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An outer membrane porin protein modulates phage susceptibility in *Edwardsiella ictaluri*

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Bacteriophages ΦeiAU and ΦeiDWF are lytic to the catfish pathogen *Edwardsiella* (*Edw.*) *ictaluri*. The *Edw. ictaluri* host factors that modulate phage–host interactions have not been described previously. This study identified eleven unique *Edw. ictaluri* host factors essential for phage infection by screening a transposon mutagenized library of two *Edw. ictaluri* strains for phage-resistant mutants. Two mutants were isolated with independent insertions in the *ompLC* gene that encodes a putative outer membrane porin. Phage binding and efficiency of plaquing assays with *Edw. ictaluri* EILO, its *ompLC* mutant and a complemented mutant demonstrated that OmpLC serves as a receptor for phage ΦeiAU and ΦeiDWF adsorption. Comparison of translated OmpLCs from 15 *Edw. ictaluri* strains with varying degrees of phage susceptibility revealed that amino acid variations were clustered on the predicted extracellular loop 8 of OmpLC. Deletion of loop 8 of OmpLC completely abolished phage infectivity in *Edw. ictaluri*. Site-directed mutagenesis and transfer of modified *ompLC* genes to complement the *ompLC* mutants demonstrated that changes in *ompLC* sequences affect the degree of phage susceptibility. Furthermore, *Edw. ictaluri* strain Alg-08-183 was observed to be resistant to ΦeiAU, but phage progeny could be produced if phage DNA was electroporated into this strain. A host-range mutant of ΦeiAU, ΦeiAU-183, was isolated that was capable of infecting strain Alg-08-183 by using OmpLC as a receptor for adsorption. The results of this study identified *Edw. ictaluri* host factors required for phage infection and indicated that OmpLC is a principal molecular determinant of phage susceptibility in this pathogen.

INTRODUCTION

Bacteriophages ΦeiAU and ΦeiDWF are lytic to the Gram-negative enteric pathogen *Edwardsiella* (*Edw.*) *ictaluri* (Walakira *et al.*, 2008), the causative agent of enteric septicaemia of catfish (ESC) (Hawke *et al.*, 1981). These two bacteriophages have similar morphology with icosahedral heads and non-contractile tails, and are classified as a member of the *Siphoviridae* (Carrias *et al.*, 2011; Walakira *et al.*, 2008). Our laboratory has previously published a comparative genome analysis of these phages, which revealed that the phage ΦeiAU and ΦeiDWF genomes are >95 % identical at a nucleotide level with some minor (albeit potentially functionally significant) changes in their predicted tail fibre proteins (Carrias *et al.*, 2011).

The dynamics of phage–host interactions are complex. Bacteriophages rely on bacterial hosts for their propagation, with the nature of the phage–host interaction being dependent upon both the bacterial host and the specific phage (Roucourt & Lavigne, 2009; Friedman *et al.*, 1984). Receptors on the bacterial cell surface are the first site of phage interaction and successful phage adsorption triggers phage genome injection into the bacterial cytoplasm (Adams, 1959). Upon entry, the phages exploit host intracellular components for their transcription and replication (Guttman *et al.*, 2005). For example, T7 and T3 phages utilize *Escherichia* (*Esch.*) *coli* RNA polymerase (Chamberlin *et al.*, 1970; Dharmgrongartama *et al.*, 1973) and thioredoxin (Mark & Richardson, 1976; Krüger & Schroeder, 1981) for the transcription of early genes and replication of phage genomic DNA, respectively. Though the host factors for phage T7 (Krüger & Schroeder, 1981) and phage λ (Friedman *et al.*, 1984) have been studied extensively, recent

Abbreviations: EOP, efficiency of plaquing; IS, insertion sequence; LPS, lipopolysaccharide.

The GenBank/EMBL/DDBJ accession numbers for the *ompLC* sequences of the 15 *Edwardsiella ictaluri* strains examined in this study are JN604516–JN604530.

A set of supplementary methods, two supplementary figures, showing multiple sequence alignments of OmpLC nucleotide and amino acid sequences from 15 *Edwardsiella ictaluri* strains, and a supplementary table, listing oligonucleotides used in this study, are available with the online version of this paper.

genome-wide screening of an *Esch. coli* K-12 in-frame, single-gene knock-out library (Baba *et al.*, 2006) provided a more complete analysis of T7 (Qimron *et al.*, 2006) and λ phage (Maynard *et al.*, 2010) dependencies on host factors. In addition to finding specific host gene products directly involved in phage infection and development, genome-wide screening also enables the identification of host factors that may be indirectly involved in phage infection, such as regulatory factors. *Edw. ictaluri* host factors for phage ΦeiAU and ΦeiDWF infection have not been previously investigated. This study will facilitate application of *Edw. ictaluri*-specific phages as a specific diagnostic assay of primary disease isolates from channel catfish, and more broadly contribute to our knowledge of phage–host molecular interactions.

The cell surface components of Gram-negative bacteria such as outer-membrane proteins (Datta *et al.*, 1977), lipopolysaccharides (LPS) (Lindberg, 1973), pili (Chibeu *et al.*, 2009) and flagella (Schade *et al.*, 1967) serve as receptors for phage adsorption and subsequent infection processes. Bacteriophages K2, SSI, T5 and H8 adsorb to OmpA (Datta *et al.*, 1977), OmpC (Beher & Pugsley, 1981), TonA (Menichi & Buu, 1983) and FepA (Rabsch *et al.*, 2007), respectively, for their initiation of infection. In addition to binding to primary protein receptors, some bacteriophages also utilize secondary receptors, mostly LPS, for their adsorption. For instance, bacteriophages K20 (Silverman & Benson, 1987), Ox2 (Sukupolvi, 1984), TuII (Datta *et al.*, 1977), T2 (Lenski, 1984) and T4 (Yu & Mizushima, 1982) can utilize outer membrane porin proteins and LPS of *Esch. coli* as a receptor for phage adsorption and infection. Moreover, bacteriophage Ox2 uses OmpA and OmpC of *Esch. coli* K-12, though these two proteins are very different with respect to their primary structures and functions, as a receptor for adsorption and infection (Morona & Henning, 1984). The lack of expression or altered structure of those bacterial surface components modulates phage susceptibility of different bacterial strains to their specific phages. In a previous study, *Edw. ictaluri* strains were observed to have varying degrees of phage susceptibility (Walakira *et al.*, 2008). This study was initiated to determine the molecular determinants of phage infection in *Edw. ictaluri*.

METHODS

Bacterial strains and culture conditions. *Edw. ictaluri* strains were grown on trypticase soy broth or agar (TSB/TSA) at 28 °C with aeration. *Esch. coli* strains were grown at 37 °C in Luria–Bertani (LB) medium or 2 × yeast tryptone (YT), with aeration. Commercially prepared electrocompetent *Esch. coli* strain DH10B (Invitrogen) was routinely used for electroporation of plasmid constructs for cloning purposes. All of the primers used in this study are listed in Supplementary Table S1 (available with the online version of this paper).

When required, growth medium was supplemented with kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}), colistin (10 μg ml^{-1}), chloramphenicol (12.5 μg ml^{-1}) and/or $CaCl_2$ (500 μM). For propagation of phages ΦeiAU, ΦeiDWF or ΦeiAU-183, *Edw. ictaluri* strains EILO, ML-08-116 or Alg-08-183 were used routinely, respectively. Phage lysates were prepared by double-layered soft agar overlay (Fortier & Moineau, 2009).

Transposon mutagenesis and screening for phage-resistant mutants. Transposon mutagenesis of *Edw. ictaluri* EILO and ML-08-116 was carried out by filter mating experiments according to methods described previously (Maurer *et al.*, 2001). Briefly, the donor *Esch. coli* SM10λpir containing pLOF-Km (Herrero *et al.*, 1990) was grown to OD_{600} 1.0. The recipient *Edw. ictaluri* EILO was also grown to OD_{600} 1.0. Cultures were mixed in a ratio of 4 : 1 (recipient : donor) in a 15 ml polypropylene conical tube containing 10 mM $MgSO_4$. The mixture was vacuum filtered through a 0.45 μm MicroFunnel filter unit (Pall Corporation). This membrane filter was transferred to a TSA plate and incubated for 4 h at 24 °C. After 4 h of incubation, the filter was transferred to LB plates containing 1 mM IPTG and incubated for 16 h for the induction of transposase. Filters were then suspended in 3 ml 10 mM $MgSO_4$ and vortexed to dislodge cells. Cell suspensions were then mixed with a high titre of relevant phages (~5.0×10^{12} p.f.u. ml^{-1}) and incubated for 15 min at room temperature to allow for phage adsorption. Phage-inoculated cells were plated and selected on TSA plates containing kanamycin and colistin for the selection of phage-resistant *Edw. ictaluri* mutants. The phage-resistant mutants were further verified by determining their efficiency of plaquing (EOP) according to the methods described below.

Phage EOP and adsorption assays. EOP assays were performed as described previously (Walakira *et al.*, 2008) with minor modifications. Briefly, freshly prepared phage stocks of known titre were serially diluted with SM buffer [5.8 g NaCl, 20 g $MgSO_4.7H_2O$, 50 ml of 1 M Tris/HCl (pH 7.5) and 5 ml 2 % gelatin; to 1 l] and optimum dilutions that provided plaques within the range of 30–300 on tested *Edw. ictaluri* strains were selected. Phage dilutions were spotted on *Edw. ictaluri* strains embedded in TSA-top agar and incubated for 16 h at 28 °C to determine the number of phage plaques. EOPs were calculated by determining the ratio of the phage titre on the respective strain to the phage titre on the phage-sensitive *Edw. ictaluri* strain 219.

Phage adsorption rates were determined by using the methods described by Fineran *et al.* (2009) with minor modifications. Briefly, *Edw. ictaluri* strains were grown in TSB supplemented with 500 μM $CaCl_2$ and the OD_{600} of the cultures were adjusted to 0.975 to achieve ~1.0×10^9 c.f.u. ml^{-1}. Before the addition of phage, the *Edw. ictaluri* culture was supplemented with chloramphenicol (12.5 μg ml^{-1}) to stop the growth of the cells and inhibit the production of progeny phages. Each culture was inoculated with relevant phages at an m.o.i. of 0.01 and incubated at 28 °C with shaking at 250 r.p.m. Phages were added to TSB without bacteria to serve as a control. Samples were collected at 0 and 35 min and were subjected to centrifugation at 21 000 ***g*** for 5 min to obtain unbound phages from the supernatant. Serially diluted supernatants were spotted on indicator bacterial strains embedded in TSA-top agar and incubated for 16 h at 28 °C to determine the titre of unbound phages in p.f.u.. The adsorption rate was determined by calculating the percentage decrease of phage titre on the supernatant from 0 to 35 min.

