that indicated that the phage regions upstream and downstream of \textit{stx\textsubscript{1a}} were present. Lysogens were also tested for integration of the phage into locus S1742 by use of the primers illustrated in Figure 4. In 2457T lysogens, φPOC-J13 inserted into S1742 and produced a PCR product of the expected size. Similarly, in lysogens of MG1655, φPOC-J13 inserted into \textit{ynfG}, the \textit{E. coli} S1742 homologue. These findings demonstrate that the integration of φPOC-J13 is site specific, as has been shown for other lambdoid prophages (9).

**Virulence Phenotypes of Shiga Toxin 1a–producing \textit{S. flexneri}**

We wanted to determine if the presence of φPOC-J13 altered the virulence phenotypes associated with \textit{S. flexneri}. Invasion and plaquing efficiencies of BS937, BS938, and BS974 were compared with those of laboratory strain 2457T (Table 3). BS938 and BS974 exhibited similar invasion efficiency as 2457T; however, invasion with BS937 was significantly higher. All 3 Stx1a–producing \textit{S. flexneri} isolates showed plaquing efficiency comparable to that of 2457T, and the plaque diameters were consistent among all strains. Although it is unclear why BS937 was more invasive, the comparable level of cell-to-cell spread suggests that φPOC-J13 does not appreciably alter the virulence properties of \textit{S. flexneri}.

**Discussion**

Bacteriophages are recognized for their contribution to the genetic diversity of bacteria and for their capacity to transfer virulence factors (28). It was first noted in the early 1980s that \textit{stx} in \textit{E. coli} was encoded by a lambdoid bacteriophage (29,30). We have identified a new \textit{stx\textsubscript{1a}}-encoding bacteriophage, φPOC-J13, from clinical isolates of \textit{S. flexneri}. Generally, the acquisition of toxin genes is thought to increase the virulence of a bacterial species. However, according to the available clinical data and our in vitro virulence assays, the production of Stx1a in \textit{S. flexneri} does not seem to increase pathogenicity within the host.

Characterization of φPOC-J13 determined that it behaves similarly to \textit{stx}-encoding phages found in STEC; however, some differences are notable. First, although φPOC-J13 responded to DNA damaging treatment by inducing the lytic cycle and induction was RecA-dependent, \textit{recA} mutants of \textit{S. flexneri} Stx1a–producing strains still maintained a level of Stx1a production and release comparable to that of the noninduced parental strains. In contrast, \textit{recA} mutants of STEC produce a very low level of Stx, and the toxin that is present remains largely cell associated rather than being released into the supernatant (24,26,27). One explanation for the differences in phenotype between Stx1a-producing \textit{S. flexneri} \textit{recA} mutants and the STEC mutants might be that all STEC \textit{recA} mutants examined encoded \textit{stx\textsubscript{2}}; thus, the regulation of \textit{stx\textsubscript{1a}}- and \textit{stx\textsubscript{2}}-encoding phages might vary. Additionally, although Stx1a was produced in \textit{S. flexneri} \textit{recA} mutants, viable phage particles were not recovered. \textit{stx\textsubscript{1a}} has an upstream promoter that is not dependent on induction of the phage lytic cycle (31). A similar promoter might be responsible for the baseline level of Stx1a production in the \textit{S. flexneri} \textit{recA} mutants and would explain the lack of infectious phage in the mutants.

φPOC-J13 lysogenized laboratory strains of \textit{E. coli} and \textit{S. flexneri}. Viable phage particles were recovered from the supernatants of 2457T lysogens but not from those of MG1655 lysogens, even though Stx1a was produced and released by lysogens of both species. This result might suggest that the stability and/or assembly of φPOC-J13 varies according to the host bacterium. Host differences in the regulation of φPOC-J13 might also account for the discrepancies between the \textit{recA} mutants of Stx1a-producing...
Shiga Toxin 1a–producing Shigella flexneri

S. flexneri and STEC. Our future studies will compare the differences in regulation of stx₁a in φPOC-J13 with that of known STEC phages.

Another aspect of these stx₁a–encoding S. flexneri isolates is their potential epidemiological link to Hispaniola. Although some patients reported no travel, ≈60% reported travel to this region or interaction with a traveler returning from this region. Most of our clinical isolates came from public health laboratories in the eastern United States, suggesting a possible focus in that area. However, the large number of isolates from the eastern United States might simply reflect the large Haitian immigrant population in this region and the resultant frequent travel to Haiti (32). Nevertheless, further surveillance of Stx₁a-producing S. flexneri is warranted to determine the extent of their emergence in Hispaniola.

