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# *Escherichia coli* O157:H7 Shiga Toxin-Encoding Bacteriophages: Integrations, Excisions, Truncations, and Evolutionary Implications

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**As it descended from *Escherichia coli* O55:H7, Shiga toxin (Stx)-producing *E. coli* (STEC) O157:H7 is believed to have acquired, in sequence, a bacteriophage encoding Stx2 and another encoding Stx1. Between these events, sorbitol-fermenting *E. coli* O157:H$^-$ presumably diverged from this clade. We employed PCR and sequence analyses to investigate sites of bacteriophage integration into the chromosome, using evolutionarily informative STEC to trace the sequence of acquisition of elements encoding Stx. Contrary to expectations from the two currently sequenced strains, truncated bacteriophages occupy *yehV* in almost all *E. coli* O157:H7 strains that lack $stx_1$ ($stx_1$-negative strains). Two truncated variants were determined to contain either GTT or TGACTGTT sequence, in lieu of 20,214 or 18,895 bp, respectively, of the bacteriophage central region. A single-nucleotide polymorphism in the latter variant suggests that recombination in that element extended beyond the inserted octamer. An $stx_2$ bacteriophage usually occupies *wrbA* in $stx_1^+/stx_2^+$ *E. coli* O157:H7, but *wrbA* is unexpectedly unoccupied in most $stx_1$-negative/$stx_2^+$ *E. coli* O157:H7 strains, the presumed progenitors of $stx_1^+/stx_2^+$ *E. coli* O157:H7. Trimethoprim-sulfamethoxazole promotes the excision of all, and ciprofloxacin and fosfomycin significantly promote the excision of a subset of complete and truncated *stx* bacteriophages from the *E. coli* O157:H7 strains tested; bile salts usually attenuate excision. These data demonstrate the unexpected diversity of the chromosomal architecture of *E. coli* O157:H7 (with novel truncated bacteriophages and multiple $stx_2$ bacteriophage insertion sites), suggest that $stx_1$ acquisition might be a multistep process, and compel the consideration of multiple exogenous factors, including antibiotics and bile, when chromosome stability is examined.**

Shiga toxins 1 and 2 (Stx1 and Stx2) are cardinal virulence factors of *Escherichia coli* O157:H7. Stx1 is nearly identical to Stx, the principal extracellular cytotoxin of *Shigella dysenteriae* serotype 1 (8). Stx2 has 56% identity to Stx1 (36). The $stx_1$ and $stx_2$ A and B subunit genes exist as tandem open reading frames (ORFs) in the central portion of lambdoid bacteriophages in *E. coli* O157:H7 (19). In Sakai and EDL933, the two *E. coli* O157:H7 strains that have been completely sequenced, the bacteriophage that encodes Stx1 is integrated into *yehV* (57), which encodes a protein that positively regulates curli expression (7), and is flanked by duplications of CCCTGT CACGTTACGCGCGTG. The bacteriophage that encodes Stx2 is integrated into *wrbA* (32, 40), which encodes a novel multimeric flavodoxin-like protein (13), and is flanked by duplications of GACATATTGAAAC. Almost all *E. coli* O157:H7 strains possess $stx_2$, and approximately three-quarters contain, in addition, $stx_1$ (strains lacking $stx_1$ are referred to hereafter as $stx_1$-negative strains) (23, 38, 45). Most human non-O157:H7 Stx-producing *E. coli* (STEC) strains possess $stx_1$ but lack $stx_2$ (5, 6, 28, 51). Except for an Stx2e-encoding bacteriophage which is known to be integrated into *yecE* in STEC ONT:H$^-$ (42), the insertion sites of *stx* bacteriophages have not yet been identified in the chromosomes of non-O157:H7 STEC strains (25, 29, 47).

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Multilocus enzyme electrophoresis analysis (53), colony hybridizations, Southern blotting, and PCRs with primers specific for *stx* genes teach that *E. coli* O157:H7 descended from *E. coli* O55:H7 or from a similar common progenitor (11). *E. coli* O55:H7 and STEC belonging to serogroup O157 form a clade termed the STEC 1 (or enterohemorrhagic *E. coli* 1) group (52). The currently accepted evolutionary model suggests that in its descent from *E. coli* O55:H7, *E. coli* O157:H7 lost the O55 *rfb-gnd* cluster and acquired the $stx_2$ bacteriophage and the O157 *rfb-gnd* cluster (4, 46, 50). Subsequently, sorbitol-fermenting $stx_2^+$ *E. coli* O157 (11, 15) separated from this lineage. After these two lineages diverged, *E. coli* O157:H7 acquired $stx_1$ (11), presumably via acquisition of the bacteriophage that contained this gene, and lost the ability to ferment sorbitol, while the sorbitol-fermenting $stx_2^+$ *E. coli* O157 evolved into nonmotile *E. coli* O157:H$^-$. Most non-O157:H7 STEC strains associated with human diseases are distantly related to *E. coli* O157:H7 and form a clade termed the STEC 2 (or enterohemorrhagic *E. coli* 2) group (54).

The bacteriophage insertion sites in the STEC 1 group have not been examined systematically to confirm the validity of or to refine our presumed understanding of the sequence of acquisition of the elements containing the *stx* genes. The recent releases of the sequences and insertion sites of the *stx* bacteriophages from two different *E. coli* O157:H7 strains (16, 39) prompted us to investigate these sites in diverse STEC 1 organisms. This study was performed to determine if, as predicted, *stx* bacteriophages occupied *wrbA* and *yehV* consecutively as this lineage evolved and to determine if *stx* bacteriophages utilize these two integration sites outside the STEC 1 lineage. Additionally, we assessed the stability of the

