J. Membrane Biol. 187, 65–70 (2002)
DOI: 10.1007/s00232-001-0152-8

The Journal of
Membrane
Biology

Functional Expression of the *Vibrio parahaemolyticus* Na^+/Galactose (vSGLT) Cotransporter in *Xenopus laevis* Oocytes

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Received: 31 August 2001/ Revised: 21 January 2002

Abstract. We have successfully expressed a bacterial cotransporter in a functional form in the *Xenopus laevis* oocyte expression system. The goals were to compare the kinetics and selectivity of the cotransporter expressed in oocytes with those obtained in bacteria and in proteoliposomes, and to determine if it is possible to measure the electrical properties of the bacterial cotransporter expressed in oocytes. The *Vibrio parahaemolyticus* Na^+/galactose cotransporter (vSGLT) expressed in oocytes has functional properties that are similar to those expressed in bacteria and those of the purified cotransporter reconstituted into liposomes. vSGLT is a Na^+-dependent transporter that is saturable with Na^+ ($K_{0.5}$ = 17 mM) and D-galactose ($K_{0.5}$ = 237 μM) and is sensitive to both D-fucose and phlorizin. In addition, vSGLT in oocytes shows a sugar specificity in the order of D-galactose > D-fucose > D-glucose, distinguishing it from the animal members of the Na^+/glucose cotransporter family. The level of transport by vSGLT in oocytes is lower overall ($V_{max} \sim 10$ pmol/oocyte/hour) compared to other plant and animal cotransporters ($V_{max} \sim 1000$ pmol/oocyte/hour). The low level of expression does not permit us to carry out electrophysiological studies of the bacterial cotransporter. This study shows the potential and unique advantages of utilizing a eukaryotic oocyte expression system to study bacterial cotransporters.

Key words: vSGLT — Na^+/galactose cotransport — Cotransporters — *Xenopus* oocytes

Introduction

The family of Na^+-coupled transporters includes over 55 members found in vertebrates, invertebrates, yeast, and bacteria (Turk & Wright, 1997). Sodium cotransporters are integral membrane proteins that utilize electrochemical potential gradients to drive substrates into cells. Subsequent expression of many of these cotransporters in *Xenopus laevis* oocytes has been instrumental in the characterization of protein function. The human Na^+/glucose cotransporter (hSGLTl), for example, has been shown to couple the uptake of glucose, galactose, water, and urea to the movement of Na^+ (Wright et al., 1998; Loo et al., 1996, 1999; Leung et al., 2000). While animal and plant cotransporters have been well studied in oocytes in this and other laboratories, the expression of bacterial members has not been reported. Inherent differences between prokaryotic and eukaryotic systems compromise the ability for oocytes to traffic functional bacterial transporters and channels to the plasma membrane. To our knowledge, only two cloned bacterial channels have been characterized utilizing the oocyte expression system (Wolters et al., 1999; Weeks et al., 2000) and there is a report on the expression of a myobacterial divalent cation transporter in these cells (Agranoff et al., 1999).

A bacterial member of the Na^+/glucose family, *Vibrio parahaemolyticus* Na^+/galactose symporter (vSGLT), has been cloned and characterized by *E. coli* expression and reconstitution into liposomes (Sarker et al., 1996, 1997; Turk et al., 2000; Xie, Turk & Wright 2000). In light of our successful expression of a bacterial urea channel in oocytes (Weeks et al., 2000), we investigated whether vSGLT could be similarly expressed. One goal was to study the electrical properties of a bacterial cotransporter. We find that vSGLT can be functionally expressed in oocytes with properties similar to those reported for this transporter in bacteria and proteoliposomes, demonstrating the feasibility and utility of expressing bacterial cotransporters in oocytes.

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Materials and Methods

Construction of the vSGLT Plasmid and cRNA Transcription

The cRNA for expression of vSGLT (Turk et al., 2000) was modified to promote its stability in oocytes by appending, to its encoding DNA, the 3′-untranslated region (UTR) and poly(A) tail of the original hSGLT1 cDNA (GenBank M24847). The vSGLT1 coding region (GenBank AF255301) was inserted into pBluescript plasmid vector immediately downstream of a T3 RNA polymerase transcription site, and its 3′-UTR was ligated to that of hSGLT1 (with its 51-mer poly(A) tail via the respective single *Nco* I site occurring in each 3′-UTR. The fidelity of the construct was confirmed by restriction digests and sequencing. cRNAs for oocyte expression of vSGLT or hSGLTl were transcribed *in vitro* with T3-RNA polymerase (Ambion T3 MEGAscript kit, Austin, TX).