The epidemiological link to Hispaniola generates many questions about what has led to the emergence of these strains. The earliest Stx₁a-producing S. flexneri isolates pre-date the earthquake that struck Haiti in January 2010. Thus, this natural disaster is not linked to the presence of stx₁a–encoding S. flexneri in the region. One possibility is that the ecosystem in Hispaniola is favorable for the acquisition of φPOC-J13 by S. flexneri and possibly other Shigella species. An environmental reservoir of Shigella spp. has never been identified; therefore, it is tempting to speculate that production of Stx₁a might give S. flexneri a survival advantage in the aquatic environment. In accordance with this hypothesis, studies on the survival of Shigella spp. in amebae indicate that S. dysenteriae 1 can persist longer than S. flexneri within Acanthamoeba castellanii (33, 34). In addition, Stx-producing bacteria can kill the protozoan Tetrahymena thermophila to avoid consumption by this predator (35). Thus, Stx₁a might benefit S. flexneri by providing a defense against eukaryotic predators.

It will be important to study clinical isolates of other Shigella species and bacterial genera to determine whether they also harbor φPOC-J13; we expect that the occurrence of this stx₁a–encoding phage will be more widespread. Although toxin production in S. flexneri did not suggest an increase in pathogenicity, the consequences of the emergence of such Stx₁a–producing strains are impossible to

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**Figure 3.** Infectious phage in the supernatants of Shiga toxin 1a gene (stx₁a)–encoding Shigella flexneri are induced with mitomycin C (mito C) treatment. Supernatants were collected from exponential cultures of BS937, BS938, and BS974 grown with or without 0.5 µg/mL mito C for 3 h. The number of infectious phage particles was determined by a soft agar overlay method that used Escherichia coli MG1655 as the recipient. Plaque forming units (PFUs) of phage lysate were counted after 24 h incubation. Data are averages of 3 independent experiments. Error bars indicate standard error.

**Figure 4.** Schematic of PCR designed to determine that Shiga toxin 1a gene (stx₁a) is phage encoded and inserted into the S1742 locus of Shigella flexneri. The genetic map shows the insertion of φPOC-J13 into locus S1742 of stx₁a–encoding S. flexneri. Location and direction of the primers used for PCR analyses are indicated. The top part of the figure indicates the genes flanking the stx₁a operon. Q, antiterminator; A and B, Stx₁a subunits that form the assembled toxin (A is the stx₁aA subunit and B is the stx₁aB subunit); yshS, a hypothetical protein that shares homology with yjhS from Escherichia coli K-12; orf, open reading frame; S, gene that encodes the phage holin; R, gene that encodes the phage endolysin.
Figure 5. PCR results from representative clinical isolates illustrate that the Shiga toxin 1a gene (stx1a) is phage encoded and inserted into the S1742 locus of Shigella flexneri. PCRs based on the primer scheme detailed in Figure 4 are shown for 6 stx1a-positive strains (BS937, BS938, BS942, BS954, BS974, BS980) and 1 stx1a-negative isolate (BS952). To show that stx1a is phage encoded, we used primer pairs Stx1R2/Phage_stxR2 (A) and Phage_stx1F2. Stx1F2 (B). To analyze the insertion of Stx1F2, we used primer pairs S1742_up/Stx_phage_up (C) and Stx_phage_dn/S1742_dn (D).

Table 3. Virulence properties of Shigella flexneri strains

| Strain | Invasion, % ± SE* | Plaquing efficiency, % ±SE† |
|--------|------------------|-----------------------------|
| 2457T  | 0.35 ± 0.04      | 4.34 ± 0.63                 |
| BS937  | 1.24 ± 0.27      | 3.22 ± 0.56                 |
| BS938  | 0.22 ± 0.03      | 4.34 ± 0.79                 |
| BS974  | 0.40 ± 0.09      | 3.24 ± 0.44                 |

*Number of colony-forming units (CFUs) recovered from HeLa cells after gentamicin protection divided by the input CFUs
†Number of plaques formed on L2 monolayers divided by the input CFUs.

