A novel strategy to isolate cell-envelope mutants resistant to phage infection: bacteriophage mEp213 requires lipopolysaccharides in addition to FhuA to enter Escherichia coli K-12

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We have developed a direct and efficient strategy, based on a three-step method, to select bacterial cell-envelope mutants resistant to bacteriophage infection. Escherichia coli K-12 strain W3110 underwent classical transposon mutagenesis followed by replica plating and selection for mutants resistant to infection by coliphage mEp213. To verify that phage resistance was due to mutations in the cell envelope, we transformed host cells with the viral genome using electroporation and selected those in which virions were subsequently detected in the supernatant. Among the nine mutants resistant to coliphage infection that we selected, six were in the fhuA gene, two were mutated in the waaC gene, and one was mutated in the gmhD gene. The latter two gene products are involved in the synthesis of lipopolysaccharide (LPS). The efficiency of plating and adsorption of phage mEp213 was affected in these mutants. We verified that LPS is required for the efficient infection of phage λ as well. We propose that this mutation-and-selection strategy can be used to find host factors involved in the initial steps of phage infection for any cognate pair of phage and bacteria.

INTRODUCTION

Bacteriophages that infect Gram-negative bacteria have to overcome the host cell envelope, which comprises three physical barriers, in order to reach the cytoplasm. These are: (1) the outer membrane (OM), with a negative polarity and characterized in the outer leaflet by lipopolysaccharide (LPS) and in the inner leaflet by a phospholipid layer; (2) the cell wall, formed by a mesh of glycopeptides and nucleases embedded in the periplasm; and (3) the hydrophobic inner membrane (IM), composed of a bilayer of phospholipids (Dreiseikelmann, 1994).

The initial steps of phage infection occur in two stages. First is the adsorption of the phage to the bacterial surface, a reversible process in which the phage spikes sense the cell surface, locating the outer membrane receptor (OMR) (Molineux, 2006). The second stage involves the irreversible attachment between the phage receptor-binding protein (RBP) and the host OMR, which ultimately triggers the unidirectional translocation of the viral genome from the capsid into the bacterial cytoplasm (Letellier et al., 2007).

Various cell surface components have been adopted by different phages as the OMR to which they bind in order to initiate infection. For example, the binding of the maltose receptor LamB of Escherichia coli K-12 by phage λ (Hazelbauer, 1975); the osmoporin OmpC of E. coli B by phage T4 (Yu & Mizushima, 1982; Goldberg et al., 1994; Rossmann et al., 2004); the ferrichrome-Fe^{3+} receptor FhuA by coliphages T1, T5, φ80, HK022, mEp167, mEp213 and other mEp phages (Killmann et al., 1995; Böhm et al., 2001; Uc-Mass et al., 2004; Hernández-Sánchez et al., 2008; Braun, 2009); the LPS core of Klebsiella spp. by phage CSF-10 (Campbrubi et al., 1991); the inner core (IC) region of LPS by coliphage T7 (Molineux, 2001; Chang et al., 2010); and the antigenic O chain of LPS of Yersinia enterocolitica serotype O3 by phage φYeO3-12 (Pajunen et al., 2000). It has also been reported that several phages, including T5,
K20, O2x and T2, require a secondary receptor, such as LPS, to efficiently invade the host (Heller & Braun, 1979; Sukupolvi, 1984; Lenski, 1984; Silverman & Benson, 1987). The molecular mechanism of the infective process through the cell envelope for phages T4, T5, T7, fd and λ has been described in great detail (Glaser-Wuttke et al., 1989; Guihard et al., 1992; Goldberg et al., 1994; Molineux, 2001). Although several OMRs are well characterized, there are no direct or efficient strategies to select cell-envelope mutants resistant to phage infection.