Oocyte Preparation

The vSGLT transporter was expressed in *X. laevis* oocytes as previously described (Hediger et al., 1987; Eskandari et al., 1997; Loo et al., 1999). Mature oocytes were isolated from adult female frogs anesthetized with 0.1% Tricaine, defolliculated, and injected with 50 ng of capped cRNA or not injected (control). Oocytes were incubated at 18°C in Barth's medium (in mM) (88 NaCl, 1 KCl, 0.8 $MgSO_4$, 0.4 $CaCl_2$, 0.3 $Ca(NO_3)_2$, 2 $NaHCO_3$, 10 HEPES-Tris; pH 7.4) for 4 days prior to use in experiments. Reagents were purchased from Sigma (St. Louis, MO) unless stated otherwise.

SDS-PAGE/Western Blot Analysis

Five oocytes were solubilized in a 0.8% Triton X-114 buffer (0.8% Triton X-114, 150 mM NaCl, 1 mM EDTA, 10 mM Tris-Cl pH 7.5) on ice and then centrifuged at 4°C to remove the insoluble yolk and lipids. Supernatants were underlaid with a sucrose solution (250 mM sucrose, 1 mM EDTA, 0.06% Triton X-114, 10 mM Tris-Cl pH 7.5), warmed briefly at 37°C, and centrifuged at room temperature to isolate the greasy pellets. Incubation at higher temperatures allows for a phase separation of Triton X-114 and the isolation of membrane proteins from globular proteins. The greasy pellets were mixed with sample buffer and volumes equivalent to 1.5 oocytes were loaded onto an 8% Tricine SDS-PAGE gel. Samples from control oocytes and vSGLT-expressing *E. coli* cell lysates were loaded as controls. After the gel was electroblotted onto a nitrocellulose membrane, the blot was immunostained for vSLGT using a 1:700 dilution of an anti-peptide antibody, Ab8792 (Hirayama et al., 1991). There is a close match between 8 amino acids in vSGLT (residues 375–382) and the peptide used to raise this antibody, and the antibody specifically recognizes vSGLT expressed in *E. coli* (Xie et al., 2000). A secondary antibody, goat anti-rabbit IgG peroxidase conjugate (Calbiochem, La Jolla, CA) was used at a dilution of 1:10,000. Immunoreactive proteins were visualized by SuperSignal chemiluminescence (Pierce, Rockfort, IL) on Hyperfilm ECL (Amersham, Piscataway, NJ).

Functional Assays

Sugar uptakes into oocytes were measured at 22°C using radioactive tracers (Ikeda et al., 1989). ^{14}C-D-galactose (56 mCi/mmol) and ^{14}C-D-glucose (310 mCi/mmol) (Amersham Life Sciences, Elk Grove, IL) uptakes were carried out at final sugar concentrations of 61 μM unless stated otherwise. Radiotracer experiments were performed on oocytes incubating in either 100 mM NaCl or 100 mM CholineCl medium (in mM: 100 NaCl or 100 CholineCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Hepes-Tris, pH 7.4). Experiments were repeated at least twice on oocytes isolated from different donor frogs. Uptakes are expressed as pmol/oocyte/hour (mean ± SEM).

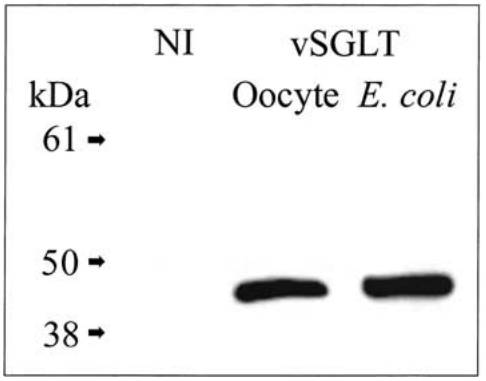


Fig. 1. Western blot of vSGLT. The signal at ~46 kDa on the blot corresponds to the vSGLT protein expressed in *Xenopus laevis* oocytes and in *E. coli*. Non-injected oocytes and vSGLT- expressing oocytes were collected and solubilized in a 0.8% Triton X-114 reducing buffer. A crude cell lysate of *E. coli* expressing vSGLT was also obtained. Protein samples were loaded on an 8% SDS-polyacrylamide gel. After the protein bands were separated, the gel was electroblotted to nitrocellulose. The Western blot was probed with antibody 8792 (Ab8792).