Identification of transposon insertion sites in phage-resistant mutants. Transposon insertion sites in the chromosome of *Edw. ictaluri* phage-resistant mutants were determined by inverse PCR (Ochman *et al.*, 1988). Genomic DNA was isolated from an overnight culture of each mutant according to the methods described previously (Ausubel *et al.*, 1999), digested with *Hin*dIII and self-ligated with T4 DNA ligase. A 1 μl volume of the ligation mix was used as template for PCR with PyroPhage 3173 DNA polymerase (Lucigen) using transposon-specific primers (see Supplementary Table S1). Amplicons were purified with a Promega Wizard SV PCR purification kit.

If inverse PCR failed to amplify the transposon-flanking regions, subcloning was performed to identify the transposon insertion sites. A 1 μg sample of genomic DNA was digested with *Sph*I and ligated into a *Sph*I-digested and dephosphorylated pUC19. The ligation mix was then transformed into *Esch. coli* strain DH10B and subclones were selected on 2× YT containing kanamycin. Plasmid DNA was purified using the Promega Wizard SV miniprep kit. Inverse PCR amplicons and plasmid inserts from subclones were sequenced with nested transposon-specific primers (Supplementary Table S1). Sequencing was performed by Lucigen using an ABI 3730xl sequencer. The transposon insertion sites and genes were compared to genes within the GenBank nr/nt database at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTX and BLASTN algorithms.

Complementation of selected phage-resistant mutants. To confirm the involvement of *Edw. ictaluri* genes required for phage infection, a complementation study of selected phage-resistant mutants was performed by introducing the wild-type copy of the disrupted gene. Phage-resistant mutants R-165, ML-17, ML-41 and ML-64 were complemented with *ompLC*, hypothetical gene NT01EI_2881, *psoB* gene and porin thermoregulatory gene, *envY*, respectively, from their corresponding wild-type strains. In addition to complementation of the R-165 mutant with *ompLC* from *Edw. ictaluri* EILO, the R-165 mutant was also complemented with *ompLC* from *Edw. ictaluri* Alg-08-183, ML-08-116, ML-08-117 and Alg-08-199. The *ompLC* gene with its predicted promoter region was amplified by PCR from *Edw. ictaluri* strains EILO, Alg-08-183, ML-08-116, ML-08-117 and Alg-08-199 using the high-fidelity TaKaRa Ex *Taq* polymerase and cloned into the pCR2.1 vector (Invitrogen), a plasmid with a pUC19 origin of replication that has a low copy number at reduced growth temperatures (Lin-Chao *et al.*, 1992), resulting in pOmpLC-EILO, pOmpLC-183, pOmpLC-116, pOmpLC-117 and pOmpLC-199, respectively. Complementation of the ML-17 mutant was accomplished by cloning the hypothetical gene NT01EI_2881 from *Edw. ictaluri* ML-08-116 into plasmid pCR2.1 with its native promoter. The *psoB* and *envY* genes from *Edw. ictaluri* ML-08-116 were amplified by PCR with PyroPhage 3137 Polymerase (Lucigen). The PCR-amplified DNA fragments were purified by Wizard SV PCR purification kit, digested with *Hin*dIII and ligated with *Hin*dIII-digested pUC19. Ligation mixtures were electroporated into *Esch. coli* strain DH10B. The recombinant clones selected after blue–white screening were confirmed by PCR and sequencing to contain the correct insert sequence. These constructs were then introduced into the appropriate *Edw. ictaluri* mutants by electroporation using procedures described previously (Russo *et al.*, 2009). The phage sensitivity of each complemented mutant was determined as described above and compared with that of its respective isogenic parental mutant strain containing the empty vector.

Modelling of the OmpLC protein. The 3D structural model of *Edw. ictaluri* OmpLC was predicted by using the SWISS-MODEL protein structure homology-modelling server (Bordoli *et al.*, 2009). The osmoporin OmpK36 of *Klebsiella pneumoniae* was used as a template model due to its high degree of similarity with OmpLC from *Edw. ictaluri* (Dutzler *et al.*, 1999). Amino acids 23–360 of *Edw. ictaluri* OmpLC were included in the model. The resulting homology structure generated by SWISS-MODEL was visualized and reproduced by PyMOL (version 1.3r) (DeLano, 2004).

Isolation of mutant phage ΦeiAU-183 lytic to *Edw. ictaluri* strain Alg-08-183. Mutant phage ΦeiAU-183, which is lytic to *Edw. ictaluri* strain Alg-08-183, was isolated by serially passaging wild-type phage ΦeiAU on *Edw. ictaluri* strain Alg-08-183, a wild-type strain resistant to the original phage ΦeiAU. *Edw. ictaluri* strain Alg-08-183 was grown from a primary glycerol stock (not serially passaged) and challenged with phage ΦeiAU by double-layered soft-agar overlay. Phages were collected from the top agar and subsequently used for challenging another primary culture of *Edw. ictaluri* strain Alg-08-183. The experiment was repeated until plaques were observed on the top agar. Once plaques were observed, phages were doubly purified and stored in 7.0 % DMSO at −80 °C until future use.

Construction and complementation of *Edw. ictaluri* Alg-08-183 *ompLC*::Tn*5* mutant resistant to ΦeiAU-183. The *ompLC* gene in pOmpLC-183 was disrupted *in vitro* by EZ-Tn*5* <Tet >insertion kit (Epicentre Biotechnologies) and electroporated into *Esch. coli* strain DH10B. Colonies isolated from 2× YT plates containing tetracycline, kanamycin and ampicillin were screened by PCR to find a clone with an appropriate Tn*5* insertion within the *ompLC* coding sequence. Transposon insertion sites were mapped by sequencing of the PCR product with the transposon-specific primer Tn5out (Supplementary Table S1). Extracted plasmid DNA from desired clones was used as a template for the amplification of the Tn*5* transposon and its 500 bp flanking *ompLC* sequences with primer $165F_{BglII}$ and $165R_{BglII}$ (Supplementary Table S1). Amplicons were subjected to gel electrophoresis to separate template plasmid from PCR products and an excised DNA band was purified with the Wizard SV PCR purification kit. *Bgl*II-digested amplicons were ligated into a *Bgl*II-digested and dephosphorylated suicide vector pGP704 (Miller & Mekalanos, 1988) using T4 DNA ligase and the resulting vector, pGP183, was electroporated into SM10λpir. The pGP183 plasmid in SM10λpir was delivered to *Edw. ictaluri* strain Alg-08-183 by conjugation according to the methods described previously (Maurer *et al.*, 2001). *Edw. ictaluri* strain Alg-08-183 transconjugants resistant to phage ΦeiAU-183, due to the disruption of the *ompLC* gene by allelic exchange, were selected by challenging with a high titre ($\sim 5.0 \times 10^{12}$ p.f.u. ml^{-1}) of phage ΦeiAU-183 on 2× YT supplemented with tetracycline and colistin. The site of the *ompLC* gene disruption on the genome of the *Edw. ictaluri* Alg-08-183 *ompLC*::Tn*5* mutant was verified by PCR followed by sequencing.

Edw. ictaluri strain Alg-08-183 *ompLC*::Tn*5* was complemented with *ompLC* from strain Alg-08-183 cloned into plasmid pCR2.1 (pOmpLC-183) with its native promoter. The *Edw. ictaluri* strain Alg-08-183 *ompLC*::Tn*5* mutant was also complemented with *ompLC* from strain EILO cloned into plasmid pCR2.1 (pOmpLC-EILO). The EOPs of the complemented mutants were determined with phage ΦeiAU and ΦeiAU-183 according to the methods described in the Supplementary Methods.

Nucleotide sequence accession numbers. The *Edw. ictaluri ompLC* genes reported in this study were sequenced from the wild-type *Edw. ictaluri* strains listed in Table 1 and submitted to GenBank under accession numbers JN604516–JN604530.

RESULTS

Transposon mutagenesis and isolation of phage-resistant mutants of *Edw. ictaluri*

Bacterial host factors play significant roles in the growth and propagation of bacteriophages (Hashemolhosseini *et al.*, 1994; Qimron *et al.*, 2006; Friedman *et al.*, 1984). To identify the *Edw. ictaluri* host factors required for phage infection, we screened mini-Tn*10* transposon-mutagenized libraries of *Edw. ictaluri* strains EILO (approximately 25 000 mutants) and ML-08-116 (approximately 30 000 mutants) that are susceptible to infection with both ΦeiAU and ΦeiDWF. The transposon-mutagenized libraries were challenged with a high titre of phages ΦeiAU or ΦeiDWF, respectively, to isolate phage-resistant mutants. A total of

12 mutants were confirmed as unique phage-resistant mutants by a soft agar overlay assay. The growth rate of the mutants was tested and none of the mutants showed an impaired growth rate in TSB compared with wild-type strains (data not shown).

Identification of genes interrupted in phage-resistant mutants

With either inverse PCR (mutant ML-43, ML-44 and ML-100) or subcloning (the remainder of the mutants) followed by sequencing with primers specific to the Tn*10* transposon, the transposon insertion sites were identified within the genome of phage-resistant mutants. The list of predicted ORFs that were disrupted by transposon insertion is provided in Table 2.

All of the transposon insertions that resulted in a phage-resistant phenotype were within predicted ORFs of *Edw. ictaluri*. We identified 11 unique genes from 12 phage-resistant mutants. The predicted outer membrane porin protein LC gene, *ompLC*, was interrupted in two phage-resistant mutants (R-6 and R-165) and analysis of transposon-insertion sites in these two mutants indicated independent insertion events in different sites within the *ompLC* gene. In mutant ML-64, the transposon was inserted within an insertion sequence (IS) element that was adjacent (1695 bp from the ATG start codon) to a putative porin thermoregulatory protein gene, *envY*. Three different hypothetical proteins with unknown functions were disrupted in three separate phage-resistant mutants (ML-17, ML-100 and R-148). The mutant ML-42, containing an insertion within a predicted ATP-dependent RNA helicase gene, *deaD*, was the only mutant that demonstrated a partially phage-resistant phenotype (Table 2).

