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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 (Camprubí *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,

Abbreviations: IM, inner membrane; IS, insertion sequence; Km, kanamycin; OM, outer membrane; OMR, outer-membrane receptor.

K20, Ox2 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 anti-phage antibodies (Viscardi *et al.*, 2003), 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 miniTn10Km^R transposon. *E. coli* DH5α was used in the transformation of the plasmid constructions derived from pPROEXd, and *E. coli* C600 (*fhuA*$^-$) 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), 50 μg chloramphenicol ml^{-1} (Cm)

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

Strain, bacteriophage or plasmid	Genotype or relevant markers	Reference or source
Strains		
W3110	F$^-$ λ$^-$ *rph*$^-$	Bachmann (1972), Jensen (1993)
C600	*leuB6 thi-1 lacY1 supE44 thr-1 rfbD1 fhuA21*	Appleyard (1954)
LE392	F$^-$, *e14*$^-$, *hsdR514*(r$^-$m$^-$) *supE44 supF58 lacY1*	Silhavy *et al.* (1984)
DH5α	*endA1 hsdR17*(r$^-$m$^+$) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacIZYA-argF*) U169 *deoR* [ϕ80d*lac*Δ(*lacZ*)M15]	Hanahan (1983)
W3110(mEp213)	W3110 with mEp213 prophage	Kameyama *et al.* (1999)
PRM4	W3110 $waaC_{265}$::miniTn10Km^R	This study
PRM12	W3110 $waaC_{212}$::miniTn10Km^R	This study
PRM11	W3110 $gmhD_7$::miniTn10Km^R	This study
Bacteriophages		
λ	Wild-type	CSH Collection*
λNK1316	λ (miniTn10::Km^R cI_{857} Pam_{80} *nin5* b522 *att*$^-$)	Kleckner *et al.* (1991)
mEp213	Lambdoid phage$_{imm\ ix}$	Kameyama *et al.* (1999)
mEp167	Lambdoid phage$_{imm\ iv}$	Kameyama *et al.* (1999)
mEp021		Kameyama *et al.* (1999)
T5		Killmann *et al.* (1995)
T7		CSH Collection
mEp213_C	Lytic phage	This study
mEp213_V	Virulent phage	This study
Plasmids		
pUCJA	*cat fhuA*$^+$	Uc-Mass *et al.* (2004)
pPROEXd	*bla lacI*^{Q} *p*Trc Δ(80 bp)	Uc-Mass *et al.* (2004)
pWaaC	*bla waaC*$^+$	This study
pGmhD	*bla gmhD*$^+$	This study

CSH: Cold Spring Harbor.

(Sigma-Aldrich) or 1 mM IPTG (Fermentas), when required. Restriction enzymes, T4 DNA ligase and T4 polynucleotide kinase used in this study were purchased from New England Biolabs, and AmpliTaq DNA Polymerase was from Applied Biosystems.

Random transposon mutagenesis. Phage λNK1316 (miniTn10::Km^R cI_{857} P_{am80} nin5 b522 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 recombination 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 °C for 1 h, then 100 µl of the culture was poured onto LB agar with Km and the plates were incubated overnight at 37 °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 10^9 p.f.u. per plate on the other plate. The host mutants that were able to grow on both plates were chosen as 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 °C; then, 0.5 ml of the culture was mixed with 3 ml of TB soft agar (at 40 °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 °C to quantify the p.f.u.

Bacteriophage propagation, DNA extraction, and electroporation. Phage propagation was according to Martínez-Peñafiel *et al.* (2012), and DNA extraction was as reported in Sambrook *et al.* (1989). The DNA pellet was resuspended in 50 µl 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 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 µg phage DNA with ~10^6 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 µF; voltage, 2.5 kV; low resistance. The transformed cells were immediately cultured in 3 ml LB broth for 4 h at 37 °C. Then, 300 µl 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 *Nla*III, at 37 °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 °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 TCG ATG AG-3′) were used to amplify the transposon-flanking sequence.

Reactions were performed using a Flexigen PCR system (Flexigen Techne) under the following conditions: a denaturing step at 94 °C/2 min, followed by 30 amplification cycles of 94 °C/30 s, 56 °C/60 s, 72 °C/60 s for each cycle, and a final extension step of 72 °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 °C/2 min, followed by 30 cycles of amplification of 96 °C/30 s, 53 °C/20 s, 60 °C/2 min for each cycle, and a final extension step of 60 °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). *Eco*RI and *Hin*dIII 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 GCC TGA CGG ATG-3′) and the Rev was waaC-HindRev (5′-CCC AAG CTT TAA AGG ATG TTA GCA TGT TTT ACC-3′). For *gmhD*, the primers were gmhD-EcoFwd (5′-GGA ATT CGA AGG TTA CAG TTA TGA TC-3′) and gmhD-HindRev (5′-CCC AAG CTT CAT GCA GAG CTC TTA TGC-3′). The PCR conditions were similar for both genes: a denaturing step at 94 °C/2 min, followed by 30 amplification cycles of 94 °C/30 s, 55 °C/60 s and 72 °C/60 s, and a final extension step of 70 °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 *Eco*RI and *Hin*dIII enzymes at 37 °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 (10^5 c.f.u.) culture and 0.01 ml of phage (10^4 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:

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 miniTn10Km^R, and from >100 independent assays, we obtained ~25 000 Km^R 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 *fhuA* gene, we transformed the nine PRMs with pUCJA (*fhuA*$^+$) and tested them in a complementation assay. Six of the mutants, as well as the control strain *E. coli* C600 (*fhuA*$^-$), 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 *fhuA*.

