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Survival of uropathogenic *Escherichia coli* in the murine urinary tract is dependent on OmpR

William R. Schwan

Department of Microbiology, University of Wisconsin-La Crosse, 1725 State St., La Crosse, WI 54601, USA

Correspondence
William R. Schwan
schwan.will@uwlax.edu

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Uropathogenic *Escherichia coli* (UPEC) can grow in environments with significantly elevated osmolarities, such as murine and human urinary tracts. OmpR is the response regulator part of a two-component OmpR–EnvZ regulatory system that responds to osmotic stresses. To determine the role of OmpR in UPEC survival, a Δ*ompR* mutant was created in the UPEC clinical isolate NU149. The Δ*ompR* mutant had a growth defect compared with the wild-type strain under osmotic stress conditions; this defect was complemented by the full-length *ompR* gene on a plasmid, but not with a mutant OmpR with an alanine substitution for aspartic acid at the phosphorylation site at position 55. Furthermore, the Δ*ompR* mutant displayed up to 2-log reduction in bacterial cell numbers in murine bladders and kidneys compared with wild-type bacteria after 5 days of infection. The ability of the bacteria to survive was restored to wild-type levels when the Δ*ompR* mutant strain was complemented with wild-type *ompR*, but not when the alanine-substituted *ompR* gene was used. This study has fulfilled molecular Koch's postulates by showing the pivotal role OmpR plays in UPEC survival within the murine urinary tract.

Escherichia coli is a major cause of urinary tract infections (UTIs) in humans, resulting in billions of dollars in healthcare-related costs each year (Foxman & Brown, 2003; Litwin *et al.*, 2005). The subgroup responsible for these UTIs has been called uropathogenic *E. coli* (UPEC). For more than two decades, a murine UTI model that mimics UTIs in humans has been used to examine UPEC pathogenesis (Hagberg *et al.*, 1983; Schaeffer *et al.*, 1987). The murine urinary tract (Loeb & Quimby, 1989) has many similarities to the human urinary tract (Ross & Neely, 1983); these similarities include wide ranges in pH and osmolarity.

Several virulence factors have been associated with the ability of UPEC to survive in the urinary tract (Johnson, 1991). Type 1 pili, haemolysins and capsule production are used by UPEC strains causing human and murine UTIs. Molecular Koch's postulates (Falkow, 1988) have been applied to virulence factors expressed by UPEC; these factors include: type 1 pili (Connell *et al.*, 1996); the DegS and DegP proteins that regulate sigma E during extra-cytoplasmic stress (Redford & Welch, 2006; Redford *et al.*, 2003); the iron transport protein TonB (Torres *et al.*, 2001); PhoU, a negative regulator of the Pho regulon (Buckles *et al.*, 2006); the D-serine deaminase DsdA (Haugen *et al.*, 2007); and flagella (Lane *et al.*, 2007; Schwan, 2008; Wright *et al.*, 2005). Other virulence factors needed by the UPEC strains to maintain a UTI may be those involved in acid tolerance and osmotic stress survival.

To initiate and sustain a UTI, UPEC bacteria must be able to survive in environments with diverse pH values and wide fluctuations in the osmolarity. We have previously reported that the *fimB* and *fimE* genes are regulated by OmpR (Schwan *et al.*, 2002), which is a protein that is part of a two-component signal transduction system that serves as a global regulator within *E. coli* (Oshima *et al.*, 2002). Two-component regulatory systems generally consist of a response regulator, and a sensor kinase that spans the inner membrane. The EnvZ–OmpR tandem represents a two-component regulatory system that is affected by osmotic changes (Mizuno & Mizushima, 1990). During an osmotic shock, some unidentified signal linked to the osmolarity of the external environment triggers autophosphorylation of EnvZ at a conserved histidine residue, using ATP to donate the phosphate group (Forst *et al.*, 1989; Igo & Silhavy, 1988). Once EnvZ is phosphorylated, EnvZ-P donates the phosphate group to OmpR at a conserved aspartic acid residue at position 55 (Forst *et al.*, 1989). OmpR-P can then serve as a transcriptional activator or repressor of various genes within the *E. coli* cell.

In this study, we deleted the *ompR* gene from strain NU149, which is a UPEC clinical isolate (Schaeffer *et al.*, 1987), and examined what effect this had on the survival of the isolate *in vitro*, as well as within a murine UTI model. We demonstrate that, compared with the wild-type, a Δ*ompR* mutation leads to: (i) a growth defect in media with osmolyte concentrations greater than or equal to 400 mM, (ii) attenuated growth in human urine, and (iii) a reduction of 2 logs in viable counts within murine urinary tracts. These results suggest that OmpR is critical for UPEC survival in mammalian urinary tracts.

