The CI Repressors of Shiga Toxin-Converting Prophages Are Involved in Coinfection of *Escherichia coli* Strains, Which Causes a Down Regulation in the Production of Shiga Toxin 2

R. Serra- Moreno, J. Jofre, and M. Muniesa*

Department of Microbiology, Faculty of Biology, University of Barcelona, Diagonal 645, E-08028 Barcelona, Spain

Received 14 January 2008/Accepted 28 April 2008

Shiga toxins (Stx) are the main virulence factors associated with a form of *Escherichia coli* known as Shiga toxin-producing *E. coli* (STEC). They are encoded in temperate lambdoid phages located on the chromosome of STEC. STEC strains can carry more than one prophage. Consequently, toxin and phage production might be influenced by the presence of more than one Stx prophage on the bacterial chromosome. To examine the effect of the number of prophages on Stx production, we produced *E. coli* K-12 strains carrying either one Stx2 prophage or two different Stx2 prophages. We used recombinant phages in which an antibiotic resistance gene (*aph, cat, or tet*) was incorporated in the middle of the Shiga toxin operon. Shiga toxin was quantified by immunoassay and by cytotoxicity assay on Vero cells (50% cytotoxic dose). When two prophages were inserted in the host chromosome, Shiga toxin production and the rate of lytic cycle activation fell. The CI repressor seems to be involved in incorporation of the second prophage. Incorporation and establishment of the lysogenic state of the two prophages, which lowers toxin production, could be regulated by the CI repressors of both prophages operating in trans. Although the sequences of the CI genes of the phages studied differed, the CI protein conformation was conserved. Results indicate that the presence of more than one prophage in the host chromosome could be regarded as a mechanism to allow genetic retention in the cell, by reducing the activation of lytic cycle and hence the pathogenicity of the strains.

Shiga toxin-producing *Escherichia coli* (STEC) strains are considered food- and waterborne pathogens, although person-to-person transmission has also been reported (25, 35). STEC strains cause bloody diarrhea and hemolytic-uremic syndrome and can lead to severe complications (35, 37, 49). One of the best known serotypes is O157:H7, which is the most common virulent serogroup in the United States and Canada, while other serotypes are frequently reported in European outbreaks (4, 49). STEC strains are normally found in cattle, goat, and sheep (5), which act as a reservoir, releasing STEC in their feces.

Shiga toxins (Stx) are the main virulence factors in STEC. They are highly related to the toxin produced by *Shigella dysenteriae* serotype I (36). Currently, two Stx have been described: Stx1 and Stx2, along with their variants (22). Some of these subtypes are found only in certain reservoirs (14). Generally, Shiga toxins are characterized by their hexameric conformation. They are formed by five B subunits, which allow toxin attachment to their enterocyte receptor; Gb3, which is also present in endothelial cells of glomerular capillaries (21); and one A subunit, which is catalytically active and blocks translation of mRNA to protein, leading to cell death (14).

Shiga toxins are encoded in the genomes of temperate phages (19). They are considered members of the lambdoid family, since they share a common genome arrangement that conserves the relative positions of the genes with similar activities and associated regulatory signals (7). Although Shiga toxin-encoding bacteriophages are a heterogeneous group in terms of morphology and their genetic organization (24, 33, 46, 54), the location of stx genes is conserved, being found next to the lytic genes and downstream of the Q antiterminator (36). Therefore, Shiga toxin production is basically linked to the induction or progression of the phage lytic cycle after activation of the SOS response in the bacterial host. The toxin is released through cell lysis (55). Following the lytic burst, Stx phages act as vectors in the stx horizontal transmission to other members of the family *Enterobacteriaceae* (1, 2, 19, 33, 44, 46, 51).

Previous studies with STEC O157:H7 strains isolated from a single outbreak showed that isolates harboring two different Stx prophages produced less toxin than strains from the same clone carrying only one Stx prophage (34). In accordance with other authors (20), we hypothesized that the expression of phage genes may be regulated when other temperate phages are present. We examined in an *E. coli* K-12 background the effect on phage and toxin production when the same host strain was converted by one or two Stx prophages. We also identified the genes implicated in the acquisition of a second prophage.

