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Adaptive Radioresistance of Enterohemorrhagic *Escherichia coli* O157:H7 Results in Genomic Loss of Shiga Toxin-Encoding Prophages

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ABSTRACT Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a foodborne pathogen producing Shiga toxins (Stx1 and Stx2), which can cause hemorrhagic diarrhea and life-threatening infections. O157:H7 strain EDL933 carries prophages CP-933V and BP-933W, which encode Shiga toxin genes (stx_1 and stx_2, respectively). The aim of this work was to investigate the mechanisms of adaptive resistance of EHEC strain EDL933 to a typically lethal dose of gamma irradiation (1.5 kGy). Adaptive selection through six passages of exposure to 1.5 kGy resulted in the loss of CP-933V and BP-933W prophages from the genome and mutations within three genes: *wrbA*, *rpoA*, and Wt_02639 (*molY*). Three selected EHEC clones that became irradiation adapted to the 1.5-kGy dose (C1, C2, and C3) demonstrated increased resistance to oxidative stress, sensitivity to acid pH, and decreased cytotoxicity to Vero cells. To confirm that loss of prophages plays a role in increased radioresistance, clones C1 and C2 were exposed to bacteriophage-containing lysates. Although phage BP-933W could lysogenize C1, C2, and *E. coli* K-12 strain MG1655, it was not found to have integrated into the bacterial chromosome in C1-Φ and C2-Φ lysogens. Interestingly, for the *E. coli* K-12 lysogen (K-12-Φ), BP-933W DNA had integrated at the *wrbA* gene (K-12-Φ). Both C1-Φ and C2-Φ lysogens regained sensitivity to oxidative stress, were more effectively killed by a 1.5-kGy gamma irradiation dose, and had regained cytotoxicity and acid resistance phenotypes. Further, the K-12-Φ lysogen became cytotoxic, more sensitive to gamma irradiation and oxidative stress, and slightly more acid resistant.

IMPORTANCE Gamma irradiation of food products can provide an effective means of eliminating bacterial pathogens such as enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, a significant foodborne pathogen that can cause severe disease due to the production of Stx. To decipher the mechanisms of adaptive resistance of the O157:H7 strain EDL933, we evolved clones of this bacterium resistant to a lethal dose of gamma irradiation by repeatedly exposing bacterial cells to irradiation following a growth restoration over six successive passages. Our findings provide evidence that adaptive selection involved modifications in the bacterial genome, including deletion of the CP-933V and BP-933W prophages. These mutations in EHEC O157:H7 resulted in loss of stx_1 and stx_2, loss of cytotoxicity to epithelial cells, and decreased resistance to acidity, critical virulence determinants of EHEC, concomitant with increased resistance to lethal irradiation and oxidative stress. These findings demonstrate that the potential adaptation of EHEC to high doses of radiation would involve elimination of the Stx-encoding phages and likely lead to a substantial attenuation of virulence.

KEYWORDS *Escherichia coli*, Shiga toxins, acid stress, bacteriophages, food safety, irradiation, lysogen, oxidative stress, radioresistance

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Enterohemorrhagic (EHEC) *Escherichia coli* O157:H7 and other Shiga-toxinogenic *E. coli*/Vero-toxinogenic *E. coli* (STEC/VTEC) are an important risk to public health and food safety. EHEC O157:H7 can cause life-threatening diseases that range from mild gastroenteritis to hemorrhagic colitis and, in extreme cases, cause hemolytic-uremic syndrome and kidney failure (1, 2). EHEC O157:H7 is proposed to have evolved from a nontoxigenic *E. coli* O55:H7 strain possessing a locus of enterocyte effacement (LEE). This evolution involved four sequential steps: (i) acquisition of Shiga toxin 2 (Stx2)-encoding genes, (ii) acquisition of plasmid pO157, (iii) acquisition of Stx1-encoding genes, and (iv) loss of beta-glucuronidase activity and the capacity to ferment D-sorbitol (3).

Bacteriophages are viruses that infect bacteria. They also contribute in several manners to the evolution of their hosts, such as by lysogenic conversion and horizontal gene transfer (4), whereby phage genes integrate into bacterial genomes and can thus increase bacterial gene content. Lysogeny may also result in gain or loss of function and potentially increased fitness or virulence. For example, the virulence of some bacteria relies on bacteriophage-encoded genes acquired through this process (5, 6). *E. coli* O157:H7 strain EDL933 possesses 1,387 more genes than the nonpathogenic *E. coli* K-12 laboratory strain MG1655. This increased repertoire includes genes encoding alternative metabolic capacities, virulence factors, several phage proteins, and hypothetical proteins (7). *E. coli* O157:H7 strain EDL933 carries both stx_1 and stx_2 genes located on prophages PV-933V and BP-933W, respectively. These prophages are considered beneficial to their bacterial hosts by providing new functions during lysogenic conversion that can be considered a bacterium-phage coevolution (8).

Stx-encoding prophages integrate into the bacterial genome by site-specific recombination through a tyrosine recombinase integrase that binds to the *att* sites to promote recombination and phage integration (9, 10). Usually, The BP-933W prophage is integrated adjacent to the *wrbA* gene, involved in the oxidative stress response, and the PV-933V phage is integrated adjacent to the *mlrA* (*yehV*) gene, which encodes a regulator involved in curli and cellulose production (11).

To maintain lysogeny, lambdoid prophages constitutively express the *cI* gene, which represses expression of genes involved in the lytic cycle. DNA-damaging agents such as UV irradiation and antibiotics, for example mitomycin C, activate the SOS response, which leads to a proteolytic cleavage of the CI protein by RecA, resulting in the induction of the lytic cycle (12–16). The same recombination reaction may promote excision of prophages by excisionase (9, 17). In some cases, prophages can inactivate the expression of a bacterial gene via their integration into the open reading frame or the adjacent regulatory region (18). The prophage can also be excised when bacterial cells require this gene function to counter a stress encountered while the bacteriophage is integrated at this site. Active lysogeny is reversible when the excised prophage can remain in the cell as an episome and may reintegrate into the bacterial chromosome in the absence of conditions that favor the lytic cycle (12, 13, 19). In other cases, excision of the prophage may be followed by phage loss without triggering lytic production or bacterial lysis. DNA damage by exposure to oxidative stress (and mitomycin C) can induce the SOS response and promote a switch from the lysogenic to the lytic state in *E. coli* O157:H7 and can also result in prophage excision (9, 17). Gamma irradiation can also induce the SOS response (14). LexA is a key regulator of the SOS response, and regulation of the phage temperate/lytic cycle in *E. coli* and other bacteria (20) and DinI inhibits RecA and suppresses the SOS response (21).

Gamma irradiation is an established means of prolonging the shelf life of products such as fresh fruits and vegetables, meat, fish, and cereals by eliminating pathogens and by reducing the total bacterial count that can lead to food spoilage (22, 23). Gamma irradiation treatments generate an oxidative stress that kills bacterial cells through generation of reactive oxygen species (ROS) from the radiolysis of water. This oxidative stress causes structural damage and physical dysfunction in bacteria, including DNA damage and modifications, disruption of the cellular envelopes, ribosome alterations, and alteration of selective permeability of the membrane (24).

However, many bacteria, including *E. coli* O157:H7, can acquire radioresistance (25).