METHODS

Bacterial strains and plasmids. The UPEC strain NU149 was used in this study. It has been well characterized, and used extensively in a murine UTI model (Schaeffer *et al.*, 1987; Schwan *et al.*, 2002). Plasmids pFR29* (Russo & Silhavy, 1991) and pD55A (V. K. Tran & L. J. Kenney, unpublished data) were a kind gift from Linda Kenney, the University of Illinois at Chicago, Chicago, IL, USA. The pFR29* plasmid has the full-length *ompR* gene cloned in front of an IPTG inducible promoter, whereas the pD55A plasmid has a mutated *ompR* gene, resulting in an alanine substituting for aspartic acid at amino acid position 55 cloned in front of an IPTG-inducible promoter. Strain DH5α MCR was used for transformations. Luria–Bertani (LB) broth and LB agar were used with the addition of the following antibiotics: kanamycin (40 μg ml^{-1}), or ampicillin (100 μg ml^{-1}) (Sigma). The λ Red recombinase system, with plasmids pKD4 (used for amplifying a kanamycin-resistance gene with flanking gene sequences to allow recombination and creation of the deletion mutation), pKD46 (containing the λ Red recombinase), and pCP20 (carrying a Flp recombinase to allow excision of the kanamycin-resistance gene once the deletion mutation had been created), was used for construction of the Δ*ompR* mutation, as described by Datsenko & Wanner (2000). We have previously used this system successfully to create a *fliC* mutation in strain NU149 (Schwan, 2008).

Construction of the Δ*ompR* mutant strain in a UPEC clinical isolate. The λ Red recombinase system (Datsenko & Wanner, 2000) was used to construct the Δ*ompR* mutation in the UPEC strain NU149. Primers OmpR1 (5′-ACGCTTACAAATTGTTGCGAACCT-TTGGGAGTACAAACATGTGTAGGCTGGAGCTGCTTCG-3′) and OmpR2 (5′-GGGCAAATGAACTTCGTGGCGAGAAGCGCAATCG-CCTCATCATATGAATATCCTCCTTAG-3′) were synthesized (IDT DNA Technologies), and used in PCR amplifications, with pKD4 plasmid DNA as a template, under the following conditions: initial denaturation at 95 °C for 5 min, then 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. The resulting amplified DNA was concentrated with a Microcon 30 filter, gel purified on a 0.8 % agarose gel, and then electroporated into competent NU149 cells, as described previously (Schwan, 2008), selecting for transformants on LB agar with kanamycin. Several transformants were screened for the lack of *ompR* gene amplification using a PCR-based system with the OmpR3 (5′-TGCTGACTCGTGAATCTTTCC-3′) and OmpR4 (5′-CGTACCGAGGTTAAGTTTGAA-3′) primer pair (319 bp product). The conditions for the PCR amplifications were as follows: initial denaturation 95 °C for 5 min, 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. One transformant, NU149 OmpR, which did not amplify a 319 bp *ompR* gene fragment, was chosen for further analysis. Removal of the kanamycin-resistance gene in strain NU149 OmpR using the pCP20 plasmid was done as noted (Datsenko & Wanner, 2000). Transformants were selected on LB agar containing 40 μg kanamycin ml^{-1}. One clone was heated to 42 °C to cure the strain of the pKD46 plasmid. The resulting UPEC strain was labelled NU149 OmpR1. To complement the strain NU149 OmpR1, electroporation of either the pFR29* or the pD55A plasmid was performed, and transformants were selected on LB agar containing 100 μg ampicillin ml^{-1}. One clone representing a successful transformation with each plasmid was chosen for additional analysis.

Growth in buffered LB broth with differences in osmolarity and pH. To test the strains under different *in vitro* environmental conditions, the pH of LB medium was adjusted by using 0.1 M sodium phosphate buffer combined with 1 % (v/v) glycerol, as previously described (Schwan *et al.*, 2002). The osmolarity of LB broth was also adjusted, as previously noted (Schwan *et al.*, 2002), using urea, sucrose or NaCl. The broths were labelled low osmolarity ($\leqslant$200 mM NaCl), moderate osmolarity (400 mM NaCl), and high osmolarity ($\geqslant$600 mM NaCl). OD_{600} readings were done after incubation for 24 h.

RT-PCR analysis. To assess whether there was a difference in *envZ* expression in the wild-type versus the *ompR* strain, a RT-PCR procedure was used. Total RNAs were isolated from wild-type and *ompR* strains grown in pH 7.0 LB broth, with and without the addition of 400 mM NaCl, to mid-exponential phase, using an RNeasy kit (Qiagen) with the addition of a 10 μl volume of 10 mg lysozyme ml^{-1}. After elution, nucleic acid concentrations were determined by spectrophotometry (Shimatzu). The RNAs were treated with RNase-free DNase (Roche) for 30 min at 37 °C to remove residual contaminating DNA. A 6 μg quantity of total RNA was used to create cDNAs using the SuperScript II reverse transcriptase kit (Invitrogen). All of the cDNAs were PCR amplified in a multiplex amplification using the EnvZ1 (5′-ACCACCATATTCGCCACCGC-3′) and EnvZ2 (5′-CTGCGTGAATATGGCGCTTC-3′) primer pair to amplify *envZ* cDNA, and the EcFtsZ1 and EcFtsZ2 primer pair that has been used to amplify *ftsZ* (Schwan *et al.*, 2002, 2007). Amplification conditions included an initial denaturation at 95 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The PCR products were loaded on 1.5 % agarose gels. Expression of the *envZ* gene was determined by comparing the expression of the *envZ* product to the *ftsZ* product, using ImageQuant software (Molecular Dynamics).