**MATERIALS AND METHODS**

**Bacterial strains, bacteriophages, and growing conditions.** The bacteriophages, strains, and plasmids used in this study are described in Table 1. Phages *ΦA9, ΦA75, ΦA312, ΦA534, ΦA549, Φ557, and ΦVTB55* were obtained from STEC isolated from cattle. They were characterized in previous studies (2, 33, 44, 45, 46). Characterization included analysis of morphology and host infectivity. Moreover, genetic characterization comprised the determination of their genome size, restriction fragment length polymorphism and location of *stx*, in the restricted phage DNA, sequences of the *stx* and integrase genes, and insertion sites used to integrate within the chromosomes of different host strains. These phages were used to convert laboratory *E. coli* strains generating lysogens carrying one Shiga toxin-encoding prophage (Stx prophage). This set of phages and phage 933W were modified by the incorporation of an antibiotic resistance gene.
### TABLE 1. Bacterial strains, bacteriophages, and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strain, lysogen, phage, or plasmid</th>
<th>Characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Shigella sonnet</em> 866</td>
<td>Used as host strain</td>
<td>33</td>
</tr>
<tr>
<td><em>E. coli</em> C600</td>
<td>Used as host strain</td>
<td>57</td>
</tr>
<tr>
<td><em>E. coli</em> LMG194</td>
<td>Used for evaluation of CI expression</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td>Used for evaluation of CI expression</td>
<td>Invitrogen</td>
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<tr>
<td><strong>Lysogens</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>E. coli</em> MC1061(Φ24_{th})</td>
<td>Lysogen of phage Φ24_{th}</td>
<td>2</td>
</tr>
<tr>
<td><em>E. coli</em> C600(Φ3538)</td>
<td>Lysogen of phage Φ3538</td>
<td>44</td>
</tr>
<tr>
<td><em>E. coli</em> C600(933W)</td>
<td>Lysogen of phage 933W</td>
<td>36</td>
</tr>
<tr>
<td>LysΦA9, LysΦA9Tc, LysΦA9Cm, LysΦA75, LysΦA75Tc, LysΦA75Cm, LysΦA312, LysΦA312Tc, LysΦA312Cm, LysΦA534, LysΦA534Tc, LysΦA534Cm, LysΦA549, LysΦA549Tc, LysΦA549Cm, LysΦA557, LysΦA557Tc, LysΦA557Cm, LysΦVMB55, LysΦVMB55Tc, and LysΦVMB55Cm</td>
<td>Lysogen 933Wαcro Lysogen 933WΔcΔcro Lysogen C600(933W)</td>
<td>This study This study</td>
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<tr>
<td><strong>Phages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΦA9, ΦA75, ΦA312, ΦA534, ΦA549, ΦA557, and ΦVMB55</td>
<td>Stx phages previously induced from STEC strains and serotypes O157:H7 and O2:H27 isolated from cattle</td>
<td>33</td>
</tr>
<tr>
<td>ΦA9Tc, ΦA9Cm, ΦA75Tc, ΦA75Cm, ΦA312Tc, ΦA312Cm, ΦA534Tc, ΦA534Cm, ΦA549Tc, ΦA549Cm, ΦA557Tc, ΦA557Cm, ΦVMB55Tc, and ΦVMB55Cm</td>
<td>Stx phage derivatives containing a fragment of the stx₂ operon replaced by an antibiotic resistance gene (tet or cat)</td>
<td>46</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pGEM-T Easy</td>
<td>Plasmid used for complementation experiments</td>
<td>Promega</td>
</tr>
<tr>
<td>pGcl1</td>
<td>pGEM-T Easy harboring a 879-bp band containing the cI gene</td>
<td>This study</td>
</tr>
<tr>
<td>pGcl1-cro</td>
<td>pGEM-T Easy harboring a 1,017-bp band containing the fragment between cI and cro genes</td>
<td>This study</td>
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<td>pGcro</td>
<td>pGEM-T Easy harboring a 287-bp band containing the cro gene</td>
<td>This study</td>
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<td>pKD46</td>
<td>Plasmid with the Red recombinase system</td>
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<tr>
<td>pACYC184</td>
<td>Plasmid carrying the tet gene for tetracycline resistance</td>
<td>40</td>
</tr>
<tr>
<td>pKD3</td>
<td>Plasmid carrying the cat gene for chloramphenicol resistance</td>
<td>10</td>
</tr>
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<td>pBAD-TOPO vector</td>
<td>pBAD-TOPO vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pBAD:2x1</td>
<td>pBAD-TOPO vector carrying a 711-bp fragment (cI gene) downstream of the pBAD promoter</td>
<td>This study</td>
</tr>
</tbody>
</table>

**PCR techniques.** PCRs were performed with a GeneAmp PCR system 2400 (Applied Biosystems, Barcelona, Spain). Purified DNA (0.5 ng) was used for PCR amplification. The oligonucleotides used in this study are described in Table 2.

**Reverse transcription-PCR (RT-PCR).** Total RNA was isolated using the Qiagen RNeasy RNA isolation kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. The digoxigenin-labeled probes were prepared as described above.

**Hybridization techniques.** Colony and plaque blot analyses were performed with Nylon-N⁺ membranes (Hybond N⁺; Amersham Pharmacia Biotech, Barcelona, Spain) (42). Colony hybridization was performed as previously described (17). Stringent hybridization was achieved with the DIG DNA detection kit (Roche Diagnostics, Barcelona, Spain) according to the manufacturer’s instructions. The digoxigenin-labeled probes were prepared as described above.

*(cat and/or tet)* in the middle of the stx₂ genes (Fig. 1) using the Red recombinase system as described previously (45).

**Bacterial strains** (Table 1) were grown in Luria-Bertani (LB) broth and on LB agar. The LB medium was supplemented with kanamycin (50 μg ml⁻¹), chloramphenicol (30 and 5 μg ml⁻¹), and tetracycline (3 μg ml⁻¹) when needed.