Currently, host mutants resistant to phage infection are obtained by using physical, chemical or genetic agents, and selection is by replica plating or secondary culture (Guglielmotti et al., 2006). Strategies for selecting mutants at the level of the cell envelope are scarce. These latter methods include adsorption tests (Stoddard et al., 2007), analysis of bacterial surface components (Nesper et al., 2000), immunoselection by cell sorting using mouse antiphage antibodies (Viscardi et al., 2000), and PCR to detect known viral genome sequences in the bacterial cytoplasm (Chen et al., 2009). The first method has limited utility and the others are both laborious and time-consuming.

In this study we describe a simple and efficient strategy, based on the combination of three methods that allowed us to differentiate the host factors involved in the initial process of infection from those associated with phage development in the cytoplasm. The strategy was tested using E. coli K-12 strain W3110 as host and the lambda-like phage mEp213 (Kameyama et al., 1999). All of the mutants resistant to phage infection that we selected were related to the cell envelope. We confirmed that FhuA is the OM receptor, and we additionally found that the waaC and gmhD (earlier named rfaC and rfaD; Heinrichs et al., 1998) gene products are involved in phage infection.

### METHODS

**Bacteria, bacteriophages and media.** Relevant characteristics of bacterial strains, bacteriophages and plasmids are shown in Table 1. Lambda-like phage mEp213 and its derivatives were grown in E. coli strain W3110. The E. coli strain LE392 was used to grow phage λNK1316 containing the miniTn10KmR transposon. E. coli DH5x was used in the transformation of the plasmid constructions derived from pPROEXd, and E. coli C600 (fruA-) was used as a negative control for mEp213 phage infection. Luria–Bertani (LB), tryptone broth (TB) and SM phage-dilution media were prepared as described by Silhavy et al. (1984). The competent cells were grown in SOB medium (Hanahan et al., 1991). All media were supplemented with 100 µg ampicillin ml⁻¹ (Amp) (Bristol-Myers Squibb), 50 µg kanamycin ml⁻¹ (Km) (Roche Diagnostics), 30 µg chloramphenicol ml⁻¹ (Cm)

### Table 1. E. coli strains, bacteriophages and plasmids

<table>
<thead>
<tr>
<th>Strain, bacteriophage or plasmid</th>
<th>Genotype or relevant markers</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W3110</td>
<td>F⁻ λ⁻ rph⁻</td>
<td>Bachmann (1972), Jensen (1993)</td>
</tr>
<tr>
<td>C600</td>
<td>leuB6 thi-1 lacY1 supE44 thr-1 rfd1 fruaA21</td>
<td>Appleyard (1954)</td>
</tr>
<tr>
<td>LE392</td>
<td>F⁻ e14 ΔhsdR514 r' m' supE44 supF8 lacY1</td>
<td>Silhavy et al. (1984)</td>
</tr>
<tr>
<td>DH5x</td>
<td>endA1 hsdR17(r⁻ m⁺) supE44 thi-1 recA1 gyrA relA1 Δ(lacZ YA-argF)</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>W3110 with mEp213 prophage</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>PRM4</td>
<td>W3110 waaC265::miniTn10KmR</td>
<td>This study</td>
</tr>
<tr>
<td>PRM12</td>
<td>W3110 waaC212::miniTn10KmR</td>
<td>This study</td>
</tr>
<tr>
<td>PRM11</td>
<td>W3110 gmhD2::miniTn10KmR</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Bacteriophages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>λ</td>
<td>Wild-type</td>
<td>CSH Collection†</td>
</tr>
<tr>
<td>λNK1316</td>
<td>λ (miniTn10::Km R Δas57 Pam90 nin5 b522 att⁻)</td>
<td>Kleckner et al. (1991)</td>
</tr>
<tr>
<td>mEp213</td>
<td>Lambdoid phageinmem ix</td>
<td>Kameyama et al. (1999)</td>
</tr>
<tr>
<td>mEp167</td>
<td>Lambdoid phagentmem iv</td>
<td>Kameyama et al. (1999)</td>
</tr>
<tr>
<td>T5</td>
<td></td>
<td>Killmann et al. (1995)</td>
</tr>
<tr>
<td>T7</td>
<td></td>
<td>CSH Collection</td>
</tr>
<tr>
<td>mEp213_C</td>
<td>Lytic phage</td>
<td>This study</td>
</tr>
<tr>
<td>mEp213_V</td>
<td>Virulent phage</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td>cat fruaA⁺</td>
<td>Uc-Mass et al. (2004)</td>
</tr>
<tr>
<td>pPROEXd</td>
<td>bla lacO⁺ PTrc Δ(80 bp)</td>
<td>Uc-Mass et al. (2004)</td>
</tr>
<tr>
<td>pWaaC</td>
<td>bla waaC⁺</td>
<td>This study</td>
</tr>
<tr>
<td>pGmA_D</td>
<td>bla gmhD⁺</td>
<td>This study</td>
</tr>
</tbody>
</table>

