Pore-forming Activity of the Tsx Protein from the Outer Membrane of Escherichia coli

DEMONSTRATION OF A NUCLEOSIDE-SPECIFIC BINDING SITE*

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Christl Maier and Erhard Bremer†
From the Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz, Federal Republic of Germany

Angela Schmid and Roland Benz
From the Lehrstuhl für Biotechnologie, Universität Würzburg, D-8700 Würzburg, Federal Republic of Germany

The Tsx protein from the outer membrane of Escherichia coli is known to be involved in the permeation of nucleosides across the outer membrane under limiting substrate conditions. We purified Tsx from an E. coli strain that overproduces Tsx. The purified protein was still functional since it could neutralize the Tsx-specific bacteriophage T6 in vitro. When the purified Tsx was reconstituted into a lipid bilayer, there was a large increase of the membrane conductance, indicating pore-forming activity of Tsx in vitro. This increase could be strongly blocked with adenine and to a much lesser extent with cytidine. Titration of the pore conductance with adenine or cytidine suggested the presence of a binding site for nucleosides in the Tsx pore. One of these, the maltose-inducible and colicin E. coli by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† To whom correspondence should be addressed.

EXPERIMENTAL PROCEDURES

Materials—DEAE-Sephacel was from Pharmacia LKB Biotechnology, Inc. A low molecular weight protein calibration kit (Sigma Chemie, München, Federal Republic of Germany) was used as molec-

1 E. Bremer, P. Gerlach, and A. Middendorf (1988) J. Bacteriol. 170, in press.

The outer membrane of Escherichia coli acts as a molecular filter for hydrophilic substrates (for reviews see Refs. 1–4). Its diffusion properties are due to the presence of a major class of proteins called porins (5). Normally, porins such as OmpF and OmpC show little specificity for solutes (6, 7) and sort the molecules primarily according to their molecular weight. However, under certain growth conditions, the outer membrane also contains porins with marked specificity (for reviews, see Refs. 8 and 9). One of these, the maltose-inducible LamB protein, functions as a general transmembrane diffusion channel and, in addition, preferentially mediates the permeation of maltose and maltodextrins across the outer membrane at low substrate concentrations (<0.1 mM) (10, 11). This specificity results from the previously reported maltose- and maltodextrin-binding site of LamB (12–15). The Tsx protein is a component of the outer membrane of E. coli (8, 9) and serves as the receptor for bacteriophage T6 and colicin K (16–18). Expression of its structural gene, tsx, is under double negative control of the deoR- and cytR-encoded repressors and is regulated positively by the CAMP complex (19–21). The DeoR and CytR proteins also control expression of the nucleoside-uptake systems NupC and NupG located in the cytoplasmic membrane (22–25) and several nucleoside-catabolizing enzymes (26). The coregulation of Tsx synthesis with the systems for nucleoside uptake and metabolism (for reviews, see Refs. 27 and 28) is of functional importance, since the Tsx protein is involved in the permeation of nucleosides across the outer membrane. This was demonstrated first by Hantke (29), who found that in tsx mutants the uptake of several nucleosides is impaired. Further analysis (19, 25, 30) revealed a remarkable specificity for the Tsx-mediated permeation of nucleosides across the outer membrane. The rate of uptake for adenine and thymidine is strongly reduced in tsx mutants, while the rate of uptake of cytidine in such a strain is almost identical to that in a tsx− strain. The importance of the Tsx protein for nucleoside uptake becomes apparent only at low (<1 μM) substrate concentrations (19, 25). At higher concentrations of substrate, the Tsx protein becomes dispensable, and the nucleosides permeate the outer membrane by diffusion through the nonspecific porins (31). Furthermore, in ompB mutants that lack the major pore-forming proteins OmpC and OmpF, the Tsx protein seems to facilitate the diffusion of serine, glycine, and phenylalanine across the outer membrane (32). Tsx protein has been purified and its amino acid composition analyzed (21, 33), but its primary structure is unknown.

