

OmpR regulation of the uropathogenic *Escherichia coli* *fimB* gene in an acidic/high osmolality environment

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Uropathogenic *Escherichia coli* (UPEC) causes more than 90% of all human urinary tract infections through type 1 piliated UPEC cells binding to bladder epithelial cells. The FimB and FimE site-specific recombinases orient the *fimS* element containing the *fimA* structural gene promoter. Regulation of *fimB* and *fimE* depends on environmental pH and osmolality. The EnvZ/OmpR two-component system affects osmoregulation in *E. coli*. To ascertain if OmpR directly regulated the *fimB* gene promoters, gel mobility shift and DNase I footprinting experiments were performed using OmpR or phosphorylated OmpR (OmpR-P) mixed with the *fimB* promoter regions of UPEC strain NU149. Both OmpR-P and OmpR bound weakly to one *fimB* promoter. Because there was weak binding to one *fimB* promoter, strain NU149 was grown in different pH and osmolality environments, and total RNAs were extracted from each population and converted to cDNAs. Quantitative reverse-transcriptase PCR showed no differences in *ompR* transcription among the different growth conditions. Conversely, Western blots showed a significant increase in OmpR protein in UPEC cells grown in a combined low pH/high osmolality environment versus a neutral pH/high osmolality environment. In a high osmolality environment, the *ompR* mutant expressed more *fimB* transcripts and Phase-ON positioning of the *fimS* element as well as higher type 1 pili levels than wild-type cells. Together these results suggest that OmpR may be post-transcriptionally regulated in UPEC cells growing in a low pH/high osmolality environment, which regulates *fimB* in UPEC.

Received 20 March 2012

Revised 29 August 2012

Accepted 15 November 2012

INTRODUCTION

More than 90% of the 7 million human urinary tract infections occurring in the United States annually (Foxman *et al.*, 2000; Hooton & Stamm, 1997) are caused by uropathogenic *Escherichia coli* (UPEC) at a cost of \$1.6 billion per year (Foxman, 2003; Hooton & Stamm, 1997). The ability of UPEC to bind to bladder epithelial cells is an important step in pathogenesis (Keith *et al.*, 1986; van der Bosch *et al.*, 1980; Virkola *et al.*, 1988). Once the UPEC attach, they are able to grow in human urine with extreme fluctuations in both pH (5.0–8.0) and osmolality (50–1400 mOsm kg⁻¹) (Kunin & Chambers, 1989; Ross & Neely, 1983).

Type 1 pili are the major adherence factor for UPEC in the lower urinary tract (Abraham *et al.*, 1985a; Hultgren *et al.*,

1985; Klemm, 1986; Ofek & Beachey, 1978; Schaeffer *et al.*, 1979, 1987; van der Bosch *et al.*, 1980), and expression is affected by phase variation that allows switching from a fully piliated state (Phase-ON) to a non-piliated state (Phase-OFF). This process involves the inversion of a 314 bp region of DNA (called *fimS*), containing the promoter for the main structural subunit *fimA* (Abraham *et al.*, 1985b).

The *fimB* and *fimE* genes located upstream of the *fimA* gene code for site-specific recombinases that position the *fimS* invertible element, controlling type 1 pili phase variation (Blomfield *et al.*, 1991; Freitag *et al.*, 1985; Klemm, 1986; McClain *et al.*, 1991, 1993; Pallesen *et al.*, 1989). The FimB protein flips the invertible element in both the Phase-ON and the Phase-OFF orientations (McClain *et al.*, 1991), whereas the FimE protein predominantly rearranges the *fimS* region in the Phase-OFF orientation (Pallesen *et al.*, 1989; Stentebjerg-Olesen *et al.*, 2000). One promoter has been identified for *fimE* (Olsen & Klemm, 1994), whereas 1–3 *fimB* promoters have

Abbreviations: HA, haemagglutination; OmpR-P, phosphorylated OmpR; UPEC, uropathogenic *Escherichia coli*.

One supplementary table and two supplementary figures are available with the online version of this paper.

been identified (Donato *et al.*, 1997; El-Labany *et al.*, 2003; Olsen & Klemm, 1994; Schwan *et al.*, 1994).

Previously, high osmolality and low pH were shown to reduce type 1 fimbriae expression in UPEC in part by repressing *fimB* expression and flipping the *fimS* region to a Phase-OFF orientation (Schwan *et al.*, 2002). An *ompR* mutant strain derepressed *fimB* expression in an acidic/high osmolality environment, suggesting that OmpR may be directly regulating *fimB* transcription in this environment.

OmpR is part of the EnvZ–OmpR two-component regulatory system that is affected by changes in osmolalities. Two-component systems have a histidine kinase (e.g. EnvZ) and a response regulator (e.g. OmpR). The EnvZ sensor protein monitors environmental osmotic changes. Under hypoosmotic conditions, EnvZ is autophosphorylated using ATP as its phosphoryl donor at the highly conserved histidine residue 243 located in the bacterial cytoplasm (Forst *et al.*, 1989; Igo & Silhavy, 1988; Kenney *et al.*, 1995). The OmpR response regulator is an autokinase that now uses the phosphorylated EnvZ histidine kinase as its phosphoryl donor to transfer the phosphate to aspartate residue 55 on the cytoplasmic OmpR protein (OmpR-P). OmpR-P then binds to a promoter region to either activate or repress transcription (Delgado *et al.*, 1993; Forst *et al.*, 1989; Mattison & Kenney, 2002; Rampersaud *et al.*, 1994). In high osmotic stress environments, phosphorylation of OmpR by acetyl phosphate (Liu & Ferenci, 2001; Matsubara & Mizuno, 1999; McCleary & Stock, 1994; Prohinar *et al.*, 2002; Wolfe, 2005) or alternative histidine kinase donors (Forst *et al.*, 1988; Matsubara & Mizuno, 1999) may occur. OmpR regulates approximately 125 *E. coli* genes (Oshima *et al.*, 2002).

To date, no studies have been performed, to our knowledge, to investigate whether OmpR or OmpR-P protein binds to the *fimB* gene promoters of UPEC. In this study, gel mobility shift and DNase I footprinting assays have shown unphosphorylated OmpR binding to one of the *fimB* promoter regions. Western blots demonstrated OmpR protein concentration changes in a clinical strain of UPEC when grown in different environments. The presence of more OmpR protein may influence *fimB* regulation when UPEC cells are growing in an acidic/high osmolality environment.