**Preparation of digoxigenin-labeled gene probes.** DNA fragments of the stx₂, cI (tetracycline resistance), cI (chloramphenicol resistance), and aph (kanamycin resistance) genes were produced by amplification with the respective primers (Table 2), labeled with digoxigenin, and used as probes as previously described (33). The aph, tet, and cat probes hybridize only with the recombinant phages, while the stx₂ probe detects only the Stx2 phages, since this fragment is replaced by the antibiotic resistance gene in the recombinant phages.
FIG. 1. Schematic map of the generation of the recombinant phages (cat or tet) harboring the antibiotic resistance genes in the stx gene, indicating the precise positions where the cassettes were incorporated, the sizes of fragments replaced by the antibiotic cassettes, and the primers used to generate the fragments.
were differences in the incorporation of a second prophage in the mutant strain harboring the pBAD::cI construct at two levels of CI expression. The recombinant phages were used for infection of the mutant strain harboring pBAD::cI as described above. Strains C600(933W) and C600 containing pBAD::cI or pBAD without cI were included as controls.

Incorporation of a second prophage in lysogens carrying a Stx prophage. To obtain E. coli strains carrying two Stx2 phages, E. coli K-12 lysogens of each Stx phage were used (33, 46). Phage suspensions of the recombinant Stx2 phages (including ΦS538 and Φ2Δ40) containing from 10^3 to 10^6 PFU ml⁻¹ were used to infect a culture of the respective lysogen (10^8 CFU ml⁻¹) to the exponential growth phase (3 to 6 h). The mixture was then incubated and plated onto LB agar with the appropriate antibiotic to produce plaques and to convert the lysogens carrying one Stx prophage were evaluated. The plaque assay was performed as described previously (34) using Shigella sonnei 866 as the host, and plaques were hybridized with the appropriate probe to check that the lytic effect was caused by the Stx2 phage or the recombinant phage.

**Evaluation of toxin production.** The differences in toxin production between the E. coli strains harboring one Stx phage and those harboring two prophages were determined for this purpose. Bacteria were grown from single colonies in LB at 37°C to the exponential growth phase (3 to 6 h), and the cultures were analyzed with Vero cell assay. The enzyme immunoassay (Premier EHEC; Meridian Diagnostics Inc., Cincinnati, OH) was used to determine the concentration of Stx2 produced by the lysogens. Dilution of each sample was tested as described by the manufacturer, and results were compared to a standard curve constructed with purified Stx2 (Toxin Technology, Inc., Sarasota, FL). Results obtained were analyzed spectrophotometrically at two wavelengths (450 and 630 nm) and processed as indicated by the manufacturer. Cytotoxicity assays were done on Vero cells (30). Serial dilutions of the supernatants of the lysogens, obtained as described above, were incubated with 4 × 10^4
10^4 Vero cells ml^-1 at 37°C in a 5% CO_2 atmosphere. Purified Stx2 from Toxin Technology, Inc. (Sarasota, FL) served as a positive control. Cells were incubated for 72 h and evaluated every 12 h. The amount of toxin contained in the last 10-fold dilution of the sample in which 50% of the Vero cells detached from the plastic, as assessed by direct microscope observation and confirmed by A_620, was considered to be the 50% cytotoxic dose (CD_50) (56).

To discard the possibility that the diminished Stx expression observed in lysogens harboring truncated phages or both phages was a transport problem from the cell, some experiments were performed with sonicated cultures. For this purpose, the cultures were sonicated at 50% power for a total of 3 min in 15-s bursts with a model 300 sonic dismembrator (Fisher Bioblock Scientific, Strasbourg, France). Sonic lysates were clarified by centrifugation at 8,000 g for 10 min, filter sterilized as described above, and used for evaluation of cytotoxicity onto Vero cells.

E. coli O157:H7 strain ATCC 43889 and E. coli C600 carrying a 933W prophage were used as Stx-producing controls. E. coli O157:H7 strain ATCC 43888, E. coli K-12 strain C600, LB broth with mitomycin C (0.5 μg · ml^-1), and phosphate-buffered saline were used as negative controls.

RESULTS

Generation of E. coli strains harboring two Shiga toxin-encoding prophages. A group of previously characterized Stx phages was used to produce laboratory lysogens (Table 1). To generate lysogens carrying two Stx prophages, a second recombinant Stx prophage was used to infect these laboratory lysogens harboring truncated phages or both phages was a transport problem from the cell, some experiments were performed with sonicated cultures. For this purpose, the cultures were sonicated at 50% power for a total of 3 min in 15-s bursts with a model 300 sonic dismembrator (Fisher Bioblock Scientific, Strasbourg, France). Sonic lysates were clarified by centrifugation at 8,000 x g for 10 min, filter sterilized as described above, and used for evaluation of cytotoxicity onto Vero cells.

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FIG. 2. Generation of Lys933WΔcro::cat and Lys933WΔΔcro::tet strains. Schematic map of the E. coli 933W cro-cI locus indicating the positions of the promoters and the cro and cI genes. (A) Schematic diagram of plasmids pGcro, pGclcro, pGcl and pBAD::cI indicating the orientation of the genes and promoters. (B) Sizes of the genes and substitution by the antibiotic cassettes to construct the mutants Lys933Δcro and Lys933ΔΔcro.