It has been hypothesized, but not directly shown, that the Tsx protein introduces a permeability pathway into the outer membrane by forming a pore (19, 25, 29, 30). To test this hypothesis and to investigate further the nucleoside specificity of the Tsx pore, we purified the Tsx protein and reconstituted it in vitro into lipid bilayers. We show here that the incorporation of Tsx into this membrane results in a strong increase in conductance. The increase can be blocked by the addition of adenine and, to a much smaller extent, by cytidine. Our results are consistent with the view that the Tsx protein forms a pore in the outer membrane, and they provide evidence for the presence of a nucleoside-specific binding site within the pore that is essential for the facilitated permeation process.
ular weight standard. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad, München, Federal Republic of Germany). All salts were of analytical grade and were obtained from Merck (Darmstadt, Federal Republic of Germany). Adenosine and cytidine were purchased from Sigma. 

Electrophoresis and Protein Assay—Plates were stained with Coomassie Brilliant Blue or silver nitrate (42, 43). The gel system used does not resolve the OmpC and OmpF proteins. The following molecular weight marker proteins were used: bovine 

\[ \text{buffer} \] was changed twice. 100 µl of a phage lysate (approximate 10^2 plaque-forming units) was mixed with increasing concentrations (2.8–21 µg) of the dialyzed Tsx protein solution (70 µg/ml in a final volume of 500 µl) and incubated for 2 h at room temperature. To determine the number of unadsorbed phages, we added 50 µl (10^6 cells/ml) of a freshly grown culture of strain P400 to the reaction mixture and incubated it for 30 min at room temperature. 3 ml of LB top agar was then added, and the mixture was plated onto a LB plate. After incubation for 16 h at 32 °C, the number of phage plaques was determined.

Membrane Experiments—The methods used for black lipid bilayer experiments have been described previously (44). The instrumentation consisted of a Teflon chamber with two aqueous compartments. Circular holes in the wall separating the two compartments had an area of either 1 mm² (for macroscopic conductance measurements) or about 0.1 mm² (for single-channel experiments). Membranes were formed across the holes by painting on a 1% solution of diphytanoyl phosphatidylcholine (Avanti Biochemicals, Birmingham, AL) in n-decane. The temperature was kept at 25 °C throughout the experiment. The aqueous solutions of adenosine and cytidine were unbuffered (pH about 6). The Tsx protein was inactivated rapidly in aqueous salt solutions. To prevent this inactivation, we added the protein to the aqueous phase either immediately prior to membrane formation or after the membranes had turned completely black. The membrane current was measured at different voltages by using a pair of matched carbon electrodes with salt bridges, which were inserted into the aqueous solutions on both sides of the membrane. The macroscopic conductance measurements were performed with a Keithley model 602 electrometer. The current through the membrane in the single-channel experiments was boosted with a current amplifier (Keithley 427), monitored with a storage oscilloscope (Tektronix 5115), and recorded on a strip chart recorder.

RESULTS

Isolation of a Tsx-overproducing Strain—Under standard laboratory growth conditions, Tsx is only a minor protein component of the outer membrane of E. coli (8, 9). For unknown reasons, the E. coli K-12 strain P400 overproduces Tsx to the extent that it comprises about 8% of the membrane

2The abbreviations used are: pfu, plaque-forming units; SDS, sodium dodecyl sulfate; S, siemens.
Tsx Protein of E. coli

To facilitate the purification of Tsx, we isolated a derivative of strain P400 that lacks or is severely deficient in the major outer membrane proteins OmpA, OmpC, OmpF, and LamB. This strain was constructed by stepwise selection for mutants resistant to bacteriophages K3h30, hym2, K20, and λvir, which use these outer membrane proteins as part of their receptor (35-38). Consequently, the outer membrane of strain CH8 contained the Tsx protein in greatly enriched amounts. Fig. 1 shows the outer membrane protein profile of CH8 (lane 3) together with those of its parent P400 (lane 1) and P407, a tsx derivative of P400 (lane 2). The membrane proteins were solubilized in SDS at 100°C and electrophoresed on a 12% SDS-polyacrylamide gel. From such gels we estimated the apparent molecular weight of the Tsx protein to be 28,000 (Fig. 1). The same molecular weight was found when the membrane proteins were solubilized for 30 min at room temperature prior to electrophoresis (data not shown).

