Functional characterization of *Actinobacillus pleuropneumoniae* urea transport protein, ApUT

Geeta Godara, Craig Smith, Janine Bosse, Mark Zeidel, and John Mathai

1Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania; 2Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts; 3Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom; 4Molecular Infectious Disease Group, Department of Paediatrics, Imperial College London, St. Mary’s Campus, London, United Kingdom

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Godara G, Smith C, Bosse J, Zeidel M, Mathai J. Functional characterization of *Actinobacillus pleuropneumoniae* urea transport protein, ApUT. Am J Physiol Regul Integr Comp Physiol 296: R1268–R1273, 2009. First published January 14, 2009; doi:10.1152/ajpregu.90726.2008.—Urea transporters (UTs) effect rapid flux of urea across biological membranes. In the mammalian kidney, UT activity is essential for effective urine concentration. In bacteria, UT-mediated urea uptake permits intracellular urease to degrade urea to ammonia and CO₂, a process that either buffers acid loads or provides nutrient nitrogen. We have characterized the urea transport channel protein ApUT from *Actinobacillus pleuropneumoniae*. Kinetic analysis of bacterial inside-out membranes enriched in ApUT showed ~28-fold increase in urea permeability (3.3 ± 0.4 × 10⁻⁴ cm/s) compared with control vesicles (0.11 ± 0.02 × 10⁻⁴ cm/s). In addition to urea, ApUT also conducts water. Urea and water transport across the channel was phloretin and mercury inhibitable, and the site of inhibition may be located on the cytoplasmic side of the protein. Glycerol and urea analogs, such as methylamine, dimethylurea, formamide, acetamide, methylurea, propanamide, and ethylamine did not permeate across ApUT.

ApUT, urea permeability; water permeability; bacterial vesicles; urea analogs

**UREA, AN END PRODUCT OF NITROGEN metabolism, is widespread in nature. Elasmobranchs and mammals use urea as an osmotocant, permitting the elasmobranch to maintain its osmolality and the mammalian kidney to effect maximal concentration of urine (9). Microbes metabolize urea as a nitrogen source or as a mechanism to combat an acidic environment. Urea transporters mediate bacterial acquisition of urea and are considered to be critical for survival.**

Because urea is a small electroneutral molecule, it exhibits appreciable permeability across lipid membranes. Although this passive urea permeability has been thought to be sufficient for its biological roles, several specific urea transporter proteins have been identified in prokaryotes and eukaryotes. In mammals UT-A (6 isoforms) and UT-B (2 isoforms) genes encode members of a specialized family of urea transporters that play an important role in the renal urinary concentrating mechanism and potentially other processes, including spermatogenesis (9).

In bacteria, two different types of transporters are known to exist for urea uptake. Under conditions of nitrogen starvation, in *Methylphilus methylotropus* and *Corynebacterium glutamicum*, an ATP-dependent ABC type transporter is responsible for urea uptake into the cell (2, 24). Passive or facilitated movement of urea is seen in *Helicobacter pylori* where urea uptake is mediated by an acid-activated urea channel, UreI, which was shown to be essential for survival in vivo (33). UreI is a single-component protein with six transmembrane-spanning domains and does not share sequence homology with mammalian urea transporters. Several ureI type genes have subsequently been identified in other microbes, such as *Streptococcus salivarius*, *Brucella melitensis*, and *H. hepaticus* (3, 25). As an example, in *H. pylori*, the gastric pathogen responsible for much of human peptic ulcer disease, activity of a urease system maintains a near neutral periplasmic and cytoplasmic pH in the face of an ambient pH 2 in the gastric lumen (26). Following its uptake into the organism, urease breaks down urea to produce ammonia and carbon dioxide; the buffering capacity of ammonia helps protect the organism from the gastric acid environment. Recent studies also suggest that the conversion of CO₂ to HCO₃⁻ by periplasmic γ-carboxy anhydrase is also essential for acid acclimation (20).