After the infection of a lysogen carrying one Stx prophage by a second incoming phage (Δstx::cat or Δstx::tet), the most common event was the incorporation of the second phage in a secondary insertion site of the host chromosome (46) (Table 3). Incorporation of a second prophage generated more lysogens (from 0.5 to 1.5 logarithmic units more) than the control
significant (replicate experiments, in all of which the differences were prophage). This observation was confirmed in the independent case (the host strain lysogenized only by the recombinant E.

show the average of the most common effect. Those cases where more lysogens harboring two phages (Stx and incoming phage) were generated than lysogens in K-12 (harboring only the incoming phage) were generated are shown in bold type. PS, phage substitution, the incoming phage replaced the Stx prophage present according to their inability to infect other lysogens in a lytic or them (Fig. 3). The same results were observed for that only some of the phages were able to generate plaques on occur.

occurred frequently. In this study, this phenomenon has been conditionally prophage, we also observed that insertion of the second separate viral particles, which converted new host strains in-
tegrated in tandem. The two prophages occupied different insertion sites in the host chromosome, and both produced integrated prophages cannot produce Stx, and consequently, no toxin was produced by the wild-type prophage, since the recombinant prophages cannot produce Stx, and consequently, no toxin was detected. The control assays performed confirmed that the commercial kit used did not detect the product of the truncated stx harbored by the recombinant phages. To discard the possibility that the diminished expression observed was a transport problem from the cell, we used sonicated cultures of Lys933W, lysogens carrying the 10 recombi-
nated phages, and the set of double lysogens containing both 933W and a recombinant phage. The supernatant of sonicated cultures showed the same results observed for the nonsoni-
cated cultures showed the same results observed for the nonsoni-
cated cultures (data not shown).

Besides the reduction in toxin production, strains with two phages also produced fewer plaque particles (Fig. 4 and Table 4). This was observed on the basis of the optical density measurements of the cultures and by plaque counts. Plaques enumerated by plaque blot hybridization using stx2 and the cat-, tet-, or aph-specific probes suggested that approximately 70% of the plaques corresponded to the Stx2 phage and the rest of the plaques corresponded to the recombinant phage (for example, see Fig. 5 with LysA549/3538).

The order of infection of the two phages does not seem to be relevant, since for example, similar results were obtained with strain Lys(24B/933W) and Lys(933W/24B). The results obtained in cytotoxicity (CD50, 5 × 10^3) and phage induction (3 × 10^10 PFU ml^-1) were comparable with results obtained with Lys933W/24B presented in Table 4 and Fig. 4.

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Some experiments performed with infection of two phages harboring antibiotic cassettes (cat or tet) were conducted to evaluate whether the number of phages produced upon induction vary if the order of conversion varied as well. These assays allowed only evaluation of phage production, without consid-

### TABLE 3. Comparison of the number of lysogens generated when the incoming phage infects a lysogen (Lys) already harboring one Stx prophage versus the number of lysogens generated when the same incoming phage infects E. coli K-12 (phage-free).

<table>
<thead>
<tr>
<th>Incoming phage</th>
<th>No. of replicate expts a</th>
<th>LysA9 vs K-12</th>
<th>LysA75 vs K-12</th>
<th>Lys312 vs K-12</th>
<th>Lys534 vs K-12</th>
<th>Lys549 vs K-12</th>
<th>Lys557 vs K-12</th>
<th>LysVTB55 vs K-12</th>
<th>LysVTB55 vs K-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>3538</td>
<td>6</td>
<td>7.50 vs 7.38</td>
<td>5.40 vs 5.38</td>
<td>4.20 vs 4.18</td>
<td>3.00 vs 2.92</td>
<td>1.80 vs 1.72</td>
<td>0.60 vs 0.58</td>
<td>2.40 vs 2.32</td>
<td>1.20 vs 1.12</td>
</tr>
<tr>
<td>933W</td>
<td>6</td>
<td>8.34 vs 7.73</td>
<td>6.88 vs 6.35</td>
<td>5.21 vs 4.72</td>
<td>3.60 vs 3.12</td>
<td>2.00 vs 1.52</td>
<td>0.80 vs 0.72</td>
<td>1.60 vs 1.12</td>
<td>0.40 vs 0.32</td>
</tr>
<tr>
<td>24B</td>
<td>5</td>
<td>8.90 vs 8.40</td>
<td>6.40 vs 6.30</td>
<td>4.80 vs 4.30</td>
<td>3.20 vs 2.80</td>
<td>1.60 vs 1.20</td>
<td>0.80 vs 0.70</td>
<td>1.40 vs 1.12</td>
<td>0.40 vs 0.32</td>
</tr>
</tbody>
</table>

a Number of independent replicate experiments.

b Those cases showing no consistent results in the replicate experiments are shown in italic type (in some cases, infection by the recombinant phage failed); results show the average of the most common effect. Those cases where more lysogens harboring two phages (Stx and incoming phage) were generated than lysogens in E. coli K-12 (harboring only the incoming phage) were generated are shown in bold type. PS, phage substitution, the incoming phage replaced the Stx prophage present in the Lys strain. NI, no infection (lysogens harboring two phages were not generated after several attempts).

The immunity relationship among the 10 phages showed that only some of the phages were able to generate plaques on single-lysogen lawns, while others were only able to convert them (Fig. 3). The same results were observed for cat or tet recombinant phages. The group of phages studied appeared mostly heteroimmune, although some phages can be grouped according to their inability to infect other lysogens in a lytic or lysogenic way. Group 1 consisted of φA75, φA312, and φVTB55. Group 2 was made up of φA312, φA549, and φVTB55. Group 3 contained φA549, φA557, and φVTB55, and group 4 consisted of φA534 and φVTB55. Group 5 consisted of phage φVTB55, which can be considered a new group because it belongs to the first four groups (Fig. 3).