The chromosomal sequences adjacent to the insertion site of miniTn10Km^R 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

S, Sensitivity; R, resistance to Km.

E. coli strain	Km	Infection by phage mEp213*	Phage progeny in supernatant after electroporation†	mEp213 infection on strains complemented with pUCJA*
W3110	S	+	+	+
C600	S	−	+	+
PRM1	R	−	+	+
PRM2	R	−	−	−
PRM3	R	−	+	+
PRM4	R	+/−	+	+/−
PRM5	R	−	+	+
PRM6	R	−	−	−
PRM7	R	−	−	−
PRM8	R	−	+	+
PRM9	R	−	+	+
PRM10	R	−	+	+
PRM11	R	+/−	+	+/−
PRM12	R	+/−	+	+/−

*(+) Sensitive (e.o.p.=1) or (−) resistant (e.o.p. $<10^{-7}$) or (+/−) partially resistant (e.o.p. $\leqslant 10^{-2}$) to phage infection.
†+, Presence; −, absence of viral progeny.

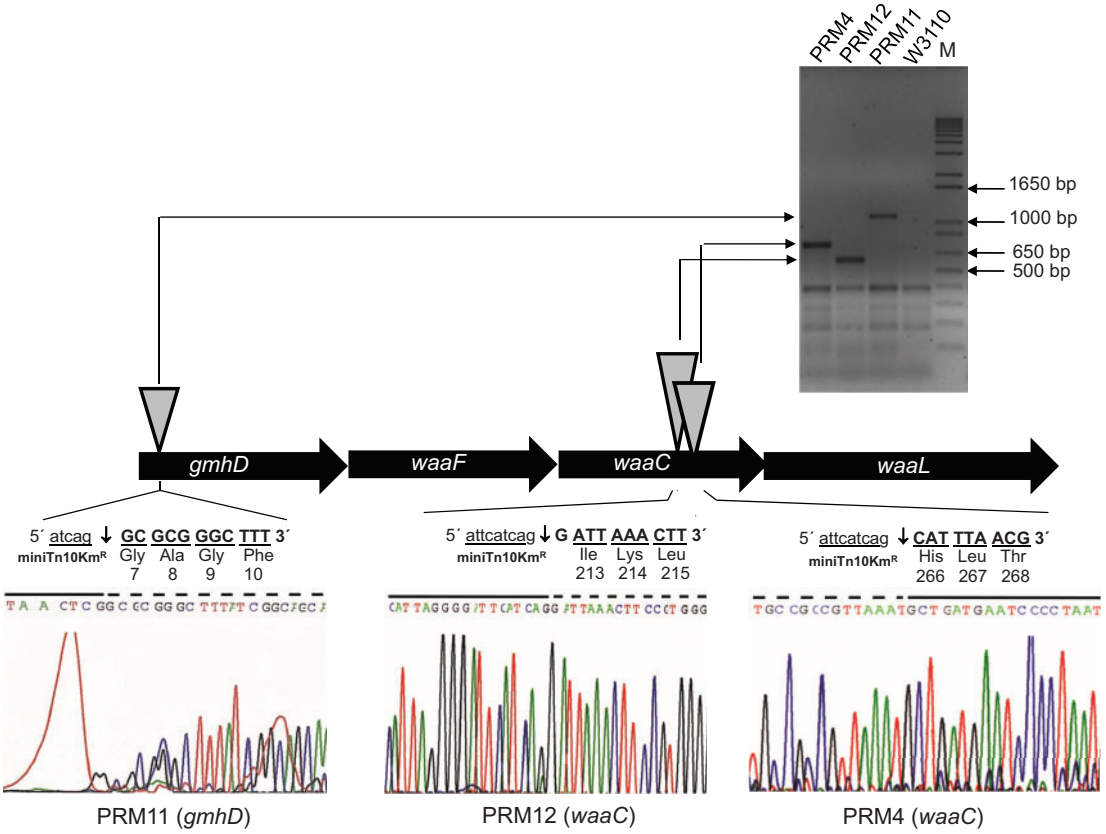

Fig. 1. Amplification and identification of the bacterial chromosomal region linked to the miniTn10Km^R transposon. Specific PCR products of the mutants PRM4, PRM12 and PRM11 are shown in the 2 % agarose gel. M, molecular mass marker 1 kb Plus (Invitrogen). Wide black arrows represent the four genes of the *gmhD* operon: *gmhD* (933 bp), *waaF* (1047 bp), *waaC* (960 bp) and *waaL* (1260 bp). Inverted triangles (grey) show the transposon insertion site. In the PRM4 mutant, the miniTn10Km^R insertion was between codons 265 and 266 of *waaC*. In the PRM12 mutant, the insertion was at codon 212 of *waaC*. In the PRM11 mutant, the insertion was at codon 7 of the *gmhD* gene. Below the *gmhD* and *waaC* genes, the miniTn10Km^R sequence is shown in lower-case type and the sequence of the interrupted gene, which is separated by a vertical arrow, is shown in upper-case type. At the top of the electropherograms, the solid line denotes the transposon IS and the dashed line the gene sequence.