Complementation of *Edw. ictaluri* phage-resistant mutants

To confirm that the phage-resistant phenotype observed in *Edw. ictaluri* mutants was due to a transposon insertion, selected mutants (R-165, ML-17, ML-41 and ML-64) were complemented with their wild-type genes to test for the restoration of a phage-sensitive phenotype. The complementation of other genes identified in this study (e.g. *fimD*) will be described in future research. Among these mutants the gene with a transposon insertion in mutant R-165 was targeted first for complementation due to the known involvement of outer membrane porins in phage infectivity in other bacterial species. Mutant R-165, in which the outer membrane porin protein gene (*ompLC*) was inactivated, was complemented with the *ompLC* gene from *Edw. ictaluri* strain EILO. The introduction of *ompLC* to mutant R-165 *in trans* fully restored the phage-sensitive phenotype and exhibited an EOP similar to wild-type strain EILO (Fig. 1). This finding, together with bioinformatic analyses suggesting that OmpLC serves as a putative outer membrane porin protein, led to the hypothesis that OmpLC acts as a receptor for phage ΦeiAU/ΦeiDWF adsorption and initiation of infection.

Mutant ML-41, in which the *psoB* gene was inactivated by transposon insertion, was complemented with the wild-type *psoB* gene. The *psoB* gene is predicted to encode a GDP-fucose synthetase that is involved in LPS biogenesis in many Gram-negative bacteria (Barua *et al.*, 2002; Moran *et al.*, 1994; Skurnik & Zhang, 1996). As expected, the introduction of *psoB in trans* restored the phage-sensitive phenotype of mutant ML-41 strain (Table 2).

Mutant ML-17 had a transposon insertion within a gene that had a top BLAST hit indicating a hypothetical protein in *Edw. ictaluri* strain 93-146 (NT01EI_2881), with additional BLAST hits (with similarly low E values) indicating that this gene may encode a Tn*10* transposase. Since this identification of a putative transposase associated with phage infectivity was an unexpected result, complementation was attempted for mutant ML-17. Initial attempts to amplify this gene by PCR were unsuccessful, probably due to the lack of available genome sequences for this strain. A larger PCR amplicon containing downstream sequences was eventually obtained that also included another downstream ORF (accession number YP_002934278.1) with a predicted gene product that has significant sequence similarity (84 % similar) to a hydrolase-oxidase encoded by *Pectobacterium wasabiae*. The introduction of this plasmid pML17 into mutant ML-17 and restoration of a phage-sensitive phenotype confirmed the involvement of this genetic region in phage infection (Table 2). Further experiments are necessary to determine any specific role for a putative Tn*10* transposase in phage infectivity.

The first attempt at complementation *in trans* was not successful for mutant ML-64, which had a transposon insertion within an ORF designated as an IS element. However, inclusion of an ORF that encodes a putative porin thermoregulatory protein EnvY, which is downstream of that IS element, in the complementing plasmid restored the phage-sensitive phenotype of mutant ML-64 (Table 2). This regulatory protein is predicted to have been inactivated due to a polar effect of the transposon insertion. The predicted EnvY protein has a homologue in *Esch. coli* K-12 that modulates the temperature-dependent expression of several porin proteins, most notably OmpF and OmpC and lambda phage receptor, LamB (Lundrigan & Earhart, 1984). Further experiments are required to clarify the role of *envY* in the putative regulation of expression of the *ompLC* gene in *Edw. ictaluri*.

OmpLC is the receptor for phage ΦeiAU adsorption

To test the hypothesis that OmpLC serves as a receptor for phage ΦeiAU adsorption, phage binding assays were performed with wild-type *Edw. ictaluri* strain EILO, its *ompLC* mutant R-165 and its complemented mutant. Phage ΦeiAU was able to bind to *Edw. ictaluri* strain EILO

Table 1. Summary of bacterial strains, phages and plasmids used in this study

Ap^r, Ampicillin resistance; Cam^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tet^r, tetracycline resistance. WT – C, Wild-type strain isolated from diseased catfish.

Bacterial strains, phages and plasmids	Relevant characteristics	Reference or source
***Esch. coli* strains**		
SM10λpir	*thi-1 thr leu tonA lacY supE recA*::RP4-2-TcT::Mu Km^r λ*pir*	Simon *et al.* (1983)
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *endA1 recA1* ϕ80*dlac*ZΔM15 Δ*lac*X74 *araD139* Δ(*ara,leu*)7697 *galU galK rpsL nupG* λ- *tonA*	Invitrogen
XL10-Gold	TetrΔ (*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r) Ap^r Cam^r]	Stratagene
***Edw. ictaluri* strains**		
EILO	WT – C	Walakira *et al.* (2008)
ML-08-116	WT – C	This study
Alg-08-183	WT – C	This study
Alg-08-221	WT – C	This study
S97-773	WT – C	Williams *et al.* (2003)
Alg-08-195	WT – C	This study
Alg-08-190	WT – C	This study
ML-08-113	WT – C	This study
Alg-08-200	WT – C	This study
Alg-08-192	WT – C	This study
Alg-08-117	WT – C	This study
R4383	WT – C	Williams *et al.* (2003)
C91-162	WT – C	Walakira *et al.* (2008)
Alg-08-199	WT – C	This study
219	WT – C	Walakira *et al.* (2008)
Alg-08-183 *ompLC*::Tn*5*	*ompLC* mutant of *Edw. ictaluri* Alg-08-183	This study
R-6	*ompLC*::Tn*10*, phage-resistant mutant of EILO	This study
R-11	Phage-resistant mutant of EILO	This study
R-137	*fimD*::Tn*10*, phage-resistant mutant of EILO	This study
R-148	Phage-resistant mutant of EILO	This study
R-165	*ompLC*::Tn*10*, phage-resistant mutant of EILO	This study
ML-17	Phage-resistant mutant of ML-08-116	This study
ML-41	*psoB*::Tn*10*, phage-resistant mutant of ML-08-116	This study
ML-42	*deaD*::Tn*10*, phage-resistant mutant of ML-08-116	This study
ML-44	*dtrA*::Tn*10*, phage-resistant mutant of ML-08-116	This study
ML-64	*envY*::Tn*10*, phage-resistant mutant of ML-08-116	This study
ML-82	*ptrA*::Tn*10*, phage-resistant mutant of ML-08-116	This study
ML-100	*ybaJ*::Tn*10*, phage-resistant mutant of ML-08-116	This study
Phages		
ΦeiAU	Wild-type *Edw. ictaluri*-specific phage	Walakira *et al.* (2008)
ΦeiDWF	Wild-type *Edw. ictaluri*-specific phage	Walakira *et al.* (2008)
ΦeiAU-183	Mutant phage derived from phage ΦeiAU	This study
Plasmids		
pUC19	Cloning vector, Ap^r	
pOmpLC-EILO1	*ompLC* gene from EILO cloned into pUC19	This study
pCR2.1	TOPO-TA cloning vector, Ap^r and Km^r	Invitrogen
pOmpLC-EILO	*ompLC* gene from EILO cloned into pCR2.1	This study
pOmpLC-116	*ompLC* gene from ML-08-116 cloned into pCR2.1	This study
pOmpLC-117	*ompLC* gene from ML-08-117 cloned into pCR2.1	This study
pOmpLC-183	*ompLC* gene from Alg-08-183 cloned into pCR2.1	This study
pOmpLC-199	*ompLC* gene from Alg-08-199 cloned into pCR2.1	This study
pOmpLC-174	Change of residue 174 (Gln⟶Tyr) of OmpLC in pOmpLC-EILO1	This study
pOmpLC-343	Change of residue 343 (Ala⟶Val) of OmpLC in pOmpLC-EILO1	This study
pOmpLC-ΔL8	14 amino acids (loop 8) of OmpLC deleted from pOmpLC-EILO1	This study
pPsoB	*psoB* gene from ML-08-116 cloned into pUC19	This study

Table 1. cont.

Bacterial strains, phages and plasmids	Relevant characteristics	Reference or source
pML17	Genes encoding hypothetical proteins NT01EI_2881 and NT01EI_2882 from *Edw. ictaluri* ML-08-116 cloned into pCR2.1	This study
pML64	IS element from ML-08-116 cloned into pUC19	This study
pEnvY	*envY* from ML-08-116 cloned into pUC19	This study
pLOF-Km	Tn*10*-based delivery plasmid, Km^r and Ap^r	Herrero *et al.* (1990)
pGP704	Suicide vector, Ap^r	Miller & Mekalanos (1988)
pGP183	Tn*5*-disrupted *ompLC* gene cloned into suicide vector pGP704, Tet^r, Km^r and Ap^r	This study

and its complemented *ompLC* mutant with much higher efficiency (adsorption rate >97 %) as compared with its *ompLC* mutant R-165 (EILO *ompLC*::Tn*10*), which exhibited a binding rate less than 4 % (Fig. 2). Since the preliminary results obtained from EOP and binding assays of phage ΦeiAU and ΦeiDWF on *Edw. ictaluri* strain EILO were very similar, the plaquing and binding efficiency of phage ΦeiDWF were not examined in further detail.

Results obtained from the phage binding assay were in agreement with the results from EOP assays carried out with strain EILO, *ompLC* mutant R-165 and its complemented mutant R-165 (pOmpLC-EILO) (Fig. 1). Strain EILO and its complemented *ompLC* mutant had very similar EOP (~1.0) whereas phage ΦeiAU was not able to produce any plaques on mutant R-165 at a phage titre of $\sim 1.0 \times 10^{12}$. Together these results indicated that OmpLC protein of *Edw. ictaluri* EILO serves as a receptor for phage ΦeiAU adsorption.

