Osmoregulatory transporter Prop influences colonization of the urinary tract by *Escherichia coli*

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Osmoregulatory transporters Prop and ProU mediate the use of betaines as osmoprotectants by *Escherichia coli*. Glycine betaine and proline betaine are present in mammalian urines. Betaine uptake may therefore facilitate the growth of *E. coli* in the urinary tract, an environment of fluctuating osmolality. Prop transporter activity was approximately threefold higher in a pyelonephritis isolate, *E. coli* HU734, than in *E. coli* K-12. The growth rate of *E. coli* HU734 in aerated minimal salts medium was reduced twofold by 0.2 M NaCl in the absence and by 0.55 M NaCl in the presence of glycine betaine. Maximal growth rate stimulation was achieved when glycine betaine was added at a concentration as low as 25 μM. Deletion of the prop locus impaired the growth rate of *E. coli* HU734 in human urine but not in minimal medium supplemented with NaCl (0.4 M), with or without glycine betaine (0.1 mM). The expression of pyelonephritis-associated (P) pili was reduced when *E. coli* HU734 was cultured in a rich culture medium (LB) of elevated salinity. The prop lesion had no influence on P pilus expression in vitro or on the recovery of bacteria from the kidneys of inoculated mice. However, it did reduce their recovery from the bladders of inoculated mice 100-fold. These data provide the first direct evidence that osmoprotective betaine accumulation and transporter Prop are pertinent to both growth in human urine and colonization of the murine urinary tract by uropathogenic *E. coli*.

**Keywords**: osmoregulation, glycine betaine, urinary tract infection, *Escherichia coli*, Prop

**INTRODUCTION**

Kunin (1987) described urine as a 'variable, but generally good, culture medium', indicating that poor iron availability, high organic acid levels, low pH and variable osmolality may limit the survival and growth of *Escherichia coli* in the human urinary tract. Since *E. coli* causes most ascending infections of the unobstructed human urinary tract (Warren, 1996) and mice are used in an experimental model for human urinary tract infection (Johnson & Russell, 1996), the ability of *E. coli* to grow in the human and murine urinary tracts has clinical importance.

The osmolality of human urine may vary from approximately 0.04 to 1.4 mol kg⁻¹ (0.5–0.8 mol kg⁻¹ with a normal diet and fluid intake) (Ross & Neely, 1983). Urine osmolalities up to 3 mol kg⁻¹ have been detected in rats and mice (Schmidt-Nielsen et al., 1983; Loeb & Quimby, 1989). *E. coli* is moderately salt-tolerant, the most salt-tolerant human isolates growing in minimal medium supplemented with up to 0.7 M NaCl (hence at an osmolarity in excess of 1.4 mol kg⁻¹) (Kunin et al., 1992). Although they varied in salt tolerance, no correlation between urinary tract origin and salt tolerance was found among 301 clinical *E. coli* isolates. Salt tolerance was defined as the maximum NaCl concentration at which there was 50% growth (optical density after 48 h) compared to a control culture in minimal medium without added NaCl (Kunin et al., 1992). These observations suggest that the osmolality of human urine varies within the range tolerated by most

**Abbreviations**: AC, L-azetidine-2-carboxylic acid; Amp, ampicillin; DHP, 3,4-dehydro-D,L-proline; TTC, triphenyltetrazolium chloride.
E. coli isolates in vitro whereas the osmolality of rodent urine may exceed the osmotolerance of E. coli. However, it remains possible that modulation of growth rate by urine osmolality influences the population density of E. coli in the urinary tract.

In E. coli K-12, osmoregulatory transporters and channels modulate cytoplasmic osmolality by mediating the active accumulation or release of osmoprotectants, available in the external medium, which are particularly compatible with the functions of cellular macromolecules (Csonka & Epstein, 1996). The osmoprotectants used by E. coli K-12 include proline, glycine betaine (N-trimethyl glycine), proline betaine (N-dimethyl proline or stachydrine) and ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid). Uptake of these compounds is mediated by transporters ProP and ProU. Transcription of proP and proU is induced when E. coli is cultured in hypertonic media and both transporters are activated when cells are exposed to osmotic upshifts.

Current research on the role of bacterial osmoadaptation in urinary tract infection was triggered by the observation that urinary glycine betaine and proline betaine provide osmoprotection to E. coli (Chambers & Kunin, 1987). Glycine betaine raises the salt tolerance of E. coli to 1 M (according to the criterion described above; Kunin et al., 1992). Defects in genetic loci proP and proU impaired (1) the accumulation of diverse betaines by E. coli K-12 (Randall et al., 1995), (2) the ability of glycine betaine to stimulate the growth (extent) of E. coli K-12 in artificial urine (Culham et al., 1994) and (3) the growth of E. coli K-12 in authentic (presumably betaine-containing) urine (Kunin et al., 1992). However, E. coli strains isolated from the human urinary tract were not distinguished from other clinical isolates by their ability to utilize betaines as osmoprotectants in vitro (Kunin et al., 1992; Culham et al., 1994). Culham et al. (1994) examined the distribution and expression of genes encoding osmoregulatory transporters ProP and ProU in 61 different E. coli strains, 28 from the human urinary tract and 33 with other human origins. All contained and expressed the proU locus; evidence for expression of proP by one (infantile diarrhoea) isolate was equivocal. Thus, betaine uptake via ProP and/or ProU can contribute to the osmotolerance of clinical E. coli isolates. However, the cited observations do not indicate whether ProP or ProU is active in uropathogenic bacteria within their mammalian hosts or whether the activities of these transporters contribute to bacterial growth within the urinary tract.

In addition to directly influencing bacterial growth in the urinary tract, osmoregulatory mechanisms may indirectly influence urinary tract infection by affecting the expression of virulence determinants. P (pyelonephritis-associated) pili are expressed by 81% of E. coli isolates from pyelonephritis patients whose risk of infection is not increased by underlying medical or anatomical conditions. They are implicated in bacterial adhesion to urinary tract tissues (Donnenberg & Welch, 1996). The effects of hypertonic media on pap (P pilus operon transcription) have not been reported despite extensive analyses of that system (van der Woude et al., 1996). However, Kunin et al. (1994) used electron microscopy of negatively stained preparations and haemagglutination to detect a reduction in P pilus expression when pyelonephritis isolates of E. coli were cultured on rich medium (trypticase soy agar) supplemented with 0.4-0.8 M NaCl.