Purification of the Tsx Protein—Outer membranes of strain CH8 were isolated by sucrose density gradient centrifugation. The outer membrane proteins were solubilized in the nonionic detergent Triton X-100 containing EDTA, and the proteins were separated chromatographically on a DEAE-Sephacel column. After washing the column with buffer and a solution of 0.1 M NaCl, we applied a linear salt gradient between 0.1 and 0.2 M NaCl (“Experimental Procedures”). Fig. 2 shows the elution profile of the separated proteins. The protein content of individual fractions was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Tsx protein was found in the second major peak and eluted from the DEAE column between approximately 0.15 and 0.18 M NaCl (Fig. 2). Highly purified Tsx protein was recovered as shown in Fig. 1, lane 4 (gel stained with Coomassie Brilliant Blue) and in Fig. 3, lanes 6-8 (gel stained with silver nitrate). In the main fractions containing pure Tsx protein, the protein concentration was estimated to be 70 µg/ml; thus we obtained approximately 1 mg of pure Tsx protein from a 4-liter culture of strain CH8.

Purified Tsx Protein Shows Receptor Activity for Phage T6—It is known that Tsx solubilized in Triton X-100 is able to interact with phage T6.

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**FIG. 3.** Protein profile of individual fractions after DEAE-Sephacel chromatography. From each fraction, 60-µl samples were mixed with 10 µl of sample buffer containing 4% SDS, boiled for 10 min, and electrophoresed on a 12% SDS-polyacrylamide gel. The gel was stained with silver nitrate. Lane 1 shows a sample of the outer membrane proteins from strain CH8 applied to the DEAE-Sephacel column. Samples from the following column fractions were analyzed: fraction 104 (lane 2), fraction 121 (lane 3), fraction 133 (lane 4), fraction 146 (lane 5), fraction 152 (lane 6), fraction 162 (lane 7), fraction 173 (lane 8), fraction 180 (lane 9), fraction 190 (lane 10), and fraction 196 (lane 11). These fractions were used to determine the specific membrane conductance shown in Fig. 2.

**FIG. 2.** DEAE-Sephacel chromatography of outer membrane proteins of strain CH8 and measurement of specific membrane conductance. A 10-ml solution of outer membrane proteins was applied to a DEAE-Sephacel column. The column was washed with column buffer and 0.1 M NaCl, and the proteins retained on the column were eluted with a linear salt gradient. Fractions of 0.7 ml were collected and their protein contents (C) were determined with the Bio-Rad protein assay. The reaction was monitored with a Gilford spectrophotometer at a wavelength of 595 nm. The specific membrane conductance (M) was measured by lipid bilayer experiments. 50-µl portions from indicated fractions were diluted 1:1 in 1% Triton X-100, and 20 µl of this mixture were added to an aqueous solution of 10 ml of 1 M KCl 'satiating' a lipid bilayer. The bilayer was formed from diphytanoyl phosphatidicholine dissolved in n-decane. The specific membrane conductance was recorded after 20 min, and each point shown (■) represents the average of two to three independent measurements.
to neutralize phage T6 \textit{in vitro} and that the presence of SDS destroys this phage receptor activity (21). Thus, the ability to neutralize phage T6 is a sensitive test for a functional Tsx protein. We tested our purified protein in the following manner. Increasing amounts (2.8–21 µg) of purified Tsx protein were incubated for 2 h with approximately 10³ pfu of phage T6, and the number of unadsorbed bacteriophages was determined by plating the mixture onto a lawn of strain P400. As shown in Fig. 4, the purified protein has receptor activity for phage T6. This neutralizing activity is phage-specific since \(\lambda\)uir, which uses the LamB protein as receptor, was not neutralized by the Tsx protein (Fig. 4). The ability of phage T6 to bind to the purified protein suggested that we had recovered Tsx in a functional form. Neutralization of phage T6 \textit{in vitro} requires an active receptor protein and lipopolysaccharide (33), indicating that our Tsx preparation contained still lipopolysaccharide. However, lipopolysaccharide plays no role in the primary adsorption of phage T6 \textit{in vivo} but is apparently required in a later step of the infection process (19, 48). Such differences in the \textit{in vivo} and \textit{in vitro} requirements for the neutralization of phage T6 could account for the low T6 receptor activity (approximately 10 µg of Tsx was required to inactivate approximately 500 phages) of our Tsx preparation (Fig. 4). Alternatively, some of the Tsx protein could be inactive as a phage receptor. The T6 receptor activity of our preparation cannot be due to undetected contaminating polypeptides since it has clearly been established that Tsx is the receptor protein for phage T6 (18, 21).