OmpLC variability among *Edw. ictaluri* strains mapped to a 3D protein model

Edw. ictaluri strains have varying degrees of susceptibility to phage ΦeiAU infection (Walakira *et al.*, 2008). The reduced phage susceptibility among some strains might be due to the lack of a receptor(s), inhibition of phage DNA injection, restriction modification of phage nucleic acids and/or abortive infection. To investigate the role of OmpLC protein in contributing to the varying degrees of phage susceptibility, the *ompLC* genes from 15 different *Edw. ictaluri* strains were sequenced and variations in their translated protein sequences were identified. Comparison of *ompLC* gene sequences from 15 different *Edw. ictaluri* strains demonstrated that the promoter and upstream regulatory regions are strikingly identical and differences were primarily observed in the 3′ coding region (see Supplementary Fig. S1, available with the online version of this paper).

A 3D model of the OmpLC protein structure was obtained by using the SWISS-MODEL using the structure of the OmpK36 porin from *K. pneumoniae* as a template (Bordoli *et al.*, 2009; Dutzler *et al.*, 1999). The predicted structure of the OmpLC protein demonstrated a typical porin structure with 16 antiparallel β strands and eight extracellular loops (Fig. 3). The comparison of the amino acid sequences among 15 different *Edw. ictaluri* OmpLC demonstrated that the amino acid variations, if present in OmpLC, are on the surface-exposed loop 8 (amino acid residues 336–349) of the predicted OmpLC protein (Fig. 4 and Supplementary Fig. S2, available with the online version of this paper).

The OmpLC loop 8 domain is critical for phage infectivity

The complementation of *ompLC* mutants of *Edw. ictaluri* strains EILO and Alg-08-183 with loop 8-deleted OmpLC, pOmpLC-ΔL8, completely abolished phage ΦeiAU susceptibility (EOP $< 1.0 \times 10^{-11}$) (Table 3), whereas pOmpLC-EILO, a plasmid construct with a wild-type *ompLC*, complemented *ompLC* mutants of *Edw. ictaluri* strains EILO, and Alg-08-183 were highly susceptible to phage ΦeiAU infection (Figs 1 and 5). Four *Edw. ictaluri* strains (ML-08-113, ML-08-116, Alg-08-195 and Alg-08-200) with an aspartate to alanine substitution on residue 344 of OmpLC demonstrated reduced phage susceptibility compared with *Edw. ictaluri* strain EILO (Fig. 3). *Edw. ictaluri* strains ML-08-117 and Alg-08-199, with two amino acids (isoleucine and serine) inserted at residue 346 and a single amino acid (glutamate to aspartate) substitution at residue 339, respectively, were resistant to phage ΦeiAU infection (Fig. 3). The importance of the amino acid sequence on loop 8 of OmpLC for determining the degree of phage susceptibility was confirmed by introducing plasmid constructs pOmpLC-116 (*ompLC* which shared identical sequence with *Edw. ictaluri* strains ML-08-116, ML-08-113, Alg-08-195 and Alg-08-200), pOmpLC-117 (*ompLC* from strain ML-08-117) or pOmpLC-199 (*ompLC* from Alg-08-199) to *ompLC* mutant *Edw. ictaluri ompLC*::Tn*10* (mutant R-165). These complementations resulted in reduced phage ΦeiAU susceptibility compared with the wild-type strain that correlated with the phage susceptibility of their corresponding strains (Table 3).

The comparison of OmpLC sequences from phage-sensitive *Edw. ictaluri* strain EILO and phage-resistant *Edw. ictaluri* strain Alg-08-183 demonstrated that two amino acid substitutions were present in OmpLC-183, a

Table 2. The identity of transposon-interrupted ORFs in *Edw. ictaluri* phage-resistant mutants

ND, Not determined. The EOPs of ΦeiAU and ΦeiDWF were calculated by determining the ratio of phage titre on tested strains to the phage titre on phage-sensitive *Edw. ictaluri* strain 219. The EOP was $<10^{-5}$ for mutant ML-42 and $<10^{-12}$ for all other mutants.

Mutant ID	Phage resistance status	Complemented mutation	GenBank accession no.*	Percentage identity†	Top BLAST hit
R-6	Completely resistant	ND (see mutant R-165)	AEQ59645	100	Outer membrane porin protein LC (OmpLC)
R-11	Completely resistant	ND	YP_002934783	100	Orn/Lys/Arg decarboxylase family protein
R-137	Completely resistant	ND	YP_002932963	100	Outer membrane fimbrial usher protein (FimD)
R-148	Completely resistant	ND	YP_002933763	95	Hypothetical protein NT01EI_2357
R-165	Completely resistant	Complemented	AEQ59645	100	Outer membrane porin protein LC (OmpLC)
ML-17	Completely resistant	Complemented with additional downstream ORF	YP_002934277	100	Hypothetical protein NT01EI_2881 (transposase)
ML-41	Completely resistant	Complemented	YP_002933798	100	PsoB
ML-42	Partially resistant	ND	YP_002931944	93	ATP-dependent RNA helicase (DeaD)
ML-44	Completely resistant	ND	YP_002933915	98	Drug resistance transporter (DtrA)
ML-64	Completely resistant	Complemented	YP_002935136	100	Porin thermoregulatory protein (EnvY)
ML-82	Completely resistant	ND	YP_002932263	99	Protease 3 (PtrA)
ML-100	Completely resistant	ND	YP_002932543	100	Hypothetical protein NT01EI_1097 (YbaJ)

*GenBank accession nos of all identified proteins except OmpLC correspond to the orthologues of *Edw. ictaluri* strain 93-146, the only *Edw. ictaluri* strain whose genome sequences are available in GenBank.

†Percentage identity of the identified proteins corresponds to the proteins from the *Edw. ictaluri* strain 93-136.

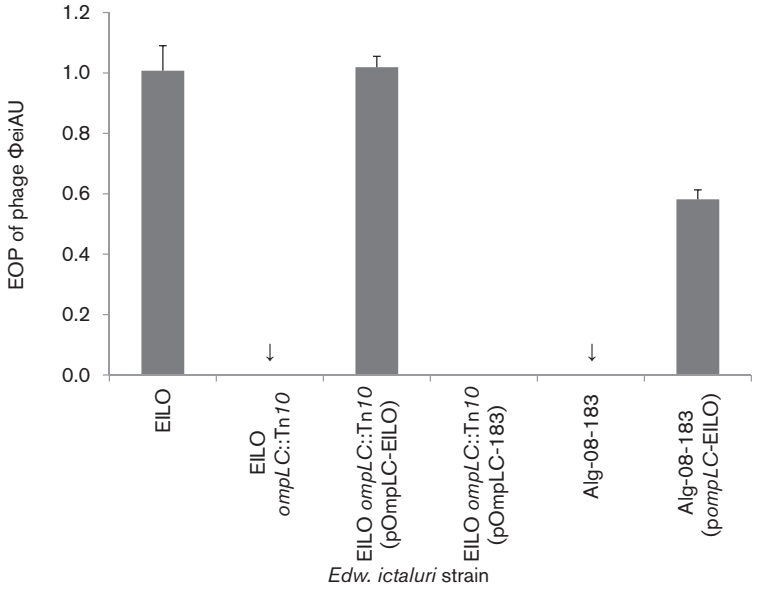

Fig. 1. EOP of phage ΦeiAU on different *Edw. ictaluri* strains. EOPs were calculated by determining the ratio of phage titre on tested strains to the phage titre on phage-sensitive *Edw. ictaluri* strain 219. The means ± SDs of EOPs were from at least three independent experiments. The designations of the strains are as follows: EILO, *Edw. ictaluri* EILO; EILO *ompLC*::Tn*10*, *ompLC* mutant of *Edw. ictaluri* EILO; EILO *ompLC*::Tn*10* (pOmpLC-EILO), EILO *ompLC*::Tn*10* mutant complemented with *ompLC* from *Edw. ictaluri* EILO; EILO *ompLC*::Tn*10* (pOmpLC-183), EILO *ompLC*::Tn*10* mutant complemented with *ompLC* from *Edw. ictaluri* Alg-08-183; Alg-08-183, wild-type *Edw. ictaluri* Alg-08-183; Alg-08-183 (pOmpLC-EILO), wild-type *Edw. ictaluri* Alg-08-183 supplemented with *ompLC* from *Edw. ictaluri* EILO. An arrow (↓) indicates that no plaques were observed after applying ~1.0×10^{12} p.f.u. on the tested strains (EOP $<1.0\times10^{-12}$).

tyrosine to glutamine substitution on residue 174 located on the predicted transmembrane β7 strand and a valine to alanine substitution on residue 343 predicted to be located on loop 8 of the C-terminal end of the OmpLC protein (Fig. 4). Based on these data it was hypothesized that the single amino acid substitution on residue 343 of OmpLC protein in *Edw. ictaluri* strain Alg-08-183 is localized on a surface-exposed loop and is an important site for phage attachment. To address this hypothesis, the OmpLC of *Edw. ictaluri* EILO was modified by site-directed mutagenesis to introduce the amino acids tyrosine and valine at residues 174 and 343, respectively, in separate plasmid constructs pOmpLC-174 and pOmpLC-343. The *ompLC* gene of *Edw. ictaluri* strain Alg-08-183 was interrupted by a Tn*5* transposon, resulting in an Alg-08-183 *ompLC*::Tn*5* mutant. The complementation of this