Our goal is to identify direct and indirect effects of environmental osmolality and of osmoregulatory betaine accumulation on the ability of uropathogenic E. coli strains to infect the urinary tract. Pyelonephritis isolate E. coli HU734 was used to develop the murine model for ascending urinary tract infection and to demonstrate that P pilus can facilitate colonization of the murine urinary tract (Hagberg et al., 1983a, b). It expresses genetic loci analogous to the proP and proU loci of E. coli K-12 (Culham et al., 1994). This paper describes deletion of the proP locus from E. coli HU734 and analyses of the effects of osmolality, glycine betaine and the proP lesion on bacterial growth, P pilus expression and colonization of the murine urinary tract.

**METHODS**

**Media and growth measurements.** Bacterial culture media included LB (Miller, 1972), LB-N (LB medium from which NaCl was omitted) and MOPS minimal medium (Neidhardt et al., 1974). MOPS medium was supplemented, unless otherwise stated, with D-glucose (0.2%, w/v) as carbon source, NH₄Cl (9.5 mM) as nitrogen source, amino acids (50 µg ml⁻¹) to meet auxotrophic requirements and thiamin (1 µg ml⁻¹). TTC (triphenyltetrazolium chloride) indicator medium for proline utilization was prepared as described by Bochner & Savageau (1977) and supplemented with proline (200 mM) and cysteine (50 µg ml⁻¹). Saline was 0.85% (w/v) NaCl. The osmolalities of selected media were determined with a vapour pressure osmometer (Wescor) to be: LB, 0.4 mol kg⁻¹; LB plus 0.3 M NaCl, 1.0 mol kg⁻¹; LB plus 0.6 M NaCl, 1.6 mol kg⁻¹; MOPS medium, 0.20 mol kg⁻¹; MOPS medium plus 0.2 M NaCl, 0.56 mol kg⁻¹; MOPS medium plus 0.4 M NaCl, 0.94 mol kg⁻¹; MOPS medium plus 0.6 M NaCl, 1.41 mol kg⁻¹; MOPS medium plus 0.8 M NaCl, 1.77 mol kg⁻¹; MOPS medium plus 1.0 M NaCl, osmolality exceeded the detection limit of the osmometer.

Rates of bacterial growth in shaken cultures were determined as follows. Overnight LB cultures were added as inocula (0.5 ml) to 25 ml LB medium in a 125 ml flask and incubated overnight. The cells were harvested by centrifugation, washed with saline and resuspended in 10 ml saline. The resulting suspension was used to inoculate 25 ml test cultures in 125 ml sidearm flasks (sidearm internal diameter, 1 cm) to an OD₅₀₀ of 0.1. Cultures were incubated at 37°C with rotary shaking at 200 r.p.m. and the OD₅₀₀ was recorded at 30 min intervals until it exceeded 1.0 (or stationary phase was reached at a lower optical density). Growth rates were determined by linear regression analysis of data defining the exponential portion of each growth curve (log absorbance versus time). Each growth condition was tested in triplicate and each experiment was performed at least twice. The growth of bacteria in stationary cultures was monitored as described...
above with the following exceptions. Cultures were incubated at 37 °C without agitation. At 3–4 h intervals after inoculation cultures were sampled, OD₆₀₀ values were determined and LB plates were inoculated with culture dilutions to determine viable counts. No culture was sampled more than once, each determination was made in duplicate and each experiment was performed twice.

For measurements of bacterial growth in urine, concentrated, first-voided morning urine was collected from six healthy adult volunteers following overnight (14 h) fluid and food deprivation. The pooled urine was centrifuged at 7000 r.p.m. at 4 °C for 10 min (Sorvall GSA rotor) and sterilized by filtration through 0.45 µm pore size filters (Gelman Sciences). Bacteria from overnight LB cultures were inoculated into 25 ml aliquots of urine in 125 ml culture flasks (three replicates per strain tested) at initial densities of approximately 10⁸ c.f.u. ml⁻¹. Cultures were incubated at 37 °C with rotary shaker at 150 r.p.m. Growth was monitored in two ways: (1) cell density was measured by monitoring OD₆₀₀ at 30 min intervals during exponential-phase growth (optical density range approximately 0–1 to 1, see Fig. 2), and (2) viable counts were monitored at 60 min intervals by inoculating LB plates with appropriate culture dilutions. After 24 h, the purity of each culture was verified by inoculating phenotype-specific media.

Bacterial strains. The E. coli strains used for this study are listed in Table 1. Strain HU734, a lacZ derivative of acute human pyelonephritis isolate GR12, has the following properties: streptomycin and spectinomycin resistance and cysteine auxotrophy, serotype O75:K5, possession of both type 1 and P pili (the latter encoded by a single pap operon), carriage of a ColV plasmid, resistance to killing by human and mouse serum, and failure to produce haemolysin (Hagberg et al., 1983a; Mamelen, 1994). Plasmid pAGK2 consists of a 10 kb ClaI fragment including the pap-3 operon from E. coli GR12 (HU734) inserted into the ClaI site in the tet gene of vector pACYC184 (Klann et al., 1992; S. Hull, personal communication). The identity and purity of each culture was tested by culturing bacteria on the following media: LB medium; LB medium supplemented with ampicillin (50 or 100 µg ml⁻¹), chloramphenicol (50 µg ml⁻¹), streptomycin (100 µg ml⁻¹) or tetracycline (25 µg ml⁻¹) (as indicated by the bacterial genotype); MOPS medium with and without required amino acids (50 µg ml⁻¹); TTC medium and lactose MacConkey agar. Cultures were also tested with the radial streak assay using the toxic proline analogues ~-azetidine-2-carboxylic acid (AC) and 3,4-dehydro-D,L-proline on MOPS medium with and without supplemental NaCl (0-3 M) as described by Grothe et al. (1986).