Protein extraction and immunoblotting. SDS-PAGE was performed with 12.5 % separating gels, and 20 μg *E. coli* cell lysates of NU149, NU149 OmpR1, NU149 OmpR1/pFR29* and NU149 OmpR1/pD55A grown in pH 7.0 LB broth. The lysates were prepared by suspending stationary-phase cultures in cell lysis buffer, as described (Malitschek *et al.*, 1994), and incubating with a 10 μl volume of 10 mg lysozyme ml^{-1} for 30 min at 37 °C, followed by sonication. Proteins were electrotransferred to nylon membranes (Amersham). Membranes were blocked with blocking buffer [1 % dried milk and 0.1 % BSA (Sigma) in Tris-buffered saline containing 0.05 % Tween 20 (TBS-T)] for 2 h, washed with TBS-T, and then allowed to react with a rabbit polyclonal antibody to OmpR protein (1/500 dilution; provided by Linda Kenney) for 2 h at room temperature. Membranes were washed three times with TBS-T, and horseradish-peroxidase-conjugated anti-rabbit antibody (Amersham) was added at a 1/10 000 dilution, and the membranes were incubated at room temperature for 1 h. After several more washes, bound antibodies were detected by the enhanced chemiluminescence system (Amersham).

Murine UTI model. A murine UTI model was used to assess the role the *ompR* mutation may play in *E. coli* cells infecting murine urinary tracts (Schaeffer *et al.*, 1987). Mid-exponential-phase bacteria at a concentration of 10^8 c.f.u. ml^{-1} in PBS were used as the inocula. Female Swiss Webster mice were transurethrally inoculated with either 50 μl per mouse of each *E. coli* strain to mimic the ascending model (total of five mice per strain), or 200 μl bacteria per mouse to allow infection of more murine kidneys (total of 8–12 mice per strain). For the two injection schemes, the Δ*ompR* mutant (strain NU149 OmpR1), the complemented *ompR* mutant with the native OmpR (strain NU149 OmpR1/pFR29*), the complemented *ompR* mutant with the mutated OmpR (strain NU149 OmpR1/pD55A), and wild-type NU149 cells, were used. For the 50 μl injection scheme, mouse organs were collected after 24 h; for the 200 μl injection volume, each organ was collected 1–5 days post-inoculation. Mouse urine was collected before the mouse was killed, and the pH and specific gravity were measured for each sample. Mouse organs were processed by adding PBS (pH 7.4) to each organ. The volume of PBS for each bladder was adjusted based on the mass of each organ, in order to standardize the bladder preparations between mice. Since there was a difference of $<$10 % in kidney masses, a 1 ml volume of

PBS was used for processing kidneys, and no compensation was necessary. The organs were homogenized with a tissue grinder, aliquots were 10-fold serially diluted in PBS, and 100 µl aliquots plated onto LB agar to ascertain viable counts. Both kidneys from an animal were collected at the appropriate time point, and they were processed together as one sample.

Growth in human urine. Two human urine specimens were collected to perform growth measurements. The pH of each specimen was measured using a pH meter (Orion), and the osmolarity was determined by using specific gravity measurements and freezing point depression (Ross & Neely, 1983).

Statistics. To determine differences in *in vitro* growth, Student's *t* test was used. With the murine UTI model results, an ANOVA, with a Bonferroni correction, was used to assess probability. *P* values <0.05 were considered significant.

RESULTS

Creation of a Δ*ompR* mutation in the UPEC strain NU149

A λ Red recombinase system (Datsenko & Wanner, 2000) was used to create a near-complete deletion mutation in the clinical isolate NU149. Because there is a 3 bp overlap between the *ompR* and *envZ* genes, this part of the *ompR* gene was not deleted to allow proper transcription of the *envZ* gene. The final deletion mutant, named NU149 OmpR1, was confirmed by PCR to be missing the *ompR* gene (data not shown). In addition, the mutant strain was shown to have no polar effect on *envZ* transcription using RT-PCR on cDNA generated from broth-grown cultures of NU149 OmpR1 and NU149 (Fig. 1).