CSH: Cold Spring Harbor.
Random transposon mutagenesis. Phage \( \tau \)NK1316 (miniTn10::Km\(^R\) drs57 \( \Delta \)aro8 nin5 b222 att\(^+\)), neither replicates nor integrates into \( E. \) coli W3110 due to a mutation in gene \( P \) involved in replication, and by deletion of the site-specific recombinase site (att\(^+\)) (Kleckner et al., 1991). This strain was used to generate host mutants resistant to Km. Phages were adsorbed with \( E. \) coli W3110 for 10 min with an m.o.i. of 1:1. The remaining phages suspended in supernatant were removed by centrifugation at 13,500 g for 10 min. Infected cells were grown in LB broth at 37\(^\circ\)C for 1 h, then 100 \( \mu \)l of the culture was poured onto LB agar with Km and the plates were incubated overnight at 37\(^\circ\)C.

Construction of phage mEp213\_V and selection of host mutants resistant to mEp213. For construction of phage mEp213\_V, the temperate phage mEp213 was mutagenized with N-methyl-N-nitro-N-nitrosoguanidine (MNNG) following the protocol described by Dhillon & Dhillon (1974), generating the lytic phage mEp213\_C. A second round of mutagenesis with MNNG was performed with mEp213\_C under the same conditions, and the clear plaques were selected in the lysogenic W3110 (mEp213) strain, generating the virulent phage mEp213\_V.

Host mutants resistant to phage mEp213 infection were selected using the classic replica plating technique first described by Lederberg & Lederberg (1952), with some modifications. In this assay, host mutants resistant to Km were replicated on LB supplemented with Km on one plate, and LB with Km spread with the virulent phage (mEp213\_V) at 10\(^8\) p.f.u. per plate on the other plate. The host mutants that were able to grow on both plates were potential phage-resistant mutants.

Sensitivity to phage infection. The sensitivity to phage infection was evaluated using a double-layer assay. First, each mutant was grown overnight in LB broth supplemented with Km at 37\(^\circ\)C; then, 0.5 ml of the culture was mixed with 3 ml of TB soft agar (at 40\(^\circ\)C) and was poured onto LB agar plates with Km. When the mixture solidified, serial dilutions of bacteriophage mEp213 were dispensed as drops onto the plates. The plates were incubated overnight at 37\(^\circ\)C to quantify the p.f.u.

Bacteriophage propagation, DNA extraction, and electroporation. Phage propagation was according to Martinez-Perianael et al. (2012), and DNA extraction was as reported in Sambrook et al. (1989). The DNA pellet was resuspended in 50 \( \mu \)l Tris-EDTA buffer.