Determination of Shiga toxin production from lysogens harboring one or two Stx2 prophages. Shiga toxin generation was evaluated after the induction of the lytic cycle of the lysogens. Figure 4 shows representative cases in which the toxin produced from a strain carrying one Stx2 prophage is compared with the toxin produced from strains harboring two prophages. Lysogens with two prophages produced less toxin than those carrying only one prophage. The cytotoxicity assays done with the toxin produced by a lysogen carrying only one prophage showed stronger cytotoxicity than those performed with the toxin produced by the respective strains with two prophages (Table 4). Toxicity was normally observed after 24 to 48 h of incubation.

In these experiments, we detected only the Shiga toxin produced by the wild-type prophage, since the recombinant prophages cannot produce Stx, and consequently, no toxin was detected. The control assays performed confirmed that the commercial kit used did not detect the product of the truncated stx harbored by the recombinant phages.

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eration to Stx, which can be produced only from a wild-type Stx2 prophage.

Lys933W was compared to Lys933Tc; after induction, they produced 9 PFU ml\(^{-1}\) versus 14 PFU ml\(^{-1}\). Lys3538/933Tc versus Lys933Tc/3538 produced 4 \times 10^5 versus 2.6 \times 10^5 PFU ml\(^{-1}\), respectively, and Lys549Cm/549Tc versus Lys549Tc/549Cm produced 1.4 \times 10^5 versus 2.3 \times 10^5 PFU ml\(^{-1}\), respectively (results are representative of three independent replicate experiments). Although some variations can be observed, the results do not show consistent differences that can lead to strong conclusions. The main differences are observed in comparisons of the double lysogens with the single lysogens, with less phage produced by the double lysogens than by the single lysogens, as previously reported (Fig. 4 and Table 4).

**Implied role of cI and cro in the incorporation of a second prophage.** The lysogen carrying the 933W Stx2 prophage was selected for these studies because it was the only lysogen that allowed lysogenic infection with all the other phages and no cases of phage substitution or noninfection were observed (Table 3). A cro mutant (Lys933W\(_{\Delta cro}\):cat) was obtained by the incorporation of a cat gene using the Red recombinase system (Fig. 2) and used as host for infection with the \(\Delta cro\) phages.

After several attempts, a stable mutant with only the cI gene knocked out was not obtained, probably because the lack of cI regulator implies a constitutive activation of the lytic cycle and a loss of 933W prophage (39). Nevertheless, a stable mutant lacking the fragment between the cI and cro genes was successfully generated (Lys933W\(_{\Delta cI}\):cat) and used as host for infection with the \(\Delta cro\) phages.

The lack of expression of cI and/or cro in the mutant strains was confirmed by RNA extraction of the strains, followed by RT-PCR. In contrast, positive RT-PCR amplification was achieved with RNA from the strains harboring pGcI, pGcIcro, and pGcro, confirming that all constructs expressed cI (see Fig. 7A) or cro. Strains C600 and C600(pGEM) were used as negative controls, while C600(933W) was the positive control.

There was no significant difference between the Lys933W \(\Delta cro\) mutant and the wild-type C600(933W) in the number of colonies incorporating a second prophage (Fig. 6A). The C600 control did not show differences either. Complementation with pGEMcro did not affect the generation of double lysogens.

**Implied role of cI and cro in the incorporation of a second prophage.**

<table>
<thead>
<tr>
<th>Recombinant phages</th>
<th>Lys933A9</th>
<th>Lys933A75</th>
<th>Lys933A312</th>
<th>Lys933A534</th>
<th>Lys933A549</th>
<th>Lys933A557</th>
<th>LysVTB55</th>
<th>Lys933W</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Phi 9)</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>+/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>+/-</td>
</tr>
<tr>
<td>(\Phi 75)</td>
<td>+/-</td>
<td>-/-</td>
<td>-/-</td>
<td>+/-</td>
<td>-/-</td>
<td>-/-</td>
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<td>+/-</td>
</tr>
<tr>
<td>(\Phi 312)</td>
<td>+/-</td>
<td>-/-</td>
<td>-/-</td>
<td>+/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>+/-</td>
</tr>
<tr>
<td>(\Phi 534)</td>
<td>+/-</td>
<td>-/+</td>
<td>-/-</td>
<td>+/-</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
</tr>
<tr>
<td>(\Phi 549)</td>
<td>+/-</td>
<td>-/+</td>
<td>-/-</td>
<td>+/-</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
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<tr>
<td>(\Phi VTB55)</td>
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<tr>
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**Table 1.** Evaluation of the sensitivity of lytic versus lysogenic infection (lytic/lysogenic) of each recombinant phage on each E. coli lysogen. Results of lytic infection were obtained by spot test and hybridization with the specific probe. Results of lysogenic infection were obtained by colony blotting and confirmed by PCR. The sensitivity of lytic versus lysogenic infection (lytic/lysogenic) are shown as follows: for lytic infection, - , no lysis; + , lysis; for lysogenic infection, - , no lysogenic conversion in the lysogen or no substitution; + , lysogenic conversion with the recombinant phage. Those cases in which lytic infection was achieved but lysogenic infection was not or vice versa are indicated by gray shading. The different immunity groups (groups 1 to 5) established are shown at the bottom of the figure.