TABLE 1. Strains used in this study

| Organism | Reference or source | *stx*$_1$[f] | *yehV*[g] | *stx*$_2$[f] | *wrbA*[g] |
|---|---|---|---|---|---|
| STEC 1 lineage | | | | | |
| O55:H7 | | | | | |
| TB156A, TB182A[a,b] | 6 | − | I | − | I |
| 5A, 5B, 5C, 5D | 4 | | | | |
| 5E[c] | 4 | − | O | − | I |
| O157:H7 | | | | | |
| 84-01,[a,b] 87-01, 87-20[a,b] | 43 | + | O | + | O |
| 93-111 | 3 | | | | |
| EK3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 20, 21, 22, 24, 25, 26 | 26 | | | | |
| U3, 4, 9, 10, 12, 13, 14, 15, 19, 21, 22, 34, 26, 28, 30, 31, 34, 35, 36, 40, 41, 43, 44, 46, 47, 48, 49, 50, P1, 4, 6, 9, 10, H2 | 8 | | | | |
| 86-24,[d] 86-28,[d] 86-17,[e] 87-07[e] | 43 | − | O | + | O |
| EK15, 28 | 26 | | | | |
| U39 | 8 | | | | |
| 87-14 | 43 | − | O | + | I |
| EK1,[b] 2, 18,[b] 19,[b] 23, 27 | 26 | | | | |
| U2, 5, 7, 11, 16, 17, 18, 20, 24, 29, 32, 33, 42, 45,[d] 51, 52, P3, 7, H4, 5 | 8 | | | | |
| P8 | 8 | − | I | + | O |
| Sorbitol-fermenting O157:H$^-$ | | | | | |
| CB2755[b] | Lothar Beutin | − | I | + | O |
| 493/89 | 13 | | | | |
| Non-STEC 1 lineage | | | | | |
| Non-O157:H7 | | | | | |
| TB352A (O26:NM), TB334C (O85:NM), TB285A (O126:H2), TB154A (O103:H6) | 6 | + | I | − | I |
| EK30, 31, 32, 33 (O103:H2), EK34 (O111:nonmotile), EK35 (O111:H8), EK36, 37 (O118:H16), EK39 (Orough:H11) | 26 | | | | |
| EK38 (O121:H19) | 26 | − | I | + | I |
| TB226A (O111:HN) | 6 | + | I | + | I |

[a] Amplicons in *yehV* in this strain have been sequenced.
[b] Amplicons in *wrbA* in this strain have been sequenced.
[c] Left end of bacteriophage occupies *yehV*; right end of bacteriophage not detected at this junction.
[d] Strain has Δ20,214 bacteriophage.
[e] Strain has Δ18,895 bacteriophage.
[f] +, present; −, absent.
[g] I, intact; O, occupied.

integration of the *stx* bacteriophages into the *E. coli* O157:H7 chromosome in selected strains.

## MATERIALS AND METHODS

**Bacteria and growth conditions.** Organisms studied are listed in Table 1. Frozen bacterial stocks were inoculated directly into Luria broth (LB; 4 ml) (43). Broths were prewarmed to 37°C if they were used as starter cultures, until an optical density at 600 nm of 0.8 was attained, at which time 300 μl was added to 3 ml of prewarmed LB with or without subinhibitory concentrations of ciprofloxacin (2 μg/liter; Bayer Corporation, West Haven, Conn.), trimethoprim (4 μg/liter)–sulfamethoxazole (20 μg/liter) (TMP-SMX; Elkins-Sinn, Cherry Hill, N.J.), or ampicillin (200 μg/liter; Sigma, St. Louis, Mo.). Fosfomycin assimilation by *E. coli* depends on induction of the glycerol phosphate or hexose phosphate transport system (24). Therefore, we used LB containing an inducer of the former system, glucose-6-phosphate (G-6-P; 50 mg/liter), with and without added

TABLE 2. Primer pairs used in this study

| Primer pair | Sequence (5′ → 3′) | Comment |
|---|---|---|
| A | AAGTGGCGTTGCTTTGTGAT | Amplicon spans $stx_1$ bacteriophage insertion site *yehV* |
| B | AACAGATGTGTGGTGAGTGTCTG | |
| C | AGGAAGGTACGCATTTGACC | Amplicon spans $stx_2$ bacteriophage insertion site *wrbA* |
| D | CGAATCGCTACGGAATAGAGA | |
| A | AAGTGGCGTTGCTTTGTGAT | Amplicon spans bacteriophage-*yehV* right junction |
| E | GATGCACAATAGGCACTACGC | |
| F | CACCGGAAGGACAATTCATC | Amplicon spans bacteriophage-*yehV* left junction |
| B | AACAGATGTGTGGTGAGTGTCTG | |
| C | AGGAAGGTACGCATTTGACC | Amplicon spans bacteriophage-*wrbA* right junction |
| G | ATCGTTCGCAAGAATCACAA | |
| H | CCGACCTTTGTACGGATGTAA | Amplicon spans bacteriophage-*wrbA* left junction |
| D | CGAATCGCTACGGAATAGAGA | |
| I | GTTATCCGTCAGCACCTTGTAGA | Primer sites span truncations in $stx_1$ bacteriophage |
| J | ATATCCGTACTCCTGGCCTTAAC | |
| K[a] | CGCTTTGCTGATTTTTCACA | $stx_1$ |
| L | GTAACATCGCTCTTGCCACA | |
| M[a] | GTTCCGGAATGCAAATCAGT | $stx_2$ |
| N | CGGCGTCATCGTATACACAG | |
| O | TGACCACACGCTGACGCTGACCA | *malB* |
| P | TTACATGACCTCGGTTTAGTTCACAGA | |

[a] Primer pair described in reference 28.

fosfomycin (1.6 mg/liter; Sigma), to study this antibiotic's effects. The influences of all antibiotics on bacteriophage integrations were studied in broths with or without bile salts (1.5 g of an equal mix of sodium cholate and sodium deoxycholate/liter [item no. B8756; Sigma]). For chromosomal structural analyses, bacteria from broths grown overnight after direct inoculation from frozen stocks were studied. All broths were incubated (16 h, 37°C) without agitation before genomic DNA was extracted with the DNeasy tissue kit (Qiagen GmbH, Hilden, Germany).

**PCR conditions.** We amplified fragments shorter than 1.5 kb in 50-μl volumes containing 10× PCR buffer (5 μl; Promega, Madison, Wis.), 10 ng of bacterial or 5 μl of reconstituted phage pellet DNA, $MgCl_2$ (1.5 mM), deoxynucleoside triphosphate (dNTP; final concentration of each nucleotide, 200 μM), primers (final concentration of each, 1 μM) (Table 2), and *Taq* DNA polymerase (1.25 U; Promega) in an iCycler (Bio-Rad, Richmond, Calif.). The following cycling conditions were employed: 4 min at 94°C, followed by 30 cycles of 30 s (94°C), 30 s (58°C), and 90 s (72°C) and a final 7-min elongation step (72°C). We amplified longer segments by using Herculase Enhance *Taq* DNA polymerase (Stratagene, La Jolla, Calif.) in 50 μl containing 300 ng of target DNA in 5 μl of 10× Herculase buffer (Stratagene), dNTP (400 μM [each]), and primers (final concentration of each oligonucleotide, 2 μM) (Table 2). For these amplifications, we used the following conditions: 10 cycles at 92°C (30 s), 58°C (30 s), and 68°C (1 min per kb of target) followed by 20 cycles at 92°C (30 s), 58°C (30 s), and 68°C (1 min per kb of target with an increment of 10 s each cycle) and a final elongation step (68°C, 10 min). Amplification products were separated in Tris-borate-EDTA agarose gels, ethidium stained, and photographed.