DNA concentration was calculated by spectrophotometric measurement of absorbance at 260 nm, and the purity index was determined by the 260/280 nm absorbance ratio. Phage DNA integrity was evaluated by electrophoresis in 1\% agarose gels stained with 0.5 mg/ml ethidium bromide ml\(^{-1}\) (Sambrook & Russell, 2001; Sambrook et al., 1989). Competent cells were prepared as described in Silhavy et al. (1984). The electroporation assay was performed by mixing 1 \( \mu \)g phage DNA with \( \sim \)10\(^5\) competent bacterial cells, and was carried out using the Cell-Porator Pulse Control and Power Supply, Series 1600 (Gibco Life Technologies) with the following settings: capacitance, 25 \( \mu \)F; voltage, 2.5 kV; low resistance. The transformed cells were immediately cultured in 3 ml LB broth for 4 h at 37\(^\circ\)C. Then, 300 \( \mu \)l chloroform was added. The culture was mixed and centrifuged at 13,000 g for 5 min at room temperature, and serial dilutions of the supernatant were analysed by the double-layer assay.

Sequencing of the chromosome region adjacent to the transposon. To identify the chromosomal sequences adjacent to the transposon insertion site, we used the Y-linker method and linkers 1 and 2 described by Kwon & Ricke (2000). DNA extraction was performed using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich). Two micrograms of genomic DNA were digested with 20 U restriction enzyme NdeIII, at 37\(^\circ\)C for 3 h. Linker 2 was phosphorylated using T4 polynucleotide kinase and mixed with linker 1 to form the Y-linker. The digested chromosomal DNA was ligated with the Y-linker, using T4 DNA ligase at 24\(^\circ\)C for 16 h. AmpliTaq DNA polymerase, the adaptor primer (5\'-CTG CTC GAA TTC AAG CTT CT-3\') and the specific primer designed for the Km resistance cassette of the transposon (5\'-TTC ATT TGA TGC ATG AG-3\') were used to amplify the transposon-flanking sequence.

Results were performed using a Flexigen PCR system (Flexigen Techne) under the following conditions: a denaturing step at 94\(^\circ\)C/2 min, followed by 30 amplification cycles of 94\(^\circ\)C/30 s, 56\(^\circ\)C/60 s, 72\(^\circ\)C/60 s for each cycle, and a final extension step of 72\(^\circ\)C/2 min. The amplified fragments were separated by electrophoresis in a 2\% agarose gel, and then purified using the MinElute PCR Purification kit (Qiagen), according to the manufacturer’s specifications. The fragments were amplified and sequenced with an ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems), using the adaptor primer and the specific primer (5\'-TGA CAA GAT GTG TAT CCA CCT TAA C-3'), designed for the insertion sequence (IS) region of miniTn10Km\(^R\), in a Perkin Elmer ABI PRISM 310 automatic sequencer (Applied Biosystems). The reaction conditions were: a denaturing step of 94\(^\circ\)C/2 min, followed by 30 cycles of amplification of 96\(^\circ\)C/30 s, 53\(^\circ\)C/20 s, 60\(^\circ\)C/2 min for each cycle, and a final extension step of 60\(^\circ\)C/2 min. Sequencing was performed at the facilities of the Genetics and Molecular Biology Department (CINVESTAV-IPN, Mexico). The obtained gene sequences were aligned against the \( E. \) coli W3110 genome reference sequence (GenBank accession no. AP009048.1, GI: 85674274), using the BLAST program and the GenBank database at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Plasmid construction and complementation test. Wild-type \( waaC \) and \( gmhD \) genes were amplified from the \( E. \) coli W3110 genome and directly cloned into the expression vector pPROExd (Polayes & Hughes, 1994; Uc-Mass et al., 2004). EcoRI and HindIII enzyme sites were included in forward (Fwd) and reverse (Rev) primers, respectively. For \( waaC \), the Fwd primer was \( waaC\)-EcoFwd (5\'-GGA ATT CAA GAG GAA GGC TGA CGG ATG-3\') and the Rev was \( waaC\)-HindRev (5\'-CCC AAG CTT CAT GCA GAG TTC TTA-3\'). For \( gmhD \), the Fwd primer was \( gmhD\)-EcoFwd (5\'-GGA ATT CAG GAA AGG TTA CAG TG-3\') and the Rev was \( gmhD\)-HindRev (5\'-CCC AAG CTT CAT GCA GAG TTC TTA-3\'). The PCR conditions were similar for both genes: a denaturing step at 94\(^\circ\)C/2 min, followed by 30 amplification cycles of 94\(^\circ\)C/30 s, 55\(^\circ\)C/60 s and 72\(^\circ\)C/60 s, and a final extension step of 70\(^\circ\)C/2 min. The amplified fragments of the \( waaC \) and \( gmhD \) genes were purified using a QiAquick Gel Extraction kit (Qiagen), and were restricted with 20 \( U \) of EcoRI and HindIII enzymes at 37\(^\circ\)C for 3 h. Each amplicon was cloned into the pPROExd vector, restricted with the same enzymes. The plasmids generated were \( pWaaC \) and \( pGmhD \). These were transformed into their respective resistant mutants for the complementation assay. Chemically competent cells were prepared according to Hanahan (1983).