\textit{Tsx-induced Increase in Macroscopic Membrane Conductance of Lipid Bilayer Membranes}—To test the pore-forming properties of Tsx, we reconstituted the purified protein into lipid bilayers (44). When the Tsx protein was added in small

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Neutralization of phage T6 by purified Tsx protein. Increasing amounts of a solution of the purified Tsx protein (about 70 µg/ml in 0.1% Triton X-100, 10 mM Tris-HCl (pH 8.0)) were incubated for 2 h with approximately 10³ pfu of a high titer lysate of phage T6 (C) and \(\lambda\)uir (C). In each assay, the number of unadsorbed phages was determined by plating these mixtures onto lawns of strain P400. As a control, both phage T6 (O) and phage \(\lambda\)uir (■) were incubated without added Tsx protein in the same buffer.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5}
\caption{Titration of Tsx-induced membrane conductance with adenosine. The membrane was formed from diphytanoyl phosphatidylcholine dissolved in \(n\)-decane. The aqueous phase contained 200 ng of Tsx/ml, 1 M KCl, and adenosine at the concentrations shown at the top of the figure. The applied voltage was 50 mV, and the temperature was 25 °C.}
\end{figure}
quantities (100 ng/ml) to the aqueous solution bathing a lipid bilayer membrane, the specific conductance of the membrane increased by many orders of magnitude. The time course of this change was similar to that described previously for other bacterial porins, including the sugar-specific LamB channel (1, 15, 45, 46); i.e. the increase in conductance was rapid for the first 15–20 min and then continued at a much slower rate. The conductance increase occurred regardless of whether Tsx was added to only one side or to both sides of the membrane. The addition of the detergent Triton X-100 alone at the same concentration used in the experiments with the protein did not lead to a significant increase in the membrane conductance. Since a steady state conductance level could not be reached in the experiments with the Tsx protein, the dependence of the specific membrane conductance on the protein concentration in the aqueous phase was somewhat difficult to determine. However, meaningful comparison was possible when we used the conductance value at a fixed time (20 min) after the addition of Tsx, when most of the conductance increase had occurred (data not shown). We found a linear relationship between the protein concentration in the aqueous phase and the membrane conductance. These results therefore suggest, that Tsx can form a pore in vitro.

**Binding of Nucleosides to Tsx**—To test the specificity of the Tsx channel for nucleosides, we performed multichannel experiments with membranes of large surface in the following way. Tsx protein was added to a black lipid bilayer membrane formed from diphytanoyl phosphatidylcholine at a concentration of about 200 ng/ml. 20 min after the addition of Tsx, when the rate of conductance increase had slowed considerably, small amounts of different adenosine solutions at final concentrations of between 0.2 and 10 mM were added to the aqueous solutions with stirring to allow equilibration. As shown in Fig. 5, the membrane conductance decreased as a function of the adenosine concentration. By assuming that during the binding of a nucleoside molecule inside the Tsx channel no ions can pass through, we were able to calculate the binding constant from a Lineweaver-Burk plot (Fig. 6). The straight line in Fig. 6 corresponds to a half-saturation constant of 0.6 mM (i.e. a stability constant of 1,500 M$^{-1}$). Similar experiments were also performed with the nucleoside cytidine (Fig. 7), and the half-saturation constant was found to be 20 mM (a stability constant of 50 M$^{-1}$). The difference in the half-saturation constants corresponds to results of in vivo studies, which showed that Tsx facilitated the permeation of adenosine, but not cytidine, across the outer membrane at low substrate concentration (19, 25, 29). It should be noted that the binding of adenosine and cytidine to the channel (i.e. the blocking of the channel) was fully reversible. Removal of the adenosine in experiments similar to those described above led to a restoration of the initial membrane conductance (before the addition of the nucleosides).