Table 1. E. coli strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source and/or derivation</th>
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<tbody>
<tr>
<td>Strains</td>
<td></td>
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</tr>
<tr>
<td>DH5α</td>
<td>K-12 F- φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (pir') supE44 thi-1 gyrA relA1</td>
<td>BRL</td>
</tr>
<tr>
<td>HU734</td>
<td>GR12 lacZ</td>
<td>G. Reid (Hagberg et al., 1983a)</td>
</tr>
<tr>
<td>HU742</td>
<td>HU734 pap</td>
<td>S. Hull (Hagberg et al., 1983a)</td>
</tr>
<tr>
<td>S17-1 ipir</td>
<td>thp pro hsdR hsdM* recA1 rp4-Tc:Mu-Km::Tn7 ipir</td>
<td>P. Goodwin (Herrero et al., 1990)</td>
</tr>
<tr>
<td>SK288I</td>
<td>K-12 F- gal leuC thi aroD T1Δ hsdR4 recA1 sbc15 endA</td>
<td>S. Hull (Klann et al., 1994)</td>
</tr>
<tr>
<td>SY327 ipir</td>
<td>K-12 Δ(lac pro) argE(Am) rif nalA recA56 ipir</td>
<td>J. Kaper (Miller &amp; Mekalanos, 1984)</td>
</tr>
<tr>
<td>WG444</td>
<td>K-12 Δ(papPA)101 srl-300::Tn10</td>
<td>Culham et al. (1994)</td>
</tr>
<tr>
<td>WG445</td>
<td>K-12 Δ(papPA)101 Δ(proP-mef)212 srl-300::Tn10</td>
<td>Culham et al. (1994)</td>
</tr>
<tr>
<td>WG484</td>
<td>K-12 srl-300::Tn10</td>
<td>Culham et al. (1994)</td>
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<td>WG541</td>
<td>HU734 Δ(papPA)566</td>
<td>This work</td>
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<tr>
<td>WG671</td>
<td>WG541 proP218</td>
<td>This work</td>
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<tr>
<td>Plasmids</td>
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<td>pAGK2</td>
<td>pACYC184 pap</td>
<td>Klann et al. (1992)</td>
</tr>
<tr>
<td>pCVD442</td>
<td>oriR6K mobRP4 Gm* sacB</td>
<td>J. Kaper (Donnenberg &amp; Kaper, 1991)</td>
</tr>
<tr>
<td>pDC10</td>
<td>pGEM4 proP*</td>
<td>Culham et al. (1993) and see text</td>
</tr>
<tr>
<td>pDC60</td>
<td>pGEM4 proP218</td>
<td>This work</td>
</tr>
<tr>
<td>pDC62</td>
<td>pJQ200mp18 proP218</td>
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</tr>
<tr>
<td>pDC66</td>
<td>pCVD442 proP218</td>
<td>This work</td>
</tr>
<tr>
<td>pJQ200mp18</td>
<td>oriR15A mobRP4 Amp* sacB</td>
<td>M. Hynes (Quandt &amp; Hynes, 1993)</td>
</tr>
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</table>

E. coli WG541 [HU734 Δ(papPA)566] was isolated essentially as described by Wood (1981). After overnight growth in MOPS medium, cells of strain HU734 were harvested by centrifugation, washed with saline and resuspended in saline to a density of approximately 2x10⁸ cells ml⁻¹. Aliquots (0.1 ml) of the resulting suspension were spread on MOPS medium plates supplemented with t-cysteine (50 µg ml⁻¹) and AC (25 µg ml⁻¹). After incubation of the plates for 48 h at 37 °C, 500 of the resulting colonies were transferred to TTC indicator medium. Strain WG541 was one of three isolates which yielded only white (put) colonies after restreaking on TTC medium. All three were devoid of proline dehydrogenase activity as indicated by the o-aminobenzaldehyde assay (Graham et al., 1984) and sensitive to inhibition by 3,4-dehydro-D,L-proline (DHP) as indicated by the radial streak test (Wood, 1981; Grothe et al., 1986). Unlike that of its parent, chromosomal DNA from strain WG541 failed to support amplification of a 201 bp DNA fragment internal to putP, a 461 bp fragment of the put intergenic region or a
628 bp DNA fragment internal to puvA (PCR performed as described by Culham et al., 1994).

Molecular biological manipulations were carried out as described by Sambrook et al. (1989). Transformations were done in E. coli strain DH5α and transformants were selected on LB plates containing 100 μg Amp ml−1 (plasmids based on pGEM4 or pCVD442) or 15 μg gentamicin ml−1 (plasmids based on pJQ200mp18) unless otherwise indicated. Deletion prop218 was created by digesting plasmid pDC10 isolated in the same manner as pDC15 (Culham et al., 1993) with restriction endonucleases EcoRI and MfeI, blunt-ending and religating the larger 5 kb fragment to create pDC60. This removed a DNA fragment extending from 59 bp upstream to 44 bp downstream of prop. Plasmid pDC60 was digested with restriction endonucleases SmaI and NruI and the 2.1 kb fragment containing chromosomal DNA flanking the prop deletion was isolated and ligated into the SmaI site of vector pJQ200mp18 to create plasmid pDC62. This plasmid was then digested with SstI and the 2 kb fragment (containing 747 bp upstream and 1253 bp downstream of the prop deletion) was ligated into the SstI site of vector pCVD442 to create plasmid pDC66 (transformation into E. coli S17-1 ipir).

Deletion prop218 was introduced to strain WG541 by allelic replacement essentially as described by Mobley et al. (1993). Strains WG541 and S17-1 ipir(pDC66) were inoculated from overnight LB cultures into 10 ml LB to an OD600 of 0.2 and incubated at 37 °C (WG541) or 30 °C (S17-1 ipir(pDC66)) with aeration to an OD600 of 0.8. Amp (100 μg ml−1) was added to cultures of the latter strain. Cells were collected by centrifugation and washed twice in an equal volume of LB. Aliquots (1 ml) of each culture were added to 9 ml LB and filtered through a 47 mm diameter, 0.45 μm pore size nitrocellulose filter (Gelman Sciences). The resulting filter was transferred to an LB plate, incubated for 3 h at 30 °C and transferred to a tube containing 2 ml saline. Cells were washed from the filter by vortex mixing and 100 μl aliquots were spread on MOPS medium plates containing L-cysteine (50 μg ml−1) and Amp (100 μg ml−1). The single resulting transconjugant was grown in Amp-supplemented LB medium overnight, subcultured to a density of approximately 1010 c.f.u. ml−1 in five aliquots LB (10 ml each) and incubated at 37 °C for 5 h to a density of approximately 1012 c.f.u. ml−1. Dilutions of each culture (0.1 ml) were spread on LB-N medium containing sucrose [5% (w/v)]. After incubation at 30 °C for 18 h, 36 colonies were selected and screened for loss of Amp resistance (30 were Amp-sensitive). Chromosomal DNA was isolated from 10 of the Amp-sensitive colonies and analysed by DNA amplification for the presence of prop218. Primers corresponding to nucleotides 1302–1321 and 1557–1539 within prop (numbering from the sequence with accession number M83089) directed PCR amplification of a 256 bp DNA fragment when wild-type proP DNA was used as PCR template. Primers corresponding to sequences flanking proP (nucleotides 124–150 and 2037–2012) directed amplification of a 311 bp DNA fragment when prop218 DNA was used as template. Strain WG671 was one of six among the ten tested isolates which carried prop218 and not the wild-type allele.