Adsorption assay. The adsorption assay was performed according to Garvey et al. (1996), with some modifications. Adsorption was determined by mixing 0.1 ml of bacterial (10\(^8\) c.f.u.) culture and 0.01 ml of phage (10\(^8\) p.f.u.). The phage/host mixture was incubated for 5, 10 and 15 min at room temperature. The mixture was centrifuged at 3000 g for 3 min. The supernatant was analysed for phage quantification using the double-layer assay. The adsorption percentage was calculated using the equation:

\[
\text{Adsorption percentage} = \left( \frac{\text{PFU before adsorption} - \text{PFU after adsorption}}{\text{PFU before adsorption}} \right) \times 100
\]
Adsorption (%) = [(initial phage titre – phage titre in the supernatant)/(initial phage titre)] × 100

RESULTS

Selection of E. coli mutants resistant to mEp213 infection

E. coli strain W3110 was infected with phage λNK1316 containing miniTn10KmR, and from >100 independent assays, we obtained ~25 000 KmR mutants. Using a replica plating method, we selected 12 mutants resistant to mEp213 infection (Table 2). In addition, these mutants resistant to Km and mEp213 infection were not lysogenic for prophage λNK1316 and mEp213 (data not shown). This latter condition is a prerequisite to avoid the selection of mutants with prophage(s). We named these mutants PRM1–PRM12 (phage-resistant mutant) (Table 2).

Selection and characterization of phage-resistant mutants in the cell envelope

All 12 PRMs were transformed with the mEp213 genome by electroporation, to bypass the physical barriers of the cell envelope, and then incubated for 4 h to ensure the production of viral progeny. Mutants PRM1, PRM3–PRM5 and PRM8–PRM12 released mature virions into the supernatant, strongly suggesting that the host resistance factor was at the level of the cell envelope (Table 2). In contrast, for mutants PRM2, PRM6 and PRM7, virions were not detected in the supernatant, suggesting that the mutation affected one or more genes involved in phage development in the bacterial cytoplasm. These three mutants were not considered in further studies.

It has been reported that phage mEp213 requires the host receptor FhuA for infection (Uc-Mass et al., 2004; Hernández-Sánchez et al., 2008). To identify the mutants altered in the fluuA gene, we transformed the nine PRMs with pUCJA (fluuA+) and tested them in a complementation assay. Six of the mutants, as well as the control strain E. coli C600 (fluuA−), restored the susceptible phenotype (Table 2). Complementation failed for PRM4, PRM11 and PRM12, suggesting that the transposon was inserted in a cell-envelope gene other than fluuA.