Therefore, urea metabolism and urea activity, in particular, have been implicated in the ability of certain bacteria to infect and cause disease (4, 8, 38). Examination of the urease gene cluster of the swine pathogen *A. pleuropneumoniae* (5) resulted in the identification and cloning of a putative urea transporter cDNA encoding a 300-amino acid protein ApUT (GenBank accession no. AAD49729). ApUT does not show any sequence homology to the acid-activated bacterial urea transporter UreI or the energy-dependent urea uptake systems in some bacteria. However, it shows 26% identity to the mammalian urea transporter UT-A2 (GenBank accession no. Q62668) (34) and 21% identity to mammalian members of the UT-B subfamily (GenBank accession no. Q13336) (27). Given the lack of detailed functional data on mammalian urea transporters, and our ability to reconstitute activity of ApUT in bacteria, we have examined ApUT function in detail as a model transporter for the UT-A/UT-B family. ApUT was overexpressed in bacteria and by using inside-out membrane vesicles enriched in ApUT, a comprehensive kinetic and functional characterization was performed.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *A. pleuropneumoniae* strain Shope 4074 (serotype 1) was propagated at 37°C with 5% CO₂ on brain-heart infusion agar (DIFCO) supplemented with 0.01% β-nicotinamide adenine dinucleotide (Roche). *Escherichia coli* strains

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Top 10 and BL21-AI (Invitrogen) were cultured in Luria-Bertani broth or on Luria-Bertani agar, supplemented with 50 μg/ml ampicillin when appropriate.

**Plasmid construction.** Genomic DNA was prepared from A. pleuropneumoniae Shope 4074 by using a QIAamp mini-DNA kit (Qiagen). Polymerase chain reaction (PCR) was performed according to standard procedures (29). Primers ApUt_F (5'-ATGAAAGCTGCT-TAATCCACCCCTA-3') and ApUt_R (5'-GACGCTTTTGAAGATCCAGCA-3') were used to amplify a 901-bp fragment containing the 5'-bp ApUt gene. The resulting PCR product was cloned into pGEM-T (Promega) to create pJApUt, which was transformed into E. coli Top10. Orientation of the insert was determined by restriction digestion with PstI and PvuI (Roche). ApUt CDNA was subsequently subcloned into an isopropyl β-d-thiogalactoside-inducible overexpression plasmid, pRSET-A (Invitrogen) downstream of the 6-His tag. The resulting plasmid, pRSET-A-ApUT, was transformed into E. coli BL21-AI for expression.

**Bacterial expression studies.** BL21-AI, E. coli strain (Invitrogen) was cultured at 37°C in Luria-Bertani broth at pH 7.4 supplemented with 50 μg/ml ampicillin and washed by centrifugation in buffer without lysozyme and EDTA, 0.1 mg/ml DNAse (Promega) to create pJApUt, which was transformed into E. coli Top10. Orientation of the insert was determined by restriction digestion with PsI and PvuI (Roche). ApUt CDNA was subsequently subcloned into an isopropyl β-d-thiogalactoside-inducible overexpression plasmid, pRSET-A containing ApUt cDNA. Cells were grown up to OD_{600} of 0.8 and transcription from T7 promoter was switched on by the addition of 0.2 mM isopropyl β-d-thiogalactoside at 10°C. After a 10-h induction period, cells were harvested and washed by centrifugation for 10 min at 3,000 g.

**Inside-out vesicle preparation.** The bacterial pellet was resuspended in buffer containing 100 mM NaCl, 100 mM sucrose, and 50 mM Tris-HCl, pH 7.4 (buffer A). Cells were centrifugally washed once in buffer A and resuspended in the buffer A containing 20 mM carboxyfluorescein (CF), 1 tablet/20 ml protease inhibitor cocktail, and 10 μg/ml DNAse. The cells were lysed by passing the bacterial solution through a French press at 4°C and centrifuged at 10,000 g for 10 min. The supernatant was collected, and the pellet containing the nuclei, unbroken cells, and debris was discarded. The supernatant was centrifuged at 70,000 g for 45 min, and the pellet was washed twice by centrifugation and resuspended in 1 ml of buffer A. The vesicles were immediately used for transport studies (13).

**Spheroplast or right-side out vesicles.** E. coli spheroplast were prepared by the method of Kaback (11) with minor modifications. Briefly, bacteria was harvested and washed as mentioned above for preparation of inside-out vesicles. Bacteria was resuspended and washed by centrifugation in a buffer made of 20% sucrose and 30 mM Tris·HCl, pH 8.0. The pellet was resuspended in the above buffer containing lysozyme (500 μg/ml) and 10 mM EDTA. After 30 min of incubation with gentle swirling at 25°C, spheroplasts were harvested by centrifugation at 13,000 g for 10 min. The pellet was washed twice by centrifugation in the above buffer without lysozyme and EDTA, and, after a final wash, the pellet was resuspended in the same buffer and used immediately for transport measurements.