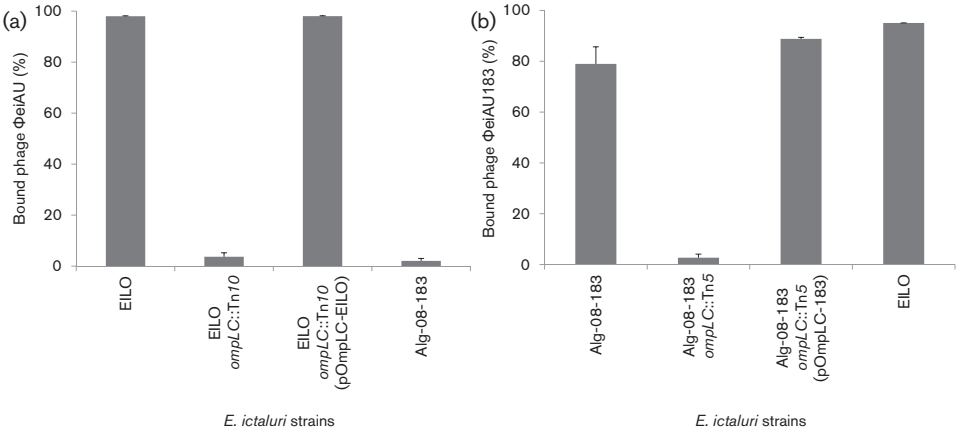

Fig. 2. Adsorption of phage ΦeiAU (a) and ΦeiAU-183 (b) to different *Edw. ictaluri* strains. For phage adsorption assays, bacterial cells (~1.0×10^{9} c.f.u. ml^{-1}) were grown until OD_{600} was ~0.975 in TSB supplemented with 500 μM $CaCl_2$. After incubating for 35 min at 28 °C with ~1.0×10^{7} p.f.u. ml^{-1} of phage, bacterial cells with their attached phages were removed by centrifugation in a microcentrifuge and supernatant was tested for its phage titre using *Edw. ictaluri* strain EILO. The percentage of adsorption was calculated as follows: [(phage titre of a control reaction without cells − phage titre of supernatant after cells were removed)/(phage titre of a control reaction without cells)]×100. The designations of the strains are as listed in the legend for Fig. 1 and as follows: Alg-08-183 *ompLC*::Tn*5*, *ompLC* mutant of Alg-08-183. Strains EILO and EILO *ompLC*::Tn*10* (pOmpLC-EILO) are highly susceptible to phage ΦeiAU and strains EILO *ompLC*::Tn*10* and Alg-08-183 are completely resistant to phage ΦeiAU. Phage ΦeiAU-183 is highly lytic to Alg-08-183, EILO and their complemented *ompLC* mutants.

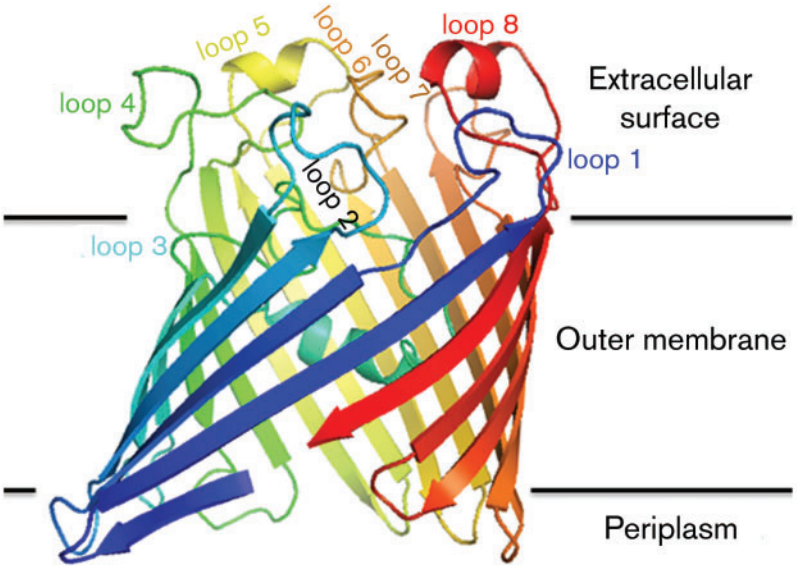

Fig. 3. 3D model of OmpLC from *Edw. ictaluri* strain EILO. This model was determined by SWISS-MODEL based on the X-ray crystallography structure of *Klebsiella pneumoniae* OmpK36 (Dutzler *et al.*, 1999). Surface-exposed loops are labelled loop 1–8. Alignment of OmpLC proteins from 15 different *Edw. ictaluri* strains indicated that all of the variability in amino acid sequences is present on loop 8 of OmpLC (see Fig. 4). The extracellular loops are located on the upper part of the structure and the periplasmic loops are located at the bottom part of the structure. Diagonal strands produce a barrel-like structure with 16 anti-parallel β strands. Loop 3 is inserted within the lumen of the OmpLC porin. Horizontal lines indicate the position of the membrane lipid bilayers.

ompLC mutant with pOmpLC-174 and pOmpLC-343, separately, showed that OmpLC with a valine substitution at residue 343 gave resistance to phage ΦeiAU infection, whereas a glutamine substitution at residue 174 caused susceptibility to phage infection (Table 3). These results demonstrated that an altered OmpLC with a point mutation predicted to be localized on loop 8 at reside 343, and not at residue 174, results in reduced phage susceptibility.

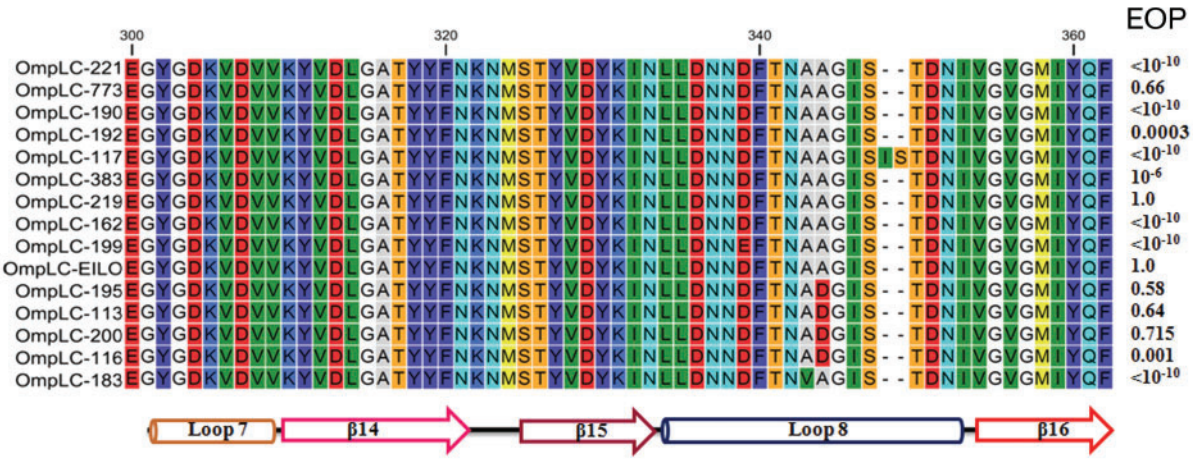

Fig. 4. Multiple sequence alignment of OmpLC protein from 15 different *Edw. ictaluri* strains. The C-terminal region of OmpLC proteins is shown in this alignment (amino acid residues from 300 to 360 for all 15 OmpLC proteins except for residues 300 to 362 for OmpLC-117). OmpLC sequences were aligned using CLC Genomics Workbench as described in Methods. Predicted beta strands and loops of OmpLC proteins are indicated by arrows and cylinders, respectively. The variations in the amino acid sequences of OmpLC proteins (OmpLC-195, OmpLC-113, OmpLC-200, OmpLC-116, OmpLC-199, OmpLC-183 and OmpLC-117) were clustered on loop 8 of OmpLC proteins. The sources of aligned OmpLC proteins are as follows. OmpLC-221, *Edw. ictaluri* Alg-08-221; OmpLC-773, *Edw. ictaluri* S97-773; OmpLC-190, *Edw. ictaluri* Alg-08-190; OmpLC-192, *Edw. ictaluri* Alg-08-192; OmpLC-117, *Edw. ictaluri* ML-08-117; OmpLC-383, *Edw. ictaluri* R4383; OmpLC-219, *Edw. ictaluri* 219; OmpLC-162, *Edw. ictaluri* C91-162; OmpLC-199, *Edw. ictaluri* Alg-08-199; OmpLC-EILO, *Edw. ictaluri* EILO; OmpLC-195, *Edw. ictaluri* Alg-08-195; OmpLC-113, *Edw. ictaluri* ML-08-113; OmpLC-200, *Edw. ictaluri* Alg-08-200; OmpLC-116, *Edw. ictaluri* ML-08-116; OmpLC-183, *Edw. ictaluri* Alg-08-183. The EOPs of the respective wild-type *Edw. ictaluri* strains are indicated on the right of the OmpLC alignment. EOPs were calculated by determining the ratio of phage titre on tested strains to the phage titre on phage-sensitive *Edw. ictaluri* 219.

Table 3. Effect of alterations on loop 8 of OmpLC on the infectivity of phage ΦeiAU against *Edw. ictaluri*

ND, Not determined.

Type of OmpLC*	*Edw. ictaluri* strain	Source of OmpLC	EOP† of ΦeiAU	
			EILO *ompLC*::Tn*10* (pOmpLC‡)	Alg-08-183 *ompLC*::Tn*5* (pOmpLC‡)
Empty vector	–	–	$<10^{-11}$	$<10^{-11}$
Typical OmpLC	EILO, 219, Alg-08-221, S97-773, Alg-08-190, Alg-08-192, R4383, C91-162	Cloned *ompLC* from *Edw. ictaluri* EILO	0.99 ± 0.08	0.66 ± 0.01
Asp344Gly	ML-08-116, Alg-08-195, ML-08-113, Alg-08-200	Cloned *ompLC* from *Edw. ictaluri* ML-08-116	0.63 ± 0.09	ND
346→IleSer	ML-08-117	Cloned *ompLC* from *Edw. ictaluri* ML-08-117	$<10^{-11}$	ND
Glu339Phe	Alg-08-199	Cloned *ompLC* from *Edw. ictaluri* Alg-08-199	$<10^{-11}$	ND
Tyr174Gly, Val343Ala	Alg-08-183	Cloned *ompLC* from *Edw. ictaluri* Alg-08-183	$(7.54 \pm 0.25) \times 10^{-5}$	$(1.27 \pm 0.71) \times 10^{-6}$
Tyr174Gly	–	Site-directed mutagenesis§	ND	0.20 ± 0.05
Val343Ala	–	Site-directed mutagenesis	ND	$(5.99 \pm 4.49) \times 10^{-5}$
Δ336-349	–	Site-directed mutagenesis	$<10^{-11}$	$<10^{-11}$

*Alterations within the mature OmpLC sequence.
†EOPs were calculated by determining the ratio of phage titre on tested strains to the phage titre on phage-sensitive *Edw. ictaluri* strain 219. EOPs are the means and SDs from at least three experiments.
‡*ompLC* from corresponding *Edw. ictaluri* strains cloned into a vector of pUC19 origin.
§Site-directed mutagenesis of selected OmpLC residues was carried out directly on pOmpLC-EILO1, a pUC19 vector in which *ompLC* from *Edw. ictaluri* EILO was cloned.