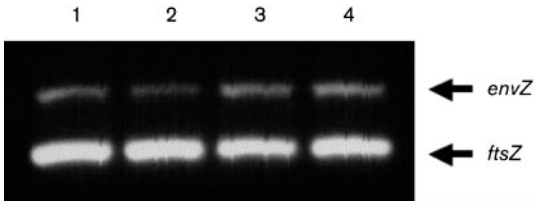

Fig. 1. Transcriptional analysis of *envZ* expression in wild-type and *ompR* strains. The analysis was done on cDNAs from cultures grown in pH 7.0 LB broth, with either no added NaCl or 400 mM added NaCl. Multiplex PCRs were set up with EnvZ1 and EnvZ2 primers to amplify a 424 bp *envZ* product, and EcFtsZ1 and EcFtsZ2 primers to amplify a 302 bp *ftsZ* product (Schwan *et al.*, 2002). Each multiplex was run three separate times. The lanes were loaded as follows: 1, NU149 (pH 7.0); 2, NU149 OmpR1 (pH 7.0); 3, NU149 (pH 7.0, 400 mM NaCl); and 4, NU149 OmpR1 (pH 7.0, 400 mM NaCl). Quantification of the data was done using ImageQuant software (Molecular Dynamics), and the number of pixels for each band was quantified. For each lane, the intensities of the *envZ* product were corrected to the intensity of the *ftsZ* band.

Confirmation of OmpR expression in the *ompR* strain transformed with pFR29* or pD55A

To demonstrate that the *ompR* strain transformed with either pFR29* or pD55A expressed OmpR protein, a Western blot was performed on lysates from NU149, NU149 OmpR1, NU149 OmpR1/pFR29* and NU149 OmpR1/pD55A grown in pH 7.0 LB broth. Strain NU149 OmpR1 did not display an OmpR protein band, whereas the wild-type strain and both plasmid-complemented strains showed an OmpR protein band on the blot (data not shown).

Growth of the *ompR* mutant strain in buffered LB broth, with differences in osmolarity and pH

Because OmpR is important in osmotically stressed *E. coli* cells, the *ompR* strain was compared with the wild-type strain grown in pH 7.0 buffered LB broth, with variations in the osmolarity. The *ompR* strain grew better than wild-type bacteria in medium with no added NaCl, and in medium with 200 mM added NaCl (Fig. 2a). However, the *ompR* strain was significantly attenuated for growth in medium supplemented with 400 mM NaCl (OD_{600} 0.38; $P<0.003$). In medium with 600 mM (OD_{600} 0.05; $P<0.01$) and 800 mM NaCl (OD_{600} 0.05; $P<0.03$), the *ompR* strain failed to grow, whereas the wild-type strain grew. Standard plate counts on selected broth samples following growth confirmed the lower viable bacterial counts for the *ompR* strain compared with the wild-type (data not shown). When the *ompR* strain was complemented with the native *ompR* gene, bacterial growth resembled that of the wild-type strain. On the other hand, complementation with the mutated *ompR* gene with an amino acid substitution at position 55 produced growth measurements similar to those of the *ompR* strain.

Growth in medium with variations in the concentration of urea was also tested. In LB broth with no added urea, all four strains had similar OD_{600} readings (Fig. 2b). When the osmolyte concentration was increased to 200 mM urea, the growth of the *ompR* strain was lower (OD_{600} 1.269) compared with the wild-type (OD_{600} 1.397; $P<0.042$). By raising the osmolarity to 400 mM, the *ompR* strain grew less well (OD_{600} 0.826) compared with the wild-type (OD_{600} 1.204; $P<0.035$). A further increase in the osmolarity caused a wider separation in the growth of the two strains (OD_{600}: *ompR* 0.308 versus wild-type 0.875; $P<0.009$). When growth was compared at the highest urea osmolarity, a greater difference in growth was noted between the mutant and the wild-type strain (OD_{600}: *ompR* 0.171 versus wild-type 0.586; $P<0.006$). Complementation of the *ompR* cells with the pFR29* plasmid resulted in OD_{600} readings similar to those of the wild-type, in all concentrations of urea, whereas complementation with pD55A failed to restore the growth of the *ompR* strain to wild-type levels.

A third osmolyte, sucrose, was then tested for its effect on growth of the four strains. Little growth difference was observed between the four strains grown in LB with no