METHODS

Bacterial strains, plasmids and growth conditions. *E. coli* NU149, a UPEC clinical isolate (Schaeffer *et al.*, 1987), was used in the PCR, quantitative real-time reverse transcriptase PCR (qRT-PCR), Western blot analyses, haemagglutination (HA) assays and electron microscopy. The NU149 OmpR1 strain with an *ompR* deletion has been described before (Schwan, 2009). Strains MC4100 (wild-type), MH1160 (*ompR*) and JMS6210 (*envZ*) were a kind gift from L. Kenney, University of Illinois-Chicago, and used to screen *lacZ* fusions. The *E. coli* strains were grown with shaking at 37 °C in media described previously (Schwan *et al.*, 2002) for β -galactosidase assays as well as RNA and protein extractions. Plasmid pWRS2-10

(Schwan *et al.*, 1992) containing the *fimB* and *fimE* genes from UPEC strain J96 was used as the template DNA for gel mobility shift and DNase I footprinting. Plasmid pJB5A (*fimB::lacZ*) has been described previously (Schwan *et al.*, 2002). For the *ompR* complementation experiments, plasmids pFR29* (Russo & Silhavy, 1991) containing the full-length unmutated *ompR* gene and pD55A (Schwan, 2009) containing a mutated *ompR* gene that does not allow phosphorylation, provided by L. Kenney, were used. The organisation of the *fimB* ORF is shown in Fig. S1, available with the online version of this paper.

β -Galactosidase assays. The β -galactosidase activity of mid-exponential-phase bacteria permeabilized with SDS and CHCl₃ was determined by using the method of Miller (1972). Assays were performed at least three times on different days, and the data were expressed as means \pm SD based on the values obtained.

Gel mobility shift assays. The gel mobility shift assays were done as previously described (Schwan *et al.*, 1994) using end-labelled *fimB* 445 bp FIMB2/PEXFIMB (Table S1) or end-labelled *fimE* 340 bp FIMEPE4/PEXFIME2 (Table S1) PCR products. Purified OmpR protein, a gift from L. Kenney, was mixed with both end-labelled DNA PCR products.

DNase I footprinting. Footprinting reactions were performed using pWRS2-10 DNA as a template for the PCR amplifications. A 445 bp fragment that contained *fimB* promoters 1 and 2 was generated by PCR using Platinum *Taq* (Invitrogen) and primer pair FIMB2/PEXFIMB (Table S1). The DNase I procedure followed Walthers *et al.* (2007). Images were acquired with a Molecular Dynamics Storm 860 Phosphorimager using the ImageQuant 5.3 software package.

Extraction of total RNA and conversion to cDNA. Total RNA was extracted from *E. coli* NU149 cells grown with shaking to mid-exponential phase (OD₆₀₀ 0.5–0.8) at 37 °C in pH 5.5/low osmolality Luria–Bertani (LB) medium, pH 5.5/high osmolality (800 mOsm NaCl) LB medium, pH 7.0/low osmolality medium or pH 7.0/high osmolality (800 mOsm NaCl) LB medium using TRIzol Reagent (Invitrogen) with 50 μ l lysozyme (10 mg ml⁻¹).

For each RNA sample, 5 μ g RNA was converted to cDNA using a SuperScript First-Strand Synthesis kit (Invitrogen) following the protocol recommended by the manufacturer.

Amplification and detection of *ftsZ*, *ompR* and *fimB* transcripts. RT-PCRs were performed as previously described (Schwan, 2009). The *ftsZ* gene was used to standardize between the samples (Table S1; Schwan *et al.*, 2002, 2007). For detection of *ompR* transcripts, a 319 bp *ompR* gene product was amplified using the primer pair OmpR3 and OmpR4 (Table S1). Detection of *fimB* transcripts was performed with the FimB5 and FimB6 (Table S1) primer pair, generating a 380 bp product. RT-PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and visualized with FOTO/Analyst PC Image Software (FOTODYNE Inc.).

qRT-PCR was performed using a Stratagene Mx3000P QPCR system (Agilent Technologies). One microlitre of a 1/10 dilution of cDNA was used in each qRT-PCR. A Power SYBR Green mastermix (Applied Biosystems) was used and products were detected with an FAM setting. The *ftsZ* gene was used to standardize between the samples (Table S1; Schwan *et al.*, 2002, 2007). A 71 bp fragment of the *ftsZ* gene was amplified using the primer pair FtsZfwd and FtsZrev (Table S1). The 96 bp *ompR* gene segment was amplified using the primer pair OmpRfwd and OmpRrev (Table S1). A 380 bp *fimB* product was amplified using the primer pair FimB5 and FimB6 (Table S1). The real-time RT-PCR parameters were as follows: initial denaturation for 10 min at 95 °C followed by 38 cycles of 15 s at 95 °C and 1 min at 58 °C. Data were analysed by using the

Table 1. Effects of *ompR* and *envZ* mutations on *fimB* expression compared with expression in wild-type, *ompR*, *envZ* and *ompR* complemented strainsValues are mean \pm SD

| Strain | Plasmid | β -Galactosidase activity (Miller units) | | | |
|--------------------------|----------------------------|--|--------------------|---------------------|----------------------|
| | | pH 7 [†] | pH 7+ [‡] | pH 5.5 [†] | pH 5.5+ [‡] |
| MC4100 (wild-type) | pJB5A (<i>fimB-lacZ</i>) | 325 \pm 50 | 182 \pm 38 | 198 \pm 68 | 131 \pm 35 |
| MH1160 (<i>ompR</i> -) | pJB5A | 406 \pm 97 | 296 \pm 48 | 161 \pm 89 | 325 \pm 56 |
| JMS6210 (<i>envZ</i> -) | pJB5A | 302 \pm 98 | 234 \pm 36 | 227 \pm 29 | 144 \pm 19 |
| MC4100 | pJB5A/pFR29* | 222 \pm 15 | 180 \pm 35 | 199 \pm 49 | 157 \pm 39 |
| MH1160 | pJB5A/pFR29* | 185 \pm 21 | 147 \pm 29 | 218 \pm 56 | 136 \pm 27 |
| MC4100 | pJB5A/pD55A | 207 \pm 50 | 157 \pm 49 | 234 \pm 53 | 115 \pm 38 |
| MH1160 | pJB5A/pD55A | 225 \pm 32 | 147 \pm 26 | 221 \pm 47 | 87 \pm 23 |

[†]LB growth (low osmolality) medium.[‡]LB growth medium containing 800 mOsm NaCl (high osmolality).

$2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). Both the *ompR* and the *fimB* transcript levels were standardized to *ftsZ* transcript levels.

PCR for *fimS* orientation determination. To determine the orientation of the *fimS* invertible element, previously described PCR techniques were used and products visualized with FOTO/Analyst PC Image Software (Schwan *et al.*, 1992, 2007). To quantify the percentage of Phase-ON or Phase-OFF bacteria, a standard curve was prepared as described by Teng *et al.* (2005) using locked-ON (DH5 α /pAON-1; Schwan *et al.*, 2002) and locked-OFF bacteria (NU149 cells passaged five times on agar shown to be 100 % Phase-OFF; Schwan *et al.*, 1992) as PCR templates and the ImageQuant 5.2 software.