**Single-channel Experiments**—We tried to perform single-channel experiments with the Tsx protein similar to those performed earlier with the general diffusion pores OmpF and OmpC and the sugar-specific LamB channel (6, 15, 45). However, we found that the single-channel conductance of the Tsx pore was considerably smaller than that of these other pores (6) and was therefore difficult to determine precisely. We estimated the single-channel conductance of Tsx to be about 10 pS in 1 M KCl. The permeation of ions through LamB is blocked completely by the presence of its specific substrates maltose and maltodextrins (15). To determine whether the same phenomenon occurs in Tsx, we measured the influence of adenosine on single-channel conductance. No single channel could be detected when 10 mM adenosine was added, indicating that the binding of nucleosides to the Tsx protein blocks the permeation of ions through Tsx.

**Pore-forming Activity Apparently Unrelated to Tsx**—We tested individual fractions eluted from the DEAE-Sephacel column for pore-forming activity in macroscopic membrane conductance experiments (Fig. 2). Fractions from the first major protein peak showed no pore-forming activity (Fig. 2; Fig. 3, lanes 3 and 4). Samples from the second major protein peak, containing primarily Tsx protein, led to a strong increase in the specific membrane conductance in proportion to the amount of Tsx protein used (Fig. 2; Fig. 3, lanes 5–9); this membrane conductance could be blocked by the addition of adenosine (data not shown). We also observed a second increase in the membrane conductance (Fig. 2) that could not be reduced by the addition of adenosine, indicating that the pore-forming activity was not due to Tsx. These fractions contained some Tsx and two minor proteins with an apparent molecular weight of 47,000 and 52,000 (Fig. 3, lanes 10 and 11).
brane of protein in the outer membrane of which show temperature-dependent mobility changes on SDS

Tsx by purification of Tsx; but, their procedures have either been tion took place at room temperature or 100 °C. This behavior is also an oligomer. In any case, it is known that the phage protein is only a minor polypeptide component of the outer membrane as judged by examination of SDS-polyacrylamide gels as a consequence of their SDS-resistant trimeric structure (21). From its mobility on a 12% SDS-polyacrylamide gel we have estimated an apparent molecular weight for the Tsx protein have been estimated (21, 29, 32). From its mobility on a 12% SDS-polyacrylamide gel we have estimated an apparent molecular weight of 29,000 daltons. Various values (25,000 and 26,000 daltons) for the apparent molecular weight for the Tsx protein have been described (21, 29, 32). From its mobility on a 12% SDS-polyacrylamide gel we have estimated an apparent molecular weight of 28,000 for Tsx after solubilization at 100 °C for 10 min in 2% SDS. This estimate is in close agreement to the results of Yamato and Hinz (33) who reported a molecular mass of 29,000 ± 2,000 daltons after sedimentation equilibrium ultracentrifugation in the presence of a detergent. Our molecular weight estimate was the same whether solubilization took place at room temperature or 100 °C. This behavior differs from that of other pore-forming proteins of E. coli which show temperature-dependent mobility changes on SDS gels as a consequence of their SDS-resistant trimeric structure (1-5). So far it is uncertain whether the active Tsx channel is also an oligomer. In any case, it is known that the phage receptor function of purified Tsx protein is destroyed by SDS (21). Therefore, our inability to detect Tsx multimers might simply reflect a strong sensitivity of Tsx oligomers to SDS.