DNA sequence analysis. Templates for sequencing of the prop locus in E. coli HU734 were synthesized by PCR amplification using synthetic oligonucleotide primers based on the known sequence of the E. coli K-12 prop locus (accession number M83089). Sequence analysis was then conducted using the same primers by Mobix (Hamilton, ON, USA). Overlapping fragments extending from 282 bp upstream to 79 bp downstream of the prop ORF were sequenced, yielding a full sequence of one DNA strand. PCR and sequencing were repeated to confirm the observed differences between E. coli K-12 and E. coli HU734.

Transport assays. The activities of transporters PutP, ProP and ProU were estimated by measuring the uptake of osmoprotectant proline. Bacteria were cultured for transport measurements and initial rates of l-proline uptake were estimated essentially as described by Milner et al. (1988). Bacteria were cultured in MOPS medium with glycerol (4 mg ml−1) as carbon source, NH4Cl (9.5 mM) as nitrogen source, cysteine (50 μg ml−1), thiamin (1 μg ml−1) and other supplements as specified. Reaction mixtures (final volume 0.5 ml) included MOPS medium (devoid of NH4Cl and amino acids), glucose (11 mM), supplemented NaCl (as indicated), uniformly labelled L-[14C]proline (concentration as specified) and 0.1–0.3 mg cell protein. Samples (0.15 ml) were removed 20 s, 40 s and 60 s after initiation of the assay with [14C]proline. Wash solutions were the same as the corresponding transport assay media except that they lacked glucose and the radiolabelled substrate. Mean uptake rates, with standard errors, were determined for triplicate uptake assays and each experiment was performed at least twice.

Haemagglutination assays. Haemagglutination assays were conducted essentially as described by Old (1985). Erythrocytes were harvested from sheep blood by low-speed centrifugation (5000 r.p.m., 4 °C, 10 min, Sorvall SS34 rotor). The pellet was washed three times with saline and erythrocytes were suspended to a final concentration of 6% (v/v) in Alsever’s solution [containing D-glucose (20.5 g l−1), sodium citrate dihydrate (8 g l−1) and NaCl (4.2 g l−1)]. Bacteria to be tested for haemagglutinating activity were suspended in saline to an OD600 of 15, yielding titres of approximately 1016 c.f.u. ml−1 as determined by dilution plating on LB medium. That suspension and serial twofold dilutions in saline (20 μl) were mixed with erythrocytes (20 μl) and saline or 2% (w/v) d-mannose (20 μl) in ceramic, deep-well dishes and haemagglutination was assessed visually. Erythrocytes failed to agglutinate in the absence of bacteria and in the presence of E. coli strain HU742 (pap).

Pili extraction and SDS-PAGE. Pili were extracted from E. coli strain HU734 (and control preparations were made from E. coli HU742) as follows. Bacteria were cultured by inoculating 25 LB plates with 100 μl (each) of an overnight LB culture and incubating for 24 h at 37 °C. Five millilitres Na MOPS buffer (5 mM, pH 7) and a sterile paper clip were added to each plate and the bacteria were resuspended by agitation of the paper clip with a magnetic stirring platform. The cell suspensions were pooled, processed with a Warin Blender (2 × 2.5 min) and centrifuged (7000 r.p.m., 4 °C, 20 min, Sorvall GSA rotor). The supernatant was centrifuged (18000 r.p.m., 4 °C, 30 min, Beckman 45Ti rotor) to remove cellular debris and pili were harvested from the resulting supernatant by centrifugation (40000 r.p.m., 4 °C, 60 min, Beckman 45Ti rotor). The resulting pellet was resuspended in water, lyophilized and solubilized for electrophoresis according to the procedure of McMichael & Ou (1979). SDS-PAGE was performed as described by Laemmli (1970) using 15% T and 2.6% C and staining with Coomassie brilliant blue R.

Colonization of the murine urinary tract. The ability of E. coli strains to ascend the urinary tracts of female, CBA mice (Jackson Laboratories) was tested essentially as described by Hagberg et al. (1983a, b). Inocula were prepared by inoculating LB cultures in Erlenmeyer flasks (culture volume 20% of flask
volume) for approximately 17 h at 37 °C without shaking to yield an OD₆₆₀ value of 0.5–0.7. Cells were harvested by centrifugation (4000 g, 10 min, room temperature) and gently resuspended at 1/200 of the culture volume in saline. The purity of the culture was checked by inoculating appropriate media and the expression of P pil was verified, during selected experiments, by testing for mannose-resistant haemagglutination. Mice, aged 6–8 weeks, were individually anesthetized by exposure to methoxyflurane (Janssen Pharmaceuticals) in a closed glass jar for 2–2.5 min. Fifty microlitres of the inoculum (approximately 10⁶ cfu) was instilled into the urinary tract of each mouse over a period of at least 20 s through soft polyethylene tubing (Intramedic Clay Adams Brand; outer diameter, 0.61 mm, inner diameter 0.28 mm) (Becton Dickinson) attached to a 30 gauge needle on a 1 ml tuberculin syringe. After injection the catheter was immediately withdrawn and the mice were placed in a recovery chamber prior to being placed back in their cages. Twenty-four hours post-inoculation the mice were euthanized by exposure to excess methoxyflurane. The bladder and kidneys from each mouse were extracted and separately homogenized in 0.5 ml sterile saline. The tissue was disrupted with 10 strokes, a cooling period on ice, and 10 additional strokes using a Potter–Elvehjem tissue grinder. The grinder included a sintered glass vessel with a PTFE-tipped steel pestle, the latter mounted in a variable speed drill which was operated at 50–75% of its maximum speed. The total volume of each homogenate was recorded. Dilutions of each homogenate were spread on LB and/or TTC medium (the two media yield the same estimates of cf.u. ml⁻¹ homogenate). Colonies were counted after 24 h (LB) or 48 h (TTC) incubation of the plates at 37 °C and colonization was estimated as numbers of cf.u. per tissue. The statistical significance of differences in the recovery of bacteria from tissues infected with different E. coli strains was evaluated with the Mann–Whitney test (Snedecor & Cochran, 1980). No bacteria were recovered from the tissues of mice inoculated with sterile saline and no put⁺ revertants or contaminants were detected with TTC medium.