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.

Strain	mEp213	mEp167	T5	λ	mEp021	T7
W3110	1	1	1	1	1	1
C600	0	0	0	1	1	1
PRM11 (*gmhD*)	0.004	0.11	0.34	0.00004	0.0004	0
PRM4 (*waaC*)	0.068	0.03	0.20	0.005	0.0004	0
PRM12 (*waaC*)	0.045	0.03	0.41	0.003	0.0004	0

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-D-glycero-D-manno-heptose instead of ADP-L-glycero-D-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,

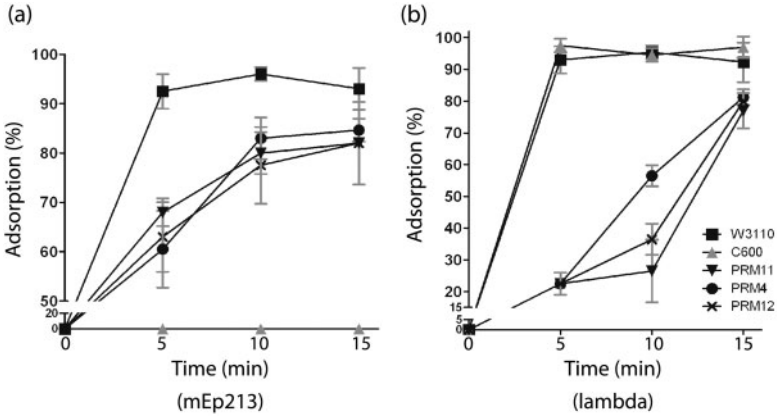

Fig. 2. Phage adsorption rates. (a) mEp213 phage adsorption and (b) λ phage adsorption. The phage adsorption was evaluated in *E. coli* W3110, C600 and the resistant mutants PRM4, PRM12 and PRM11. Bacteria were mixed with phages at an m.o.i. of 0.1 at 37 °C. After 5, 10 and 15 min incubation, aliquots were taken and the amount of free phage was tested using the double-layer assay. Results are the mean of three independent assays; error bars, SD.

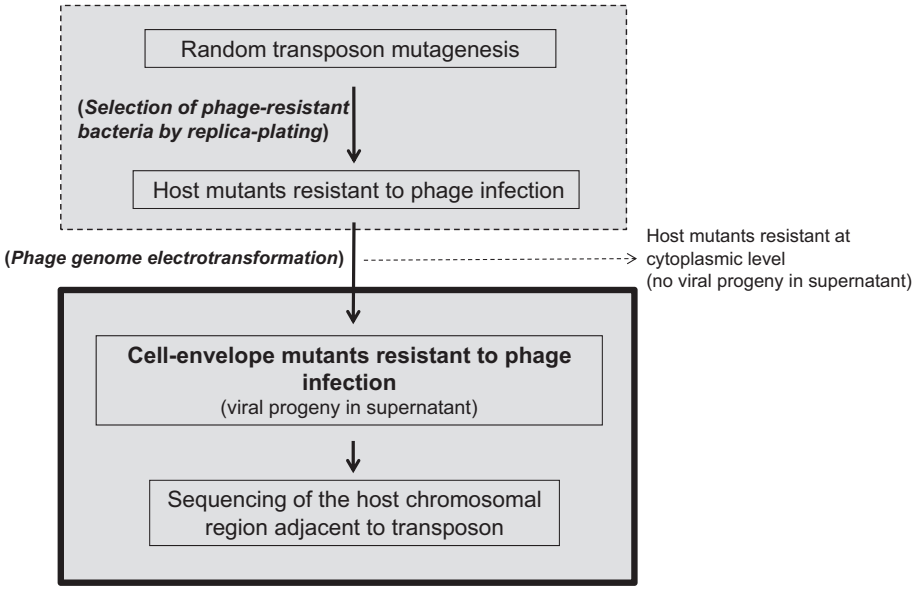

Fig. 3. Strategy proposed for selection of host mutants resistant to phage infection at the level of the cell envelope. The top panel shows the two steps used to generate mutants using a transposon followed by replica plating to screen the mutants resistant to phage infection. The lower panel represents 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.

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 LPS (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 (*fhuA*$^-$), 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 *waaC* 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.

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^{Man}–IID^{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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