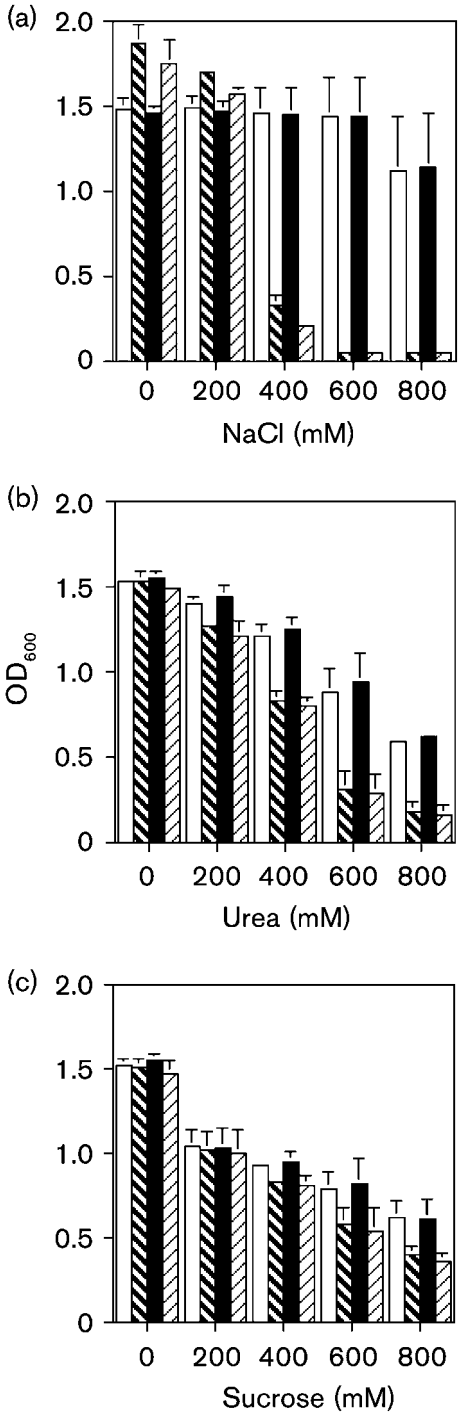

Fig. 2. Effects of osmolarity on bacterial growth of wild-type cells (NU149, open bar), an *ompR* mutant (NU149 OmpR1, bold hatched bar), an *ompR* mutant complemented with the native *ompR* gene (NU149 OmpR1/pFR29*, filled bar), and an *ompR* mutant complemented with a mutated *ompR* gene (NU149 OmpR1/pD55A, hatched bar). Osmolarity effects were tested by using (a) NaCl, (b) urea and (c) sucrose as the osmolytes, at concentrations ranging from 0 to 800 mM. Growth was measured as the OD at 600 nm, and the values represent the means (±SD) from at least three separate runs.

added sucrose or when 200 mM sucrose was added (Fig. 2c). In LB with 400 mM sucrose, the *ompR* strain grew less well than the wild-type (OD_{600} 0.829 versus 0.926, respectively), but the decrease was not significant ($P<0.112$). An increase in the sucrose concentration to 600 mM resulted in an even lower OD_{600} reading for ompR (OD_{600} 0.574) compared with the wild-type (OD_{600} 0.781); the difference was significant ($P<0.007$). Growth of the *ompR* strain in LB broth containing the highest sucrose concentration showed an OD_{600} of 0.392 compared with 0.611 for the wild-type ($P<0.017$). Complementation with the native *ompR* gene fully restored the growth of the *ompR* strain, but complementation with the mutated *ompR* gene did not. Thus, all of the osmolytes that were tested affected growth of the *ompR* strain at a high concentration.

To further assess how a combination of changes in pH and differences in osmolarity affected *in vitro* growth, the *ompR* strain was grown in pH 5.5 and pH 7.0 LB broth, with and without the addition of 400 mM NaCl. The *ompR* strain grew less well in the pH 5.5 (OD_{600} 0.35; $P<0.0002$) and pH 7.0 broths (OD_{600} 0.56; $P<0.00002$) supplemented with 400 mM NaCl compared with the wild-type strain grown in pH 5.5 LB containing 400 mM NaCl (OD_{600} 1.79) or pH 7.0 medium (OD_{600} 1.75) (Fig. 3). Growth was restored to wild-type levels by complementing with the native *ompR* in pH 5.5 LB broth with 400 mM NaCl (OD_{600} 1.46), and in pH 7.0 LB broth with 400 mM NaCl (OD_{600} 1.63); wild-type levels of growth were not restored by complementing with the mutated *ompR* gene (pH 5.5 LB broth with NaCl, OD_{600} 0.35; pH 7.0 LB broth with

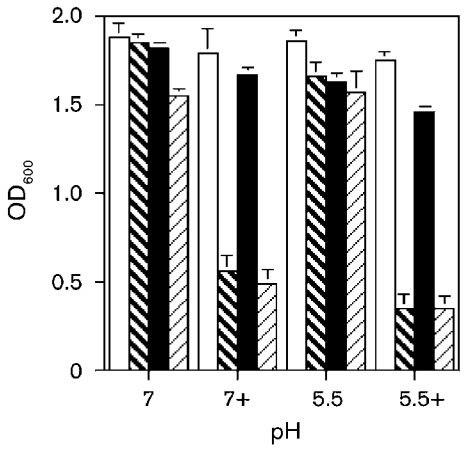

Fig. 3. Effects of a combination of osmolarity and pH on bacterial growth of wild-type cells (NU149, open column), an *ompR* mutant (NU149 OmpR1, bold hatched bar), an *ompR* mutant complemented with the native *ompR* gene (NU149 OmpR1/pFR29*, filled bar), and an *ompR* mutant complemented with a mutated *ompR* gene (NU149 OmpR1/pD55A, hatched bar). Growth at pH 5.5 and pH 7.0 with 400 mM NaCl (+) and without NaCl was measured at an OD of 600 nm, and the values represent the means (±SD) from at least three separate runs.