RESULTS

Construction of PutPA- and ProP-deficient mutants

E. coli K-12 (like Salmonella typhimurium) possesses three genetic loci pertinent to the uptake and accumulation of osmoprotectants: putPA, proP and proU. All three loci are present and expressed in E. coli HU734 (Culham et al., 1994). The put operon includes divergently transcribed genes putP and putA. PutP is a Na⁺-proline symporter which does not accept glycine betaine as substrate (Milner et al., 1987). PutA is the bifunctional enzyme which catalyses the oxidation of proline to glutamic acid (Maloy, 1987). Lesions in putP and putA were without effect on osmoprotection of S. typhimurium by proline (Csonka, 1981). The put locus was deleted from E. coli HU734 since defects in genetic loci proP and proU are cryptic to simple phenotypic tests in put⁺ strains of E. coli K-12. Derivatives of E. coli K-12 defective at putP have increased resistance to the toxic proline analogue AC. Those defective at putP, but not those defective at both putP and putA, are also resistant to analogue DHP (Wood, 1981). We therefore selected bacteria that were resistant to AC, screening to identify those which were sensitive to DHP, unable to utilize proline and devoid of proline dehydrogenase activity (see Methods). The presence of the desired deletion [Δ(putPA)566] in the resulting isolate, strain WG541, was verified by PCR (see Methods).

The sequence of the proP locus from E. coli HU734 (accession no. U75904) was determined to assess its similarity with the E. coli K-12 locus. That analysis revealed only three predicted amino acid differences for ProP, each falling within a different hydrophilic segment of the (putative) protein (the predicted changes were Ala-193 to Glu-193, Tyr-257 to His-257 and Phe-380 to Ser-380). Four single nucleotide differences were found in the 282 bp DNA sequence upstream of proP, one (C→A) at the −47 position with respect to proP promoter P1 and another (G→A) at the −31 position with respect to promoter P2. To remove locus proP from strain WG541, deletion proP218 (extending from 59 bp upstream to 44 bp downstream of the ProP ORF) was created in vitro and a segment of E. coli K-12 DNA encompassing that deletion was inserted in vector pCVD44 (Donnenberg & Kaper, 1991). The resulting plasmid, pDC66, was used to create strain WG671 (WG541 proP218) by allelic replacement. The proP deletion was verified by PCR-based DNA amplification (see Methods).

Activities of transporters PutP, ProP and ProU in E. coli HU734

In E. coli K-12, proline serves as a substrate for transporters PutP, ProP and, to a limited extent, ProU. The Kₘ values of transporters PutP and ProP for proline are approximately 0.3 µM (Wood & Zadworny, 1979) and 100 µM (R. T. Voogle, personal communication), respectively. Proline uptake via transporter ProU is barely detectable in E. coli K-12 (Grothe et al., 1986). AC and DHP are toxic for E. coli and they serve as substrates for transporters PutP, ProP and ProU. The zone of growth inhibition caused by each compound in the radial streak test is thus directly related to transporter activity (Grothe et al., 1986). DHP is detoxified by proline dehydrogenase (PutA), so putA defects enhance DHP toxicity (Wood, 1981).

As was anticipated on the basis of prior experience with E. coli K-12 derivatives, strains HU734, WG541 and WG671 could be distinguished from one another by examining their patterns of sensitivity to AC and DHP on low and high NaCl media with the radial streak test (Table 2). The patterns of AC and DHP sensitivity observed for these strains and the corresponding E. coli K-12 derivatives were qualitatively similar. However, larger zones of growth inhibition were attributable to ProU activity (putPA proP strains) and to ProP plus ProU activity (putPA pro strains) in the derivatives of E. coli HU734 than in the derivatives of E. coli K-12.

The proline uptake activities of strains HU734 and WG541 were examined under conditions designed to enhance detection of transporter PutP (low osmolality medium, 10 µM proline as transport assay substrate)
Table 2. AC and DHP sensitivities of E. coli K-12, E. coli HU734 and their derivatives

Zones of growth inhibition (mm) were determined by the radial streak test using 0.5 mg test compound (AC or DHP) per plate containing MOPS medium with glucose (11 mM) as carbon source, with or without NaCl (0.3 M). The E. coli K-12 strains were WG484 (wild-type), WG444 [WG484 Δ(putPA)101] and WG445 [WG444 Δ(proP-mel)212]. The E. coli HU734 strains were HU734 (wild-type), WG541 [HU734 Δ(putPA)566] and WG671 (WG541 proP218). The results are means of 6 determinations (K-12 derivatives) or 18 determinations (HU734 derivatives) ± SEM.

<table>
<thead>
<tr>
<th>Test compound and medium</th>
<th>Genotypes of K-12 strains:</th>
<th>Genotypes of HU734 strains:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>putPA</td>
</tr>
<tr>
<td>AC (−NaCl)</td>
<td>17 ± 2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>DHP (−NaCl)</td>
<td>14 ± 1</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>AC (+NaCl)</td>
<td>23 ± 1</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>DHP (+NaCl)</td>
<td>15 ± 2</td>
<td>21 ± 2</td>
</tr>
</tbody>
</table>

Table 3. Proline uptake activities of E. coli HU734 and its derivatives

E. coli strains were HU734 (wild-type), and derivatives WG541 [HU734 Δ(putPA)566] and WG671 (WG541 proP218). The results [in nmol min⁻¹ (mg protein)⁻¹] are means of three determinations ± SEM for representative experiments. NT, not tested.