FIG. 3. Evaluation of the sensitivity of lytic versus lysogenic infection (lytic/lysogenic) of each recombinant phage on each E. coli lysogen. Results of lytic infection were obtained by spot test and hybridization with the specific probe. Results of lysogenic infection were obtained by colony blotting and confirmed by PCR. The sensitivity of lytic versus lysogenic infection (lytic/lysogenic) are shown as follows: for lytic infection, - , no lysis; + , lysis; for lysogenic infection, - , no lysogenic conversion in the lysogen or no substitution; + , lysogenic conversion with the recombinant phage. Those cases in which lytic infection was achieved but lysogenic infection was not or vice versa are indicated by gray shading. The different immunity groups (groups 1 to 5) established are shown at the bottom of the figure.
that spontaneous phage induction was not the cause of the low number of double lysogens generated with the mutant.

When the mutated strain was complemented with pGcI, the difference with the wild type was removed (Fig. 6C). Negative controls with empty vectors (pGEM) were examined and did not show relevant differences compared with the noncomplemented mutant. Strain C600 alone or complemented with pGcI was also used as a control for a prophage-free strain, and although not significant, a small increase in the number of colonies which incorporated a new prophage was observed in those carrying pGcI (data not shown).

The pBAD::cI construct was used to evaluate the influence of CI concentration in the generation of lysogens with two phages. RT-PCR confirmed cI expression in the strains harboring this construct (Fig. 7A).

### Table 4. Cytotoxic effect on Vero cells and number of phage

<table>
<thead>
<tr>
<th>Lysogen</th>
<th>Cytotoxic effect (CD50/ml)</th>
<th>Phage count (PFU/ml)</th>
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<tbody>
<tr>
<td>Lys(933W)</td>
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<td>4.9 × 10^6</td>
</tr>
<tr>
<td>Lys(933W/VTB55)</td>
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</tr>
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<td>Lys(933W/924A)</td>
<td>5 × 10^4</td>
<td>6.6 × 10^4</td>
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<td>5 × 10^2</td>
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</tr>
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</tr>
<tr>
<td>Lys(FA549/FA538)</td>
<td>10 × 10^2</td>
<td>4.1 × 10^4</td>
</tr>
</tbody>
</table>

a The lysogens harboring only one phage are shown in bold type.

b Cytotoxic effect on Vero cells (CD50) of the supernatants of the induced cultures of lysogens carrying one or two prophages. Values of CD50 were measured on Vero cells and correspond to the dilution of the supernatant of the induced cultures that produces a 50% reduction of the cells.

c Number of phage present in the supernatants of the induced lysogens detected on S. sonnei strain 866.

**FIG. 4.** Comparison of toxin and phage production between lysogens carrying one Stx prophage and lysogens carrying two prophages (Stx2 phage and a recombinant phage). Phages are shown below the x axis without the initial F symbol. Toxin production was evaluated in the supernatant of the cultures after mitomycin C induction. The concentration was calculated with a standard curve performed with purified Stx2. Values on the z axis correspond to the phage counts on S. sonnei strain 866 in the supernatants of the cultures after mitomycin C induction. The results are the averages plus standard deviations (error bars) from three independent trials.

**TABLE 4. Cytotoxic effect on Vero cells and number of phage**

![FIG. 5. Plaque assay of phages induced from strain LysA549/3538 carrying two phages. Most of the plaques generated from lysogenic strains harboring two prophages correspond to the Stx2 phage. Both membranes were transferred from the same plate in the same experiment; one was hybridized with the stxA2 probe, while the other was hybridized with the cat probe to reveal the differences in plaque formation of each phage present in the E. coli strain harboring both phages.](http://jb.asm.org/)
level of expression of $cI$ at a final arabinose concentration of 0.2% in Lys933WΔ$cro$ (Fig. 7B). Similar results were observed for control strains LMG194 and C600 (data not shown). Complementation of the mutant strain with pBAD::$cI$ when the highest $cI$ expression was activated (0.2% arabinose) led to the generation of more lysogens carrying the second phage than with lower $cI$ concentrations (0.002% arabinose) (Fig. 7C). Strain C600 with the pBAD::$cI$ construct induced with 0.2% arabinose showed a higher rate of incorporation of the prophage than the naive strain did, although the difference between both sets of experiments was not as relevant as observed with the mutant (Fig. 7D). These results indicate that $cI$ seems to be involved in the incorporation of the second prophage. However, some other factors present in the 933WΔ$cro$ prophage should hinder the incorporation of the second prophage in the mutant strain, since incorporation of a single prophage in strain C600 with or without pBAD::$cI$ can be easily achieved.

Negative controls with empty vectors (pBAD) were used and did not show relevant differences compared with the non-complemented mutant.