**Sequencing.** Selected amplicons were cloned into the pCR4 TOPO vector (Invitrogen Corporation, Carlsbad, Calif.) and sequenced by using the PE Applied Biosystems (Foster City, Calif.) kit and an ABI PRISM 377 DNA sequencer (Applied Biosystems). Homology searches were performed on a National Center for Biotechnology Information BLAST server (12).

**Segment excision proportions (SEPs).** The proportions of intact bacteriophage insertion sites in DNA extracted after overnight culture were calculated for selected *E. coli* O157:H7 strains grown in various media by using a LightCycler Real Time PCR cycler (Bio-Rad). Primer pairs A-B and C-D were used to produce amplicons that spanned the insertion sites, and primer pairs A-E, F-B, C-G, and H-D were used to produce amplicons that spanned the junctions between the bacteriophages and chromosomes (Table 2). These primers were used in 1 μM final concentrations in 25 μl of PCR mixture containing bacterial DNA (10 ng), $MgCl_2$ (1.5 mM), dNTP (final concentration of each nucleotide, 200 μM), *Taq* DNA polymerase (1.25 U), and 10× SYBR Green PCR buffer (2.5 μl; PE Biosystems, Warrington, United Kingdom).

SEPs were calculated according to the following formula: $SEP = X/[X + (Y + Z)/2]$, where $X$ is the number of copies per unit of volume of the target DNA spanning the insertion sites and $Y$ and $Z$ are the numbers of copies per unit of volume corresponding to amplicons spanning the right and left bacteriophage-chromosome junctions, respectively. The number of copies per microliter was derived by dividing the product of $6.02 \times 10^{23}$ (copies per mole) and the concentration (grams per microliter) by the molecular mass, where molecular mass (grams per mole) = (number of base pairs × 660 Da/bp) and 1 mol is equal to $6 \times 10^{23}$ molecules (i.e., the number of copies). SEPs were quantified in quadruplicate on two separate days. In each experiment, a standard curve was derived by using a recombinant plasmid consisting of the pCR4 TOPO vector into which an amplicon generated by primers I and J was cloned; concentrations used in the standard curve ranged between 1 and $10^{10}$ copies/μl.

**Statistics.** We used analysis of variance to test for equality of means among the 12 conditions and Tukey's method for multiple comparisons for the pairwise comparison of means (35).

**Phage extractions from culture supernatants.** Chloroform (50 μl) was added to aspirated supernatants from centrifuged (5,000 × *g*, 4°C, 10 min) 3-ml broth cultures; resulting suspensions were shaken (120 rpm, room temperature, 10 min) and centrifuged (4,000 × *g*, room temperature, 10 min). The supernatant was removed and incubated (37°C, 30 min) after the addition of DNase I (2 U/ml; Ambion, Austin, Tex.) and RNase A1 (10 μg/ml; Ambion). Phage particles were then precipitated overnight on ice by adding NaCl (1 M final concentration) and polyethylene glycol 8000 (to 10% [wt/vol]), followed by centrifugation (10,000 × *g*, 4°C, 20 min). The pellet was suspended in 500 μl of 10 mM Tris (pH 7.5) containing $MgCl_2$ (10 mM) and NaCl (100 mM) before being extracted twice with equal volumes of chloroform. The chloroform-extracted supernatant was again treated with DNase I (10 U in 500 μl, 37°C, 30 min) to digest noncoated bacterial DNA. The phage particles were then disrupted by adding phenol (500 μl), and liberated DNA was extracted with phenol-chloroform (1:1) and chloro-

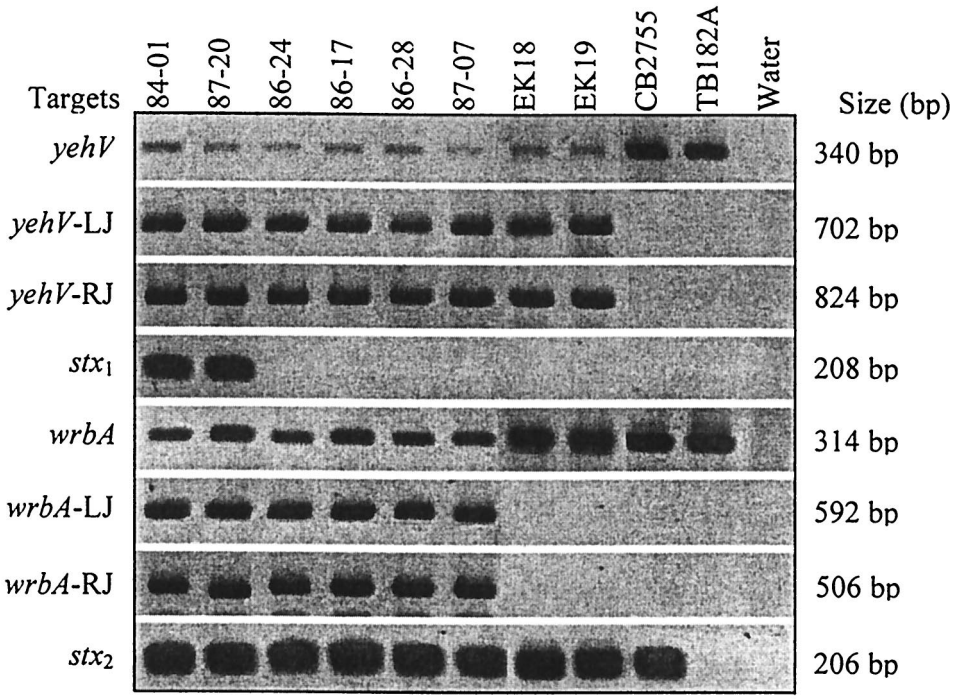


FIG. 1. Amplicons for investigation of the *yehV* and *wrbA* integration sites in *E. coli* O157:H7. Bacterial strains used, loci examined, and lengths of resulting amplicons are listed across the top and to the left and right of the rows of amplicons, respectively. LJ, left junction; RJ, right junction.

form-isoamyl alcohol (24:1) and precipitated on ice with 3 M sodium acetate (0.1 volume) and chilled absolute ethanol (2 volumes, 20 min). Precipitated DNA was then centrifuged (12,000 × *g*, 4°C, 5 min), and pellets were washed (70% ethanol), dried, and resuspended in 100 μl of Tris-EDTA (pH 8.0).