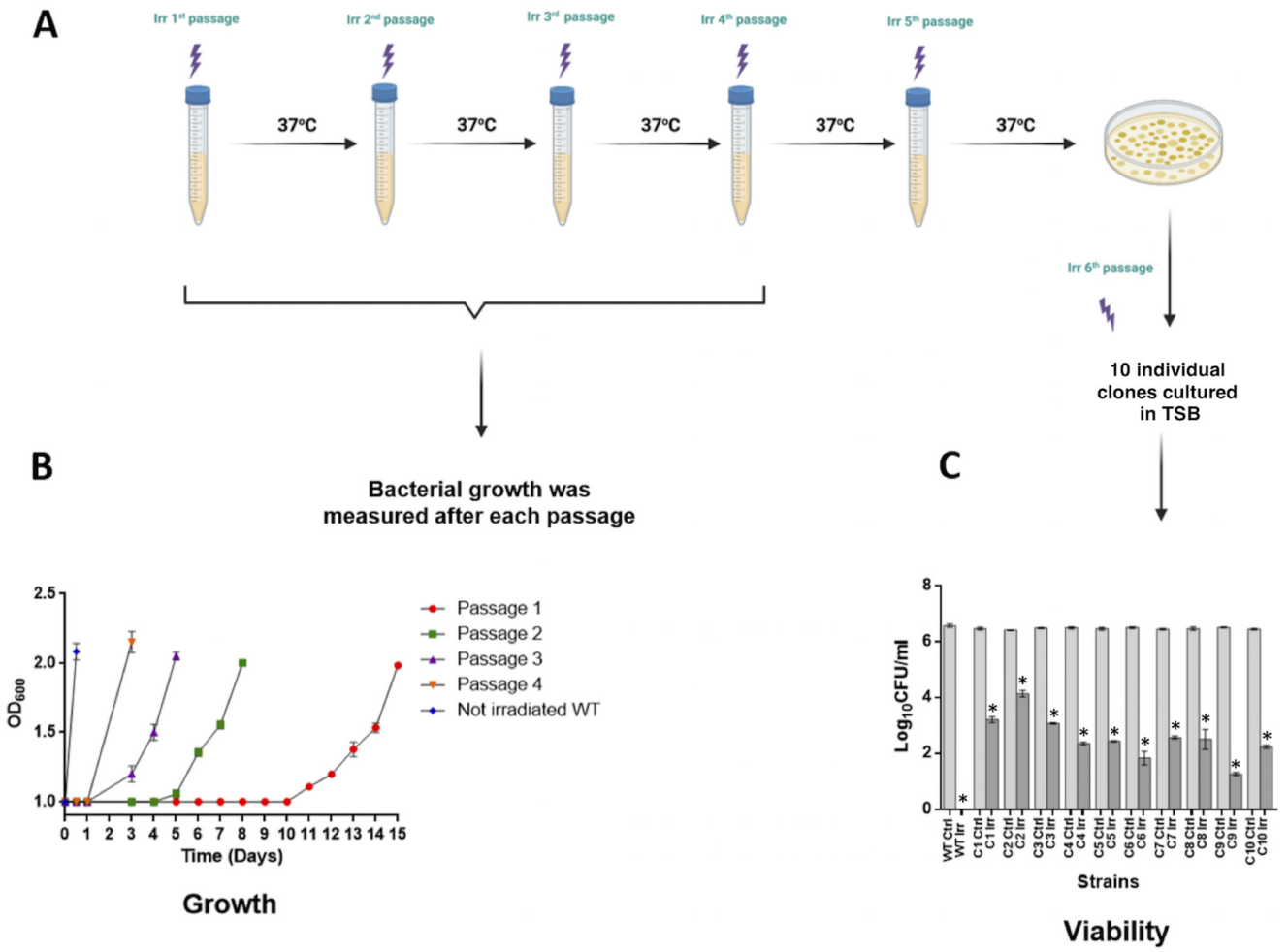

FIG 1 *E. coli* O157:H7 adaptation to an initially lethal dose of irradiation. (A) Procedure for adapting bacteria to a lethal dose (1.5 kGy). Bacteria were adapted to a 1.5-kGy gamma irradiation (Irr) dose by successive passages from the exponential phase of growth ($OD_{600} \approx 1$). After each passage, bacteria were incubated at 37°C in TSB with shaking until reaching an OD_{600} of 2. Bacterial cultures were subjected consecutively to a radiation dose of 1.5 kGy. After the 5th passage, bacterial cells ($OD_{600} \approx 1$) were irradiated, plated on TSA plates, and incubated overnight (O/N) at 37°C. (B) Bacterial growth following irradiation after each passage. A volume of 200 μL of O/N cultures was diluted to an OD_{600} of 0.05 in TSB, distributed into wells of sterile microtiter plates, and incubated at 37°C for 20 h without agitation. The OD_{600} growth measurements were performed by Bioscreen C apparatus every hour after a mixing period of 30 s. (C) Viability of 10 adapted clones after the 6th passage following irradiation. Clones culture were incubated individually O/N at 37°C with shaking, diluted 100-fold, incubated at 37°C with shaking until reaching an OD_{600} of ≈1, irradiated at 1.5 kGy, serially diluted, plated on TSA plates, and incubated overnight (O/N) at 37°C. The decrease in viability after irradiation compared to the control was significant (*, $P < 0.05$) for all tested samples. The results represent the means of replicate experiments for a minimum of three samples. Differences between the $\log_{10}$ CFU counts of the irradiated/control untreated (Ctrl) for each strain versus the wild-type (WT) parental strain were analyzed using the Student *t* test.

In this study, we investigated mechanisms underlying how *E. coli* O157:H7 can develop resistance to a normally lethal dose of gamma irradiation and determine what genetic changes can contribute to this process. *E. coli* O157:H7 strain EDL933, carrying both stx_1 and stx_2, was adapted to a 1.5-kGy dose of irradiation, which is normally considered lethal, by several passages. Genome sequencing of the radiation-adapted bacteria and stress resistance studies were performed to determine radiation resistance mechanisms of *E. coli* O157:H7 strain EDL933.

RESULTS

Genomic modification of *E. coli* O157:H7 following adaptation to a normally lethal dose of gamma irradiation. To identify adaptations allowing *E. coli* O157:H7 to become more resistant to radiation doses that are normally lethal to *E. coli*, we adapted strain EDL933 to 1.5 kGy of irradiation through several passages (Fig. 1A). The results showed that bacteria were able to survive and grow during 14 days after the first treatment and the adapted bacterial population increased with the number of passages