NaCl, OD_{600} 0.49). These results demonstrate that the *ompR* mutation causes an *in vitro* growth defect in UPEC cells grown in moderate-to-high osmolarity media.

Growth in human urine was affected by the *ompR* mutation

Since there was a difference in growth of the *ompR* strain compared with the wild-type bacteria in medium with either moderate or high osmolarity, the strains were tested for their ability to grow in human urine. Two human urine samples were collected. Urine no. 1 had a pH of 5.98, and an osmolarity of 300 mM, whereas urine no. 2 had a pH of 6.07, and an osmolarity of 400 mM. The four strains described above were inoculated into aliquots of the human urine, and incubated for 18 h at 37 °C. All of the strains grew in both urine samples. However, the *ompR* strain did not grow as well as the wild-type strain. The differences in growth were not significant for the first urine specimen [mean ($\pm$SD) OD_{600}: *ompR* 0.400 ± 0.081 versus wild-type 0.529 ± 0.051; $P<0.105$], but they were significant for urine no. 2 (*ompR* 0.300 ± 0.03 versus wild-type 4.84 ± 0.012; $P<0.045$). Complementation of the *ompR* strain with pFR29* restored the growth to wild-type levels in both urine samples (urine no. 1, 0.567 ± 0.047; urine no. 2, 0.510 ± 0.054), yet complementation with the pD55A plasmid did not restore the growth (urine no. 1, 0.417 ± 0.015; urine no. 2, 0.294 ± 0.03). These results show that *ompR* mutation is associated with a growth defect in human urine.

Murine urinary tract survival difference is associated with *ompR* mutation in UPEC

From the *in vitro* analysis, it was clear that the *ompR* strain had a growth deficiency when placed in medium with a moderate-to-high osmolarity. To assess whether this *ompR* mutation caused a similar growth defect in UPEC within the murine urinary tract, the wild-type strain NU149, the *ompR* strain NU149 OmpR1, and NU149 OmpR1/pFR29* and NU149 OmpR1/pD55A strains, were inoculated into the urinary tracts of female mice using a low-volume (50 µl) or a high-volume (200 µl) inoculum. The mouse urine samples had pH values that ranged from 5.5 to 7.0, and osmolarities that ranged from 350 to 600 mM.

For the low-volume inoculations testing true urinary tract ascension, 4/5 wild-type infected mice had kidneys that were infected. Only 2/5 *ompR*-infected mice had colonized kidneys. Complementation of the *ompR* strain with pFR29* resulted in 4/5 mice with infected kidneys, but complementation with pD55A led to 2/5 mice having infected kidneys.

Using a more aggressive UTI model with a larger volume to ensure that a greater number of murine kidneys would be infected, the viable counts were determined for each group after 1, 3 and 5 days post-inoculation. One day post-inoculation, the *ompR* strain (2.06×10^3 c.f.u. ml^{-1}, Fig. 4a) displayed a decline of nearly 2 logs in the mean viable count from the bladder as compared with the wild-type strain (1.27×10^5 c.f.u. ml^{-1}, $P<0.015$). However, there was no statistical difference when comparing the viable counts in the kidneys from either the *ompR* strain (1.28×10^3 c.f.u. ml^{-1}) or the wild-type strain (2.58×10^3 c.f.u. ml^{-1}, $P>0.400$) (Fig. 4b). Complementation with the native *ompR* gene restored the viable counts to wild-type levels in the bladder (2.57×10^5 c.f.u. ml^{-1}), but complementation with the mutated *ompR* gene did not.

The 3 day post-inoculation data also showed a statistical difference between the *ompR* strain (4.25×10^2 c.f.u. ml^{-1}) and the wild-type strain (7.2×10^3 c.f.u. ml^{-1}; $P<0.012$) in the murine bladder (Fig. 4c). Moreover, the mean viable count of NU149 OmpR1 in the kidneys (1.03×10^3 c.f.u. ml^{-1}) was approximately 1.8 logs lower than for the wild-type strain NU149 (8.15×10^4 c.f.u. ml^{-1}; $P<0.006$) (Fig. 4d). Again, complementation with the native *ompR* gene resulted in mean viable counts from the bladders and kidneys that were comparable with those of the wild-type strain, whereas viable counts remained unchanged for the *ompR* strain that was complemented with the mutated *ompR* gene.