<table>
<thead>
<tr>
<th>Growth medium supplement</th>
<th>Proline concn (assay) (µM)</th>
<th>HU734 genotypes:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>putPA</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>None</td>
<td>200</td>
<td>NT</td>
</tr>
<tr>
<td>NaCl (0.3 M)</td>
<td>200</td>
<td>NT</td>
</tr>
</tbody>
</table>

(Table 3). As for E. coli K-12, the putPA deletion reduced proline uptake by strain WG541, measured under these conditions, to a low level. The proline uptake activities of strains WG541 and WG671 were examined under conditions designed to enhance detection of transporter ProP (low and high osmolality medium, 200 µM proline as transport assay substrate) (Table 3). The residual, NaCl-inducible proline uptake activity present in E. coli WG671 was likely due to transporter ProU. For an analogous E. coli K-12 derivative cultured under similar, high-salt conditions, the activity attributable to ProU was only 0.2 nmol min⁻¹ (mg cell protein)⁻¹ (Grothe et al., 1986). The activity of transporter ProP could be computed by subtracting that measured in strain WG671 (proP) from that measured in strain WG541 (proP⁺). On that basis ProP activity rose 24-fold (from 5 nmol min⁻¹ (mg protein)⁻¹ to 121 nmol min⁻¹ (mg protein)⁻¹ when the bacteria were cultured in NaCl-supplemented medium. The ProP activity of E. coli K-12 cells cultured in the same, NaCl-containing medium with d-fructose or glycerol as carbon source was less than 50 nmol min⁻¹ (mg protein)⁻¹ (Grothe et al., 1986). These data confirmed the successful isolation of bacteria deficient in transporters PutP and ProP, also indicating that transporters ProP and ProU may be more active in E. coli HU734 than they are in E. coli K-12.

Salt tolerance of E. coli HU734 and its derivatives

The salt tolerance of E. coli HU734 was compared with that of E. coli WG484 (K-12 srl-300::Tn10) by examining their growth rates in NaCl-supplemented MOPS minimal medium (Fig. 1). Although the two strains showed similar growth rates in unsupplemented medium (0.340 ± 0.004 h⁻¹ and 0.370 ± 0.004 h⁻¹, respectively (mean ± standard deviation for four replicates), E. coli HU734 was more salt-sensitive than E. coli K-12. The NaCl concentrations required to reduce the growth rates of these strains twofold were approximately 0.2 M and 0.5 M, respectively. The corresponding values for growth in medium supplemented with the osmoprotectant glycine betaine (1 mM) were approximately 0.55 M NaCl (HU734) and 0.8 M NaCl (WG484). The effect of glycine betaine concentration (range 25 µM–1 mM) on growth rate was examined for strain HU734 in medium supplemented with 0.4 M NaCl. Full growth stimulation (from negligible growth to a rate of 0.215 ± 0.005 h⁻¹) was observed at the lowest glycine betaine concentration tested. When a similar experiment
E. coli osmoadaptation and urinary tract infection

Fig. 1. Growth of E. coli in MOPS medium as a function of osmolality and glycine betaine supplementation. E. coli HU734 (a) and E. coli WG484 (b) (a K-12 derivative) were cultured, with shaking, in MOPS minimal medium as described in Methods. The osmolality (+) of the growth medium was varied, as indicated, by addition of NaCl. Glycine betaine was absent (●) or present (1 mM) (■). Growth rates are reported as means ± SD for four replicate growth rate determinations.

was performed with strain WG484 (glycine betaine range 62.5 μM–1 mM, 0.6 M NaCl) full growth stimulation (from 0.11 ± 0.02 h⁻¹ to 0.208 ± 0.005 h⁻¹) was again observed at the lowest glycine betaine concentration tested. Strains HU734, WG541 and WG671 grew at the same rate in MOPS medium (data not shown). Their ability to grow in hypersaline medium [MOPS plus 0.4 M NaCl (osmolality 0.94 mol kg⁻¹)] with or without glycine betaine (0.1 mM) was also tested (Table 4). The growth rates of the three strains in glycine-betaine-supplemented medium were indistinguishable, indicating that mutations Δ(putPA)566 and prop218 were without effect on osmoprotection under these conditions, perhaps because transporter ProU was active.

Bacterial growth in human urine was also assessed. E. coli HU734 grew at similar rates in hypertonic human urine (Fig. 2) and in betaine-supplemented, defined medium of similar osmolality (Fig. 1, Table 4). Growth rates in human urine were 0.29 h⁻¹ for urine with osmolality of 0.80 mol kg⁻¹ and pH 5.79, and 0.27 h⁻¹ for urine with osmolality of 0.95 mol kg⁻¹ and pH 5.73, estimated from plots of log OD₆₀₀ versus time for

<table>
<thead>
<tr>
<th>Glycine betaine</th>
<th>HU734 genotypes:</th>
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<tbody>
<tr>
<td></td>
<td>Wild-type</td>
</tr>
<tr>
<td>-</td>
<td>0.070 ± 0.006</td>
</tr>
<tr>
<td>+</td>
<td>0.28 ± 0.02</td>
</tr>
</tbody>
</table>

Table 4. Stimulation of growth of E. coli HU734 and its derivatives by glycine betaine in hypertonic media

Fig. 2. Effects of transporter defects on growth of E. coli in human urine. Growth of E. coli strains HU734 (■), WG541 (putP, ●) and WG671 (putP prop, ▲) in hypertonic human urine was estimated by dilution plating (c.f.u. ml⁻¹) and measurement of OD₆₀₀ as described in Methods. (a) Pooled urine, pH 5.79, osmolality 0.80 mol kg⁻¹; (b) pooled urine, pH 5.73, osmolality 0.95 mol kg⁻¹. Insets show relationships between viable count and OD₆₀₀.
periods of exponential growth within the OD$_{600}$ range 0.1–1.0. Whereas the putPA lesion was without effect on growth in urine, the prop lesion impaired growth to a degree that was more pronounced in the urine of higher osmolality (Fig. 2). The relationship between OD$_{600}$ and viable count [c.f.u. (ml culture)$^{-1}$] for strain WG671 (putPA prop) differed from that for strains HU734 and WG541 (putPA). The OD$_{600}$ values for cultures of strain WG671 were higher than those of strains HU734 and WG541 for equivalent viable counts, again to a degree which was greater in the urine of higher osmolality (Fig. 2, insets). This difference suggests that bacteria with reduced compatible solute uptake activity are dehydrated, having a higher refractive index than their counterparts, and/or that they differ in size, being larger (longer) than their counterparts.