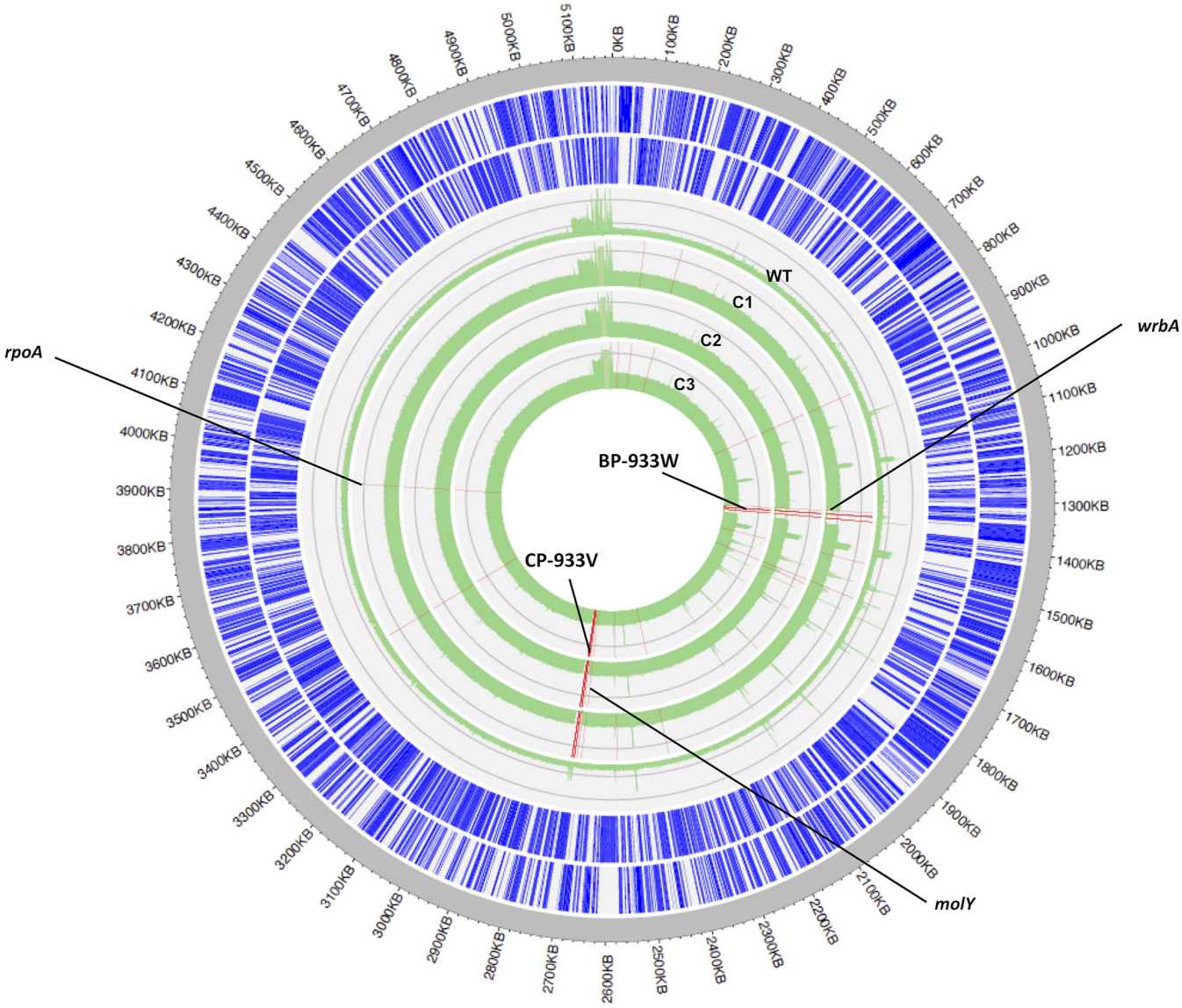

FIG 2 Circular genome maps of *E. coli* O157:H7 strain EDL933 and clones adapted to a lethal dose of irradiation (C1, C2, and C3). From outside to inside, the wild type followed by the C1, C2, and C3 clones (green circles), the circles represent the genes encoded on the forward strand, those encoded on the reverse strand, the coverage of the wild type, and that for clones C1, C2, and C3. Common mutated genes between C1, C2, and C3 are indicated by red lines.

(Fig. 1B). The viability of 10 clones was tested after the 6th irradiation passage (1.5 kGy). All of them were able to survive directly after this lethal stress (Fig. 1C). Since the viability of adapted *E. coli* O157:H7 clones C1, C2, and C3 was the highest (survival counts of 2.8, 3.71 and 2.4 log CFU/mL following irradiation, respectively), the genomes of these three clones were sequenced.

Whole-genome sequencing revealed that *E. coli* O157:H7 irradiation-adapted clones C1, C2, and C3 had deletions of the DNA sequences of two prophages, CP-933V and BP-933W (encoding the Stx1 and Stx2 toxins), after the second passage of irradiation. Further, three other genes, *rpoA*, *wrbA*, and *molY*, which encodes a hypothetical protein, contained point mutations (Fig. 2). PCR amplification and sequencing of *rpoA*, *wrbA*, and *molY* from sequential clones obtained after each passage showed that these mutations appeared after the second or third passage. For example, in *wrbA*, nucleotide G at position 25 was replaced by A (valine at position 9 by isoleucine) after the second passage (Table 1).

TABLE 1 Point mutations identified in *E. coli* O157:H7 C1, C2, and C3 radiation-adapted clones

Gene	Mutation	Passage
Wt_00837 (*wrbA*)	p.G25A; p.Val9Ile	2
Wt_02639 (*molY*)	p.C3109T; p.Arg1037Cys	3/4
Wt_03821 (*rpoA*)	p.G835T; p.Gly279Cys	1/2

Correlation between irradiation adaptation and loss of *stx* genes. Sequencing data suggested that loss of the prophages could be a beneficial adaptation to survive irradiation stress. To investigate this hypothesis, *E. coli* O157:H7 strain Sakai (isolated from a human clinical case) and four other isolates of *E. coli* O157:H7 from animals or humans were also tested to validate the correlation between adaptation to a lethal dose of gamma irradiation and loss of *stx* genes. Confirmation of identification and serotype of the four isolates, including 2 nontoxinogenic O157:H7 isolates, was performed by the Quebec Public Health Laboratory using anti-O157 and H7 antisera. The strains were subjected to molecular typing by pulsed-field gel electrophoresis (PFGE) according to procedures approved by PulseNet International (26).

Isolates 2 and 3 are *stx* free and could survive immediately following irradiation treatment at 1.5 kGy. The other strains lost their *stx* genes after the second passage of irradiation (Table 2).

Lysogeny by prophage BP-933W and gain in cytotoxicity. To further confirm the hypothesis that a gain in resistance to irradiation was due to prophage excision, each of the three adapted clones and the *E. coli* K-12 strain MG1655 (as a control) were incubated with the culture supernatant of irradiated wild-type (WT) *E. coli* O157:H7 for 18 h at 37°C to obtain lysogens that had been reinfected with prophages (27). Lysogenic clones of C1, C2, and *E. coli* K-12 strain MG1655 (C1-Φ, C2-Φ, and K-12-Φ, respectively) were isolated and the presence of Shiga toxin genes stx_{1A}, stx_{1B}, stx_{2A}, and stx_{2B} was investigated by PCR. We were unable to obtain a lysogen of clone C3. PCR demonstrated that the lysogens had gained stx_2 genes. Genomic DNA sequencing revealed the absence of the phages in the C1-Φ and C2-Φ bacterial genomes. In contrast, BP-933W was integrated in *E. coli* K-12-Φ at the *wrbA*-specific CATCGTTTCAATATGTC site. This site has previously been recognized for the integration of bacteriophage 933W in *E. coli* K-12 (28, 29).