The chromosomal sequences adjacent to the insertion site of miniTn10KmR in PRM4, PRM11 and PRM12 were amplified for sequencing. The PCR products for each mutant showed a single amplification band by electrophoresis (Fig. 1). The chromosomal sequence in PRM4 showed that the transposon insertion was in the waaC gene, between codon AGC (Ser) and CAT (His) near the C terminus (codons 265 and 266, respectively). The mutant PRM12 transposon insertion was in the waaC gene as well, but between the second and third nucleotide of CGG (Arg) at codon 212 (Fig. 1). The waaC gene has 960 bp and encodes a peptide of 319 aa, ADP-heptose: LPS heptosyltransferase I (Chen & Coleman, 1993; Kadrmas & Raetz, 1998; GenBank accession no. BAE77671). In PRM11, the transposon is inserted between the guanines of GGC (Gly) of codon 7 of the gmhD gene, which is 933 bp and encodes the ADP-L-glycero-D-mannoheptose-6-epimerase, a protein of 310 aa (Pegues et al., 1990; BAE77673). The products of these genes are involved in the synthesis of LPS, and the three mutants showed mucoid phenotype.

Table 2. Characteristics of bacterial mutants resistant to phage infection

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Km</th>
<th>Infection by phage mEp213*</th>
<th>Phage progeny in supernatant after electroporation†</th>
<th>mEp213 infection on strains complemented with pUCJA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>S</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C600</td>
<td>S</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PRM1</td>
<td>R</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PRM2</td>
<td>R</td>
<td>–</td>
<td>–</td>
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<td>PRM3</td>
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</tr>
<tr>
<td>PRM12</td>
<td>R</td>
<td>+/−</td>
<td>+</td>
<td>+/−</td>
</tr>
</tbody>
</table>

*(+ ) Sensitive (e.o.p. =1) or (−) resistant (e.o.p. <10−7) or (+/−) partially resistant (e.o.p. ≤10−2) to phage infection.

†,+ , Presence; −, absence of viral progeny.
LPS is required for efficient mEp213 infection

Phage mEp213 showed efficiency of plating (e.o.p.) values of 0.045, 0.068 and 0.004 in PRM12, PRM4 and PRM11, respectively (Table 3), compared with an e.o.p. of 1 for strain W3110 and zero for the negative control strain C600 (**fhuA**). e.o.p. was calculated by determining the ratio of the phage titre on the respective strain to the phage titre on the phage-sensitive strain W3110. Susceptibility to mEp213 infection was recovered completely when the mutants were complemented with pWaaC for PRM4 and PRM12 and pGmhD for PRM11, showing e.o.p. values of 1 (data not shown). These results suggest that the gene products involved in the biosynthesis of the inner core of LPS are necessary for the efficient development of mEp213. In addition, we tested the sensitivity of these three PRMs to phages mEp167, T5, λ, mEp021 and T7. All the mutants

Table 3. Phage infectivity on LPS mutants

The e.o.p. for each mutant was calculated relative to the titre of mEp213 on W3110. The results are the mean of three independent assays.

<table>
<thead>
<tr>
<th>Strain</th>
<th>mEp213</th>
<th>mEp167</th>
<th>T5</th>
<th>λ</th>
<th>mEp021</th>
<th>T7</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C600</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PRM11</td>
<td>0.004</td>
<td>0.11</td>
<td>0.34</td>
<td>0.00004</td>
<td>0.0004</td>
<td>0</td>
</tr>
<tr>
<td>(<strong>g mhD</strong>)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PRM4</td>
<td>0.068</td>
<td>0.03</td>
<td>0.20</td>
<td>0.005</td>
<td>0.0004</td>
<td>0</td>
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<tr>
<td>(<strong>w ac</strong>)</td>
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</tr>
<tr>
<td>PRM12</td>
<td>0.045</td>
<td>0.03</td>
<td>0.41</td>
<td>0.003</td>
<td>0.0004</td>
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<tr>
<td>(<strong>w ac</strong>)</td>
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</table>
exhibited partial resistance to phage infection, except to phage T7 (Table 3), to which they were completely resistant. This verifies that LPS is the unique receptor for phage T7, as has been reported before (Molineux, 2001; Chang et al., 2010). The three PRMs exhibited e.o.p. values of 0.03–0.11 for phage mEp167, 0.2–0.41 for T5, 0.0004 for mEp021, and 0.00004–0.005 for phage λ. These results suggest that phages λ, mEp167, mEp021 and T5 require LPS for efficient infection, similar to mEp213. For phages λ and T5, a requirement for LPS has been observed before (Heller & Braun, 1982; Silverman & Benson, 1987).