**Effects of osmolality on P piliation of E. coli HU734 and its derivatives**

Effects of growth medium osmolality, glycine betaine supplementation and transporter lesions on P piliation of strain HU734 were assessed with a haemagglutination assay designed to detect expression of the P pilus adhesin, PapG (see Methods). Whereas cells of strain HU734 cultured to stationary phase in LB medium (shaken or non-shaken) mediated mannose-resistant haemagglutination (MRHA), those cultured to stationary phase in MOPS minimal medium, shaken or non-shaken, did not. By analogy with a previous report (Kunin et al., 1994), the MRHA titre of E. coli HU734 decreased from 1/32 for cells cultured in non-shaken LB medium (0.4 mol kg$^{-1}$) to 1/8 for those cultured in LB plus 0.3 M NaCl (1.0 mol kg$^{-1}$). Cells cultured in LB medium plus 0.6 M NaCl (1.6 mol kg$^{-1}$) did not mediate MRHA. The MRHA titres for strains WG541 (putPA) and WG671 (WG541 prop) were 1/16 and 1/8, respectively, for LB-grown bacteria and 1/2 after culture in LB plus 0.3 M NaCl. Neither mediated MRHA after cultivation in LB plus 0.6 M NaCl. Electrophoretic analysis of crude pilus extracts revealed that major pilus protein PapA followed similar trends (Fig. 3). These data indicated only small effects of the putPA and prop deletions on pilus expression; osmoprotection mediated by Prop did not strongly attenuate the adverse effect of elevated salinity on P piliation.

**Colonization of the murine urinary tract by E. coli HU734 and its derivatives**

Mice and non-human primates provide the preferred animal models for ascending infection of unobstructed human urinary tracts (Johnson & Russell, 1996). Urine from female CBA mice was found to contain 674±93 μM proline betaine and 687±204 μM glycine betaine (means±standard deviations for determinations made on five independent samples) (M. Lever, personal communication). These levels are significantly higher than those of urine from human subjects (mean betaine levels 298±687 μM and 78±65 μM, respectively) but similar to those of urine from other mammals (Lever et al., 1994a). They confirm the availability of betaines to serve as osmoprotectants during analyses of ascending urinary tract infection performed with the murine model.

The numbers of colony forming units (as log c.f.u.) recovered from the bladders and kidneys of CBA mice inoculated with E. coli strains WG541 (prop$^+$) and WG671 (prop) are compared in Fig. 4. The numbers of c.f.u. recovered from tissues infected with strain WG541 were similar to those previously reported for infection of
CBA mice with *E. coli*HU734 (Hagberg *et al.*, 1983a), confirming our successful implementation of the murine model. Strain WG671 [Δ(putPA)566 prop218] was recovered from murine bladders at a level 100-fold lower than strain WG541 [Δ(putPA)566] (significant at the 95% confidence level according to the Mann–Whitney test) (Snedecor & Cochran, 1980; Mobley *et al.*, 1993). However, no significant difference in recovery of the two strains from murine kidneys was detected.

**DISCUSSION**

Kunin (1987) suggested that osmolar fluctuations influence the survival and growth of *E. coli* in the human urinary tract. This report extends our analyses of osmoregulation by human pyelonephritis isolate HU734 and the relationship between osmoadaptation and colonization of the urinary tract by *E. coli*. Deletion of genetic loci putPA and prop from the chromosome of *E. coli* HU734 facilitated characterization of its transporter activities and examination of their impact on colonization of the murine urinary tract. Like *E. coli* K-12, *E. coli* HU734 expresses transporters PutP, Prop and ProU. Osmotic induction yielded Prop activity in *E. coli* HU734 that was threefold higher than that of *E. coli* K-12 (Tables 2, 3). It will be of interest to determine whether that elevation is related to differences in DNA sequence between the prop loci of *E. coli* K-12 and *E. coli* HU734 and whether it is common among urinary tract isolates. The activity of transporter ProU also appeared to be elevated in *E. coli* HU734, but that conclusion must be confirmed by deletion of the proU locus and comparison of the glycine betaine uptake activities of proU+ and proU- bacteria.

Deletion of transporters PutP and Prop had no effect on the growth rate of *E. coli* HU734 in hypertonic minimal medium with or without glycine betaine (Table 4). In analogous studies on *S. typhimurium*, lesions in both prop and proU were required to abolish osmoprotection by glycine betaine. A lesion in either locus reduced but did not eliminate osmoprotection (Dunlap & Csonka, 1985). Deletion of transporter Prop did impair bacterial growth in hypertonic human urine (Fig. 2) and it reduced the numbers of bacteria recovered from the bladders of inoculated mice 100-fold. However, recovery of bacteria from the kidneys was unaffected (Fig. 4). The effect of the prop lesion on bladder colonization suggests that transporter Prop is expressed by bacteria in the bladder and that osmoprotective betaine uptake facilitates survival and/or growth of bacteria which inhabit that tissue. If transporter ProU is also expressed and active in the urinary tract, bacteria defective at both prop and proU may be further impaired in their ability to proliferate in that environment.

Why did the prop lesion influence the recovery of bacteria from the bladders but not the kidneys of infected mice? The balance between proliferation through growth and cell division versus loss through death and micturition is expected to be more critical to population density for free than for surface-bound urinary tract bacteria (Gordon & Riley, 1992). Reductions in bacterial growth rate due to high osmolality and/or a Prop defect would influence bacterial population density in the bladder more than in the kidney if a smaller fraction of the bacteria in the bladder than the kidney were surface-associated. Adhesion of *E. coli* to uroepithelial cells is believed to be mediated, at least in part, by P pili. P pilus expression [both PapG (see Results) and PapA (Fig. 3) were impaired when the bacteria were cultured in NaCl-supplemented LB media with osmolalities in excess of 1 mol kg−1]. If the expression of P pili within the urinary tract shows a similar dependence on urine osmolality, P-pilus-mediated bacterial adhesion may also be impaired by urine osmolality within the normal murine (up to 3 mol kg−1) or human (0.04–1.3 mol kg−1) range. However, osmoprotection mediated by transporter Prop did not significantly attenuate the inhibition of P pilus expression by high salinity (Fig. 3).