Transformation of strain Alg-08-183 with ΦeiAU DNA yields phage progeny

The transformation of phage ΦeiAU genomic DNA into an otherwise phage-resistant *Edw. ictaluri* Alg-08-183 resulted in the generation of progeny phages (data not shown). This result ruled out all other potential phage-resistance mechanisms except adsorption/DNA-injection-mediated phage resistance. The introduction of *ompLC* from the

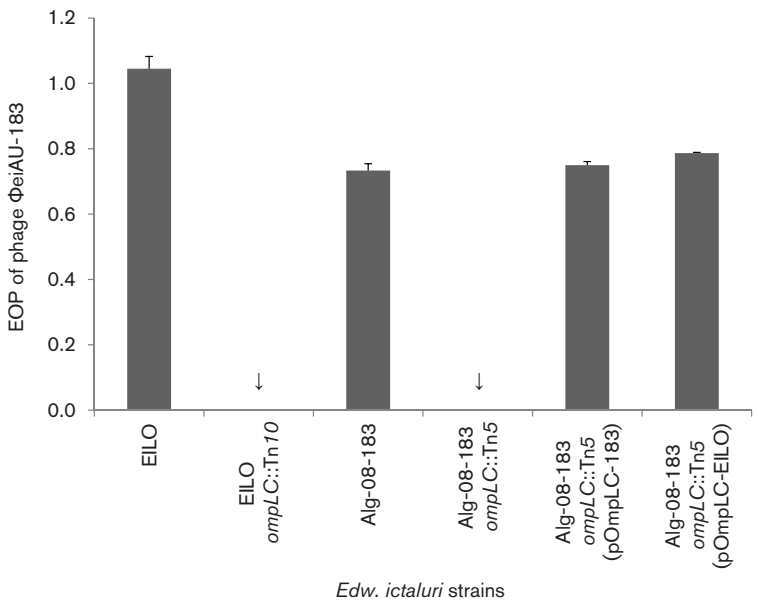

Fig. 5. The EOP of phage ΦeiAU-183 on different *Edw. ictaluri* strains. EOPs were calculated by determining the ratio of phage titre on tested strains to the phage titre on phage-sensitive *Edw. ictaluri* strain 219. The means and SDs of EOPs were from at least three independent experiments. The designations of the strains are as follows: strain EILO, *Edw. ictaluri* EILO; EILO *ompLC*::Tn*10*, *ompLC* mutant of *Edw. ictaluri* EILO; Alg-08-183, wild-type *Edw. ictaluri* Alg-08-183; Alg-08-183 *ompLC*::Tn5, *ompLC* mutant of Alg-08-183; Alg-08-183 *ompLC*::Tn*5* (pOmpLC-183), Alg-08-183 *ompLC*::Tn*5* complemented with *ompLC* from *Edw. ictaluri* Alg-08-183; Alg-08-183 *ompLC*::Tn*5* (pOmpLC-EILO), Alg-08-183 *ompLC*::Tn*5* complemented with *ompLC* from *Edw. ictaluri* EILO. Phage ΦeiAU-183 was serially passaged on *Edw. ictaluri* strain Alg-08-183, a previously phage-resistant strain. An arrow (↓) indicates that no plaques were observed after applying ~1.0×10^{12} p.f.u. on the tested strains (EOP $< 1.0 \times 10^{-12}$).

phage-susceptible *Edw. ictaluri* strain EILO to the completely phage-resistant *Edw. ictaluri* strain Alg-08-183 made this strain susceptible to phage ΦeiAU infection (Fig. 1). The EOP of phage ΦeiAU on wild-type *Edw. ictaluri* strain Alg-08-183 complemented with *ompLC* from *Edw. ictaluri* EILO was $\sim 5.82 \times 10^{11}$ times higher than that of the original strain. This finding indicated that OmpLC is the primary factor contributing to ΦeiAU infectivity in *Edw. ictaluri* strain Alg-08-183.

Passaged ΦeiAU adapts to unique OmpLC epitopes in strain Alg-08-183

A mutant phage ΦeiAU-183 was isolated by passaging phage ΦeiAU in the phage-resistant *Edw. ictaluri* strain Alg-08-183. This mutant phage ΦeiAU-183 was highly lytic to *Edw. ictaluri* Alg-08-183 with an EOP value of ~0.8 whereas no noticeable plaques were observed on this strain with the wild-type ΦeiAU using a titre of $\sim 1.0 \times 10^{12}$ p.f.u. ml^{-1} (Fig. 5). To determine whether OmpLC of *Edw. ictaluri* strain Alg-08-183 (*ompLC-183*) is required for phage ΦeiAU-183 infection, the EOP of phage ΦeiAU-183 was determined against strain Alg-0-183, the *ompLC* mutant of Alg-08-183 and its *ompLC*-183 complemented mutant Alg-08-183 *ompLC*::Tn*5* (pOmpLC-183). Interestingly, the *ompLC* mutant of strain Alg-08-183 (*ompLC*::Tn*5*) was resistant to phage ΦeiAU-183 infection and complementation of this mutant with the *ompLC* gene from *Edw. ictaluri* strain Alg-08-183 with plasmid pOmpLC-183 restored a phage-sensitive phenotype (Fig. 5).

A phage binding assay conducted with phage ΦeiAU against *Edw. ictaluri* strain EILO, its *ompLC* mutant EILO *ompLC*::Tn*10* (R-165) and *Edw. ictaluri* strain Alg-08-183 demonstrated that phage ΦeiAU can bind strongly to strain EILO whereas the adsorption rate was poor with *ompLC* mutant R-165 and *Edw. ictaluri* strain Alg-08-183 (Fig. 2a). The binding assay was also conducted with phage ΦeiAU-183 against strain Alg-08-183, its *ompLC* mutant Alg-08-183 *ompLC*::Tn*5*, complemented mutant Alg-08-183 *ompLC*::Tn*5* (pOmpLC-183) and strain EILO, and demonstrated that phage ΦeiAU-183 has a stronger adsorption rate for wild-type Alg-08-183 (>80 %), complemented mutant Alg-08-183 *ompLC*::Tn*5* (pOmpLC-183) (>85 %) and EILO (>95 %) as compared with its *ompLC* mutant (<3 %) (Fig. 2b). These findings demonstrated that the passaged phage ΦeiAU-183 can utilize the OmpLC of *Edw. ictaluri* strains Alg-08-183 and EILO for adsorption and subsequent infection. These results also demonstrated that the lack of *ompLC* gene expression is not the reason for phage ΦeiAU resistance to Alg-08-183 as it was observed that mutant phage ΦeiAU-183 was capable of using OmpLC-183 as a means for establishing a productive infection in this strain. The observation that complementation of the Alg-08-183 *ompLC*::Tn*5* mutant with *ompLC* from strain EILO resulted in sensitivity to phage ΦeiAU (Fig. 5) was in agreement with the previous observation that complementation of wild-type Alg-08-183 strain with *ompLC* from EILO resulted in sensitivity to phage ΦeiAU infection (Fig. 1).

To further confirm the role of OmpLC as a phage susceptibility determinant, the *ompLC* gene from *Edw. ictaluri* Alg-08-183 (on pOmpLC-183) was introduced into the *ompLC* mutant of EILO (R-165) and the EOP of phage ΦeiAU was determined. The EOP of the pOmpLC-183-complemented mutant R-165 was about 1.35×10^4 times less than that observed from complementation with pOmpLC-EILO (Fig. 1). These data further confirmed that phage ΦeiAU was unable to infect *Edw. ictaluri* Alg-08-183 strain due to its altered OmpLC protein. Taken together, these data demonstrate that OmpLC is a determinant of phage infection to *Edw. ictaluri*.

DISCUSSION

In this study, the *Edw. ictaluri* host factors required for phage ΦeiAU and ΦeiDWF infection were identified in order to further the understanding of phage–host interactions and allow development of a phage-based diagnostic assay for *Edw. ictaluri* primary disease isolates. Transposon-mutagenized libraries of *Edw. ictaluri* strains EILO and ML-08-116 were screened for phage-resistant mutants and 12 unique mutants were isolated that showed complete or partial resistance to phage ΦeiAU and ΦeiDWF infection. Eleven *Edw. ictaluri* genes were identified from those phage-resistant mutants. Host factors identified by this genome-wide screening of *Edw. ictaluri* are predicted to be involved in different stages of phage infection with potential roles from initiation of infection to phage morphogenesis. To the best of our knowledge, this study identified several genes that have never been reported in any bacterial species as an essential host factor for bacteriophage infection.

Deletion of the *ompLC* gene, site-directed mutagenesis and complementation assays demonstrated that the putative outer membrane porin OmpLC of *Edw. ictaluri* is important for phage adsorption and serves as a receptor for phage ΦeiAU infection. Many outer-membrane proteins of *Esch. coli* such as OmpC, OmpF, OmpT and PhoE serve as receptors for phages TulB and T4 (Yu & Mizushima, 1982), T2 (Riede *et al.*, 1985), M2 (Hashemolhosseini *et al.*, 1994) and TC45 (Chai & Foulds, 1978), respectively. The *ompLC* gene is flanked on the chromosome of *Edw. ictaluri* strain 93-146 by genes encoding a hypothetical protein (NT01EI_1358) and a putative asparaginyl-tRNA synthetase. Since neither of these adjacent genes is part of a genetic operon with *ompLC* this further confirms that the transposon insertion in *ompLC* did not result in any polar effect on adjacent genetic loci. In this study, it was also demonstrated that the putative porin thermoregulatory protein EnvY is required for phage infection in *Edw. ictaluri*. In *Esch. coli*, it has been reported that EnvY modulates the temperature-dependent expression of several porin proteins, most notably OmpF,

OmpC and the lambda phage receptor LamB (Lundrigan & Earhart, 1984). The requirement of EnvY for phage infection suggests that the expression of OmpLC is regulated by this protein and that the inactivation of the *envY* gene results in phage resistance due to the lack of OmpLC expression.