Results

The full-length *V. parahaemolyticus* Na^+/galactose cotransporter (vSGLT) protein was expressed in *X. laevis* oocytes and was identified on a Western blot (Fig. 1). Antibody 8792 (Ab8792) recognized a ~46 kDa band corresponding to the vSGLT protein expressed in *E. coli* cells. The antibody also identified a ~46 kDa band in vSGLT-expressing oocytes, but not in non-injected oocytes (NI).

Sugar uptake by vSGLT was Na^+-dependent (Fig. 2). In 100 mM CholineCl buffer, the D-galactose uptakes by vSGLT (2 ± 0.4 pmol/oocyte/hour) and the human Na^+/glucose transporter (hSGLT1) (3 ± 0.02 pmol/oocyte/hour) were over 2-fold higher than by non-injected oocytes (0.9 ± 0.04 pmol/oocyte/hour). Addition of 100 mM NaCl to the bathing medium significantly increased the sugar uptake by both transporters. The D-galactose uptake by vSGLT was 4 ± 0.3 pmol/oocyte/hour, a 2.5-fold increase, and that by hSGLTl was 254 ± 13 pmol/oocyte/hour, an 85-fold increase. Further experiments performed with 61 μM (^{14}C)-D-glucose or 61 μM (^{14}C)-α-methyl-D-glucose (αMDG) showed that there is a small Na^+-dependent D-glucose uptake (0.4 ± 0.1 pmol/oocyte/hour) but not any Na^+-dependent αMDG uptake.

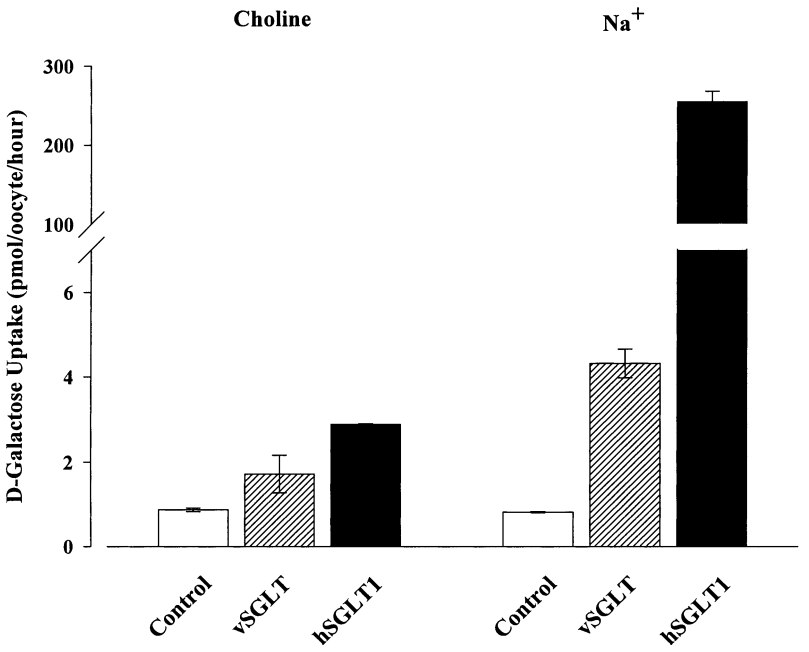


Fig. 2. Na^+-dependent D-galactose uptake by vSGLT. Oocytes obtained from the same frog were injected either with 50 ng of vSGLT cRNA, 50 ng of human Na^+/glucose cotransporter (hSGLTl) cRNA, or not injected (control). 61 μM ^{14}C-D-Galactose uptakes were performed on oocytes after 4 days incubation in Barth's medium. For the uptake experiments, oocytes were incubated in either 100 mM NaCl buffer or 100 mM CholineCl buffer and the sugar transport was measured in pmol/oocyte/hour. Each error bar in the graph represents the average D-galactose uptake by 6 oocytes. *Left*. In choline buffer, the D-galactose uptake by vSGLT-expressing oocytes is 2-fold greater than in control oocytes (2 ± 0.4 pmol/oocyte/hour). D-Galactose uptake by SGLT1 (3 ± 0.02 pmol/oocyte/hour) is 3-fold greater than in control oocytes (0.9 ± 0.04 pmol/oocyte/hour). *Right*. In the presence of Na^+ the D-galactose uptake increases in oocytes expressing vSGLT (4.3 ± 0.3 pmol/oocyte/hour) and SGLT1 (254 ± 13 pmol/oocyte/hour) over control oocytes (0.8 ± 0.04 pmol/oocyte/hour).