Protein extraction and Western blotting. SDS-PAGE was performed with 10–20 % Tris/HCl polyacrylamide gels (Bio-Rad), and 10 μ g *E. coli* NU149 cell lysates from the same shaking exponential-phase cultures used for RNA extractions and processed as previously described (Schwan, 2009). For IPTG (New England Biolabs) induction, 1 mM of IPTG was added to the cultures. Proteins were electrotransferred to PVDF Immobilon membranes (Millipore) and processed as previously noted (Schwan, 2009). Rabbit polyclonal antibody to OmpR protein adsorbed with NU149 OmpR1 cell lysates (1 : 1000 dilution; provided by L. Kenney) and goat anti-rabbit IgG fluorescein-conjugated secondary antibody (Millipore, 1 : 500 dilution) were used. Bound antibodies were detected using a Storm 860 Phosphorimager (Amersham Biosciences) and analysed with Image Quant 5.3 software (Amersham).

Haemagglutination assays. The HA assays were performed with 1 % guinea pig erythrocytes (Hardy Diagnostics) as previously described (Schwan *et al.*, 1992). The titres represent the average of three separate runs.

Electron microscopy. Samples of NU149 and NU149 OmpR1 grown in pH 7.0 LB with 800 mOsm NaCl were attached to Formvar-coated grids and processed as described by Hultgren *et al.* (1985). Grids were observed using a JEOL 1200EXII transmission electron microscope. Images were gathered using a Gatan 791 digital camera running Digital Micrograph. A rank sharpening filter was applied globally to the images to help visualize the pili on the bacteria. Between 50 and 100 cells were examined for each growth condition.

Statistics. A Student's *t*-test was used to assess probabilities. *P*-values <0.05 were considered significant.

RESULTS

Effects of growth medium and *ompR/envZ* mutations on type 1 pilus expression

Previous work suggested that OmpR repressed *fimB* expression in UPEC growing in acidic/high osmolality conditions (Schwan *et al.*, 2002). To obtain further evidence that OmpR was involved in the *fimB* gene transcriptional changes, additional experiments were performed using the same *fimB-lacZYA* (pJB5A) fusion and growth environments as the previous study. Initially, *fimB* expression in wild-type cells was examined. Wild-type *E. coli* MC4100 had the highest level of *fimB* expression when grown in pH 7.0/low osmolality medium (LB) (Table 1). This expression decreased in response to high osmolality conditions (2-fold decrease in pH 7.0/800 mOsm NaCl medium), acidic conditions (1.6-fold decrease in pH 5.5 medium) and a combination of these two conditions (2.5-fold decrease in pH 5.5/800 mOsm NaCl medium). These results substantiated our previous study (Schwan *et al.*, 2002) showing that *fimB* had its lowest expression in acidic/high osmolality medium.

The effects of *envZ* (JMS6210) and *ompR* (MH1160) mutations on *fimB* expression were then examined. First, an *envZ* mutation did not significantly alter the expression of *fimB* when compared with wild-type, suggesting that EnvZ does not play a role in regulating *fimB* transcription. However, an *ompR* mutation appeared to impact *fimB* transcription under certain growth conditions. Although no statistical difference in *fimB* transcription was observed when comparing the *ompR* mutant and wild-type strains grown in low osmolality media (regardless of pH), *fimB* expression was elevated in the *ompR* mutant strain when cells were grown in high osmolality media (Table 2). This derepression of *fimB* occurred under both pH conditions, specifically a 1.6-fold derepression in the pH 7.0/800 mOsm NaCl medium and a 2.5-fold derepression of

Table 2. Measurement of haemagglutination (HA) titre for UPEC strains NU149, NU149 OmpR1 and NU149/OmpR1/pFR29+* grown in buffered LB at pH 5.5 and pH 7 with and without 800 mOsm NaCl

Values are the mean of three runs.

| Strain | HA titre | | | |
|--------------------|----------|--------|--------|----------|
| | pH 7 | pH 7+† | pH 5.5 | pH 5.5+† |
| NU149 | 512 | 64 | 128 | 4 |
| NU149 OmpR1 | 512 | 256 | 16 | 32 |
| NU149 OmpR1/pFR29* | 512 | 64 | 128 | 8 |

†Addition of 800 mOsm NaCl.

fimB expression in the pH 5.5/800 mOsm NaCl medium (Table 1). These results confirmed the results from the previous study (Schwan *et al.*, 2002) showing OmpR negatively regulated *fimB* transcription in cells grown in a high osmolality/acidic environment.

To confirm that the loss of OmpR caused the derepression of *fimB* expression, plasmids containing either the wild-type *ompR* gene (pFR29*) or a mutated *ompR* gene (pD55A) were used to complement the *ompR* mutant. Complementation of the *ompR* mutation occurred with the pFR29* (full-length unaltered OmpR) plasmid (Table 2). Surprisingly, the pD55A plasmid that contained a D55A substitution which changed the aspartate at position 55 to an alanine and generated an unphosphorylated OmpR protein, also complemented the *ompR* mutation, implying that unphosphorylated OmpR could repress *fimB* transcription. In addition, greater repression of *fimB* transcription was observed in the cell populations complemented with either pFR29* or pD55A because of a multicopy effect that allowed more OmpR protein expression.

Unphosphorylated and phosphorylated OmpR bind weakly to *fimB* promoter region 2

From the results in Table 1, we hypothesized that unphosphorylated OmpR bound to one or more of the *fimB* promoters to repress *fimB* transcription in *E. coli* cells grown in an acidic/high osmolality environment mirroring conditions in the human urinary tract. To determine if purified unphosphorylated OmpR bound to the *fimB* promoter region, a gel mobility shift assay was performed. Purified unphosphorylated OmpR shifted the mobility of the *fimB* promoter region in a concentration-dependent manner (Fig. 1a), suggesting unphosphorylated OmpR may be directly repressing *fimB* expression. When OmpR phosphorylated with phosphoramidate was used, the *fimB* promoter region also shifted (Fig. 1b). Purified D55A OmpR protein also caused a gel shift of the *fimB* DNA fragment, paralleling the unphosphorylated OmpR result (Fig. 1c). Another gel mobility shift with the radiolabelled *fimE* promoter region DNA showed no gel shift with unphosphorylated OmpR,

demonstrating that the unphosphorylated OmpR protein did not bind non-specifically to DNA fragments (Fig. 1d). These results suggested that unphosphorylated OmpR was sufficient to repress *fimB* transcription.