**Permeability measurements.** Water permeability (P_f) was measured by exposure of vesicles to an abrupt doubling of external osmotic pressure in a stopped-flow fluorometer. The shrinkage of vesicles in response to the osmotic gradient was measured as change in fluorescence over time due to self-quenching of entrapped CF. Curves (8–12) were averaged and fit to a single exponential. A family of single exponential curves was generated by simulation of the water permeability equation where only the P_f was varied using MathCad software: \( \frac{dV}{dt} = (P_f)(SAV)(MVW)(C_{in}/V_{in}) \), where \( V_t \) is the relative volume of the vesicle at time \( t \) (i.e., volume at time \( t \) divided by the initial volume), \( P_f \) (cm/s) is the osmotic water permeability coefficient, SAV is the surface area-to-volume ratio, MVW is the molar volume of water (18 cm³/mol), and \( C_{in} \) and \( C_{out} \) are initial solute concentrations inside and outside the vesicle. Since the external solute volume is very large compared with internal vesicle volume, the \( C_{out} \) is assumed to be constant. \( P_f \) was obtained by comparing the experimental single exponential with that of single exponentials obtained from simulation (21). The permeability of urea, urea analogs, and glycerol were measured by preequilibrating the vesicles at a solute concentration of 200 mM each in buffer A for 30 min and then rapidly diluting them twofold in a stopped-flow device to create an isoosmotic urea gradient. Solute efflux results in vesicle shrinkage and CF quenching, which was recorded over time. Vesicles sizes were determined by using quasielastic light scatter using a DynaPro particle sizer. The average diameter of inside-out vesicles and spheroplast are in the range of 0.24 to 0.32 microns and 1.2 to 3.0 microns, respectively. Permeability coefficients were calculated using MathCad software as described earlier (21, 22). Urea transport inhibitor studies were done by incubation of vesicles for 30 min in buffer A containing 1.0 mM phloretin or a 10-min incubation in 0.5 mM mercuric chloride. Control vesicles were similarly treated. Kinetic studies of ApUt were performed by incubating the vesicles in various urea solutions ranging in concentrations from 100 to 1,000 mM. In the stopped-flow measurements, appropriate amounts of NaCl were added to maintain isoosmotic conditions upon dilution. Proton permeability was measured by exposure of vesicles to a gradient of 0.5 pH unit, and the internal pH decrease due to entry of protons was monitored as a decrease in entrapped CF fluorescence (22). Ammonia permeability was measured by exposure of vesicles incubated in buffer A at a pH of 6.8 to 10.0 mM ammonia gradient. Ammonia entry leads to an increase in internal pH, which is monitored as a fluorescence increase of entrapped CF in a stopped-flow device as described earlier (22). Groups were compared using unpaired Student’s t-test and \( P < 0.05 \) was considered significant.

**RESULTS**

Expression of ApUt in bacterial membrane vesicles was detected by SDS-PAGE of membrane vesicles probed using anti-His antibody. Fig. 1. Inside-out vesicles isolated from bacteria expressing ApUt show a major band of ~35 kDa. The faint higher molecular weight bands seen on the blot may represent oligomers of ApUt. Control membrane vesicles obtained from uninduced bacteria do not show any bands. Sequence analysis of ApUt including the 6-His tag predicts a molecular weight of 34 kDa. The presence of the 6-His tag either at the NH2 or COOH terminus of the protein did not affect the expression or the function of the protein (data not shown).