**Nucleotide sequence accession number.** The newly determined sequence was deposited in GenBank under accession number AY160192-5.

## RESULTS

**Bacteriophage insertions in *yehV* and *wrbA*.** As expected, the $stx_1$ bacteriophage insertion site in *yehV* was unoccupied in most (six of the seven) *E. coli* O55:H7 strains tested (Table 1 and Fig. 1); the only exception was strain 5E, in which the left junction of a bacteriophage was found in *yehV*. Also as expected, *yehV* was uninterrupted in sorbitol-fermenting *E. coli* O157:$H^-$. The sequences of the amplicons spanning this locus confirmed that neither bacteriophage nor other inserted DNA disrupts this locus, and duplicated flanking sequences are not present. Also as predicted, *wrbA* is intact in ancestral *E. coli* O55:H7 but is occupied by a bacteriophage in *E. coli* O157:$H^-$, as it is in each of the two *E. coli* O157:H7 strains that have been sequenced (16, 32, 39, 40, 57).

However, contrary to expectations, bacteriophage sequences disrupt *yehV* at the insertion site used by $stx_1$ bacteriophages in 34 of 35 $stx_1$-negative/$stx_2^+$ *E. coli* O157:H7 strains tested (Table 1 and Fig. 1). Also unexpectedly, *wrbA* is intact in 27 of these 35 isolates. Amplicons suggesting intact *wrbA* in three $stx_1$-negative/$stx_2^+$ *E. coli* O157:H7 strains were sequenced, and the gene was confirmed as ancestral and unoccupied, without duplicated GACATATTGAAAC sequences flanking the insertion site.

**Analysis of DNA occupying *yehV* in $stx_1$-negative *E. coli* O157:H7.** Four $stx_1$-negative *E. coli* O157:H7 strains were chosen for extended analysis of the occupied *yehV* site, and the structural details of the occupying DNA are provided in Fig. 2. In strains 86-24 and 86-28, GTT is present in lieu of 20,214 bp and 31 complete ORFs that exist in VT1-Sakai (32) (for purposes of simplicity, all positions are related to those in the sequenced Sakai strain, though the same findings also apply to the $stx_1$ bacteriophage in strain EDL933). In strains 86-17 and 87-07, TGACTGTT takes the place of 18,895 bp. This missing 18,895-bp segment comprises 28 complete ORFs and one partial ORF, Ecs 2960, which encodes a putative protease/scaffold protein. We have termed these truncated structures the Δ20,214 and Δ18,895 bacteriophages, respectively.

In the Δ20,214 bacteriophage, 114- and 198-bp hybrid ORFs (HOs) are created from two smaller ORFs that are unregistered in the Sakai database. The octamer in the Δ18,895 bacteriophage gives rise to three HOs that do not exist in VT1-Sakai (Table 3). The trimer in the Δ20,214 bacteriophage engenders two HOs. None of these five HOs has extensive homology to genes in the database.

The trimer and octamer shown in Fig. 2 have identical regions 3′ to their borders, including genes encoding the putative prophage repressor C1 and the regulator Cro, which are involved in phage immunity. The gene encoding regulator protein CII, a third probable immunity molecule in the complete $stx_1$ bacteriophage, is absent from both truncated forms. Homologues to putative transposases OrfA and OrfB of IS*629* are in proximity to HO-T4 in the Δ18,895 bacteriophage, but these OrfA and OrfB homologues are not found in the Δ20,214 bacteriophage. The octamer's 5′ border is adjacent to the inverted repeat at the right end of the IS*629* homologue.

The two Δ18,895 bacteriophages each contain an A→G sin-

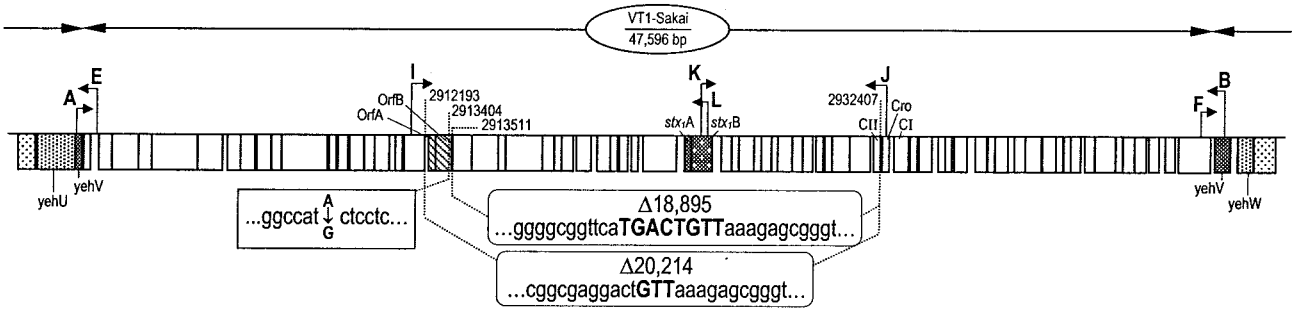


FIG. 2. Structures of two forms of truncated bacteriophages of $stx_1$-negative *E. coli* O157:H7. Inserted GTT and TGACTGTT sequences replace segments that are found in truncated $stx_1$ bacteriophages in two sequenced strains. ORF borders, proportions, and designations and nucleotide positions relate to those in reference 16. An A→G SNP 108 nucleotides 5′ to the octamer in both sequenced Δ18,895 bacteriophages and primer locations used to generate data pertaining to $stx_1$ bacteriophages are noted. A, E, I, K, L, J, F, and B are primers.

gle-nucleotide polymorphism (SNP) in ORF Ecs 2959, 108 nucleotides from the 5′ border of the TGACTGTT octamer. Interestingly, a GACT sequence in the Δ20,214 bacteriophage that is also found in VT1-Sakai is adjacent to the GTT that is found in lieu of the missing 20,214 nucleotides, producing a heptamer that is identical to seven of the eight nucleotides in the octamer.

**Analysis of *yehV* and *wrbA* in non-O157:H7 STEC.** In each of 15 non-O157:H7 strains examined, the insertion sites in *yehV* and *wrbA* are intact, and these genes do not contain bacteriophage-chromosome junctions. Thus, as those of STEC ONT:H$^-$ (42), these elements integrate into the chromosomes at positions other than those utilized in the sequenced $stx_1^+$/$stx_2^+$ *E. coli* O157:H7 strains.