In our previous study, we observed that *Edw. ictaluri* strains had varying degrees of susceptibility to phage infection (Walakira *et al.*, 2008). *Edw. ictaluri* strains recently obtained from diseased catfish also showed variability in their degree of phage susceptibility (Table 3). This study showed that OmpLC from different *Edw. ictaluri* strains varied in amino acid sequences, and in seven *Edw. ictaluri* strains (out of 15 tested) the variations in amino acid sequence were predicted to be clustered on the surface-exposed loop 8 of OmpLC. None of the *Edw. ictaluri* strains with amino acids substitutions on loop 8 of their OmpLC protein showed higher phage susceptibility compared with strain EILO that contained a 'typical' OmpLC sequence. Furthermore, the deletion of loop 8 from OmpLC completely abolished *Edw. ictaluri* phage susceptibility. These results showed striking similarities with a previous finding that demonstrated that alterations clustered in a small region near the surface-exposed carboxy terminus of Tsx protein resulted in Tsx-specific phage-resistance phenotypes (Schneider *et al.*, 1993). The involvement of other surface-exposed regions in phage attachment is not unexpected as it has been demonstrated by mutational analysis of several phage receptors (Cole *et al.*, 1983; Gehring *et al.*, 1987; Heine *et al.*, 1988).

The OmpLC proteins from *Edw. ictaluri* strains EILO and Alg-08-183 with altered phage adsorption phenotypes were studied in detail by phage binding and EOP assays with their corresponding *ompLC* mutants. Several lines of evidence indicate that the OmpLC of *Edw. ictaluri* modulates phage susceptibility. First, the introduction of phage ΦeiAU genomic DNA by electroporation to phage resistant *Edw. ictaluri* strain Alg-08-183 resulted in progeny phages by avoiding the natural route of phage infection. This transformation experiment demonstrated that phage resistance in strain Alg-08-183 is due to a phage adsorption/DNA injection deficiency. Second, this strain was capable of being infected by introducing the OmpLC from the phage-sensitive strain EILO. This result demonstrated that inefficient phage adsorption due to an altered OmpLC is responsible for the ΦeiAU phage resistance in Alg-08-183. Third, the introduction of the OmpLC from strain Alg-08-183 into the *ompLC* mutant of strain EILO (mutant R-165) resulted in reduced phage sensitivity, reflecting the poor attachment of ΦeiAU to OmpLC-183. The same consistent patterns were observed when this EILO *ompLC* mutant was complemented with altered *ompLC* from *Edw. ictaluri* strains ML-08-117, ML-08-116 or Alg-08-199 (Table 3). The OmpLC-117 (OmpLC from *Edw. ictaluri* strain ML-08-117) and OmpLC-199 (OmpLC from *Edw. ictaluri* strain Alg-08-199) completely resisted phage infectivity when introduced into the *ompLC* mutant of EILO, as observed in their wild-type strains. The OmpLC-116-complemented *ompLC* mutant of EILO showed about the same degree of phage susceptibility as observed with their wild-type strains ML-08-113, Alg-08-195 and Alg-08-200. However, the phage susceptibility of this OmpLC-116-complemented EILO mutant was higher than that of wild-type strain ML-08-116 which has identical *ompLC* sequences to *Edw. ictaluri* strains ML-08-113, Alg-08-195 and Alg-08-200. These findings suggest that in addition to OmpLC, other host factors contribute to phage infection in *Edw. ictaluri*. Other studies have shown that the sugar moieties of LPS side chains (Skurnik *et al.*, 1995; Yu & Mizushima, 1982) and the spatial orientation of LPS and outer-membrane proteins are important for phage attachment and infection (Beacham & Picken, 1981). Fourth, a phage that was serially passaged and adapted to strain Alg-08-183 (ΦeiAU-183) also utilized OmpLC-183 as a receptor for infection. It has been demonstrated that coliphage TuIa, which uses the OmpF protein as a receptor, can acquire mutational changes to exploit OmpC or LamB proteins or both as a substitute receptor (Moreno & Wandersman, 1980). Likewise, host-range mutants of phage Ox2 can exploit two different outer-membrane proteins, OmpA and OmpC, of *Esch. coli* K-12 as a receptor for infection (Morona & Henning, 1984). Phage Ox2 can switch from protein to carbohydrate receptors by altering a tail fibre protein by a single mutation (Drexler *et al.*, 1991). In this study we have demonstrated that OmpLC is a determinant of host specificity and modulates the degree of phage infectivity in *Edw. ictaluri* strains.

In addition to OmpLC there were other *Edw. ictaluri* host factors that were demonstrated to contribute to phage infection. The *psoB* gene complemented the *Edw. ictaluri* *psoB*::Tn*10* mutant and is predicted to encode a GDP-fucose synthetase that is involved in LPS biogenesis in many Gram-negative bacteria (Barua *et al.*, 2002; Moran *et al.*, 1994; Skurnik & Zhang, 1996). This result suggests that LPS might be a potential co-receptor for phage ΦeiAU infection in *Edw. ictaluri*.

The disruption of a putative outer membrane fimbrial usher gene, *fimD*, of *Edw. ictaluri* also resulted in a phage-resistant phenotype. It has been reported that an outer membrane fimbrial usher protein, which is a molecular chaperone, is a potential virulence factor in *Edw. ictaluri* required for catfish infection (Thune *et al.*, 2007). This result is in agreement with previous observations that acquisition of phage resistance in bacteria may result in a less virulent phenotype (Capparelli *et al.*, 2010; Santander & Robeson, 2007; Evans *et al.*, 2010). The studies of *Staphylococcus aureus* fitness cost associated with phage resistance showed that emerging phage-resistant bacteria provide broad immunity against *S. aureus* infection in mice (Capparelli *et al.*, 2010).

Finally, another phage-resistant mutant contained a transposon insertion in a putative ATP-dependent RNA

helicase DeaD gene (*deaD*) that resulted in partial resistance to ΦeiAU and ΦeiDWF. The orthologues of this RNA helicase have been studied in *Esch. coli* and they are involved in the dissociation of RNA duplexes (Bizebard *et al.*, 2004), mRNA processing (Py *et al.*, 1996) and ribosome biogenesis (Charollais *et al.*, 2003, 2004). However, an ATP-dependent RNA helicase has never been implicated in phage infection. The lack of DeaD protein might affect the transcription of phage-encoded mRNAs and could be responsible for reduced phage susceptibility. Further studies are required to determine the exact nature of DeaD-mediated modulation of phage infection in *Edw. ictaluri*.

In conclusion, this study has identified *Edw. ictaluri* host factors required for phage infection. OmpLC has been determined to serve as a receptor for phage infection and variation in its protein sequence modulates the nature of phage infectivity in different *Edw. ictaluri* strains. Knowledge gained from studies of phage–host interactions in *Edw. ictaluri* will further our collective knowledge of the molecular determinants of phage infection and will also result in improved results for the application of these phages in the diagnosis of enteric septicaemia of catfish.

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REFERENCES

Adams, M. H. (1959). *Bacteriophages*. New York: Interscience Publishers.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (editors) (1999). *Short Protocols in Molecular Biology*. New York: Wiley.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L. & Mori, H. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**, 2006, 0008.

Barua, S., Yamashino, T., Hasegawa, T., Yokoyama, K., Torii, K. & Ohta, M. (2002). Involvement of surface polysaccharides in the organic acid resistance of Shiga Toxin-producing *Escherichia coli* O157:H7. *Mol Microbiol* **43**, 629–640.

Beacham, I. R. & Picken, R. N. (1981). On the receptor for bacteriophage T4 in *Escherichia coli* K-12. *Curr Microbiol* **6**, 291–293.

Beher, M. G. & Pugsley, A. P. (1981). Coliphage which requires either the LamB protein or the OmpC protein for adsorption to *Escherichia coli* K-12. *J Virol* **38**, 372–375.

Bizebard, T., Ferlenghi, I., Iost, I. & Dreyfus, M. (2004). Studies on three *E. coli* DEAD-box helicases point to an unwinding mechanism different from that of model DNA helicases. *Biochemistry* **43**, 7857–7866.

Bordoli, L., Kiefer, F., Arnold, K., Benkert, P., Battey, J. & Schwede, T. (2009). Protein structure homology modeling using SWISS-MODEL workspace. *Nat Protoc* **4**, 1–13.

Capparelli, R., Nocerino, N., Lanzetta, R., Silipo, A., Amoresano, A., Giangrande, C., Becker, K., Blaiotta, G., Evidente, A. & other authors (2010). Bacteriophage-resistant *Staphylococcus aureus* mutant confers broad immunity against staphylococcal infection in mice. *PLoS ONE* **5**, e11720.

Carrias, A., Welch, T. J., Waldbieser, G. C., Mead, D. A., Terhune, J. S. & Liles, M. R. (2011). Comparative genomic analysis of bacteriophages specific to the channel catfish pathogen *Edwardsiella ictaluri*. *Virol J* **8**, 6.

Chai, T. J. & Foulds, J. (1978). Two bacteriophages which utilize a new *Escherichia coli* major outer membrane protein as part of their receptor. *J Bacteriol* **135**, 164–170.

Chamberlin, M., McGrath, J. & Waskell, L. (1970). New RNA polymerase from *Escherichia coli* infected with bacteriophage T7. *Nature* **228**, 227–231.

Charollais, J., Pflieger, D., Vinh, J., Dreyfus, M. & Iost, I. (2003). The DEAD-box RNA helicase SrmB is involved in the assembly of 50S ribosomal subunits in *Escherichia coli*. *Mol Microbiol* **48**, 1253–1265.

Charollais, J., Dreyfus, M. & Iost, I. (2004). CsdA, a cold-shock RNA helicase from *Escherichia coli*, is involved in the biogenesis of 50S ribosomal subunit. *Nucleic Acids Res* **32**, 2751–2759.

Chibeu, A., Ceyssens, P. J., Hertveldt, K., Volckaert, G., Cornelis, P., Matthijs, S. & Lavigne, R. (2009). The adsorption of *Pseudomonas aeruginosa* bacteriophage phiKMV is dependent on expression regulation of type IV pili genes. *FEMS Microbiol Lett* **296**, 210–218.

Cole, S. T., Chen-Schmeisser, U., Hindennach, I. & Henning, U. (1983). Apparent bacteriophage-binding region of an *Escherichia coli* K-12 outer membrane protein. *J Bacteriol* **153**, 581–587.