![Fig. 1. Immunoblot analysis of Actinobacillus pleuropneumoniae urea transporter (ApUt). Lysates from vesicles containing ApUt or control vesicles lacking ApUt were analyzed by 8–16% SDS-PAGE immunoblot, and protein was probed by using anti-His antibody. The band at ~35 kDa represents the ApUt monomer and the higher molecular weight band may represent ApUt trimers.](http://ajpregu.physiology.org/) by 10.220.33.3 on May 6, 2017
Urea transport studies were performed by preloading the vesicles with urea and then abruptly diluting them in the stopped-flow device into buffer of the same osmolality but half the concentration of urea as the interior of the vesicles. Efflux of urea was followed by water efflux, resulting in shrinking of the vesicles (18). This vesicle shrinkage was monitored by the self-quenching of entrapped CF. Fig. 2 shows that ApUT-containing vesicles equilibrate rapidly, while control vesicles without ApUT take a longer time to equilibrate. The urea permeability coefficient of ApUT-containing vesicles was ~28-fold higher (3.3 × 10⁻⁴ cm/s) than that of the control vesicles (P<0.001). To check whether the ApUT was also permeable to water, ApUT-containing vesicles were subjected to a doubling of extravesicular osmolarity and their volume change was monitored over time. Fig. 3 shows that ApUT-containing vesicles shrank more rapidly than control vesicles upon exposure to an osmotic gradient, which suggested that ApUT was permeable to water. Osmotic water permeability coefficients of ApUT vesicles were ~3.5-fold higher (P<0.005) compared with those of control vesicles, 0.53 ± 0.14 × 10⁻² cm/s (P<0.005). From prior work, it was unclear whether urea transporters function more as urea pores or urea carriers. These apparent mechanisms were distinguished by defining whether or not urea flux was saturable. To determine whether ApUT exhibits saturability, and, if so, to define the Km and Vmax of urea transport, vesicles containing ApUT were preloaded with various concentrations of urea and the efflux of urea over time was monitored under isoosmotic conditions. Fig. 4, shows the rate of vesicle shrinkage due to urea efflux at various substrate concentrations. At higher substrate concentrations, the rate of urea efflux tended to saturate. The data was fitted by nonlinear regression to the Michalis-Menton equation and the apparent Km and Vmax values computed using the nonlinear regression method are 182 mM and 60 s⁻¹, respectively. S/V, substrate rate; S, substrate.

To determine the specificity of ApUT for urea, the permeabilities of several urea analogs were measured by substituting the analogs for urea during preloading and measuring their efflux under conditions identical to those used in the urea efflux assays. Fig. 5a shows that the measured permeability of various analogs (methylamine, dimethylurea, formamide, acetamide, methylurea, propanamide, and ethylamine) was similar for both the ApUT-containing vesicles and control vesicles lacking urea transporter. This suggests that none of the urea analogs tested permeate through the ApUT pore. Since ammonia is a breakdown product...
of urease activity on urea, we also checked whether ApUT is permeable to ammonia. Fig. 5B shows that ammonia does not permeate across ApUT. Furthermore, ApUT was also found to be impermeable to protons, Fig. 5B and glycerol (data not shown). Together these results suggest that ApUT is specific for urea with a small water conductance.

The classical urea transport inhibitor phloretin and mercury inhibited urea transport through ApUT, Fig. 6. Interestingly, mercury was found to inhibit urea transport only in inside-out vesicles. The sidedness of urea transport inhibition by mercury suggests that the mercury-binding site is located on the intracellular side of the protein molecule. Water permeability of inside-out vesicles was inhibited up to 75% (data not shown) upon incubation with mercury. However, water permeability in spheroplast showed a very high background rate, and, therefore, mercury inhibition results for water permeability were not conclusive (unpublished observations, Mathai JCM). In addition, water permeability of inside-out vesicles containing ApUT was also significantly inhibited by phloretin (ApUT, 1.7 ± 0.2 × 10⁻² cm/s; with phloretin, 0.62 ± 0.22 × 10⁻² cm/s) (P < 0.05), indicating that both urea and water permeate through the same pathway.

**DISCUSSION**

Recent work has defined, in some detail, the genetics and molecular biology of mammalian and bacterial urea transporters (25, 26, 30, 35). Efforts to develop a functional understanding of how UTs transport urea across the membrane will require a combination of detailed structural and functional studies. Bacterial transporter proteins have, in general, proven to be better subjects than their mammalian counterparts for reconstitution and subsequent detailed functional and structural study. We have therefore performed a detailed functional characterization of a bacterial urea transporter, ApUT, from the swine pathogen *A. pleuropneumoniae*, because it bears considerable sequence identity to mammalian UTs. Although this microbe is a strict respiratory tract pathogen, urease activity has been implicated in its virulence (1). Since the pathogenetic site for this organism, the respiratory tract, does not have an acidic pH, it is not clear whether urea uptake and metabolism...
is needed as a source of nitrogen or whether urea’s breakdown products, ammonia and carbon dioxide, serve in some other capacity to establish or sustain the infection.