Assuming that the $cI$ product plays a role in the integration of a second prophage in the 933W lysogen chromosome, 933W $cI$ protein could interact with the second prophage, producing a more stable lysogenic state and causing a reduction in toxin production at the same time. We evaluated the differences in the sequences of the $cI$ genes of the 10 phages studied, and the amino acid sequences of the proteins encoded by the genes.
were compared. The CI genes of all phages, except for phage λ/H9021A9 and λ/H90213538, were sequenced successfully. For the phages studied, there were differences in the amino acid sequences of the CI proteins (Fig. 8). To evaluate the relevance of the differences in the protein structure, the CI protein conformation was analyzed to examine whether CI genes of heteroimmune phages could work in trans. All CI proteins analyzed shared two common domains (Fig. 8). The first domain corresponds to a helix-turn-helix domain in the NH2-terminal half of CI that mediates its DNA binding. The second domain corresponds to a RecA-mediated peptidase that enhances CI self-cleavage after RecA binding, breaking the CI homodimer, so that the repressor can no longer form dimers and bind DNA.

**DISCUSSION**

Shiga toxins are major virulence factors in STEC. They are encoded in the genomes of temperate lambdoid phages (19), and Stx expression is normally linked to induction of the lytic cycle (43), which allows the release of toxin. A large proportion of the STEC strains isolated from the environment usually carry more than one Stx prophage (2, 3, 17, 24, 34, 50, 54). The presence of more than one stx copy in the host chromosome can alter the expression and release of Shiga toxin. It has been reported that isolates of STEC with two copies of an stx gene produced significantly more Stx in vitro than did strains of the same serotype containing a single copy of the same stx gene (3, 11). In contrast, some evidence of Stx competition has been identified in wild-type pathogenic *E. coli* O157:H7 (9), and it has also been suggested that the presence of two phages can reduce the Stx produced and that the loss of one of the prophages consequentially produced a significant increase in Stx production (34). In order to study this effect, we generated *E. coli* K-12 strains carrying either one or two Stx2 prophages. We used Shiga toxin-encoding bacteriophages that appeared to be heterogeneous in their host infectivity, phage genome size, insertion site occupancy when converting different host strains, stx2 gene sequence, restriction fragment length polymorphism, and integrase sequence (13, 33, 46). In the present study, we also examined their immunity profiles: most of them appeared heteroimmune, although four immunity groups can be established.

Our finding that incorporation of a second prophage generated more lysogens could be explained as follows: transduction generates lysogens harboring two phages. Once produced, these lysogens show lower phage induction rates than lysogens carrying only one phage, which reduces the number of lysed colonies, thus allowing the growth of these lysogens once generated. The relatively easy generation of strains harboring two prophages could explain the abundance of STEC strains carrying more than one Stx prophage. Similar observations have
already been made in other studies, where lysogens seemed to
be good hosts for new prophages (13, 15, 23, 31).

Heteroimmune curing or substitution of one phage by the
incoming one was also a frequent event observed in our studies
and can be explained by natural immunity between the two
phages. Superinfection of a lysogen by a phage with a different
immunity but the same recombinase frequently leads to the
loss of the original prophage (heteroimmune curing) (48). No
infection by the second prophage or more colonies harboring
only one prophage were observed in only a few cases.

In those cases in which we obtained lysogens carrying two
Stx2 prophages, toxin production decreased as a result of lower
phage production after the lytic cycle was induced. This obser-
vation suggests gene regulation between both phages and con-
firms the direct link between phage induction and toxin pro-
duction (43). In consequence, the lysogenic state seems to be
stabilized by the presence of a second prophage. This finding is
consistent with our observations of certain lysogens carrying
one Stx prophage (for example, LysA75), in which sponta-
neous induction (28) of the lytic cycle was observed. In these
lysogens, spontaneous induction was not longer detected when
a second prophage was incorporated (data not shown).

Moreover, our results are consistent with previous studies in
which wild O157:H7 STEC strains isolated from the same
outbreak could harbor either one or two functional Stx2
phages (34). In that study, toxin production was also evaluated,
indicating that less toxin was generated when two prophages
coeexisted in the same cell. This indicates that toxin production
depends on the lytic cycle induction rate (which is linked to
phage production and toxin release) rather than on the num-
ber of functional stx2 genes present in the bacterial chromo-
some. The finding that our E. coli lysogens carrying two pro-
phages produce fewer infectious viral particles indicates that
these strains are less susceptible to the SOS response to acti-
vate their lytic cycle, thus producing less toxin. Despite the fact
that our results are in accordance with previous observations,
it should be noted that the experiments presented here have
been performed with phages isolated mostly from animal
strains, and thus, our results might differ from experiments
with human isolates (3, 11). In addition, our data were ob-
tained in an E. coli K-12 background under experimental con-
ditions that differ from the physiological situation (16); there-
fore, they cannot be necessarily transferred into an in vivo
situation during natural human STEC infection.

Experiments conducted to determine which genes could be
involved in the acquisition of a second prophage in a 933W
lysoegen that lowers toxin production indicate that the cI re-
pressor of the 933W phage is involved. The mutant strain
constructed lacks the whole fragment between the cI and cro
genes, including promoters pRM and pR. Deletion of the
whole fragment including the promoters avoids interference
with the incoming phage, although the mutated prophage is

FIG. 8. (A) Alignment of the CI protein sequences of the phages studied, 933W (933) and
ΦA75 (A75). The proteins were aligned using CLUSTALW (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html). Amino acids that were identical in the two
phages are indicated by asterisks and gray shading. Symbols in the sequence: *, no amino acids in this fragment in one of the sequences; ., the
amino acids of both sequences are noncoincident. Symbols below the sequence: :, amino acids of both chains are strongly similar; .., amino acids
of both chains are weakly similar. (B) Domain map of CI. The protein domains are indicated. The white boxes represent the helix-turn-helix XRE
region for DNA binding. The gray boxes represent the RecA-mediated peptidase domains which correspond to the self-cleavage of CI mediated
by RecA activity. The predicted model for each domain is indicated below the boxes.