To determine more precisely where OmpR bound, DNase I footprinting assays were performed with DNA that was amplified by PCR with primers specific for the *fimB* promoter region 2. A sequencing ladder of the *fimB* sequence was used to determine exactly where OmpR/OmpR-P bound on the *fimB* promoter region 2 (data not shown). Unphosphorylated OmpR weakly bound to a 20 bp region (+2 to -18) (Fig. 2) and protected three bases of the -10 box, which included a hypersensitive site at the A at position -7 (Figs 2 and 3). In addition, a 24 bp region (+2 to -22) of the *fimB* promoter region 2 was protected by OmpR phosphorylated with phosphoramidate (Figs 2 and S1), spanning the entire -10 box plus 17 bases downstream of the -10 box.

UPEC *ompR* transcription is constant regardless of growth conditions

As both OmpR and OmpR-P bound weakly to one of the *fimB* promoters, we hypothesized that a high OmpR protein concentration in a low pH/high osmolality environment could be responsible for the regulation of *fimB*. To test this idea, we first determined the level of *ompR* transcription using cDNAs converted from total RNAs extracted from UPEC NU149 grown in four different media [pH 5.5 (low osmolality), pH 5.5/800 mOsm NaCl (high osmolality), pH 7.0 (low osmolality) and pH 7.0/800 mOsm NaCl (high osmolality)]. We used the UPEC NU149 strain for the remainder of the experiments to have a more valid comparison. Using RT-PCR, no discernible difference in *ompR* transcription was observed for the UPEC grown in the four media (data not shown).

As PCR lacks sensitivity, qRT-PCR was subsequently performed to ascertain *ompR* transcription differences compared with an *ftsZ* housekeeping control. After applying the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001) to normalize the *ompR* transcripts to the *ftsZ* gene, no significant difference in *ompR* transcription was found in either logarithmic (Fig. 3a) or stationary phase UPEC cultures (data not shown) grown in the various media. Thus, the UPEC growth environment did not differentially regulate *ompR* transcription.

OmpR protein level increases in a combined low pH/high osmolality medium

Although *ompR* transcription did not differ in UPEC NU149 when grown in the four growth conditions, post-transcriptional regulation could affect OmpR protein levels and then *fimB* transcription. To determine whether there was a difference in OmpR protein levels in cultures from each growth condition, SDS-PAGE and Western blot analyses were performed on stationary phase UPEC NU149 cell lysates probed with absorbed polyclonal rabbit anti-OmpR antibody.

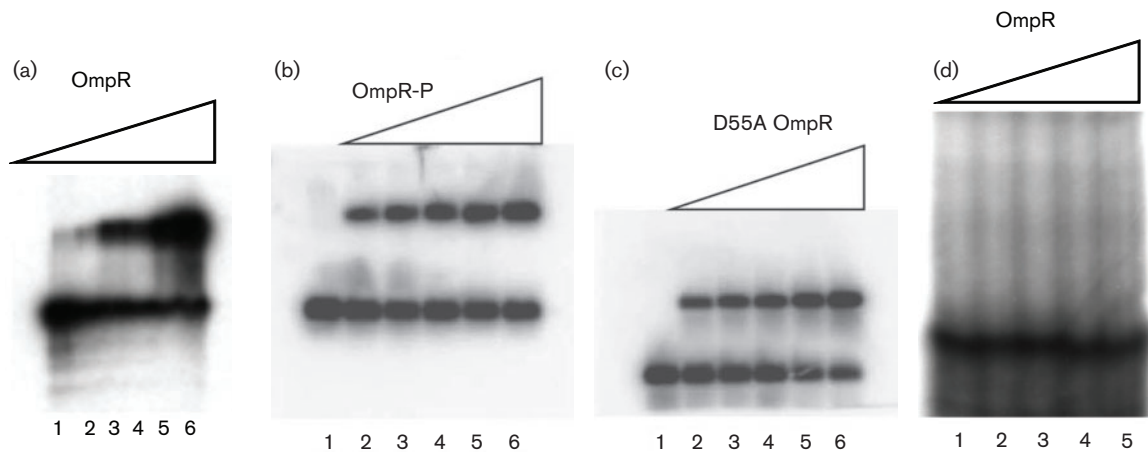


Fig. 1. Gel mobility shift assays that use OmpR protein. (a) A gel shift with the 445 bp ³²P-labelled *fimB* promoter region and OmpR. (b) A gel shift with the 445 bp ³²P-labelled *fimB* promoter region and OmpR-P. (c) A gel shift with the 445 bp ³²P-labelled *fimB* promoter region and D55A OmpR. (d) A gel shift with the 340 bp ³²P-labelled *fimE* promoter region. Lanes were loaded as follows: (1) DNA alone, (2) DNA plus 0.5 μM OmpR, (3) DNA plus 1 μM OmpR, (4) DNA plus 2 μM OmpR, (5) DNA plus 5 μM OmpR and (6) DNA plus 8 μM OmpR.

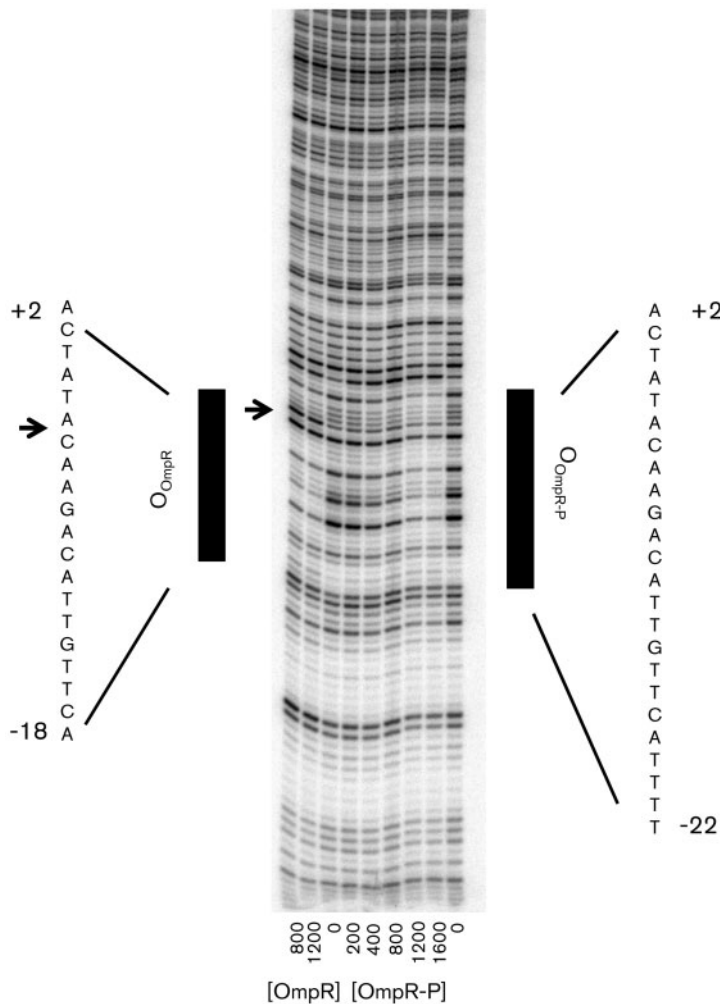


Fig. 2. DNase I footprinting analysis of unphosphorylated (OmpR) and phosphorylated OmpR (OmpR-P) at the *fimB* promoter region 2. The concentration (nM) of OmpR and OmpR-P used in each lane is indicated at the bottom. The lanes with '0' indicate the cleavage pattern by DNase I in the absence of OmpR/OmpR-P. The coordinates indicate positions relative to the *fimB* promoter 2 site. Regions of protection are indicated by black bars for OmpR (+2 to -18) and OmpR-P (+2 to -22) and a hypersensitive site is indicated by an arrow.