Several putative bacterial urea transporters have been identified by scanning the genome data bank (25). Sequence analysis studies suggest that vertebrates acquired UT genes from bacteria, and gene duplication events gave rise to putatively 10 transmembrane segments for UTs A2 to A5 and 20 for UT-A1 (25). Sequence analysis of ApUT suggests that it is probably comprised of at least seven transmembrane segments and shares ~26% sequence identity with mammalian urea transporters where most of the homologues are proposed to comprise 10 transmembrane-spanning segments. Furthermore, the tandem sequence repeats seen in eukaryotic urea transporters (LPXTXPFP) (28) are also seen in ApUT. Like UT-A or UT-B proteins from higher organisms, ApUT is not homologous to the acid-activated UreI family of urea transporters, which are proteins from higher organisms, ApUT is not homologous to this protein family. Interestingly, the water transport protein Aquaporin 1 does not transport protons due to an electrostatic barrier within the protein core. Therefore, like water transport via AQPI, it is likely that a similar barrier exists in UTs and that transport of urea or water by ApUT involves hydrogen bond breakage (12).

Having discovered that like UT-B, ApUT has the capacity to transport water, we tested whether ApUT mediated flux of ammonia or protons. This was important to evaluate so as to ascertain whether ApUT might contribute to pH regulation. In particular, since urea is a polar molecule and enters into hydrogen bonding with itself and water, there exists the potential for the formation of a “proton wire” (7). We observed that the flux of ammonia or protons was not increased by expression of ApUT, indicating that neither were transported via this protein. Interestingly, the water transport protein Aquaporin 1 does not transport protons due to an electrostatic barrier within the protein core. Therefore, like water transport via AQPI, it is likely that a similar barrier exists in UTs and that transport of urea or water by ApUT involves hydrogen bond breakage (12).

The availability of an atomic structure for ApUT would permit this suggestion to be evaluated.

Previously, it has been difficult to assess kinetic parameters of urea transporters due to a combination of the high concentration of urea required to approach saturation and the expression systems used. By applying our method to this problem we were able to show that ApUT tends to saturate, with an apparent \( K_m \) of 182 mM and a \( V_{max} \) of 60 s\(^{-1}\). In one set of experiments where inside-out vesicles showed very high ApUT-mediated urea flux, we observed \( K_m \) and \( V_{max} \) values of 357 mM and 140 s\(^{-1}\). The \( K_m \) and \( V_{max} \) numbers should be treated with caution as vesicles become unstable at higher urea concentrations. Studies performed on the mammalian erythrocyte urea transporter UT-B have estimated the \( K_m \) of UT-B to be 218 mM (16, 23). Therefore, given the phylogenetic distance and relatively low sequence homology to UT-B, our estimate of ApUT \( K_m \) seems reasonable. The measured turnover numbers for UT-B has been reported to be two to three orders of magnitude faster than the fastest known carrier (erythrocyte anion exchanger). Accordingly, a channel-mediated mechanism was suggested for UT-B mediated urea transport (19). The apparent saturation of ApUT indicates that rather than acting as a simple pore, urea is transported by this transporter either by a carrier-mediated mechanism or by a simple channel process involving a binding site for urea. To distinguish between a channel or a simple carrier mode of transport, additional specific measurements of single-channel fluxes or turnover numbers are required (19, 36). The measured single-channel fluxes of mammalian urea transporters UT-A2 and UT-A3 are 100-fold less than UT-B, the erythrocyte urea transporter, and did not show apparent saturation kinetics, which suggest a channel mode of urea transport for these transporters (18).

What can ApUT tell us about urea transporters from higher organisms? Given the lack of detailed kinetic information for the UTs from higher organisms, whether ApUT represents a true paradigm of the UT family as a whole is at present difficult to determine. ApUT is a bacterial urea transporter that functionally shares several characteristics with phylogenetically higher members of the UT-A/UT-B family in terms of urea transport, phloretin-inhibition, and transport of water. Therefore, cautionous extrapolation of the data in this paper should prove useful for future experiments addressing detailed functional analysis of mammalian UTs. Future work will involve reconstitution of ApUT into proteoliposomes to measure its single-channel permeability and crystallization.
In summary, we have experimentally and functionally reconstituted a bacterial urea transporter that bears 26% homology to mammalian kidney urea transporters. ApUT transports urea highly effectively and can transport water. Urea flux exhibits saturable kinetics and is inhibited by application of mercury to the intracellular face of the protein molecule. ApUT is highly specific for urea and excludes urea-like solutes.

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Present address of G. Godara: Maine Medical Center, Portland, Maine 04102.

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REFERENCES