For the 5 day post-inoculation time point, the mean viable count for the bladder homogenates from the *ompR* strain infected mice (3.05×10^2 c.f.u. ml^{-1}) was 1 log less than for the wild-type strain (3.3×10^3 c.f.u. ml^{-1}, $P<0.009$) (Fig. 4e). Within the kidneys, day 5 viable count for the wild-type (6.4×10^5 c.f.u. ml^{-1}) was more than 3 logs higher than for the *ompR* strain (2.00×10^2 c.f.u. ml^{-1}; $P<0.05$) (Fig. 4f). From day 1 to day 5, the mean viable count for strain NU149 in the kidneys rose by approximately 2 logs, whereas the *ompR* strain displayed a 1 log reduction in the mean viable count in this organ system over the same time period. Successful complementation of the *ompR* mutation occurred with the native *ompR*, but not with the mutated *ompR* gene. The analyses demonstrated that there was a distinct survival advantage for the wild-type strain over the *ompR* strain in the murine urinary tract.

DISCUSSION

UPEC survival in the murine or human urinary tract is dependent on many variables, including the ability to withstand variations in osmolarity that can reach as high as 800 mM in humans, and 1.5 M in mice (Loeb & Quimby, 1989; Schmidt-Nielsen *et al.*, 1983). Our results show that OmpR is critical for the survival of UPEC within a murine urinary tract. We demonstrated that a UPEC clinical isolate with an *ompR* deletion was severely crippled in its ability to grow *in vitro* in medium with moderate-to-high osmolarity as compared with the wild-type parental strain. Growth was attenuated for the NU149 OmpR1 strain in moderate-osmolarity medium (400 mM NaCl) for two out of the three osmolytes tested, and either no growth was seen when

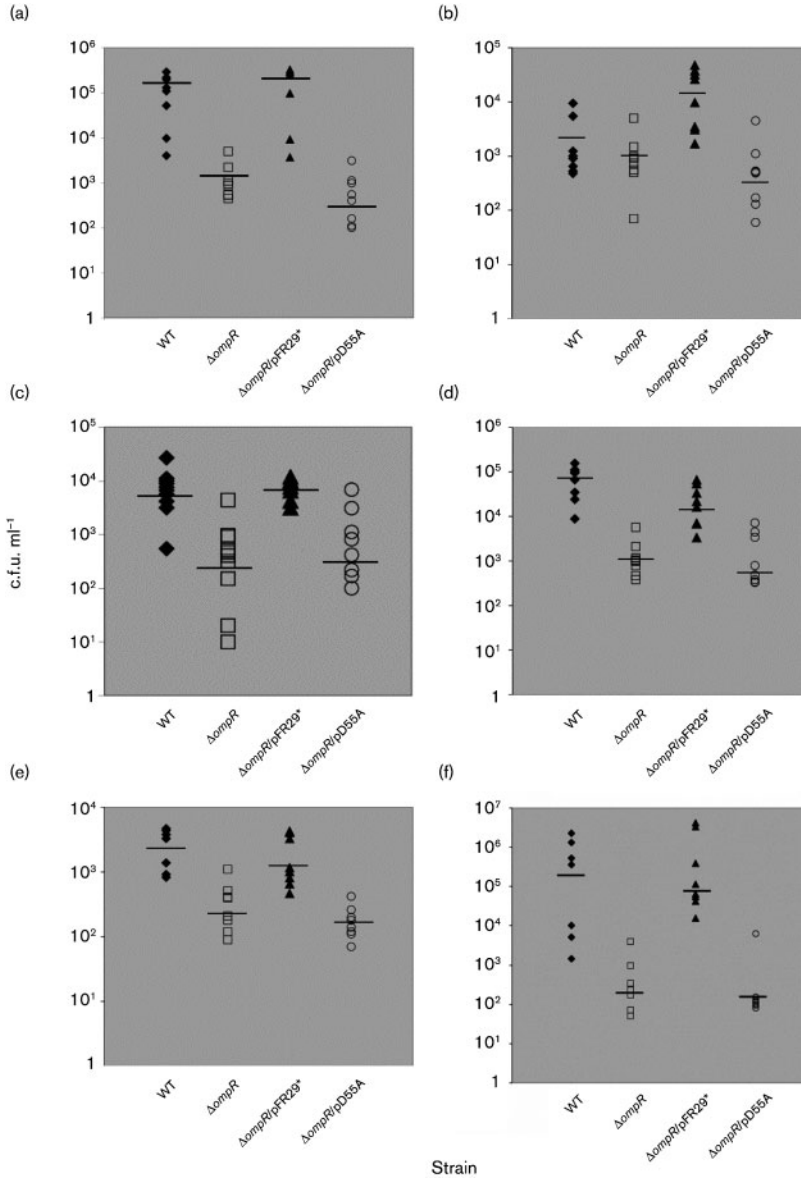

Fig. 4. Independent challenges of female BALB/c mice with *E. coli* strain NU149 (WT, ◆), an *ompR* mutant (NU149 OmpR1, □), an *ompR* mutant strain complemented with the native *ompR* gene (NU149 OmpR1/pFR29*, ▲), and an *ompR* mutant strain complemented with a mutated *ompR* gene (NU149 OmpR1/pD55A, ○). Bacterial counts are shown for: (a) the bladder, 1 day post-inoculation; (b) the kidney, 1 day post-inoculation; (c) the bladder, 3 days post-inoculation; (d) the kidney, 3 days post-inoculation; (e) the bladder, 5 days post-inoculation; and (f) the kidney, 5 days post-inoculation. Each data point represents the c.f.u. ml^{-1} for one mouse. Horizontal bars represent the median values of the bacterial concentration of the population.