Why did the prop lesion reduce the recovery of *E. coli* HU734 from the murine bladder (Fig. 4) and its growth rate in hypertonic human urine (Fig. 2) but not its growth rate in a hypertonic minimal medium (Table 4)? A number of important characteristics differentiate the conditions faced by bacteria in the bladder (*in vivo*) during urine culture (*in vitro*) and during minimal medium culture (*in vitro*). They include: (1) microaerophilic [*in vivo* (Maluszynska *et al.*, 1992)] versus aerobic (*in vitro*); (2) the presence (*in vivo*, and in urine culture *in vitro*) versus the absence (in minimal
medium \textit{in vitro}) of urea; (3) potentially variable (\textit{in vivo}) versus constant (\textit{in vitro}) osmolality; (4) a mixture of organic substituents (\textit{in vivo}, and in urine culture \textit{in vitro}) versus single carbon (glucose) and nitrogen (NH$_4$Cl) sources provided in excess (in minimal medium \textit{in vitro}); (5) continuous (\textit{in vivo}) versus batch (\textit{in vitro}) culture; and (6) a combination of surface-bound and suspension culture (\textit{in vivo}) versus suspension culture (\textit{in vitro}). During our efforts to characterize the transporter activities of \textit{E. coli} HU734 and its derivatives, the bacteria were cultured under conditions which would permit comparison of the resulting data with properties previously reported for \textit{E. coli} K-12. Relatively little information is available concerning the impact of the listed culture conditions on osmoprotective compatible solute uptake, but the potential roles of aeration and urea content merit particular attention.

Anaerobiosis attenuated the sensitivity of \textit{E. coli} K-12 to toxic proline analogues AC and DHP under both low- and high-salt conditions, implying reduced activity of transporter PutP, ProP and/or ProU (Reese et al., 1996). However, the relative impact of anaerobiosis on the activities of those transporters, or indeed on others whose activities are also expected to depend on cellular energy status, was not reported.

Urea can reach concentrations of 0.5 M and 1.5 M in human and rat urine, respectively. Since most biological membranes are urea-permeable, urea is not expected to alter the distribution of water across bacterial cell membranes. Urea, alone, did not elicit uptake of glycine betaine or proline betaine by \textit{E. coli} and neither glycine betaine nor proline betaine stimulated the growth of \textit{E. coli} in urea-supplemented media unless NaCl levels were also high (Chambers & Kunin, 1985; Randall et al., 1996). Those observations are consistent with known requirements for the activation of transporters ProP and ProU and the assumption that the cytoplasmic membrane of \textit{E. coli} is urea-permeable (Csonka & Epstein, 1996). They suggest that, in considering its capacity to elicit an osmoregulatory response from \textit{E. coli}, the osmolality of urine should be corrected for the contribution of urea. Thus normal human urine would vary in ‘corrected’ osmolality within the range of standard growth media (0.2–0.4 mol kg$^{-1}$) and the maximum ‘corrected’ osmolalities of human and murine urines would be less than 1 mol kg$^{-1}$ and 1.5 mol kg$^{-1}$, respectively. These corrected values fall within the range of the (urea-free) minimal medium used here to test effects of osmolality, glycine betaine and transporter ProP on bacterial growth rate (Fig. 1, Table 4). Although urea alone does not elicit osmoregulatory betaine uptake, cytoplasmic glycine betaine alleviates the inhibition of bacterial growth by urea (Chambers & Kunin, 1985; Randall et al., 1996). It is thus possible that the activity of transporter ProP assumes more importance for bacterial growth in the bladder than in urea-free, \textit{in vitro} culture because its activity attenuates effects of urea on bacterial growth rate.

The activities of transporters ProP and ProU respond to the osmolality of the extracellular medium; ProP activity, at least, also responds to other signals. Transcription of loci proP and proU is enhanced when bacteria are cultured in high osmolality media; proP transcription is and proU transcription may also be increased during stationary-phase growth (Csonka & Epstein, 1996). As noted above, both ProP and ProU are activated when bacteria are subjected to an osmotic upshift. Although urine osmolality is likely to be important, further investigation will be required to fully define which stimuli elicit osmoprotective activity in bacteria that colonize the murine bladder.

Lever et al. (1994a, b) examined the betaine content of serum and urine as a function of the age, sex and health status of human subjects. In normal humans urinary glycine betaine levels were 78±65 μM (median 67 μM, range 5 μM–405 μM); those of proline betaine were 298±687 μM (median 96.5 μM, range from not detectable to 514 mM). Mice and other mammals have higher urinary glycine betaine and proline betaine levels than humans (Lever et al., 1992, 1994a). Maximal stimulation of the growth rate of \textit{E. coli} HU734 in hypertonic minimal medium was observed with glycine betaine at levels as low as 25 μM (see Results). The glycine betaine levels in the urine of healthy humans (and mice) are therefore unlikely to limit the ability of \textit{E. coli} to respond to urine concentration by using glycine betaine as an osmoprotectant. It has been suggested that toxic betaine analogues recognized by bacterial but not host transporters may serve as antibacterial agents for the treatment of urinary tract infection (Chambers et al., 1987). These data suggest that particular attention should be paid to the substrate specificity of transporter ProP in pursuing that concept.

The inhibition of bacterial growth in human urine (Fig. 2) and the reduction in colonization of the murine bladder (Fig. 4), both due to a proP lesion, offer the first direct evidence that osmoprotective betaine accumulation and transporter ProP are pertinent to urinary tract colonization by \textit{E. coli}. In view of its high osmolality, rodent urine may impose a greater barrier to the growth of \textit{E. coli} than human urine. The murine model should thus be regarded as a particularly sensitive one for the detection of osmolality effects on urinary tract colonization.

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