Datta, D. B., Arden, B. & Henning, U. (1977). Major proteins of the *Escherichia coli* outer cell envelope membrane as bacteriophage receptors. *J Bacteriol* **131**, 821–829.

DeLano, W. L. (2004). Use of PYMOL as a communications tool for molecular science. *Abstr Pap Am Chem Soc* **228**, U313–U314.

Dharmgrongartama, B., Mahadik, S. P. & Srinivasan, P. R. (1973). Modification of RNA polymerase after T3 phage infection of *Escherichia coli* B. *Proc Natl Acad Sci U S A* **70**, 2845–2849.

Drexler, K., Dannull, J., Hindennach, I., Mutschler, B. & Henning, U. (1991). Single mutations in a gene for a tail fiber component of an *Escherichia coli* phage can cause an extension from a protein to a carbohydrate as a receptor. *J Mol Biol* **219**, 655–663.

Dutzler, R., Rummel, G., Alberti, S., Hernández-Allés, S., Phale, P. S., Rosenbusch, J. P., Benedí, V. J. & Schirmer, T. (1999). Crystal structure and functional characterization of OmpK36, the osmoporin of *Klebsiella pneumoniae*. *Structure* **7**, 425–434.

Evans, T. J., Ind, A., Komitopoulou, E. & Salmond, G. P. C. (2010). Phage-selected lipopolysaccharide mutants of *Pectobacterium atrosepticum* exhibit different impacts on virulence. *J Appl Microbiol* **109**, 505–514.

Fineran, P. C., Blower, T. R., Foulds, I. J., Humphreys, D. P., Lilley, K. S. & Salmond, G. P. C. (2009). The phage abortive infection system, ToxIN, functions as a protein–RNA toxin–antitoxin pair. *Proc Natl Acad Sci U S A* **106**, 894–899.

Fortier, L. & Moineau, S. (2009). Phage Production and Maintenance of Stocks, Including Expected Stock Lifetimes. In *Bacteriophages: Methods and Protocols*, pp. 203–219. Edited by M. R. J. Clokie & A. M. Kropinski. New York: Humana Press.

Friedman, D. I., Olson, E. R., Georgopoulos, C., Tilly, K., Herskowitz, I. & Banuett, F. (1984). Interactions of bacteriophage and host

macromolecules in the growth of bacteriophage λ. *Microbiol Rev* **48**, 299–325.

Gehring, K., Charbit, A., Brissaud, E. & Hofnung, M. (1987). Bacteriophage λ receptor site on the *Escherichia coli* K-12 LamB protein. *J Bacteriol* **169**, 2103–2106.

Guttman, B., Raya, R. & Kutter, E. (2005). Basic phage biology. *In Bacteriophages Biology and Application*, pp. 29–66. Edited by E. Kutter & A. Sulakvelidze. Boca Raton, FL: CRC Press.

Hashemolhosseini, S., Holmes, Z., Mutschler, B. & Henning, U. (1994). Alterations of receptor specificities of coliphages of the T2 family. *J Mol Biol* **240**, 105–110.

Hawke, J. P., Mcwhorter, A. C., Steigerwalt, A. G. & Brenner, D. J. (1981). *Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish. *Int J Syst Bacteriol* **31**, 396–400.

Heine, H. G., Francis, G., Lee, K. S. & Ferenci, T. (1988). Genetic analysis of sequences in maltoporin that contribute to binding domains and pore structure. *J Bacteriol* **170**, 1730–1738.

Herrero, M., de Lorenzo, V. & Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J Bacteriol* **172**, 6557–6567.

Krüger, D. H. & Schroeder, C. (1981). Bacteriophage T3 and bacteriophage T7 virus–host cell interactions. *Microbiol Rev* **45**, 9–51.

Lenski, R. E. (1984). Two-step resistance by *Escherichia coli* B to bacteriophage T2. *Genetics* **107**, 1–7.

Lin-Chao, S., Chen, W. T. & Wong, T. T. (1992). High copy number of the pUC plasmid results from a Rom/Rop-suppressible point mutation in RNA II. *Mol Microbiol* **6**, 3385–3393.

Lindberg, A. A. (1973). Bacteriophage receptors. *Annu Rev Microbiol* **27**, 205–241.

Lundrigan, M. D. & Earhart, C. F. (1984). Gene *envY* of *Escherichia coli* K-12 affects thermoregulation of major porin expression. *J Bacteriol* **157**, 262–268.

Mark, D. F. & Richardson, C. C. (1976). *Escherichia coli* thioredoxin: a subunit of bacteriophage T7 DNA polymerase. *Proc Natl Acad Sci U S A* **73**, 780–784.

Maurer, J. K., Lawrence, M. L., Fernandez, D. H. & Thune, R. L. (2001). Evaluation and optimization of a DNA transfer system for *Edwardsiella ictaluri*. *J Aquat Anim Health* **13**, 163–167.

Maynard, N. D., Birch, E. W., Sanghvi, J. C., Chen, L., Gutschow, M. V. & Covert, M. W. (2010). A forward-genetic screen and dynamic analysis of lambda phage host-dependencies reveals an extensive interaction network and a new anti-viral strategy. *PLoS Genet* **6**, e1001017.

Menichi, B. & Buu, A. (1983). Integration of the overproduced bacteriophage T5 receptor protein in the outer membrane of *Escherichia coli*. *J Bacteriol* **154**, 130–138.

Miller, V. L. & Mekalanos, J. J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **170**, 2575–2583.

Moran, A. P., O'Malley, D. T., Kosunen, T. U. & Helander, I. M. (1994). Biochemical characterization of *Campylobacter fetus* lipopolysaccharides. *Infect Immun* **62**, 3922–3929.

Moreno, F. & Wandersman, C. (1980). OmpC and LamB proteins can serve as substitute receptors for host range mutants of coliphage TuIa. *J Bacteriol* **144**, 1182–1185.

Morona, R. & Henning, U. (1984). Host range mutants of bacteriophage Ox2 can use two different outer membrane proteins of *Escherichia coli* K-12 as receptors. *J Bacteriol* **159**, 579–582.

Ochman, H., Gerber, A. S. & Hartl, D. L. (1988). Genetic applications of an inverse polymerase chain reaction. *Genetics* **120**, 621–623.

Py, B., Higgins, C. F., Krisch, H. M. & Carpousis, A. J. (1996). A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. *Nature* **381**, 169–172.

Qimron, U., Marintcheva, B., Tabor, S. & Richardson, C. C. (2006). Genomewide screens for *Escherichia coli* genes affecting growth of T7 bacteriophage. *Proc Natl Acad Sci U S A* **103**, 19039–19044.

Rabsch, W., Ma, L., Wiley, G., Najar, F. Z., Kaserer, W., Schuerch, D. W., Klebba, J. E., Roe, B. A., Laverde Gomez, J. A. & other authors (2007). FepA- and TonB-dependent bacteriophage H8: receptor binding and genomic sequence. *J Bacteriol* **189**, 5658–5674.

Riede, I., Degen, M. & Henning, U. (1985). The receptor specificity of bacteriophages can be determined by a tail fiber modifying protein. *EMBO J* **4**, 2343–2346.

Roucourt, B. & Lavigne, R. (2009). The role of interactions between phage and bacterial proteins within the infected cell: a diverse and puzzling interactome. *Environ Microbiol* **11**, 2789–2805.

Russo, R., Panangala, V. S., Wood, R. R. & Klesius, P. H. (2009). Chemical and electroporated transformation of *Edwardsiella ictaluri* using three different plasmids. *FEMS Microbiol Lett* **298**, 105–110.

Santander, J. & Robeson, J. (2007). Phage-resistance of *Salmonella enterica* serovar Enteritidis and pathogenesis in *Caenorhabditis elegans* is mediated by the lipopolysaccharide. *Electron J Biotechnol* **10**, 627–632.

Schade, S. Z., Adler, J. & Ris, H. (1967). How bacteriophage χ attacks motile bacteria. *J Virol* **1**, 599–609.

Schneider, H., Fsihi, H., Kottwitz, B., Mygind, B. & Bremer, E. (1993). Identification of a segment of the *Escherichia coli* Tsx protein that functions as a bacteriophage receptor area. *J Bacteriol* **175**, 2809–2817.

Silverman, J. A. & Benson, S. A. (1987). Bacteriophage K20 requires both the OmpF porin and lipopolysaccharide for receptor function. *J Bacteriol* **169**, 4830–4833.

Simon, R., Priefer, U. & Puhler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Nat Biotechnol* **1**, 784–791.

Skurnik, M. & Zhang, L. (1996). Molecular genetics and biochemistry *of Yersinia* lipopolysaccharide. *APMIS* **104**, 849–872.

Skurnik, M., Venho, R., Toivanen, P. & al-Hendy, A. (1995). A novel locus of *Yersinia enterocolitica* serotype O:3 involved in lipopolysaccharide outer core biosynthesis. *Mol Microbiol* **17**, 575–594.

Sukupolvi, S. (1984). Role of lipopolysaccharide in the receptor function for bacteriophage Ox2. *FEMS Microbiol Lett* **21**, 83–87.

Thune, R. L., Fernandez, D. H., Benoit, J. L., Kelly-Smith, M., Rogge, M. L., Booth, N. J., Landry, C. A. & Bologna, R. A. (2007). Signature-tagged mutagenesis of *Edwardsiella ictaluri* identifies virulence-related genes, including a *Salmonella* pathogenicity island 2 class of type III secretion systems. *Appl Environ Microbiol* **73**, 7934–7946.

Walakira, J. K., Carrias, A. A., Hossain, M. J., Jones, E., Terhune, J. S. & Liles, M. R. (2008). Identification and characterization of bacteriophages specific to the catfish pathogen, *Edwardsiella ictaluri*. *J Appl Microbiol* **105**, 2133–2142.

Williams, M. L., Azadi, P. & Lawrence, M. L. (2003). Comparison of cellular and extracellular products expressed by virulent and attenuated strains of *Edwardsiella ictaluri*. *J Aquat Anim Health* **15**, 264–273.

Yu, F. & Mizushima, S. (1982). Roles of lipopolysaccharide and outer membrane protein OmpC of *Escherichia coli* K-12 in the receptor function for bacteriophage T4. *J Bacteriol* **151**, 718–722.

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