The production of Stx was evaluated by enzyme-linked immunosorbent assay (ELISA). As expected, C1 and C2 did not produce Stx. In contrast, these clones produced Stx after lysogenization by phage (C1-Φ and C2-Φ) (Fig. 3A). The cytotoxicity effect of clones (C1 and C2), and lysogens (C1-Φ and C2-Φ) versus WT *E. coli* O157:H7 was determined (Fig. 3B). According to previous research, STEC strains are cytotoxic for Vero cells (30). To evaluate this phenotype, Vero cells were exposed to supernatants of bacterial cultures as described in Materials and Methods. The lactate dehydrogenase (LDH) released from damaged Vero cells was measured as described in the CytoTox96 kit (Promega, USA). The amount of LDH released is proportional to the number of lysed Vero cells. Strains producing Stx toxin gave higher cytotoxicity levels after 12 h of incubation. However, cytotoxicity was completely absent when Vero cells were infected with clone C1 or C2. Interestingly, K-12-Φ was also

TABLE 2 Correlation between passage at the lethal dose of gamma irradiation and Stx carriage of *E. coli* O157:H7 strains

Isolates or strain (our lab)	Presence of *stx* before irradiation	Time before growth following irradiation (days)	Presence of *stx* after irradiation (after passage 2)
Isolate 1 (bovine)	stx_1 and stx_2	8	No
Isolate 2 (human case)	None	1	No
Isolate 3 (bovine)	None	1	No
Isolate 4 (swine)	stx_1 and stx_2	21	No
Sakai (human case)	stx_1 and stx_2	18	No

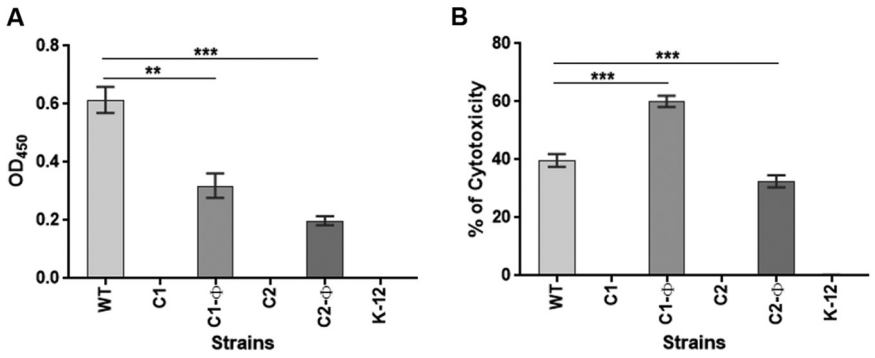

FIG 3 Cytotoxicity of *E. coli* O157:H7 clones carrying or not BP-933W prophages. (A) Shiga toxin (Stx) detection in supernatants of *E. coli* O157:H7, C1, C2, K-12 MG1655, C1-Φ, C2-Φ, and K-12-Φ by ELISA. Each strain was incubated in TSB at 37°C (O/N), diluted 1/10 in TSB supplemented with mitomycin C (50 ng/mL) and was then incubated at 37°C for 20 h. ELISA was performed in 24-well microplates using 1 mL of each supernatant, rabbit IgG, anti-Stx2 as detection antibody, and polyclonal anti-rabbit as secondary antibody coupled to horseradish peroxidase. Absorbance was read using a Biotek microplate reader at 450 nm and Gen 5 2.07 software. No Stx was detected in C1 or C2. *E. coli* K-12 was included as a negative control. (B) Cytotoxicity effect on Vero cells using LDH release detection test. Vero cells were plated into 96-well cell culture plates in Dulbecco's Modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and incubated at 37°C under a 5% CO_2 atmosphere. Cells were washed once with Hanks' balanced salt solution and bacteria were added to confluent monolayers at a multiplicity of infection of 100 for 2, 4, 9, and 16 h. The total amount of LDH released into the medium was determined using the CytoTox96 kit. The percent cytotoxicity was calculated using the following formula: percent cytotoxicity = 100 $\times$ experimental LDH release (OD_{490})/maximum LDH release (OD_{490}). The results represent the means of replicate experiments for a minimum of three samples. Differences between each strain versus WT were analyzed using the Student *t* test (**, $P < 0.01$; ***, $P < 0.001$ compared to WT levels).

able to produce Stx and had become cytotoxic. According to the sequence data, the prophage BP-933W was suspected to be present as an episome in *E. coli* C1-Φ and C2-Φ. To confirm this, extrachromosomal DNA from *E. coli* C1-Φ and C2-Φ was extracted and analyzed by pulsed-field gel electrophoresis (data not shown). A low concentration ($<$1 ng/μL) of an extrachromosomal element of 61.7 kb was extracted from the gel. PCR of the extracted band confirmed detection of stx_{2A} and stx_{2B} from the extracted extrachromosomal DNA. Cytotoxicity test results confirmed that the presence of Stx toxin could damage Vero cells, induce cell lysis, and release lactate dehydrogenase (31).

Resistance to a lethal dose of irradiation. A dose of 1.5 kGy of irradiation is known to be lethal for *E. coli* O157:H7 (32). At the same time clones C1, C2, and C3 adapted, and over time through six repeated passages and exposure to this irradiation dose, were able to become more resistant and grow following this high level of radiation stress (Fig. 1C and 4A). Interestingly, *E. coli* K-12 strain MG1655 was more resistant to irradiation than wild-type *E. coli* O157:H7 strain EDL933 and showed a survival rate of 4.0 $\log_{10}$ CFU/mL (Fig. 4C and 5). Compared to *E. coli* O157:H7 EDL933, which showed no growth at 1.5 kGy of irradiation, the C1 clone survived with viable counts decreasing to 2.86 $\log_{10}$ CFU/mL and was able to grow after an adaptive period of 8 h. In contrast, prophage reintroduction reduced the viability and growth of C1 (Fig. 4B and 5). Immediately following irradiation treatment, the lysogen C1-Φ only demonstrated survival of 0.5 $\log_{10}$ CFU/mL (Fig. 5). Similarly, prophage introduction increased sensitivity of *E. coli* K-12 to irradiation. Viable counts of K-12-Φ lysogen decreased to 0.98 $\log_{10}$ CFU/mL after exposure to 1.5 kGy (Fig. 4 and 5C).

Acid resistance. A hallmark of the pathogenicity of *E. coli* O157:H7 is its resistance to acid in the gastrointestinal tract (33). To verify if clones that lost the PV-933V and BP-933W prophages during adaptation to irradiation become more sensitive to acidic pH, we determined bacterial viability in tryptic soy broth (TSB) medium at pH 2. *E. coli* O157:H7 radioresistant clone C1 did not survive after 2 h in this acid pH environment, whereas the parental strain EDL933 was resistant to acid (pH 2), as expected. Interestingly, prophage reintroduction increased significantly the viability ($P \leq 0.005$) up to 4 h for the C1-Φ lysogen (Fig. 6). However, the viability of C1 after reintroduction