References

1. Tesh VL, O’Brien AD. The pathogenic mechanisms of Shiga toxin and the Shiga-like toxins. Mol Microbiol. 1991;5:1817–22. http://dx.doi.org/10.1111/j.1365-2958.1991.tb00805.x
2. Butler T. Haemolytic uraemic syndrome during shigellosis. Trans R Soc Trop Med Hyg. 2012;106:395–9. http://dx.doi.org/10.1016/j.trstmh.2012.04.001
3. Keusch GT, Bennish ML. Shigellosis: recent progress, persisting problems and research issues. Pediatr Infect Dis J. 1989;8:713–9. http://dx.doi.org/10.1097/00006454-198910000-00011
4. McDonough MA, Burretton JR. Spontaneous tandem amplification and deletion of the Shiga toxin operon in Shigella dysenteriae 1. Mol Microbiol. 1999;34:1058–69. http://dx.doi.org/10.1046/j.1365-2958.1999.01669.x
5. Unkmeir A, Schmidt H. Structural analysis of phage-borne stx genes and their flanking sequences in Shiga toxin-producing Escherichia coli and Shigella dysenteriae type 1 strains. Infect Immun. 2000;68:4856–64. http://dx.doi.org/10.1128/IAI.68.9.4856-4864.2000
6. Stockbline NA, Jackson MP, Sung LM, Holmes RK, O’Brien AD. Cloning and sequencing of the genes for Shiga toxin from Shigella dysenteriae type 1. J Bacteriol. 1988;170:1116–22.
7. Calderwood SB, Auclair F, Donohue-Rolfe A, Keusch GT, Mekalanos JJ. Nucleotide sequence of the Shiga-like toxin genes of Escherichia coli. Proc Natl Acad Sci U S A. 1987;84:4364–8. http://dx.doi.org/10.1073/pnas.84.13.4364
8. Newland JW, Stockbline NA, Neill RJ. Cloning of genes for production of Escherichia coli Shiga-like toxin type II. Infect Immun. 1987;55:2675–80.
9. Schmidt H. Shiga-toxin-converting bacteriophages. Res Microbiol. 2001;152:687–95. http://dx.doi.org/10.1016/S0923-2508(01)01249-9
10. Neely MN, Friedman DJ. Functional and genetic analysis of regulatory regions of coliphage H-1B: location of Shiga-like toxin and lysis genes suggest a role for phage functions in toxin function. Mol Microbiol. 1998;28:1255–67. http://dx.doi.org/10.1046/j.1365-2958.1998.00890.x
11. Gupta SK, Stockbline N, Omond M, Hise K, Fair MA, Mintz E. Emergence of Shiga toxin 1 genes within Shigella dysenteriae type 1 isolates from travelers returning from the Island of Hispanola. Am J Trop Med Hyg. 2007;76:1163–5.
12. Beutin L, Strauch E, Fischer I. Isolation of Shigella sonnei lysogenic for a bacteriophage encoding gene for production of Shiga toxin. Lancet. 1999;353:1498. http://dx.doi.org/10.1016/S0140-6736(99)00961-7
13. Strauch E, Lurz R, Beutin L. Characterization of a Shiga toxin–encoding temperate bacteriophage of Shigella sonnei. Infect Immun. 2001;69:7588–95. http://dx.doi.org/10.1128/IAI.69.12.7588-7595.2001
14. Scheutz F, Teel LD, Beutin L, Pierard D, Buvens G, Karch H, et al. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. J Clin Microbiol. 2012;50:2951–63. http://dx.doi.org/10.1128/JCM.00860-12
15. Clark CS, Maurelli AT. Shigella flexneri inhibits staurosporine-induced apoptosis in epithelial cells. Infect Immun. 2007;75:2531–9. http://dx.doi.org/10.1128/IAI.00186-06
16. Gentry MK, Dalrymple JM. Quantitative microtiter cytotoxicity assay for Shigella toxin. J Clin Microbiol. 1980;12:361–6.
17. Schmitt CK, McKee ML, O’Brien AD. Two copies of Shiga-like toxin II–related genes common in enterohemorrhagic Escherichia coli strains are responsible for the antigenic heterogeneity of the O157:H- strain E32511. Infect Immun. 1991;59:1065–73.
18. O’Brien AD, LaVeck GD. Purification and characterization of a Shigella dysenteriae 1–like toxin produced by Escherichia coli. Infect Immun. 1983;40:675–83.
19. Lech K, Brent R. Plating lambda phage to generate plaques. Curr Protoc Mol Biol. 2001;Chapter 1:Unit1.11. PMID: 18265039 doi: 10.1002/0471142727.mb0111s13.