the osmolarity was increased with the addition of 600 or 800 mM NaCl (high osmolarity), or the growth was substantially reduced in medium with high concentrations of sucrose or urea. This growth defect was successfully complemented by using the native *ompR* gene on a plasmid, but not using the mutated *ompR* gene that resulted in an amino acid substitution at position 55 in the OmpR protein. The change from an aspartic acid to an alanine would negate the transfer of a phosphate group from EnvZ (Delgado *et al.*, 1993), resulting in a potentially unphosphorylated OmpR protein that would be unable to transcriptionally regulate a number of genes within the *E. coli* cells when the bacteria were grown in an osmotically stressed environment. The phosphorylation of OmpR, in turn, regulates a number of genes within *E. coli* (Oshima *et al.*, 2002).

Medium with moderate-to-high osmolarity supported the growth of the *ompR* strain to a lesser extent than it supported growth of the wild-type strain. When moderate osmolarity was combined with an acidic pH medium, an additional growth reduction was shown for the *ompR* strain. Why are acidity and osmolarity combined important? The two human urine samples had acidic pH values and moderate osmolarities. All of the murine urine samples had moderate-to-high osmolarity, and most were acidic. Most human urine samples are in the acidic range (Ross & Neely, 1983; and personal observations). Certainly, acid conditions combined with moderate osmotic conditions adversely affected growth of the *ompR* strain more so than they affected growth of the strain in neutral pH medium. Some genes that are regulated by OmpR may play an important role in survival of the bacteria in an acidic/high

osmotic environment. In *Helicobacter pylori*, an OmpR-like protein regulates the acid-adaptation response (Bury-Moné *et al.*, 2004). OmpR is one of the proteins upregulated by acid adaptation in enterohaemorrhagic *E. coli* (Huang *et al.*, 2007), so there could be a function for OmpR in UPEC growing in an acidified human urinary tract.

In vitro growth attenuation by the *ompR* strain was also observed when the strain was inoculated into murine urinary tracts. Over a 5 day period of time, viable counts for strain NU149 OmpR1 fell by 1 log in the bladders and kidneys, and this was presumably due to the sensitivity to the high osmotic conditions found in the urinary tract. In contrast, the wild-type strain viable counts in the kidneys rose by over 2 logs. Complementation of the *ompR* mutation with the native *ompR* gene enabled the UPEC to grow nearly as well as wild-type bacteria under conditions of high osmolarity conditions; this fulfilled molecular Koch's postulates for the UPEC *ompR* gene (Falkow, 1988).

Why is the *ompR* mutation so crippling for UPEC? In terms of overall fitness, one study has shown that an *ompR* strain grows less well compared with wild-type *E. coli* in salt water (Darcan *et al.*, 2003). The poor growth in an osmotically stressed environment is likely to be related to OmpR being a global regulator that affects scores of genes within *E. coli* (Oshima *et al.*, 2002). Mutations in *ompR* have been shown to cause changes in the outer-membrane-protein profile within *E. coli* (Forst & Roberts, 1994; Pratt & Silhavy, 1995; Prohinar *et al.*, 2002; Rampersaud *et al.*, 1991). Under an osmotic stress, such as being dropped into a mammalian urinary tract, the bacteria change their outer-membrane proteins (Alteri & Mobley, 2007; Hagan & Mobley, 2007). The loss of OmpR would prevent those changes from occurring. Moreover, OmpR affects expression of surface structures such as type 1 pili (Schwan *et al.*, 2002), curli (Jubelin *et al.*, 2005; May & Okabe, 2008; Römling *et al.*, 1998) and flagella (Shin & Park, 1995). Changes in expression of the surface structures could also have an impact on the ability of UPEC to survive in a mouse or a human urinary tract. In addition, OmpR appears to regulate expression of enterochelin and the aerobactin receptor protein IutA (Oshima *et al.*, 2002; Wooldridge & Williams, 1991). Iron acquisition is critical for UPEC survival in the urinary tract. Alteri & Mobley (2007) demonstrated that IutA expression was induced when the bacteria were grown in human urine. IutA is expressed by UPEC, but not by laboratory strains of *E. coli*. Thus, an *ompR* deletion would have an impact on outer-membrane-protein expression, which is a trait shared by all *E. coli* cells, and it would also affect the expression of virulence factors, such as IutA, that are expressed by UPEC, and are critical for survival in either a murine or a human urinary tract.

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