**Excision of bacteriophages from the chromosomes of *E. coli* O157:H7.** Primers spanning complete and truncated bacteriophage insertion sites in *E. coli* O157:H7 elicit from bacterial DNA faint PCR products that are the sizes that would be expected had these genes been intact (Fig. 1). The presence of these faint bands suggests that a subset of the bacteria in these broths have chromosomes that no longer contain inserted elements. These primers do not elicit amplicons with sterile broth as a target (data not shown). Primers spanning the chromosome-bacteriophage junctions produce amplicons that are considerably more abundant than those that span the insertion sites. Amplicon sequences across the *yehV* and *wrbA* insertion sites in selected strains (Table 1) demonstrate that an intact gene is regenerated from a site that has been formerly occupied. Thus, intact and truncated bacteriophages are excised from the *E. coli* O157:H7 chromosome at discernible frequencies, and the excisions generate ancestral integration sites in the chromosome.

**Antibiotics, bile salts, and excision of *stx* bacteriophages.** TMP-SMX significantly increased the proportion of intact *yehV* and *wrbA* sites in all *E. coli* O157:H7 strains tested, whether the occupying bacteriophage was complete or truncated. Ciprofloxacin and fosfomycin significantly increased SEPs at *yehV* and *wrbA* in most strains and in some strains, respectively. Bile salts usually significantly attenuated the increased SEPs (Fig. 3 and Table 4). There was no indication that either of the chromosome-bacteriophage junctions were preferentially amplified, thereby distorting the SEP; the intra-isolate copy numbers of junctional amplicons were within 97% of each other in >99% of the determinations.

**Appearance of bacteriophage DNA in culture supernatants.** To determine whether bacteriophage excisions result in the appearance of intact bacteriophage in the culture supernatant, we subjected DNase-treated supernatants of $stx_1^+$/$stx_2^+$ *E. coli* O157:H7 strains 84-01 and 87-20 and $stx_1$-negative/$stx_2^+$ *E. coli* O157:H7 strains 86-17 and 86-24 to PCR with primer pairs specific for $stx_1$ (K-L) and $stx_2$ (M-N) or truncated $stx_1$ bacteriophages (I-J). In these experiments, organisms were grown in the absence or presence of subinhibitory concentrations of each antibiotic.

TABLE 3. HOs in truncated bacteriophages that are not present in VT1-Sakai

| Bacteriophage (strains) | HO[a] | Start codon[e] | Stop codon[e] | No. of amino acids in putative corresponding proteins |
|---|---|---|---|---|
| Δ20,214 (86-24,[a] 86-28[b]) | HO-T1 | 2912159 | 2932402 (TAA) | 37 |
| | HO-T2 | 2932460 | 2912053 (TGA) | 65 |
| Δ18,895 (86-17,[c] 87-07[d]) | HO-T3 | 2913402 | 2932408 (TAA) | 39 |
| | HO-T4 | 2913511 (+T)[f] | 2913276 (TGA) | 78 |
| | HO-T5 | 2932460 | 2913472 (TGA) | 33 |

[a] GenBank accession number A4160193.
[b] GenBank accession number A4160192.
[c] GenBank accession number A4160195.
[d] GenBank accession number A4160194.
[e] Nucleotide position based on sequence of Sakai strain.
[f] The start codon arises from the 5′ T in the TGACTGTT octamer.

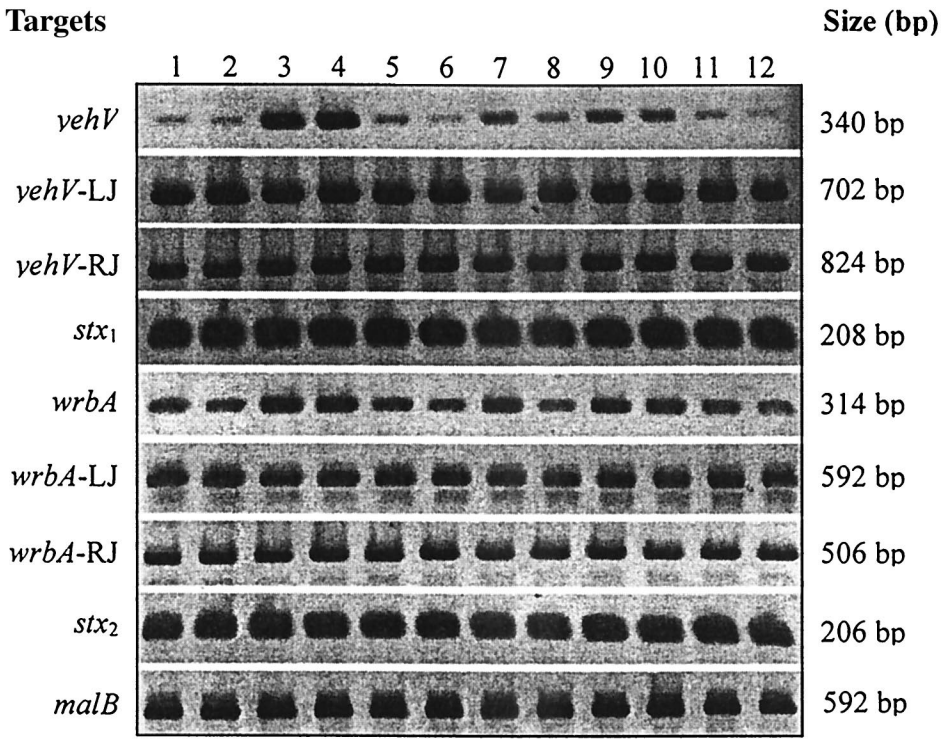


FIG. 3. Amplicons elicited across integration sites, in response to antibiotics and bile salts. *E. coli* O157:H7 strain 87-20 was grown in LB without (lanes 1 and 2) or with (lanes 3 and 4) TMP-SMX, ciprofloxacin (lanes 5 and 6), or ampicillin (lanes 7 and 8) or in LB with G-6-P without (lanes 9 and 10) or with (lanes 11 and 12) fosfomycin. Samples in even-number lanes were grown in bile salts. Loci examined and lengths of resulting amplicons are listed to the right and left of the rows. LJ, left junction; RJ, right junction.

DNase-resistant $stx_1$ and $stx_2$, presumably representing phage-coated DNA, appeared in the supernatants of the two $stx_1^+/stx_2^+$ strains. *malB* amplicons were not elicited from the supernatant, so the *stx* sequences in the supernatant cannot be attributed simply to bacterial lysis. In contrast, truncated bacteriophage sequences were not amplified from the supernatants of the two $stx_1$-negative/$stx_2^+$ *E. coli* O157:H7 strains tested (data not shown).