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20. Kane CD, Schuch R, Day WA Jr, Maurelli AT. mxiE regulates intracellular expression of factors secreted by the Shigella flexneri 2a type III secretion system. J Bacteriol. 2002;184:4409–19. http://dx.doi.org/10.1128/JB.184.16.4409-4419.2002

21. Zurawski DV, Mitsuhashi C, Muny KL, McCormick BA, Maurelli AT. OspF and OspC1 are Shigella flexneri type III secretion system effectors that are required for postinvasion aspects of virulence. Infect Immun. 2006;74:5964–76. http://dx.doi.org/10.1128/IAI.00594-06

22. Herold S, Karch H, Schmidt H. Shiga toxin–encoding bacteriophages—genomes in motion. Int J Med Microbiol. 2004;294:115–21. http://dx.doi.org/10.1016/j.ijmm.2004.06.023

23. Köhler B, Karch H, Schmidt H. Antibacterials that are used as growth promoters in animal husbandry can affect the release of Shiga-toxin-2-converting bacteriophages and Shiga toxin 2 from Escherichia coli strains. Microbiology. 2000;146:1085–90.

24. Fuchs S, Mühldorfer I, Donohue-Rolfe A, Kerenyi M, Emody L, Alexiev R, et al. Influence of RecA on in vivo virulence and Shiga toxin 2 production in Escherichia coli pathogens. Microb Pathog. 1999;27:13–23. http://dx.doi.org/10.1006/mpat.1999.0279

25. Pacheco AR, Sperandio V. Shiga toxin in enterohemorrhagic E.coli: regulation and novel anti-virulence strategies. Front Cell Infect Microbiol. 2012;2:81. http://dx.doi.org/10.3389/fcimb.2012.00081

26. Mühldorfer I, Hacker J, Keusch GT, Acheson DW, Tschape H, Kane AV, et al. Regulation of the Shiga-like toxin II operon in Escherichia coli. Infect Immun. 1996;64:495–502.

27. Muniesa M, Recktenwald J, Bielaszewska M, Karch H, Schmidt H. Characterization of a Shiga toxin 2e-converting bacteriophage from an Escherichia coli strain of human origin. Infect Immun. 2000;68:4850–5. http://dx.doi.org/10.1128/IAI.68.9.4850-4855.2000

28. Brüssow H, Canchaya C, Hardt WD. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiol Mol Biol Rev. 2004;68:560–602. http://dx.doi.org/10.1128/MMBR.68.3.560-602.2004

29. O’Brien AD, Newland JW, Miller SF, Holmes RK, Smith HW, Formal SB. Shiga-like toxin-converting phages from Escherichia coli strains that cause hemorrhagic colitis or infantile diarrhea. Science. 1984;226:694–6. http://dx.doi.org/10.1126/science.6387911

30. Scotland SM, Smith HR, Willshaw GA, Rowe B. Vero cytotoxin production in strain of Escherichia coli is determined by genes carried on bacteriophage. Lancet. 1983;322:216. http://dx.doi.org/10.1016/S0140-6736(83)90192-7

31. Wagner PL, Livny J, Neely MN, Acheson DW, Friedman DJ, Waldor MK. Bacteriophage control of Shiga toxin 1 production and release by Escherichia coli. Mol Microbiol. 2002;44:957–70. http://dx.doi.org/10.1046/j.1365-2958.2002.02950.x

32. Louis BM. Geographies of the Haitian diaspora. Transforming Anthropology. 2013;21:198–9. http://dx.doi.org/10.1111/traa.12015_1

33. Saeed A, Abd H, Edvinsson B, Sandstrom G. Acanthamoeba castellanii an environmental host for Shigella dysenteriae and Shigella sonnei. Arch Microbiol. 2009;191:83–8. http://dx.doi.org/10.1007/s00203-008-0422-2

34. Saeed A, Johansson D, Sandstrom G, Abd H. Temperature depended role of Shigella flexneri invasion plasmid on the interaction with Acanthamoeba castellanii. Int J Microbiol. 2012;1:8. http://dx.doi.org/10.1155/2012/917031

35. Lainhart W, Stolfa G, Koudelka GB. Shiga toxin as a bacterial defense against a eukaryotic predator, Tetrahymena thermophila. J Bacteriol. 2009;191:5116–22. http://dx.doi.org/10.1128/JB.00508-09

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