The kinetics of the vSGLT cotransporter were obtained by measuring the substrate concentration dependence of D-galactose uptake. Uptakes in 100 mM NaCl were first measured as a function of sugar concentration (Fig. 3*A*). vSGLT-mediated D-galactose transport was saturable at high external sugar concentrations. The $K_{0.5}$ for D-galactose was 237 μM with a V_{max} of 10 pmol/oocyte/hour. Similar uptakes performed in the presence of 60 μM D-galactose were measured as a function of external Na^+ concentration (Fig. 3*B*). D-Galactose uptake by vSGLT-expressing oocytes was also saturable with increasing concentrations of Na^+. The $K_{0.5}$ for Na^+ was 17 mM and the V_{max} was 5 pmol/oocyte/hour. The calculated Hill coefficient was 0.8.

Competition experiments were performed in the presence of 100 mM NaCl to determine the substrate specificity of vSGLT (Fig. 4). Sugars (5 mM) added to the external medium were examined for their ability to inhibit D-galactose transport by vSGLT. D-Galactose uptake by vSGLT-expressing oocytes was most strongly inhibited by D-galactose (95%) followed by D-fucose (75%). D-Galactose uptakes were reduced also by the addition of β-methyl-galactose (60%), D-glucose (55%), and by αMDG (30%). Other sugars, L-glucose, βMDG, 2-deoxyglucose, L-arabinose, and sucrose, had no effect. Competitive inhibitors of the SGLT1 cotransporter also decreased vSGLT D-galactose uptake. Addition of 1 mM phlorizin to the bathing medium inhibited D-galactose transport by 60%, while 100 μM rhapontin and 100 μM deoxyrhapontin inhibited uptake by 50 and 30%, respectively.

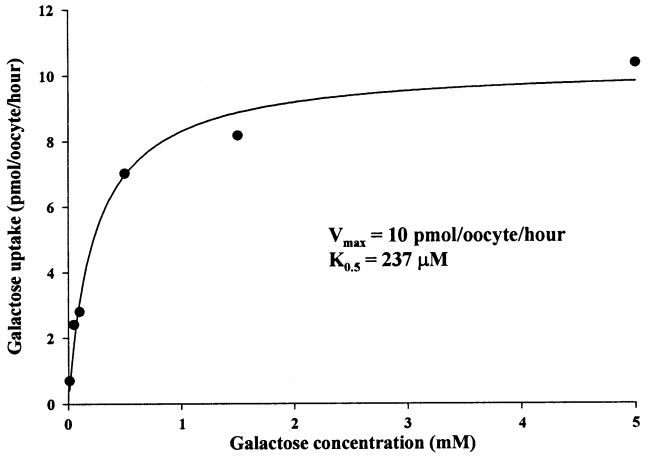
Discussion

In this study, we report the expression and functional characterization of a bacterial transport protein, utilizing the heterologous *X. laevis* oocyte expression system. Our results indicate that the *V. parahaemolyticus* Na^+ galactose cotransporter (vSGLT), when expressed in oocytes, has functional similarities corresponding to that expressed in *V. parahaemolyticus* and *E. coli*, or when the proteins were purified and reconstituted into proteoliposomes (Sarker et al., 1994, 1996, 1997; Turk et al., 2000; Xie et al., 2000). vSGLT in oocytes also retains properties that are distinct from those of other members of the Na^+/glucose family of cotransporters.

Expression of the full-length vSGLT protein in oocytes was confirmed by Western blotting. Recent findings identify vSGLT as a 59-kDa protein, determined by DNA sequencing and by mass spectrophotometric analysis (Turk et al., 2000). Like most other hydrophobic membrane proteins, vSGLT runs at a low molecular weight position (~46 kDa) on SDS-PAGE (Hirayama et al., 1992; Xie et al., 2000).

Sugar uptake by vSGLT is Na^+-dependent, saturable with Na^+ and sugar, substrate-specific, and sensitive to phlorizin. Galactose uptake is saturable with respect to both the external concentration of Na^+ ($K_{0.5,Na^+}$ of 17 mM, Hill coefficient of 1) and of D-galactose ($K_{0.5,\ D\text{-}gal}$ of 237 μM at 100 mM Na^+). The estimated $K_{0.5,Na^+}$ in oocytes is an order of magnitude lower than that determined for vSGLT in

A.



B.