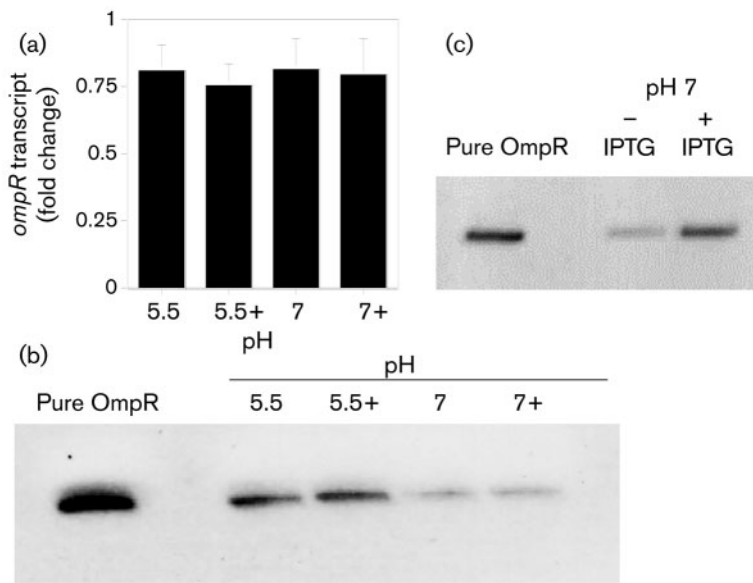


Fig. 3. Quantitative determination of *ompR* transcript and OmpR expression levels in exponentially grown UPEC NU149 cells in different environments. (a) qRT-PCR analysis of UPEC NU149 cells grown to mid-exponential phase in pH 5.5, pH 5.5 plus 800 mOsm NaCl (5.5+), pH 7.0 and pH 7.0 plus 800 mOsm NaCl (7.0+) LB. The fold change in *ompR* transcript levels that were corrected using *ftsZ* are shown as the mean \pm SD from three separate runs. (b) Western blot analysis of OmpR protein expression. One hundred nanograms of purified OmpR protein or 10 μ g of total protein isolated from NU149 grown in pH 5.5, 5.5+ (pH 5.5 LB with 800 mOsm NaCl), 7.0 and 7.0+ (pH 7.0 LB with 800 mOsm NaCl) LB. (c) One hundred nanograms of purified OmpR protein or 10 μ g of total protein isolated from NU149 grown in pH 7.0 LB with (+) or without (-) IPTG. The blots were probed with absorbed polyclonal rabbit anti-OmpR antibody.

One hundred nanograms of purified histidine-tagged OmpR protein reacted well with the antibody (Fig. 3b).

From the Western blot analysis, the steady-state levels of OmpR were higher in an acidic medium versus a neutral medium (Fig. 3b). A significant (fourfold) increase in OmpR protein expression occurred when NU149 cells grown in pH 5.5 (low osmolality) medium (29 715 pixels) were compared with the same strain grown in the pH 7.0 (low osmolality) medium (7443 pixels, $P < 0.0076$). This result did not correlate with the results from Table 1 that showed no significant difference in *fimB* transcription under these conditions when comparing the wild-type and *ompR* strains. This discrepancy suggests that another global regulator may play a role under acidic conditions. Osmolality did not appear to have a major effect on steady-state OmpR levels. There was no significant difference in OmpR expression in cells grown in pH 5.5 medium (low osmolality, 29 715 pixels) versus pH 5.5/800 mOsm NaCl medium (high osmolality, 34 898 pixels) ($P < 0.266$). A slightly significant effect was observed in cells grown in a pH 7.0 environment (low osmolality, 7443 pixels) compared with UPEC cells grown in pH 7.0/800 mOsm NaCl (10 896 pixels, 1.5-fold increase, $P < 0.02$). These OmpR expression profiles did not entirely correlate with the *fimB* results from Table 1, again suggesting that another global regulator may be a co-factor. The difference in OmpR expression in strain NU149 was the highest (4.7-fold) when comparing cells grown in pH 5.5/800 mOsm NaCl medium with cells grown in pH 7.0 medium ($P < 0.0076$). Cells grown to stationary phase also displayed significant differences in OmpR levels when comparing pH 5.5/800 mOsm with pH 7.0 grown cells (data not shown).

In addition, quantitative Western blots using different concentrations of purified OmpR protein confirmed the fold

differences observed in Fig. 3(b) (data not shown). Collectively, these results demonstrated that UPEC grown *in vitro* in an acidic/high osmolality environment express more OmpR protein through a post-transcription change than cells grown in a neutral pH/low osmolality environment.

Because there was a low level of OmpR expression in cells grown in pH 7.0 medium, less OmpR would be available to repress *fimB* transcription and this could explain the high level of *fimB* transcription in a pH 7.0 environment shown in Table 1. We hypothesized that if OmpR levels were artificially increased in cells grown in pH 7.0 medium, this change would result in decreased *fimB* expression and decreased levels of HA of guinea pig erythrocytes, used as a relative measure of type 1 pili on the surface, due to decreased type 1 pili. To test this idea, the pFR29* plasmid, containing the wild-type *ompR* gene under IPTG control, was introduced into the clinical strain NU149. NU149/pFR29* cells were grown in pH 7.0 medium, divided into aliquots that were treated or untreated with IPTG, and whole-cell lysates prepared. Western blots performed on these lysates confirmed that addition of IPTG resulted in increased levels of OmpR. While the lysate derived from cells grown without IPTG displayed a level of OmpR expression (8516 pixels) consistent with the prior blot, the lysate derived from cells grown with IPTG had elevated OmpR (20 241 pixels) (Fig. 3c). Significantly, a qRT-PCR analysis of both populations showed 2.6-fold less *fimB* expression in the IPTG-induced population compared with the population grown in standard pH 7.0 LB. When both cultures were tested for their HA, the HA titre of the population without IPTG was 524, whereas the HA titre for the population with IPTG was 128, consistent with decreased levels of type 1 pili. These results demonstrate that overexpression of OmpR under pH 7.0 growth

conditions decreases *fimB* expression and overrides post-transcriptional regulation of OmpR.