## DISCUSSION

The current model of emergence of toxigenic *E. coli* O157:H7 from its nontoxigenic, less virulent progenitor, *E. coli* O55:H7, relies on four crucial sequential events: (i) acquisition of an $stx_2$ bacteriophage in a single event and at a single site (probably *wrbA*); (ii) splitting off of the clone leading to *E. coli* O157:H$^-$; (iii) acquisition of the $stx_1$ bacteriophage in a single event and at a single site (probably *yehV*) by *E. coli* O157:H7; and (iv) loss of the ability to ferment sorbitol by *E. coli* O157:H7 (event iv might have preceded event iii during this descent). The data we present confirm this model to the point of emergence of serogroup O157 from serotype O55:H7 and to the point of divergence of the nonmotile sorbitol-fermenting lineage before an $stx_1$ bacteriophage occupied *yehV*. The only finding that is discordant with this model, prior to these two points in evolution, is our observation that the left junction of a bacteriophage occupies *yehV* in *E. coli* O55:H7 strain 5E. However, strain 5E, unlike other tested members of this serotype, possesses *iha* and other components of a tellurite resistance, adherence-conferring island (44). Thus, in light of its genetic aberrancy, the presence of bacteriophage sequences in *yehV* in strain 5E is difficult to interpret.

The parsimonious scenario described above explains the emergence of the *E. coli* O157 serogroup from its nontoxigenic progenitor, but this model must now be modified to accommodate two unanticipated findings in $stx_1$-negative/$stx_2^+$ *E. coli* O157:H7 strains. First, an $stx_2$ bacteriophage occupies *wrbA* in only a minority of these organisms. This means that the $stx_2$ bacteriophage was inserted into a site or sites other than *wrbA* in *E. coli* O157:H7 at multiple different times in history or that intrabacterial mobilizations of the bacteriophage led to vacation of the *wrbA* insertion locus and entry of the bacteriophage into other places in the chromosome. Second, bacteriophages that are truncated to the extent that they lack, at a minimum, part of $stx_1$ occupy *yehV* in almost all isolates tested.

Accordingly, we produce in Fig. 4 several new working evolutionary models to help refine present concepts of descent of the STEC 1 clade. In postulated lineage A, $stx_1$-negative/$stx_2^+$ *E. coli* O157:H7 strains in which the $stx_2$ bacteriophage is permanently integrated into a site or sites other than *wrbA* would constitute a separate branch of the STEC 1 clade, which would have diverged from other *E. coli* O157:H7 strains before the $stx_2$ bacteriophage became stably integrated into *wrbA* in postulated lineage B or C. Postulated lineage A also would have sustained independent losses of the sorbitol-fermenting phenotype and acquisitions of bacteriophages that occupy *yehV* in an evolutionary scenario that parallels the events leading to the emergence of postulated lineages B and C.

TABLE 4. SEPs across *yehV* and *wrbA* after growth in LB with various additives[a]

| Locus | Strain | Mean (standard deviation) of SEPs[b] in: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | LB | BS | TMP-SMX | TMP-SMX + BS | CIP | CIP + BS | AMP | AMP + BS | G-6-P | G-6-P + BS | FOS | FOS + BS |
| *yehV* | 84-01 | 2.8 (3.5) | 2.8 (3.5) | **16.9** (19.7) | 7.9 (9.7) | 6.8 (8.3) | 7.1 (9.2) | 2.6 (3.6) | 2.9 (3.9) | 2.6 (4.0) | 2.1 (2.8) | 6.9 (7.4) | 1.5 (1.9) |
| | 87-20 | 5.0 (9.8) | 1.3 (1.1) | **38.9** (20.7) | 31.5 (17.7) | 4.7 (3.2) | 3.9 (2.8) | 1.6 (2.0) | 2.0 (3.2) | 1.3 (1.1) | 1.6 (1.8) | 11.2 (7.4) | 6.2 (5.3) |
| | 86-17 | 3.2 (1.4) | 2.1 (1.1) | **13.6** (3.9) | 5.6 (1.2) | **6.8** (2.1) | 3.7 (0.6) | 2.3 (0.5) | 1.7 (0.5) | 2.4 (0.5) | 1.7 (0.4) | **8.9** (4.2) | 2.1 (0.8) |
| | 86-24 | 1.6 (1.7) | 0.5 (0.8) | **9.0** (5.8) | 2.3 (2.2) | **3.3** (2.9) | 1.6 (1.6) | 1.2 (1.3) | 0.8 (0.8) | 1.2 (1.4) | 0.8 (0.9) | 2.9 (2.9) | 1.2 (1.3) |
| *wrbA* | 84-01 | 29.2 (24.6) | 33.9 (30.1) | **98.3** (79.7) | 62.8 (54.9) | **51.6** (37.1) | 44.2 (37.3) | 34.6 (26.9) | 36.4 (29.3) | 33.3 (27.6) | 34.0 (28.6) | 52.3 (32.7) | 5.4[c] (5.5) |
| | 87-20 | 8.9 (6.7) | 5.0 (3.8) | **99.8** (39.9) | 64.7 (30.3) | **34.9** (14.8) | 20.6 (13.5) | 8.9 (6.3) | 5.7 (4.2) | 8.1 (5.5) | 6.1 (4.6) | **57.8** (27.0) | 23.9 (19.4) |

[a] Abbreviations: BS, bile salts; CIP, ciprofloxacin; AMP, ampicillin; FOS, fosfomycin.

[b] Proportions were multiplied by $10^3$. Values in bold are significantly ($P < 0.05$) higher than those for broth without antibiotic. Underlined values indicate that antibiotic effects are significantly ($P < 0.05$) attenuated by bile salts.

[c] The SEP in bile salts plus antibiotic was significantly ($P < 0.05$) less than those in corresponding broth with bile salts and corresponding antibiotic without bile salts even though the SEP in broth with antibiotic and without bile salts was not significantly higher than that in corresponding broth.