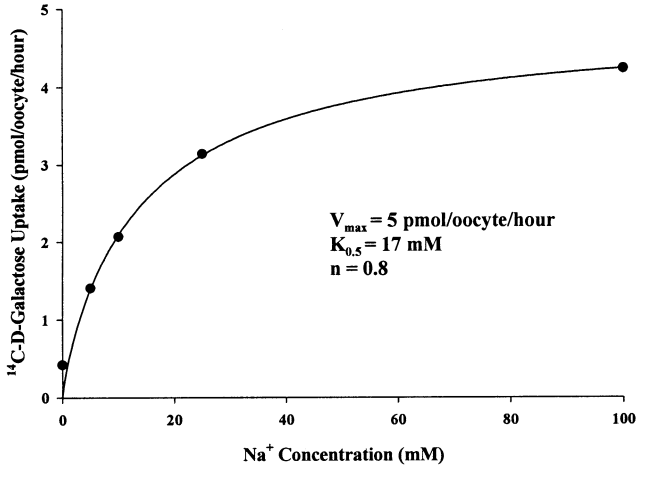


Fig. 3. Kinetics of sugar transport by vSGLT. vSGLT cRNA was injected into oocytes and uptakes of D-galactose were measured 4 days later. Each data point represents the uptake by 4–10 oocytes. Na^+ concentrations varied between 0 mM and 100 mM and D-galactose concentrations varied between 10 μM and 5 mM. All values have been corrected for endogenous sugar uptake and the standard errors of the mean were less than 10% of the mean value. The apparent affinities ($K_{0.5}$) and maximal velocities (V_{max}) are shown in the graphs. (*A*) Dependence on [^{14}C-D-galactose] in 100 mM Na^+. Data points were fitted to the equation $v = (V_{max}[S])/(K_{0.5} + [S])$ where V_{max} is the maximal velocity (pmol/oocyte/hour), [*S*] is the D-galactose concentration (mol/l), and $K_{0.5}$ is the concentration that yields half-maximal velocity (mol/l). The $K_{0.5}$ for D-galactose is 237 μM and the corresponding V_{max} is pmol/oocyte/hour. (*B*) Dependence on [Na^+] of 61 μM ^{14}C-D-galactose uptake. Data points were fitted to the equation $v = ([S]^n V_{max})/(K_{0.5} + [S]^n)$. [S] is the Na^+ concentration (mol/l), V_{max} is the maximal velocity (pmol/oocyte/hour), $K_{0.5}$ is the apparent affinity constant, and *n* is the Hill coefficient. The $K_{0.5}$ for Na^+ is ~17 mM ($n = 0.8$) and the V_{max} is 5 pmol/oocyte/hour.

proteoliposomes (129 mM with a Hill coefficient of 1, Turk et al., 2000). In bacterial cells, the D-galactose uptake mediated by vSGLT peaked at 40 mM NaCl and decreased at higher concentrations (Sarker et al., 1994). The $K_{0.5,\ D\text{-}gal}$ for vSGLT in oocytes is comparable to that obtained for vSGLT reconstituted in proteoliposimes (158 μM, Turk et al., 2000), but is 5–10 times higher than that reported for bacterial cells (Sarker et al., 1996, 1997). The variations in kinetic parameters in cells may be due to differences in the electrochemical potential gradients across the cell membranes. In the case of the protein reconstituted in liposomes, there are differences in membrane lipid composition, driving forces, and, possibly, differences in the orientation of the protein in the lipid bilayer. The vSGLT transporter is substrate-specific, transporting D-galactose, D-glucose, and D-fucose. αMDG, a sugar analog transported with high affinity by the human Na^+/glucose cotransporter (hSGLT1), inhibits D-galactose uptake by vSGLT-expressing oocytes but does not appear to be transported (Sarker et al., 2000; Turk et al., 1996; Xie et al., 2000). This is similar to the mammalian SGLTs where some hexoses are transported by one cotransporter, but are non-transported inhibitors in another, e.g., 5-thioglucose is transported by hSGLT1 but is a non-