Levels of *fimB* transcripts change in the *ompR* strain grown in a high osmolality environment

The β -galactosidase assays suggested that in an *ompR* strain, expression of *fimB* increased compared with the wild-type cells when grown in a high osmolality environment. To verify these results, qRT-PCR was performed on total RNAs isolated from NU149 and NU149 OmpR1 cells grown in the same media as described above. Data points were standardized to *ftsZ* expression. The pH 7.0 medium with no added salt was used as a baseline as there was little difference between the NU149 and NU149 OmpR1 data (Fig. 4, $P < 0.493$). When the osmolality was increased to 800 mOsm at pH 7.0, *fimB* levels dropped approximately 4.2-fold in the wild-type lane versus a 1.4-fold drop in the *ompR* lane ($P < 0.024$). In the pH 5.5 LB, *fimB* transcript levels dropped about 2.6-fold in the wild-type lane versus pH 7.0 ($P < 0.032$), but there was an 8.7-fold drop in *fimB* transcript levels in the *ompR* lane under the same growth condition as compared with pH 7.0 ($P < 0.019$). The *fimB* levels dropped further (8-fold decline compared with pH 7.0, $P < 0.013$) in wild-type cells grown in the pH 5.5 LB with 800 mOsm medium, but *fimB* transcript levels declined less (2.8-fold, $P < 0.06$) in the *ompR* strain when compared with pH 7.0. The results paralleled the β -galactosidase assay findings, demonstrating that *fimB* transcript levels are higher in the *ompR* versus wild-type strain when both were grown in a high osmolality environment.

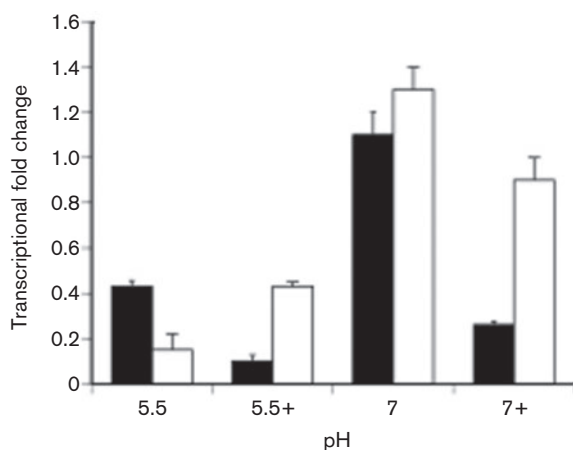


Fig. 4. Quantitative determination of *fimB* transcript expression by qRT-PCR. UPEC NU149 (solid black bars) and NU149 OmpR1 (open white bars) cells were grown to mid-exponential phase in pH 5.5, pH 5.5 plus 800 mOsm NaCl (5.5+), pH 7.0 and 7.0 plus 800 mOsm NaCl (7.0+) LB. The fold change in *fimB* transcript levels that were corrected using *ftsZ* are shown as the mean \pm SD from two separate runs.

Orientation of the *fimS* element changes in the *ompR* strain grown under high osmolality conditions

Previously, we have demonstrated that the orientation of the 314 bp *fimS* element was affected by pH and osmolality (Schwan *et al.*, 1992, 2002). Because the *fimB* transcript level changed in the *ompR* mutant strain when grown under high osmolality conditions, we investigated whether there was also an effect on the positioning of the *fimS* element. A PCR-based approach (Schwan *et al.*, 1992, 2007; Fig. S2) was used combined with a quantitative comparison of *fimS* orientation (Teng *et al.*, 2005) to measure the prevalence of Phase-ON and Phase-OFF DNA in NU149, NU149 OmpR1 and NU149 OmpR1/pFR29* populations when grown in LB with varying pH and osmolality. This analysis showed that *ompR* mutant cells grown at pH 7.0 (ratio ON/OFF=83:17%) had the invertible element positioned approximately equal to the wild-type cells (ratio ON/OFF=81:19%) and the complemented mutant (ratio ON/OFF=78:22%, Fig. 5). Under pH 7.0 with high salt growth conditions, the wild-type cells shifted significantly towards a Phase-OFF orientation (ratio ON/OFF=48:52%), but the *ompR* mutant appeared still to position the *fimS* element to favour the Phase-ON orientation (ratio ON/OFF=70:30%) compared with wild-type cells (ratio ON/OFF=48:52%). In pH 5.5 LB, the *ompR* mutant had more cells in the Phase-OFF orientation (ratio ON/OFF=18:82%) than the wild-type (ratio ON/OFF=62:38%), consistent with the observed effect on *fimB* transcription. However, more Phase-ON oriented cells were present in the *ompR* mutant in pH 5.5 LB with 800 mOsm NaCl (ratio ON/OFF=51:49%) versus wild-type cells (ratio ON/OFF=20:80%). Together, these results indicated that the derepression of *fimB* in the *ompR* mutant led to more Phase-ON oriented cells when grown in a high osmolality environment. In a low osmolality/low pH environment, less *fimB* was transcribed in the *ompR* strain compared with wild-type, suggesting another regulatory system such as an acid tolerance system regulator may be involved in regulating *fimB* in the absence of OmpR.

Production of type 1 pili is altered in the *ompR* cells versus wild-type cells

Both *fimB* transcript levels and positioning of the *fimS* invertible element changed in the *ompR* strain grown under high osmotic stress conditions. To determine if the level of type 1 pili also changed in the *ompR* mutant strain, HA assays were done using guinea pig erythrocytes. The results showed that NU149, NU149 OmpR1 (*ompR*) and NU149 OmpR1/pFR29* cells had the same HA assay titres when grown at pH 7.0 (Table 2). However, when the osmolality increased by 800 mOsm at pH 7.0, both the wild-type and the complemented mutant populations had an eightfold decline in the HA titre, whereas the *ompR* population had only a twofold drop. Furthermore, transmission electron