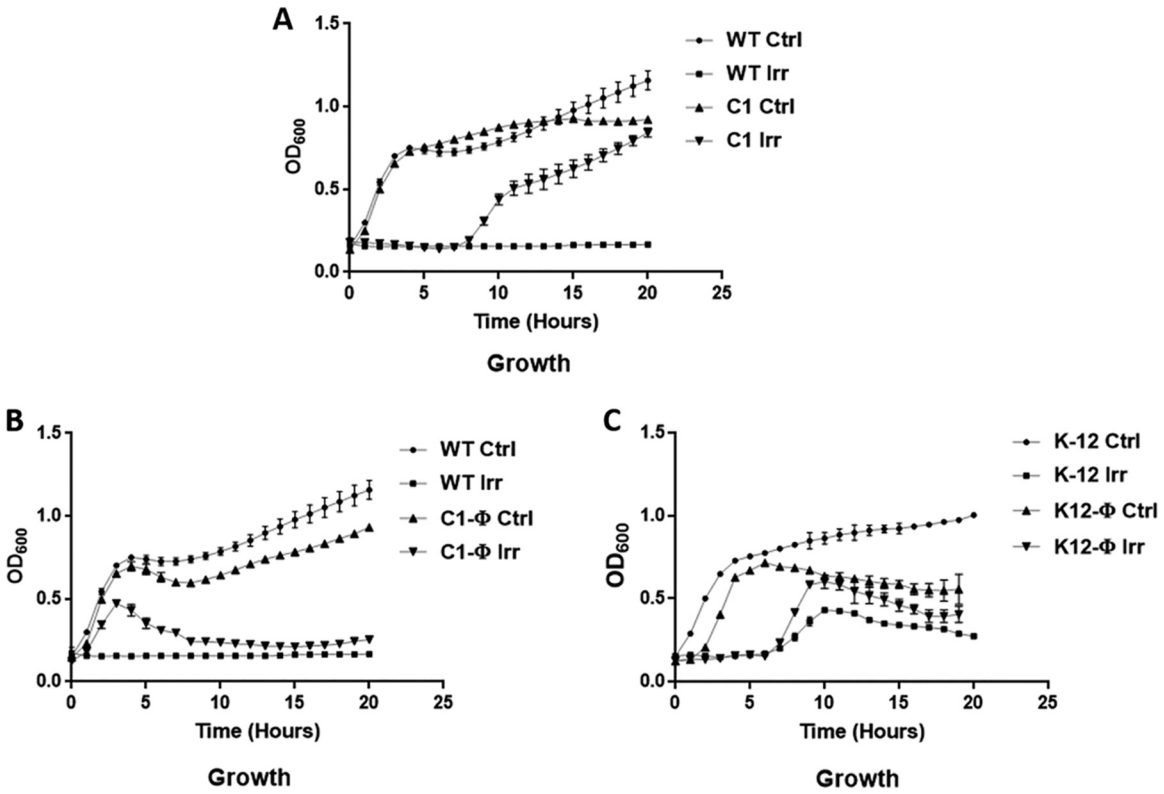

FIG 4 Growth of *E. coli* O157:H7 adapted to a lethal dose of irradiation. Growth of C1 (A), C1-Φ (B), and K-12-Φ (C) is shown. O/N bacterial culture was diluted 100 times, incubated at 37°C with shaking until reaching an OD_{600} of ≈1, and irradiated at 1.5 kGy. Volumes of 200 μL of cell culture diluted to an OD_{600} of 0.05 in TSB were distributed into wells of sterile microtiter plates and incubated at 37°C for 20 h without agitation. The OD_{600} growth measurements were performed by a Bioscreen C apparatus every hour after a mixing period of 30 s. The results represent the means of replicate experiments for a minimum of three samples. Vertical bars represent the standard errors of the means.

of prophages was still less than the viability of the EDL933 parent strain when exposed to an acidic (pH 2) environment. Introduction of phage BP-933W to *E. coli* K-12 also increased survival at pH 2, particularly after 3 to 4 h of treatment. These results suggest that bacteriophages and particularly BP-933W may contribute to increased acid resistance in *E. coli* O157:H7 EDL933 and *E. coli* K-12 strains.

Paraquat resistance. As with irradiation stress, oxidative stress produces reactive oxygen species (ROS), which can cause DNA damage and activate a bacterial stress response (34) and also induce activation of the bacteriophage lytic cycle (35). To determine if prophages contribute to aggravating an ROS response, we tested paraquat resistance of *E. coli* O157:H7, the clones that were adapted to irradiation, and clones wherein prophages were reintroduced. As shown in Fig. 7, parental strain EDL933 is sensitive to paraquat and its viability was reduced by 0.3 to 1.4 $\log_{10}$ CFU/mL. In contrast, radioresistant clones C1 and C2 were completely resistant to paraquat stress, indicating a correlation between oxidative and irradiation stress resistance. Further, reintroduction of prophage in these radiation-adapted clones (lysogenic strains C1-Φ and C2-Φ) rendered them significantly sensitive to paraquat ($P \leq 0.05$ with 0.2 μM and $P \leq 0.005$ with 0.3 and 0.5 μM) compared to the adapted clones C1 and C2. Viability of these bacteria was reduced by 1.4 to 1.8 $\log_{10}$ CFU/mL after exposure to paraquat. Introduction of prophage BP-933W to *E. coli* K-12 also resulted in an increased sensitivity to paraquat at 0.2 μM, wherein *E. coli* K-12-Φ had a 1-log-greater decrease in viability than parental strain *E. coli* K-12 strain MG1655. These results indicate that the presence of bacteriophage, such as Shiga toxin-encoding phages, in addition to rendering *E. coli* more sensitive to radiation stress, can also increase sensitivity to oxidative stress.

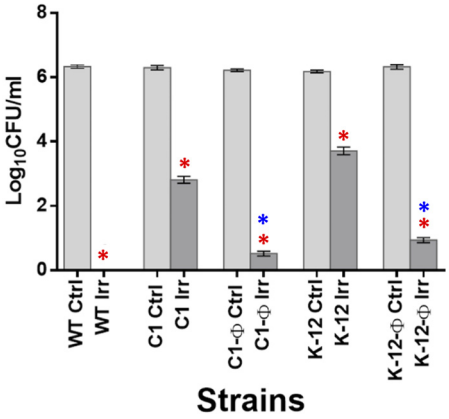

FIG 5 Viability of *E. coli* O157:H7 clones carrying or not BP-933W prophages following a lethal dose of gamma irradiation. Bacterial culture was incubated O/N at 37°C with shaking, diluted 100 times, incubated at 37°C with shaking until reaching an OD_{600} of ≈1, irradiated at 1.5 kGy, serially diluted, plated on TSA plates, and incubated O/N at 37°C. Wild-type *E. coli* O157:H7 EDL933 showed no growth at 1.5 kGy of irradiation compared to clone C1, which was able to survive and grow after an adaptive period of 8 h. However, the introduction of prophage reduced the viability and growth of C1-Φ. Similarly, prophage introduction increased sensitivity of *E. coli* K-12 to irradiation after exposure to 1.5 kGy. The results represent the means of replicate experiments for a minimum of three samples. Differences between the $\log_{10}$ CFU counts of Irr/Ctrl for each strain were analyzed using the Student *t* test (red asterisk, $P < 0.05$ for irradiated compared to control before irradiation; blue asterisk, $P < 0.05$ increased level of survival of strain C1 or K-12 without bacteriophage after irradiation compared to same strains with bacteriophage [Φ]).