**Phages mEp213 and λ show decreased adsorption to cells with mutations in waaC or gmhD**

Adsorption of phage mEp213 was examined with the three PRMs. The percentage of adsorption at 5 min was 60–70 %, and at 10 and 15 min was 75–85 %, compared with ~95 % for W3110 after 5 min (Fig. 2a). Adsorption was restored when the mutants were complemented with plasmids containing their respective wild-type genes, and showed values similar to the wild-type *E. coli* W3110 strain (data not shown). Additionally, we investigated adsorption of phage λ to these three mutants. After 5 min, ~25 % of the phages were adsorbed; after 10 min it remained at ~25 % for the gmhD mutant, but was 35–55 % for the waaC mutants. At 15 min it was ~80 % for all three mutants (Fig. 2b). These results indicated that LPS plays an important role during adsorption in the infection process of these phages.

**DISCUSSION**

The aim of this study was to propose and validate a strategy for the selection of host mutants resistant to phages at the level of the cell envelope. The procedures included random transposon mutagenesis, selection of phage-resistant mutants by replica plating, electroporation of the phage genome into the mutant host and detection of potential viral progeny in the supernatant, and sequencing of the chromosomal regions adjacent to the transposon insertions (Fig. 3). The presence of viral progeny in the culture supernatant of electroporated phage-resistant strains strongly suggests that these mutants were affected at the level of the cell envelope.

Of the nine cell-envelope mutants, six complemented the function of the receptor FhuA (Table 2), suggesting that the mutations were in the *fhuA* gene. This high percentage of *fhuA* mutants (66.67 %) could be attributed to the severe selection of the resistance phenotype. In addition, obtaining these putative *fhuA* mutants showed that the approach of this strategy was reliable.

The other three cell-envelope mutants, which were not complemented for FhuA function, showed that the transposon insertions were either in the *waaC* gene (PRM4 and PRM12) or in the *gmhD* gene (PRM11) (Fig. 1). These products came from the *gmhD* operon, which contains the *gmhD, waaF, waaC* and *waaL* genes. The first three gene products are involved in the biosynthesis of the LPS inner core. *waaF* encodes ADP-heptose: LPS heptosyltransferase II (Gronow, et al., 2000). The product of the *waaL* gene, O-antigen ligase, connects the inner core to the O antigen of LPS (Klena et al., 1992). mEp213 phage adsorption in the above three mutants showed a decrease of 25–35 % at 5 min and a decrease of ~15 % at 10–15 min when compared with the adsorption using the wild-type strain W3110 (Fig. 2a). Basically, no adsorption was observed for C600. The decrease in adsorption for mEp213 could be attributed to the difficulty in finding the primary receptor FhuA, as reported by Molineux (2006), because the *gmhD* mutant displays the stereoisomer ADP-β-glycero-β-manno-heptose instead of ADP-α-glycero-β-manno-heptose (Kneidinger et al., 2002), and *waaC* mutants display an LPS structure without the inner core (Coleman & Deshpande, 1985). Alteration or loss of the LPS inner core results in cell stress. The RpoS sigma factor activates the *gab* operon and succinate production, increasing the amount of capsular polysaccharide (Joloba et al., 2004), and this is consistent with the mucoid phenotype observed in these three mutants. Inactivation of *waaL* does not cause a detectable morphological phenotype.
because the E. coli K-12 strain lacks the O antigen (Roncero & Casadaban, 1992; Stevenson et al., 1994).