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not inducible anymore. With the exception of N, all phage-encoded functions required for lytic growth ultimately derive from transcription initiation at pR (52, 53). By deleting pR, we blocked the expression of cII, but this was not relevant, since CII directly affects cI, which was truncated. CII can also act on int, but since the phage was already integrated, this should not be a problem, and in addition we avoid excision, a process in which int is also involved together with cis. The lack of excision was experimentally confirmed. The pRE promoter, however, involved in the lytic-lysogenic pathway decision at early stages remained intact. Results of complementation with pGcI suggested that even cI or pR should be involved in generation of double lysogens. Results of complementation with pBAD::cI clearly suggested that cI plays a role in the generation of double lysogeny and suggested that different concentrations of the repressor influence the incorporation of the incoming phage differently. Our results indicate, however, that some other genes present in the mutated prophage must also be involved in blocking infection with the second incoming phage, since the mutant strain (harboring the mutated prophage) generates a lower number of double lysogens, while a high number of lysogens are produced when the incoming phage infects a naïve C600 strain (prophage-free) or C600(pBAD::cI). Nevertheless, the unknown gene must somehow be linked to cI, which is involved in a direct or indirect way in the incorporation of the incoming phage in the nonmutated strains.

We hypothesize that the lytic repressor of different phages can act in trans and regulate the lysogenic cycle of the two phages present in the same genome. For those cases in which they appeared heteroimmune and the cI sequences were unrelated, we observed conserved CI protein domains that might enhance binding to the operator regions of the second prophage, stabilizing the lysogenic state and reducing toxin production. This conclusion cannot be extrapolated to phages ΦA9 and Φ3538, since successive attempts to identify and sequence their cI gene were unsuccessful. For those phages sharing identical cI and operator regions, such as phage 933W or phage Φ24H (13, 38), or for phages of this study which shared the same regulator sequences, the explanation is not so clear. The possible presence of an antirepressor has been suggested as a mechanism to allow superinfection of phage Φ24H (13) by cleavage of the CI protein. This would reduce the concentration of CI, avoiding immunity due to repression by CI protein. However, the ant gene was not identified in our phages (data not shown) or in 933W, although the presence of another gene encoding a protein with the same antirepressor role cannot be excluded. In experiments with the lys933W mutant, our results suggest that since the CI protein of the 933W prophage cannot be produced, the CI concentration of the second phage alone is not sufficient to enhance its incorporation. The amounts of CI or CI homologues caused by the synergistic presence of two cI genes or their reduction by the action of a possible antirepressor could explain the variations observed within our group of prophages. Our experiments also suggest differences in the incorporation of the second phage depending on the CI concentration, but more accurate experiments would be necessary to identify the threshold concentration of CI and/or CI homologues necessary to decide whether the incoming phage will integrate or whether superinfection will occur. These observations are in agreement with other authors who anticipate that 933W prophages would direct the synthesis of a tightly regulated amount of repressor (26). They are also consistent with other authors who suggest that repressor differences may be responsible for the variations in toxin and phage production observed in different lysogens obtained with the same host strain (55). Wagner et al. (55) also suggest that with more than one prophage repression system present in a host strain, the control of Stx2 production may move further away from the lytic switch of the prophage and more toward a matrix of interaction between phage repressors and host factors, as confirmed in our work. Moreover, since several truncated prophages without the stx gene have been identified in wild-type pathogenic E. coli O157:H7 (29), the repressor genes present in these prophages could theoretically influence the expression of other phages (including other Stx prophages) present in the same chromosome, controlling their induction and expression of the genes encoded by these phages, including the stx gene among other genes.

The acquisition of more genetic information by the incorporation of a prophage can provide the cell with new functions. For instance, pathogenic genes, which under certain conditions could be beneficial to bacteria, are also a survival strategy for phages (6, 8, 18). Generally, without any selection, temperate phages are spontaneously induced and subsequently lost. This explains why most STEC strains do not possess two phages in their genome. However, in some cases, the presence of more than one prophage facilitates their maintenance in the bacterial chromosome. The explanation of evolutionary advantage suggested for a single phage can also be applied to a second phage. The contribution of phages (together with plasmids, transposons, or pathogenicity islands) to the evolution of bacteria is widely recognized, as they behave as vectors for gene acquisition (8, 12, 18, 47, 55). In this context, phages contribute to short-term bacterial diversification (8). Nevertheless, bacteria need to acquire and fix these genes to achieve long-term genetic diversification (8, 27). The reduction of lytic induction observed in our lysogens carrying two prophages could be considered a first step toward the stabilization of genetic information in the host strains. This could be regarded a prerequisite to fix the new genes. The reduction of lytic induction implies a reduction in toxin production, which produces a less virulent strain. This seems to be a logical strategy to colonize a new environment effectively. A fraction of the population would be able to activate its lytic cycle, causing the destruction of this fraction, but allowing the whole population to be virulent. Another fraction would keep its lysogenic state stable, which reduces the pathogenicity of this fraction, but ensures the maintenance of the population.

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