DISCUSSION

Irradiation can effectively reduce or eliminate some bacterial pathogens from foods, although some bacteria demonstrate an adaptive response and resistance to radiation treatment. How EHEC can adapt to normally lethal doses of gamma irradiation is not well understood. In addition, one of the key virulence traits of EHEC O157:H7 is the production of Stx toxins (Stx1 and Stx2) encoded, respectively, by CP-933V and BP-933W temperate bacteriophages (7, 9, 36). Oxidative stress has also been shown to induce Shiga toxin-encoding lambdoid phages, and consequently, EHEC lysogens harboring

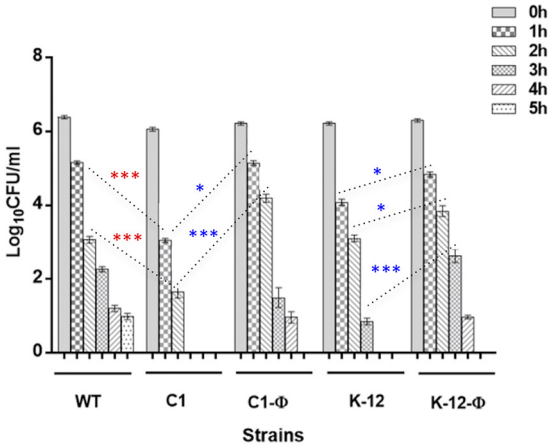

FIG 6 Viability of *E. coli* O157:H7 clones carrying or not BP-933W prophages and subjected to acid stress (pH 2) over time. O/N bacterial culture was diluted to an OD_{600} of ≈1 in TSB at pH 2 (HCl, 1 N) and incubated at 37°C under agitation for 5 h. Bacteria were serially diluted every hour, plated on TSA plates, and incubated O/N at 37°C. The results represent the means of replicate experiments for a minimum of three samples. The $\log_{10}$ CFU counts for each strain versus the WT were analyzed every hour using the Student *t* test. There is no significant difference between each strain (C1, C phage) and WT and between K-12 and K-12 phage at the beginning of the test. Red triple asterisks ($P < 0.001$) indicate significant decreases in survival of C1 compared to WT at same time post exposure. Blue single asterisks ($P < 0.05$) and triple asterisks ($P < 0.001$) indicate significant increases in survival of C1 and K-12 due to introduction of bacteriophage (Φ) compared to same strain without bacteriophage.

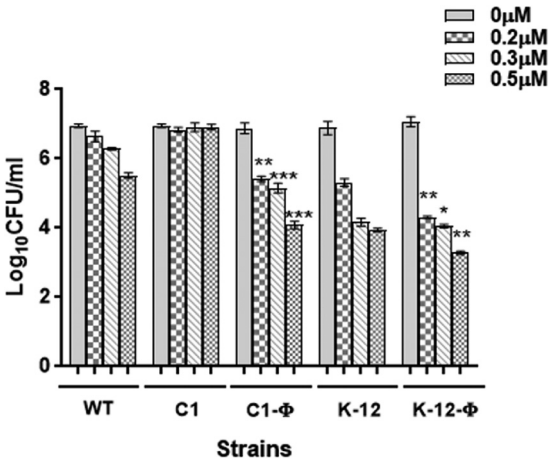

FIG 7 Viability of *E. coli* O157:H7 clones carrying or not BP-933W prophages under oxidative stress (paraquat). O/N bacterial culture was diluted to an OD_{600} of ≈1, incubated in TSB supplemented with paraquat at 0.2 μM, 0.3 μM, or 0.5 μM, incubated at 37°C under agitation for 40 min, serially diluted, plated on TSA plates, and incubated overnight (O/N) at 37°C. The $\log_{10}$ CFU counts for each strain versus WT were analyzed using the Student *t* test. The results represent the means of replicate experiments for a minimum of three samples. There is no significant difference between each strain (C1, C phage) and WT and between K-12 and K-12 phage at the beginning of the test. Single ($P < 0.05$), double ($P < 0.01$), and triple ($P < 0.001$) asterisks indicate significant decreases in survival of strains C1 or K-12 at specific paraquat concentrations following introduction of bacteriophage BP-933W (Φ) compared to the corresponding strain without bacteriophage.

these phages can be more sensitive to oxidative stress (35, 37). In this work, the potential for adaptation of O157:H7 strain EDL933 to an initially lethal dose of irradiation was investigated by repeatedly irradiating bacterial cells followed by a period of growth restoration over six cycles. It was found that selected radioresistant clones also had increased resistance to paraquat-generated oxidative stress and were more sensitive to acidity than parental strain EDL933. Loss of both prophages BP-933W and CP-933V (which also resulted in loss of the stx_1 and stx_2 genes) was responsible for the adaptation to a lethal dose of irradiation in these clones. This result is coherent with reports describing the loss of the CP-933V and BP-933W *E. coli* O157:H7 prophages from the genome following antibiotic stress from exposure to ciprofloxacin, fosfomycin, or mitomycin C, although this stress also promoted bacterial cell lysis (38).

There is increasing evidence that Stx prophage-encoded factors can modify bacterial gene expression and alter multiple phenotypes. Stx phage lysogeny has a direct effect on the global expression of bacterial genes; moreover, an increase in acid tolerance and motility has been reported when bacteria were lysogenized (39). Further, RNA sequencing (RNA-seq) studies have revealed a positive effect of phage ϕ24B carrying stx_{2A} on the gene expression of acid resistance in *E. coli* K-12 strain MC1061 mediated by the phage-encoded regulator CII. Moreover, CII also apparently represses expression of elements of the LEE-encoded type III secretion system, which is critical for EHEC virulence (40, 41).

Several studies have shown that phage integration or excision can inactivate or alter gene function through physical disruption of the genes or their promoter regions. Therefore, insertion and excision of prophages from bacterial genomes can alter bacterial physiology (42). CP-933V is integrated near the *mlrA* (*yehV*) chromosomal locus and contributes to reduced expression of curli fimbriae and biofilm formation (43). BP-933W is integrated at the *wrbA* site and alters responses to environmental stress (29, 44). Deletion of these prophages may therefore lead to changes in *mlrA* and *wrbA* expression and may restore their native function and regulation in *E. coli* (45).

In our study, CP-933V and BP-933W excision was an early adaptation to survive gamma irradiation stress, as it occurred after the second irradiation cycle. Under DNA damage stress, RecA cleaves the CI repressor promoting phage induction, which can lead to phage

release and bacterial cell lysis (19). Some bacteria may excise the prophages as an episome that may potentially reintegrate into the bacterial genome. For certain phages, the episome can also be lost without killing the bacteria (42). This is the case with phages CP-933V and BP-933W, which were excised and were eliminated from clones (C1 and C2) after 2 passages at 1.5 kGy. In our current study, it is likely that the increase in resistance of the adapted clones to gamma irradiation and oxidative stress was due to loss of the prophage genomes by excision from the bacterial chromosome.

Interestingly, in the process of adaptation of *E. coli* K-12 to a lethal dose of irradiation, among other genomic alterations, the *E. coli* K-12 prophage e14 was also deleted from the genome (46, 47). As with CP-933V and BP-933W, e14 is a lambdoid prophage that is inducible by mitomycin C treatment. The insertion site in the bacterial genome is the isocitrate dehydrogenase (*icd*) gene, which is involved in oxidative stress resistance (38, 47, 48). These data corroborate our results and indicate that increased resistance to irradiation in *E. coli* is linked to a loss of prophage elements from the genome.

In addition, the e14 prophage is excised in the response to SOS induction (49). Harris et al. (46) concluded that this phenomenon is due to mutations in the *recA* gene. In the present work, however, no mutations were identified in the *recA* gene in radiation-adapted clones C1, C2, and C3 (see Fig. S1 in the supplemental material).