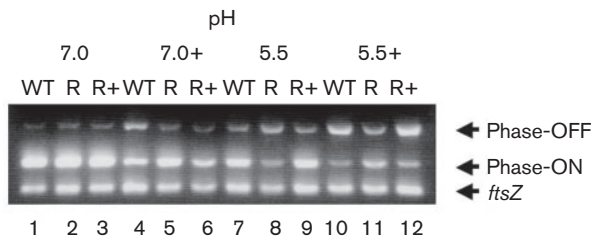


Fig. 5. Determination of the *fimS* invertible element orientation by PCR on chromosomal DNAs isolated from NU149, NU149 OmpR1 and NU149 OmpR1/pFR29* cells grown in pH 5.5 or 7.0 LB with or without added NaCl (+, added NaCl). Cells were harvested during mid-exponential phase following aerobic growth with agitation at 250 r.p.m. at 37 °C in pH 7.0 LB media with either no added NaCl (low salt) or 800 mOsm added NaCl (high salt) or in pH 5.5 LB medium with either no added NaCl (low salt) or 800 mM NaCl (high salt). Multiplex PCRs were set up with INV and FIMA primers to amplify Phase-ON-oriented DNA (ON, 450 bp product) (Schwan *et al.*, 1992), FIME and INV primers to amplify Phase-OFF-oriented DNA (OFF, 750 bp product) (Schwan *et al.*, 1992), and EcFtsZ 1 and 2 primers to amplify the *ftsZ* gene (302 bp product) (Schwan *et al.*, 2007). Each multiplex was run at least three separate times. WT, wild-type NU149; R, *ompR* strain; R+, *ompR* strain complemented with pFR29*. The lanes were loaded onto a 1.5% agarose gel as follows: lane 1, NU149 (pH 7.0, low salt); lane 2, NU149 OmpR1 (pH 7.0, low salt); lane 3, NU149 OmpR1/pFR29* (pH 7.0, low salt); lane 4, NU149 (pH 7.0, high salt); lane 5, NU149 OmpR1 (pH 7.0, high salt); lane 6, NU149 OmpR1/pFR29* (pH 7.0, high salt); lane 7, NU149 (pH 5.5, low salt); lane 8, NU149 OmpR1 (pH 5.5, low salt); lane 9, NU149 OmpR1/pFR29* (pH 5.5, low salt); lane 10, NU149 (pH 5.5, high salt); lane 11, NU149 OmpR1 (pH 5.5, high salt); and lane 12, NU149 OmpR1/pFR29* (pH 5.5, high salt). For each lane, the intensities of the OFF and ON states were quantified using ImageQuant software (Molecular Dynamics) and corrected to the intensity of the *ftsZ* band. The corrected values for both states were standardized to the respective wild-type band (lane 1).

microscopy demonstrated the *ompR* mutant strain exhibited more type 1 pili (Fig. 6a) compared with the wild-type strain (Fig. 6b).

In an acidic growth environment (pH 5.5), the wild-type and complemented mutant strains showed a fourfold drop in the HA titre versus the pH 7.0 baseline. The *ompR* strain showed an additional eightfold drop over the HA titre displayed by the wild-type strain. On the other hand, when the osmolality was increased by 800 mOsm in the same pH 5.5 environment, wild-type and complemented mutant strains had further declines in the HA titre to 4, but the *ompR* strain had a rebound in the HA titre up to 32. These results demonstrate that OmpR lowers type 1 pili expression under high osmotic stress conditions.

DISCUSSION

In the human urinary tract, UPEC survive in the differing pH/osmotically stressed environments by utilizing the EnvZ/OmpR system. Previously, we showed that the osmolality of the growth environment affects UPEC type 1 pilus expression and our results suggested that OmpR was regulating several *fim* genes (Schwan *et al.*, 2002). Our working hypothesis was that in an acidic/high osmolality environment, OmpR repressed *fimB* transcription in UPEC by binding to a *fimB* promoter. Using gel mobility shift and DNase footprinting assays, we have demonstrated that OmpR binds to a *fimB* promoter region. In addition, genetic and phenotypic data indicated that under high osmolality conditions, OmpR represses *fimB* transcription, leading to an increased Phase-OFF position for the *fimS* switch and a corresponding decrease in expression of type 1 pili.

OmpR regulates other *E. coli* genes (Oshima *et al.*, 2002). Several studies have demonstrated OmpR/OmpR-P binding upstream of the -35 and -10 boxes of the *ompF* and *ompC* promoter regions (Head *et al.*, 1998; Maeda *et al.*, 1988; Tsung *et al.*, 1989). OmpR binding to a 20 bp region in the *ompF* promoter region has been previously observed (Harlocker *et al.*, 1995; Huang & Igo, 1996), and a loose consensus sequence containing an AC at positions 1,2 and 11,12 with a central GXXXC sequence was proposed for OmpR binding (Head *et al.*, 1998). A more recent study further defined the OmpR binding site as having a core sequence comprising a central GTXTCA (5'-3' with the X being either an A or a T) (Rhee *et al.*, 2008).

Our results showed that OmpR and OmpR-P shifted the mobility of a DNA sequence spanning the *fimB* promoter 1 and 2 region. Furthermore, DNase footprinting experiments demonstrated that OmpR and OmpR-P protected 20 and 24 bp sites, respectively, at the *fimB* promoter 2. Binding of the 20 bp site by OmpR may be due to binding of an unphosphorylated OmpR monomer. The expanded 24 bp protected area might be due to another phosphorylated OmpR monomer attaching to the first OmpR monomer bound to the DNA, resulting in a dimer. Rhee *et al.* (2008) have proposed such a model to explain some of the OmpR-mediated gene regulation. In our study, both of the OmpR protected regions spanned the -10 box of the *fimB* promoter 2. Within these protected regions, a putative OmpR binding motif sequence, 5'-ACTTGTTA-CAGAAC-3', with an AC at positions 1, 2 as well as an internal GTTACA sequence, was found (Fig. 3). Although the internal sequence for the *fimB* promoter 2 does not precisely match the core GTXTCA OmpR binding sequence (Rhee *et al.*, 2008), it only differs by one base, with an A rather than a T at the fourth position. This one base mismatch may explain the relatively low affinity of OmpR/OmpR-P binding for the *fimB* promoter 2 region as compared with the high-affinity sequence for *ompF* and *ompC* promoter regions. Low-affinity OmpR-P binding has been demonstrated for the *flhDC* promoter region, but no conserved core sequence was observed (Shin & Park, 1995).

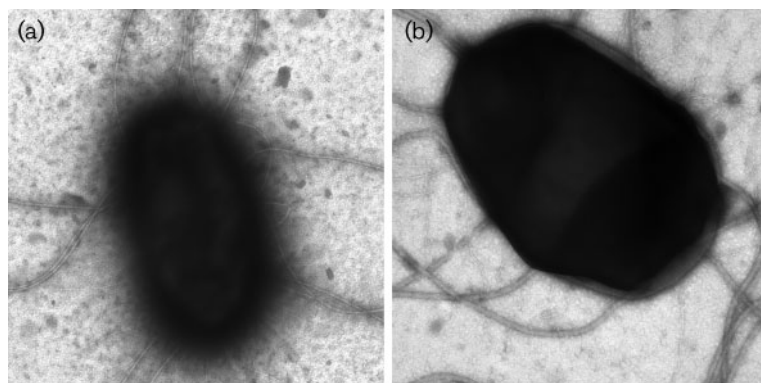


Fig. 6. Electron micrographs of NU149 OmpR1 (a) and NU149 (b) cells grown in pH 7.0 LB with 800 mOsm NaCl. Magnification, ca. $\times 100\,000$.