Serial evolutionary scenarios (postulated lineages B and C) are more likely to have occurred than are parallel scenarios, because they require fewer events leading to extant organisms. With respect to the *stx*$_2$ bacteriophage, in postulated lineages B and C this element would have entered a sorbitol-fermenting progenitor *E. coli* O157:H7 strain once and integrated into its chromosome at a single site, probably *wrbA*. Then, the sorbitol-fermenting O157:H$^-$ clone would have diverged. Subsequently, *yehV* was occupied by either *stx*$_1$ bacteriophages (postulated lineage B) or truncated variants (postulated lineage C). At a later point, the *stx*$_2$ bacteriophage vacated its initial integration site and entered one or more additional sites, leading to a common genotype of *E. coli* O157:H7 existing today but not the genotype represented by the sequenced strains. Each model proposed requires the past existence of a sorbitol-fermenting *E. coli* O157:H7 intermediate, preferably with an *stx*$_2$ bacteriophage inserted in *wrbA*. Such a strain has yet to be found and might be extinct.

Currently available data do not predict whether postulated lineage B is more likely to have occurred than postulated lineage C. In postulated lineage B, *stx*$_1$-negative/*stx*$_2^+$ *E. coli* O157:H7 descends from *stx*$_1^+$/*stx*$_2^+$ *E. coli* O157:H7, whereas postulated lineage C has a reverse descent order. In either case, the truncated bacteriophages are either progenitors to or descendants of complete *stx*$_1$ bacteriophages. The variants' sequences contain several clues that might help discern the origin or destiny of these structures. First, the GACTGTT in the octamer in the Δ18,895 bacteriophage and the GACT that is juxtaposed to the 5′ end of the inserted GTT in the Δ20,214 bacteriophage raise the possibility that one of the truncated bacteriophages descended from the other. Indeed, the mobile DNA in the Δ20,214 bacteriophage could include some or all of the four nucleotides 5′ to the GTT. Additionally, identical nonsynonymous A→G SNPs 108 nucleotides from the 5′ border of each octamer in the two Δ18,895 truncated bacteriophages that we sequenced suggest that the SNPs and the truncations in these strains did not arise independently. Thus, even though the most straightforward mechanism for acquiring or losing *stx*$_1$ would be the simple exchange of the octamer for *stx*$_1$ and contiguous DNA, or vice versa, the presence of this SNP suggests that at least one of the borders of recombination leading to the acquisition or loss of *stx*$_1$ is distal to at least one of the octamer's borders. Indeed, such instructive SNPs in *rfbE* strongly suggest interlineage cotransfer of large chromosomal segments between *E. coli* O157:H16 strain 13A81 and *E. coli* O157:H$^-$ strain 3584-91 (46). Also, the juxtaposition of the right end of IS*629* and the 5′ terminus of the octamer suggests that a transposon-mediated process has played a role in the evolution of the structures of these bacteriophages. Continued analyses of polymorphisms and genotypes in instructive *E. coli* O157:H7 strains should refine our understanding of the emergence of *E. coli* O157:H7.

While the two HOs in the Δ20,214 bacteriophage arise through the GTT trimer's bridging of parts of two ORFs in the Sakai genome, the genesis of the three HOs in the Δ18,895 bacteriophage is not so straightforward and warrants additional comment. HO-T3 results from the A→G SNP, because the G gives rise to an ATG start codon not found in the Sakai sequence. The A opposite the 5′ T in the TGACTGTT octamer, which creates a start codon that is not found in the

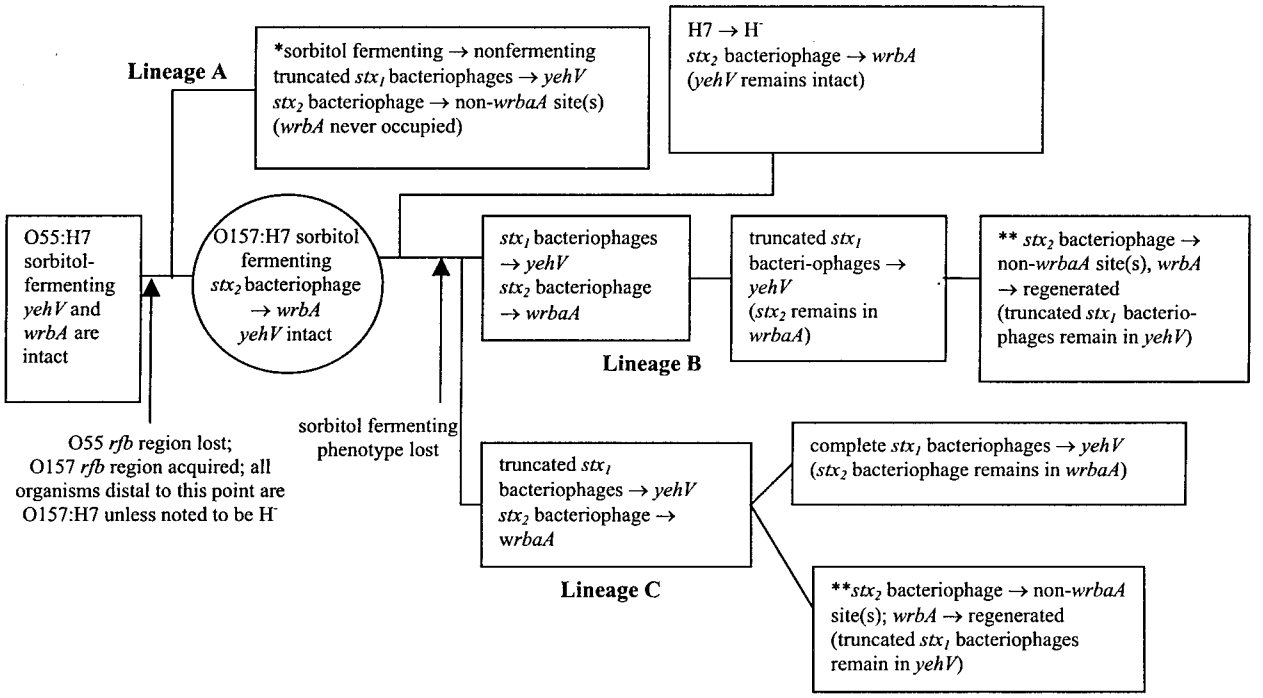


FIG. 4. Evolutionary scenarios. Serotypes, phenotypes, genotypes, critical events, and postulated intermediate form (circle) in three different scenarios leading to the five STEC 1 forms known to exist today (boxes). One asterisk indicates that the sequence of listed events is not known but is presumed to have occurred at different times during evolution. This result would obviate the need for postulated lineage B or C to produce such an organism. Two asterisks indicate that organisms in this box would not exist if lineage A exists.

Sakai strain, engenders HO-T4. The functions of these HOs are unknown.