It is possible that *E. coli* O157:H7 radiosensitivity may therefore in part be due to prophage induction, which could result in increased bacterial cell death and lysis. To test this hypothesis, CP-933V and BP-933W prophages were reintroduced into *E. coli* O157:H7 radioresistant clones C1 and C2 using the method of Hull et al. (50). It was shown that only BP-933W could be reintroduced into the bacterial clones (C1 and C2), but the phage did not reintegrate back into the bacterial genome. Moreover, to reintroduce prophages, clones and strains were incubated with the supernatant of irradiated WT *E. coli* O157:H7. It was, however, not determined whether this supernatant contained both CP-933V and BP-933W or only BP-933W phages.

It is important to highlight that, as observed in clones C1, C2 and C3, two passages at a 1.5-kGy lethal dose of irradiation also caused the loss of *stx* genes (stx_1 and stx_2) in other EHEC strains, including isolate 1, isolate 4, and the EHEC Sakai strain (Table 2). de Vargas and Landy (51) have shown that simultaneous excision of the lambdoid prophages inhibits reintegration of phages by disruption of the phage attachment site (*attP*). In this study, the bacterial *attB* site in the *wrbA* gene (29) was mutated (G25→A), suggesting that mutation of the bacterial attachment site may induce prophage excision and inhibit further integration at the same site. Also, other studies (52, 53) showed for other Stx phages an ability to integrate into multiple sites in a host bacterial strain that may be even sometimes resolved in polylysogeny and increased Stx toxin production. For example, for the Stx phage $\Phi 24_B$, which shares a considerable amount of genomic DNA sequence with Stx phage 933W, several alternate integration sites were identified when the *attB* site in the *wrbA* gene was altered or deleted. Most of these alternative sites are located in genes encoding proteins of unknown function. So, since *wrbA* was mutated following irradiation and passaging, this may explain why BP-933W could not integrate at the altered *wrbA* attachment site.

Notably, the clones which have been adapted to radiation treatment have lost the Stx-encoding genes and are now no longer cytotoxic to Vero epithelial cells. The cytotoxicity, however, is restored following reintroduction of BP-933W and expression of the Stx2 toxin. Although clone C1 is noncytotoxic, it is resistant to a lethal dose of irradiation. In contrast, the C1-Φ lysogen that regained BP-933W and stx_2 significantly lost radioresistance concomitant with a regain in the capacity to kill target host epithelial cells. Taken together, these findings indicate that while harboring the stx_2-bearing prophage can contribute to EHEC virulence/host cell toxicity, its carriage is detrimental for surviving radiation stress. Taken together, the results support the use of gamma radiation to effectively reduce and eliminate EHEC from food products but demonstrate a low but potential capacity for such strains to develop radioresistance. Importantly however, the potential adaptation of EHEC to high doses of radiation would result in elimination of the Stx-encoding phages and would likely

TABLE 3 Primers used in this study

Gene[a]	Primers[b]	Product size (bp)
rpoA	F, CAACCATTCTGGCTGAACAA; R, CAGAGACAGTCCACGGGAAG	270
moly	F, TTGGCTCATCTTGCGCATTG; R, TTGAAGCTGCCCTACGTACC	250
stx_{1A}	F (Z3345), GAAACATGGGTGCTCTGACA; R (stx_{1A}), GTAAATTCCTTCGCAACCA	300
stx_{1B}	F (stx_{1B}), CGCTTTGCTGATTTTTCACA; R (Z3342), ACTGCACCTTCTCCACCTGT	300
stx_{2A}	F (Z1460), GGTAAACGCCTTCACAAAGC; R (stx_{2A}), CGATACTCCGGAAGCACATT	300
stx_{2B}	F (stx_{2B}), TCCCGGGAGTTTACGATAGA; R (Z3342), TACACCGTCTGGTACGTGGA	300

[a]The *stx* genes were used for insertion site verification.
[b]F, forward; R, reverse.

lead to a decreased resistance to acid pH and greatly reduce the potential of such strains to cause disease.

Conclusion. In this work, *E. coli* O157:H7 adaptation to a typically lethal dose of gamma irradiation involved modifications in the genome, including deletion of CP-933V and BP-933W prophages encoding the Stx toxins. Further, loss of these prophages resulted in loss of cytotoxicity to epithelial cells and significantly decreased resistance to acidity (pH 2.0) while increasing resistance to oxidative stress. This specific adaptation to irradiation might be seen as an example of a biological trade-off where the bacterium cannot maintain virulence and resistance at the same time. Accordingly, reintegration of prophage BP-933W in radioresistant clones resulted in a regain in both cytotoxicity and acid resistance but a marked increase in radiosensitivity. Overall, the results demonstrate that although there is some potential for EHEC to develop radioresistance, the adaptation would likely engender a loss of key virulence traits such as Stx-mediated cytotoxicity and decreased resistance to acid pH, both traits that are paramount for the low infectious dose and cell and tissue damage that are hallmarks of EHEC pathogenesis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. In this study, *E. coli* O157:H7 EDL933 and Sakai, K-12 MG1655 strains (our laboratory stock), and four distinct isolates of *E. coli* O157:H7 (previously isolated from animals or humans and provided to us by the Quebec Public Health Laboratory) were used. All bacterial cultures were grown in tryptic soy broth (TSB; Difco, Becton, Dickinson, Sparks, MD, USA) with or without agitation (240 rpm) in a TC-7 roller drum (New Brunswick) or on tryptic soy agar (TSA) plates (Difco, Becton, Dickinson) at 37°C. A Bioscreen C apparatus (Growth Curves USA) was used for growth assays. Briefly, 200-μL volumes of bacterial cell cultures diluted to an optical density at 600 nm (OD_{600}) of 0.05 in TSB were distributed into wells of sterile microtiter plates and then incubated at 37°C for 20 h without agitation. The OD_{600} growth measurements were performed every hour after a mixing period of 30 s. For viability studies, bacterial cultures were serially diluted, plated on TSA plates, and incubated overnight (O/N) at 37°C. For Stx detection and cytotoxicity assays, 50 ng/mL of mitomycin C (Sigma-Aldrich, Canada) was added to bacterial cultures.

Adaptation of *E. coli* O157:H7 to a normally lethal dose of gamma irradiation. *E. coli* O157:H7 strain EDL933 was adapted to a normally lethal gamma irradiation dose by successive passages from the exponential phase of growth ($OD_{600} \approx 1$). After each passage, bacteria were incubated at 37°C with shaking until reaching an OD_{600} of 2. Bacterial cultures were transferred into microcentrifuge tubes and subjected consecutively to a radiation dose of 1.5 kGy (32). After the 5th passage, bacterial cells ($OD_{600} \approx 1$) were irradiated, plated on TSA plates, and then incubated overnight at 37°C. Ten colonies (clones) were cultivated O/N in TSB at 37°C, diluted 100 times (100 μL was added to 9,900 μL), and incubated at 37°C under agitation until reaching an OD_{600} of $\approx$1. A volume of 200 μL of each sample was then irradiated at 1.5 kGy, and the rest of the culture was stored at -80°C. Afterwards, viability and growth studies were performed. The irradiation treatments were performed at room temperature (20 $\pm$ 1°C) at the Canadian Irradiation Center as described previously (25).