A previous study by Cai & Inouye (2002) indicated that at low osmolality, phosphorylation of only 3.5% (120 nM or 70 OmpR-P molecules per cell) of total OmpR protein (2024 molecules of OmpR per cell) would be necessary for promoting *ompF* transcription. However, in a high osmolality medium, phosphorylation of 10% (580 nM or 350 OmpR-P molecules per cell) of total OmpR molecules in a cell (3500 molecules of OmpR per cell) would be enough to repress the *ompF* gene and stimulate expression of the *ompC* gene (Cai & Inouye, 2002). Our *fimB* DNase footprint assays showed protection by OmpR and OmpR-P at 800 nM (Fig. 5), equating to 466 OmpR molecules per cell, or 13.5% of total OmpR protein. Our observed binding of OmpR to the *fimB* promoter 2 region occurs at levels well within the physiological range of OmpR protein that would be present in UPEC growing in a high osmolality environment. Whether OmpR binds to either of the two remaining *fimB* promoters remains unknown and will serve as an area of further exploration.

Since both OmpR and OmpR-P bound weakly to the *fimB* promoter region 2, we investigated whether OmpR protein itself might be influenced by pH or osmolality in UPEC cells, through either transcriptional or translational changes. We hypothesized that OmpR levels would be higher in an acidic/high osmolality environment, leading to low-affinity binding to the *fimB* promoter and direct regulation of *fimB* transcription. However, the RT-PCR and qRT-PCR analyses showed no difference in *ompR* transcript levels in strain NU149 grown in the four growth conditions used previously (Schwan *et al.*, 2002). These results ruled out the possibility that transcriptional changes in *ompR* were responsible for the *fimB* regulation.

In *E. coli*, *envZ* and *ompR* are co-transcribed, but 35 times more OmpR protein is present compared with EnvZ, presumably because of differences in translational initiation (Cai & Inouye, 2002). Based on this previous study and the lack of change in our *ompR* transcriptional assays, we hypothesized post-transcriptional regulation of OmpR in response to low pH and/or high osmolality conditions. Western blots confirmed that OmpR protein levels increased (fourfold) in UPEC cells in response to acidic conditions, providing evidence for post-transcriptional

regulation of OmpR. The post-transcriptional change could be tied to greater translational initiation of the *ompR* transcript in an acidic environment; greater binding by RNA-binding proteins (e.g. Hfq-dependent stimulation of translation) under stress conditions (Nogueira & Springer, 2000); or through interactions with small, non-coding RNAs that affect gene regulation (Valentin-Hansen *et al.*, 2007). Indeed, Hfq-binding sRNA is linked to a number of target-mRNAs, including porin proteins, regulated at the post-transcriptional level within *E. coli* (Guillier & Gottesman, 2006). OmpR is regulated by at least two sRNAs, OmrA and OmrB, bound to Hfq (Guillier & Gottesman, 2008). As sRNAs derepress translation by smoothing out mRNA hairpin loops (Majdalani *et al.*, 1998), the sRNAs bound to Hfq may be allowing better translation of *ompR* transcripts when UPEC cells are grown under acidic conditions.

Levels of *fimB* expression were highest in a neutral pH/low osmolality condition and lowest in a low pH/high osmolality medium. A switch from a neutral pH/low osmolality environment that favours *fimB* activation by SlyA (a proposed activator of *fimB* transcription, McVicker *et al.*, 2011) or RcsB (another proposed activator of *fimB* transcription, Schwan *et al.*, 2007) to a low pH/high osmolality environment found in the human urinary tract that favours OmpR could have relevance in regulating *fimB* (Schwan, 2011). OmpR could displace SlyA or RcsB on the *fimB* promoter site as the OmpR level increases in UPEC growing in an acidic/high osmolality environment. These results suggest a possible physiological role for OmpR in *E. coli* growing in acidic/high osmolality environments, such as the human kidney.

Prior studies have indicated that OmpR protein expression in *Salmonella* and enterohaemorrhagic *E. coli* increased after acid shock or growth in an acidic environment compared with a neutral pH environment (Bang *et al.*, 2000; Huang *et al.*, 2007). These previous studies provide evidence that the OmpR protein is important for survival in acidic environments, and our data showing that the OmpR protein is elevated in response to low pH is consistent with these prior works.

Previously, wild-type *E. coli* grown in a high osmolality environment (added sucrose) had a 1.7-fold increase in

OmpR protein as compared with cells grown in a low osmolality medium (Cai & Inouye, 2002). Although our data indicated only subtle changes in OmpR protein levels in response to high osmolality, OmpR/OmpR-P ratios could be influenced by osmolality and may be a contributing factor *in vivo*. If OmpR/OmpR-P represses *fimB* expression, an *ompR* mutant strain should display greater levels of *fimB* transcripts and higher expression of type 1 pili in UPEC grown in a high osmolality growth environment. Indeed, *fimB* transcript levels were four- to eightfold higher in the *ompR* mutant compared with wild-type cells under high osmolality conditions, which in turn led to more Phase-ON cells and higher type 1 pili expression. Our model is that greater concentrations of OmpR and OmpR-P in UPEC growing in kidney urine (acidic/high osmolality) may allow OmpR-P to directly bind first to the higher affinity sites on the *fimB* promoter region followed by binding to lower affinity sites as the concentration of OmpR increased. This scenario would be similar to the binding affinity regulating the *ompF* gene (Huang & Igo, 1996; Qin *et al.*, 2001; Rampersaud *et al.*, 1994). OmpR and OmpR-P binding to the *fimB* promoter region would repress *fimB* transcription, thereby increasing the cellular ratio of FimE to FimB. In turn, FimE would bind to the invertible element, switching it to the Phase-OFF orientation and resulting in non-piliated bacteria in the kidneys observed in previous studies (Hultgren *et al.*, 1985; Schaeffer *et al.*, 1987). Non-piliated UPEC cells in the kidney would be less likely to be phagocytized by phagocytic cells targeting the type 1 pili (Perry *et al.*, 1983; Silverblatt *et al.*, 1979).

ACKNOWLEDGEMENTS

We thank Linda Kenney, Leslie Morgan and Don Walthers for their assistance with the DNase I footprinting, anti-OmpR antibody, purified OmpR protein and mutant strains used in this study. All of the DNase I footprints were performed in Linda Kenney's laboratory. We also thank David Anderson and Matthew Zuberbuehler for technical assistance with the RNA extractions. This study was funded by a University of Wisconsin-La Crosse graduate student Research, Service and Educational Leadership grant to A. E. R. and a National Institutes of Health grant AI065432-01A2 to W. R. S.

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Edited by: U. Dobrindt