Ohnishi et al. (37) recently reported that in six of eight unrelated $stx_2^+$ *E. coli* O157:H7 strains, the Sp5 bacteriophage (their designation for the $stx_2$ bacteriophage) does not occupy *wrbA*, its site of integration in the sequenced VT1-Sakai and 933W strains. Our data confirm these particular findings and extend them to a larger set of strains from North America. Our data differ, however, in relation to the frequencies of utilization of *yehV* by the $stx_1$ bacteriophage. Whereas *yehV* was occupied by an $stx_1$ bacteriophage in each of the 58 $stx_1^+$ *E. coli* O157:H7 strains that we studied, Ohnishi et al. provide data that the $stx_1$ bacteriophage integrates into other loci in two of the seven $stx_1^+$ *E. coli* O157:H7 strains from Japan that they examined.

Two bacteriophage immunity genes, *cro* and C1, that remain in the truncated bacteriophages might prevent replication of similar bacteriophages (10). Therefore, if the truncated bacteriophages are progenitors of the complete $stx_1$ bacteriophage, $stx_1$ and flanking regions might have been acquired from an element that is unrelated to the $stx_1$ bacteriophage. Indeed, bacteriophages plausibly acquire segments from a vast pool of related and unrelated bacteriophages (17). In 1987, Huang et al. (19) suggested that had $stx_1$ been acquired via fortuitous removal from a donor chromosome during imprecise prophage excision, this gene would have been located closer to one of the attachment sites; its location in the central portion of the bacteriophage suggests that the acquisition of $stx_1$ involved deletions and duplications during evolution. The presence of truncated $stx_1$ bacteriophages in $stx_1$-negative *E. coli* O157:H7 lends support to their proposal.

Our data also shed light on the effects of exogenous factors on chromosome stability in *E. coli* O157:H7. Mitomycin C increases the number of copies of *stx* genes (20) and $stx_1$ bacteriophages (57) in *E. coli* O157:H7, presumably via bacteriophage induction and excision. Ciprofloxacin lyses an $stx_1$-negative/$stx_2^+$ *E. coli* O157:H7 strain, an effect attributed to induction of the $stx_2$ bacteriophage (58). Antibiotics used as growth-promoting food supplements in agriculture induce Stx-encoding bacteriophages in several serotypes of STEC (30). We now demonstrate that TMP-SMX and fluoroquinolones, which increase $stx_2$ expression in vitro (26, 27), promote the excision of $stx_2$ bacteriophages as well as of complete and truncated $stx_1$ bacteriophages. We additionally demonstrate that subinhibitory concentrations of fosfomycin, which increase the release of Stx from STEC (14, 55, 56), might, at least in some strains, lead to bacteriophage excision. Though fosfomycin is active against the bacterial cell wall, exposure to this compound results in induction and excision of the bacteriophage, raising the possibility that phage excision might be a nonspecific response of *E. coli* O157:H7 to bacterial stress. However, ampicillin, another cell wall-active antibiotic, produced no such effect. It is also interesting that the excision of bacteriophage does not necessarily result in rapid lysis of the bacterial cell, as evidenced by our ability to pellet bacteria in which the chromosome has intact insertion sites at *yehV* and *wrbA*. The isolation and study of viable *E. coli* O157:H7 in which excision has occurred will be instructive in determining

the fate of the formerly integrated complete and truncated bacteriophages.

Recombination is the predominant mechanism of evolution of *E. coli* O157:H7. Its bacteriophages contain many recombination-prone regions (31), and the *E. coli* O157:H7 chromosome eliminates pseudogenes, presumably via recombination, faster than it acquires them (18). Antibiotics might also contribute to evolution as exogenous agents by inducing *stx* bacteriophages, leading to the deposition of bacteriophage DNA into the environmental genetic pool. Interestingly, chlorate and anaerobic growth lead to the duplication or deletion of the *stx* gene in *S. dysenteriae* serotype 1 (33).

The attenuation of bacteriophage excision by bile salts suggests that these ubiquitous intestinal compounds should be further investigated for their effects on bacteriophage stability and induction. Bile salts inhibit phage growth in *Salmonella enterica* serotype Enteritidis (22) and increase phage production in *Bacteroides fragilis* (1), but their effects on bacterial genomic stability and on virulence factor dissemination are largely unstudied. We should note that the analysis of bile salts and of antibiotics was confined only to pelleted, presumably nonlysed cells at the end of the incubation period. Also, we did not assess the effects of these agents on induction and lysis during the preceding 16 h of growth.

*E. coli* O157:H7 strains differ in their ability to produce Stx (9, 14), to adhere to (2, 41) and invade (49) eukaryotic cells, and to secrete proteins (34). Perhaps interstrain, and even interassay (48), phenotypic differences can be attributed, at least in part, to chromosomal instability. Indeed, it is interesting that STEC strains demonstrate differential rates of alteration of pulsed-field gel electrophoresis patterns during subculture (21). Furthermore, we wish to caution against assigning chromosomal insertion sites occupied or unoccupied statuses based solely on the generation from *E. coli* O157:H7 DNA of amplicons that are the sizes predicted from the K-12 chromosome sequence. Specifically, because a subset of chromosomal molecules from organisms grown overnight in broth culture sustained bacteriophage excisions, which would result in shorter amplicons, it is necessary to perform corroborative amplifications focusing on the junctions between the element of interest and the chromosome before proposing that an insertion site is not occupied in a particular strain. Also, categorizing an *E. coli* O157:H7 strain as $stx_1$-negative/$stx_2^+$ without further characterization fails to address the diversity of chromosomal patterns that are present, and it might be inappropriate to draw epidemiologic conclusions or to associate genotypes of infecting isolates with clinical illnesses. This heterogeneity now warrants consideration when analyzing strains, as simple *stx* genotyping fails to address the diversity of chromosomal patterns within the *E. coli* O157:H7 serotype. Finally, these data demonstrate that pathogens chosen for sequencing, even those occurring within the same serotype, might not be representative of many members of that serotype.

In summary, the architecture of the *E. coli* O157:H7 chromosome is considerably more complex and diversified than previously recognized; its evolution involves either parallel acquisition of $stx_2$ bacteriophages or, more likely, intrabacterial $stx_2$ bacteriophage insertion site changes. Truncated bacteriophages occupy *yehV* in $stx_1$-negative *E. coli* O157:H7. These truncated structures are either progenitors to or descendants of structures that contained $stx_1$. Antibiotics promote excisions of complete and truncated bacteriophages. Bile salts, previously unrecognized modifiers of bacteriophage integration stability, can attenuate these excisions. Environmental factors in the diverse milieus in which STEC exists must be considered when the dynamism of the *E. coli* O157:H7 chromosome is examined.

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