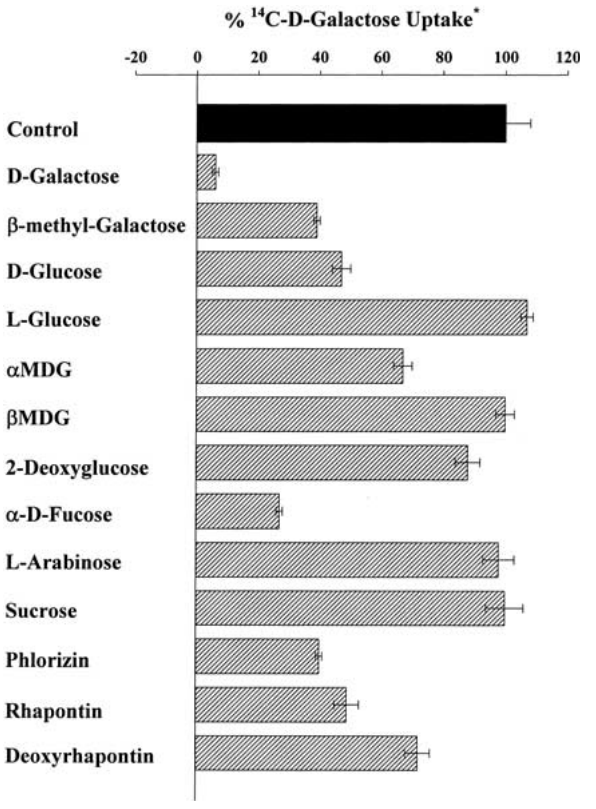


Fig. 4. Substrate specificity of vSGLT. 61 μM ^{14}C-D-galactose uptakes were measured in vSGLT-expressing oocytes in the absence (*control*) or presence of 5 mM sugars (unless noted otherwise) in the bathing medium. All values have been corrected for endogenous uptake. Uptakes were graphed as a percentage of the control uptake with error bars (errors are ≤6%). Each error bar represents the uptake by 5–8 oocytes. D-galactose radiotracer uptake by vSGLT is inhibited by 5 mM D-galactose (95%), D-fucose (75%), β-M-D-galactose (60%), 1 mM phlorizin (60%), D-glucose (55%), and α-M-D-glucose (αMDG) (30%). There is no significant inhibition of vSGLT D-galactose uptake by L-glucose, β-M-D-glucose (βMDG), 2-deoxyglucose, L-arabinose, or sucrose. The inhibitor concentrations were 1 mM (phlorizin) and 0.1 mM (rhapontin and deoxyrhapontin).

transported blocker of pSGLTS (Diez-Sampedro et al. 2001). Phlorizin, the specific inhibitor of Na^+/glucose cotransporters, also blocks sugar uptake by vSGLT in oocytes, although the K_i is higher than that reported for hSGLT1 (1 *vs.* 0.001 mM). The K_i for vSGLT-expressing oocytes is comparable to that in JM1100 cells expressing vSGLT (Sarkar et al., 1997; Xie et al., 2000). The relatively low phlorizin K_i for vSGLT is probably related to low affinity for D-glucose relative to D-galactose. These results suggest that vSGLT expressed in oocytes retains its functions as a saturable Na^+-dependent cotransporter of D-galactose, D-glucose, and α-D-fucose and which is inhibited by αMDG and phlorizin.

The vSGLT transporter has functional characteristics that distinguish it from the animal members of the Na^+/glucose cotransporter family. vSGLT appears to have a coupling ratio of 1 Na^+/1 sugar molecule (Hill coefficient ~1), and a sugar selectivity in the order of D-galactose > D-fucose > D-glucose. In contrast, the human Na^+/glucose cotransporter (hSGLT1) transports 2 Na^+ ions for each sugar molecule and transports D-galactose, D-glucose, and αMDG with comparable affinities (Wright et al., 1998).

The level of transport by vSGLT in oocytes (V_{max} ~10 pmol/oocyte/hour) is low compared to that determined for SGLT1 (V_{max} 1600 pmol/oocyte/hour; Ikeda et al., 1989) but is comparable to that for pSGLT2 (V_{max} 20 pmol/oocyte/hour; Kanai et al., 1994). Low transport levels by vSGLT in oocytes, despite high levels of vSGLT protein in oocytes as determined by Western blotting, may be due to difficulty in trafficking the bacterial protein to the plasma membrane, as is the case for another bacterial membrane cotransporter, lac permease (Kaback, H.R., personal communication). Until conditions are optimized for higher expression levels, we are unable to fully exploit the power of electrophysiological techniques to study this transporter (Parent et al., 1992; Mackenzie et al., 1996; Loo et al., 1998). However, our study shows the potential advantages of employing the heterologous oocyte expression system as an additional method with unique advantages for functionally characterizing bacterial membrane protein transporters.

We thank Dr. B. A. Hirayama for helpful advice and criticism. This study was supported by NIH grants DK19567 and DK44602.

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