Acid and oxidative stress resistance assays. *E. coli* O157:H7 wild-type strains, *E. coli* K-12 MG1655, and all clones with and without prophages were cultivated in TSB and incubated O/N at 37°C under agitation. For acid resistance assays, bacteria were diluted to an OD_{600} of $\approx$1 in TSB at pH 2 (HCl, 1 N) and incubated at 37°C under agitation for 5 h. Viability of bacteria was tested every hour as described for general culture conditions. For oxidative stress resistance, bacteria at an OD_{600} of $\approx$1 were incubated in TSB supplemented with paraquat at 0.2 μM, 0.3 μM, or 0.5 μM and incubated at 37°C under agitation for 40 min. Viability was then evaluated by plate count dilutions.

PCR. Overnight bacterial cultures were diluted at 1/1,000 and incubated in TSB at 37°C until the exponential phase of growth ($OD_{600} \approx 1$). DNA extraction was performed using a DNeasy blood and tissue kit (Qiagen, Montreal, Canada) as recommended by the supplier. Amplification of genes was carried out by PCR using the specific primers that were designed using the Primer3 plus program (Table 3). PCR amplification and sequencing of *rpoA*, *wrbA*, and *molY* from sequential clones were obtained after each passage. From each passage that was analyzed, four colonies from plates were randomly selected and

amplified by PCR for these genes. Amplified bands corresponding to each of these genes were gel purified and sent for DNA sequencing.

Bioinformatics analyses. The DNA of the EHEC EDL933 wild-type strain, each of the adapted clones, and *E. coli* K-12 and lysogens of clones were sequenced. Bacterial genomic DNA was isolated using the DNeasy blood and tissue kit (Qiagen, Montreal, Canada). The DNA libraries for sequencing were prepared at the Sequencing Platform of Genome Canada (McGill University, Canada) and sequenced at high throughput using an Illumina HiSeq apparatus. The whole-genome shotgun sequencing data set was deposited to the Sequence Read Archive of the National Center for Biotechnology Information under BioProject number PRJNA777472. The raw sequencing reads were processed using fastp version 0.19.5 (54) and ParDRe 2.2.5 (55). Read quality was assessed using FastQC version 0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The reads were *de novo* assembled using SPAdes version 3.13.0 (56) with k-mer lengths of 21, 33, 55, and 77. The resulting contigs were aligned on the reference sequence of *E. coli* EDL933 (57) using Mauve version 2015-02-13 (58) and were annotated using Prokka version 1.13.3 (59). The nucleotide differences and genome alterations among adapted clones and wild-type strains were identified using Snippy version 3.2 (https://github.com/tseemann/snippy). The macro differences were visualized by mapping the reads from the clones onto the sequence of the wild-type strain with the combined use of BWA version 0.7.17-r1188 (60), SAMtools version 1.9 (61), and shinyCircos (62).

Statistical analyses were done using Prism 5 (GraphPad Software, Inc.). A *P* value was considered to be statistically significant at ≤ 0.05 (for either analyses using the Student *t* test or Kruskal-Wallis rank sum test).

Cytotoxicity assays. Cytotoxicity assays were performed as described by Xiong et al. (30), with some modifications. Vero cells (ATCC CCL-81) were plated into 96-well cell culture plates (10^4 cells/well) in Dulbecco's Modified Eagle's medium (Wisent Bioproducts, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Wisent Bioproducts) and incubated at 37°C under a 5% CO_2 atmosphere. Before infection, the cells were washed once with Hanks' balanced salt solution (Wisent Bioproducts) and bacteria were added to confluent monolayers at a multiplicity of infection (MOI) of 100 for 2, 4, 9, and 16 h. The total amount of LDH released into the medium was determined using the CytoTox96 kit (Promega, USA) according to the manufacturer's instructions and measured using a Tecan plate reader (Tecan Group Ltd.) at 490 nm. The percent cytotoxicity was calculated using the following formula:

$$\text{Percent cytotoxicity} = 100 \times \text{experimental LDH release}\,(\text{OD}_{490})/\text{maximum LDH release}\,(\text{OD}_{490})$$

Prophage reintroduction. *E. coli* O157:H7 EDL933 was cultivated in TSB supplemented with 50 ng/mL of mitomycin C and incubated at 37°C for 18 h. Bacteria were centrifuged (5,000 $\times$ *g* for 10 min at 4°C) and the supernatant was filtered through syringe filters (0.45-μm pore size). Infection of bacteria with phages from the filtered supernatant was carried out as described by Lu and Breidt (27). A quantity of 200 μL of TSB and 5 μL of *E. coli* K-12 MG1655 or of EHEC bacteria adapted to a lethal dose of gamma irradiation (clones C1, C2, and C3) at the exponential phase of growth ($OD_{600} \approx 1$) and 45 μL of filtered supernatant were added and incubated in a 96-well microplate at 37°C for 20 h without agitation. To select lysogenic bacteria, 100 μL of cell culture from each well was centrifuged as described immediately above, and 10 μL of each supernatant was used in spot tests on a lawn of *E. coli* K-12 MG1655 and by PCR for detection of stx_{1A}, stx_{1B}, stx_{2A}, and stx_{2B} using specific primers (Table 3).

PFGE of phage DNA. Phage DNA was extracted from O/N cultures of *E. coli* K-12 MG1655-Φ using the ZymoPURE Miniprep kit (Cedarlane, Burlington, ON, Canada). Pulsed-field gel electrophoresis (PFGE) was performed with a CHEF Mapper XA from Bio-Rad with the following parameters: separation from 5 kb to 500 kb, calibration factor of 1.0, buffer of 0.5× Tris-borate-EDTA (TBE), temperature of 14°C, 1.0% pulsed field certified (PFC) agarose, gradient of 6.0 V/cm, run time of 15 h 16 min, included angle of 120°, initial switch time of 0.22 s, final switch time of 8.53 s, and linear ramping factor.

Spot test. A 5-mL volume of targeted bacteria at an OD_{600} of 0.15 to 0.2 was applied to TSA plates containing 0.5% agar for 30 s, and the culture was then discarded. A quantity of 10 μL of tested samples was spotted onto the completely dried agar as a bacterial overlay. The plates were left to dry and were inspected for lysis zones after an O/N incubation at 37°C.

Detection of Stx. Stx toxins were detected in supernatants of *E. coli* O157:H7, C1, C2, K-12 MG1655, and lysogenic strains (C1-Φ, C2-Φ, and K-12-Φ) by ELISA. The test was performed as follows. Briefly, strains were incubated in TSB at 37°C. After O/N incubation, bacterial cultures were diluted 1/10 in TSB supplemented with mitomycin C (50 ng/mL) and were incubated at 37°C for 20 h. The supernatants were filtered as described above. The ELISA was performed in 24-well microplates using 1 mL of each supernatant, rabbit IgG (Sigma-Aldrich, ON, Canada), anti-Stx2 as detection antibody, and polyclonal anti-rabbit (Thermo Fisher Scientific, Burlington, ON, Canada) as secondary antibody coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Absorbance was read using a Biotek microplate reader at 450 nm and Gen 5 2.07 software.

Data availability. The whole-genome shotgun sequencing data set from different strains/clones was deposited to the Sequence Read Archive of the National Center for Biotechnology Information under BioProject number PRJNA777472.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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