For the λ phage, the decrease in adsorption on these three mutants was even more pronounced, showing a reduction of ~70% at 5 min, a decrease between 40 and 60% at 10 min for waaC and a decrease of ~70% for gmhD at the same time point, and at 15 min adsorption was reduced ~15% for the three mutants compared with the adsorption of the wild-type W3110 or C600 strains (Fig. 2b). The difference in the adsorption values between phage λ and mEp213 could be attributed to the characteristics of each phage’s OMR. Phage λ adsorbs to the LamB receptor, which has a trimeric structure (Schirmer et al., 1995), while the FhuA protein is monomeric (Ferguson et al., 1998). In addition, the number of FhuA receptors on a cell is ~10 times greater than the number of LamB receptors (Carmel & Coulton, 1991; Chapman-McQuiston & Wu, 2008).

Similarly, the e.o.p. analysis showed a decreased value in the three LPS mutants infected with the phages λ, mEp021, mEp167, T5 or T7, compared with that of wild-type strain W3110 (Table 3). The e.o.p. values for the phages mEp213, mEp167 and T5 were similar, possibly because they use the same primary receptor: FhuA. The low value observed for phage T5 corroborated the requirement for λ (Heller & Braun, 1979). The e.o.p. values for phage λ and mEp021, although similar, were even lower than those observed for mEp213 (Table 3). The primary receptor for mEp021 has not yet been characterized, but this phage multiplies well in strain C600 (fluA^-), which indicates that it does not use the FhuA receptor. It is noteworthy that for phage λ, the e.o.p. in the gmhD mutant was lower than in the waaC mutants (Table 3). This suggests that the spatial configuration of the stereoisomer L-glycero-D-manno-heptose conferred by the epimerase GmhD could be more important than the absence of the inner core of LPS for λ infection. Studies comparing the stereoisomer D-glycero-D-manno-heptose with L-glycero-D-manno-heptose have shown that the L-configuration is important, since mutants with the D-configuration exhibit an enhanced sensitivity to low concentrations of antibiotics such as novobiocin and other hydrophobic drugs. In addition, the gmhD mutant is unable to grow at 42°C (Coleman, 1983). In the three mutants, phage exclusion was complete for T7, confirming that the LPS inner core is an essential receptor for phage T7 adsorption (Qimron et al., 2006). We can infer from this result that the gmhD and waaC products, in these three mutants, could be dysfunctional (Table 3).

A polar effect in waaF and waaC gene expression due to the mini-transposon insertion in gmhD was excluded, as the complementation assay with pGmhD restored completely the infection of phages mEp213, mEp167, T5, λ, mEp021 and T7 (data not shown). Downstream of waaF is waaL, which encodes the O-antigen ligase that links the inner core to the O antigen of LPS. It has been reported that strain W3110 lacks the O antigen (Roncero & Casadaban, 1992; Stevenson et al., 1994); thus, the possible polar effect on waaL expression of the mini-transposon insertion in waaC may not be relevant.

**Fig. 3.** Strategy proposed for selection of host mutants resistant to phage infection at the level of the cell envelope. The top panel shows the two steps used to generate mutants using a transposon followed by replica plating to screen the mutants resistant to phage infection. The lower panel represents the implementation of phage DNA electroporation to select resistant mutants at the cell-envelope level. The detection of viral progeny in the supernatants after an incubation period indicates that the resistant factors are at this level.
The fact that the mutants obtained were only affected in the OM could be largely attributed to the severe selection of the resistant phenotype. However, we cannot rule out the possible role of IM and/or periplasmic factors in mEp213 infection. In phage λ, two IM proteins have been reported to participate in the translocation of the phage genome: IIC\textsuperscript{Man}–IID\textsuperscript{Man}, which are components of the sugar phosphotransferase system (PTS) (Esquinas-Rychen & Erni, 2001). Investigation of IM or periplasmic factors involved in mEp213 infection will demand further research.

The strategy described in this study should be useful to obtain and identify host mutants resistant to phages at the level of the cell envelope, since electroporation is a technique that can be applied to almost any bacterium. The use of this strategy should contribute significantly to our understanding of bacteriophage development at the initial steps of infection.

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