It has long been suspected (19, 25, 29, 30, 32) that Tsx is a pore-forming protein in vivo. Our successful reconstitution of purified Tsx protein into lipid bilayer membranes demonstrates that this outer membrane protein forms an ion-permeable channel in vitro and thus provides strong support for its proposed in vivo role. There is clear evidence that Tsx has an important function for the permeation of nucleosides at low substrate concentration (<1 μM) across the outer membrane. In tsx mutants the uptake of nucleosides, with the exception of cytidine and deoxycytidine, is impaired (19, 25, 29, 30). Furthermore, strains lacking OmpF porin, a major outer membrane protein, show reduced transport rates of adenosine of 15-20%, while tsx mutations cause a rate reduction of 90% (49). This specificity of Tsx for nucleosides is poorly understood. We show here that the Tsx protein contains a binding site for nucleosides, and we suggest that this nucleoside-binding site is responsible for the specificity of the Tsx channel. The increased membrane conductance caused by the incorporation of Tsx pores into lipid bilayer membrane could be reduced by the addition of adenosine. Furthermore, no single channel could be detected in the presence of adenosine.

Our successful reconstitution of purified Tsx protein into lipid bilayer membranes demonstrate that this outer membrane protein forms an ion-permeable channel in vitro and thus provides strong support for its proposed in vivo role. There is clear evidence that Tsx has an important function for the permeation of nucleosides at low substrate concentration (<1 μM) across the outer membrane. In tsx mutants the uptake of nucleosides, with the exception of cytidine and deoxycytidine, is impaired (19, 25, 29, 30). Furthermore, strains lacking OmpF porin, a major outer membrane protein, show reduced transport rates of adenosine of 15-20%, while tsx mutations cause a rate reduction of 90% (49). This specificity of Tsx for nucleosides is poorly understood. We show here that the Tsx protein contains a binding site for nucleosides, and we suggest that this nucleoside-binding site is responsible for the specificity of the Tsx channel. The increased membrane conductance caused by the incorporation of Tsx pores into lipid bilayer membrane could be reduced by the addition of adenosine. Furthermore, no single channel could be detected in the presence of adenosine.

Our in vitro results are in excellent agreement with the in vivo situation, where it has been demonstrated that the uptake of adenosine is strongly dependent on Tsx while the uptake of cytidine is not reduced in tsx mutants (19, 25, 29). The physiological relevance of this different dependence on Tsx for their permeation across the outer membrane is a matter

\[ \text{C. Maier, E. Bremer, A. Schmid, and R. Benz, unpublished results.} \]
of speculation. We note however that cytidine is the effector molecule of the CytR repressor, which strongly influences ttx expression (19, 28) and also controls the synthesis of two nucleoside-specific transport systems and several nucleoside-catabolizing enzymes (27, 28). The apparent Txs-independent permeation of cytidine across the outer membrane is very low concentration may allow cytidine to alert the cell to the presence of other exogenous nucleosides and to induce the expression of genes involved in their uptake and metabolism.

The single-channel conductance previously determined in lipid bilayer experiments for the general diffusion pores OmpC and OmpF were 1.5 and 1.9 nS, respectively, and that for the maltose- and maltodextrin-specific LamB channel was 160 pS (6, 15) in a 1 M KCl solution. Under the same experimental conditions, we found a considerably smaller single-channel conductance of 10 pS for the Txs protein. As discussed by Hancock (50) and by Benz et al. (15), the diameters of a substrate-specific channel cannot be estimated from such small single-channel measurement.

In E. coli, there is another outer membrane protein (LamB) that acts as a general porin but shows a marked substrate specificity for maltose and maltodextrins (10–15). As with the ttx gene, expression of the structural gene for the LamB protein is coregulated with the system for the uptake and utilization of its specific substrates. The specificity of the LamB porin has also been attributed to the presence of a substrate-binding site. The reported K<sub>c</sub> of maltotriose (4 × 10<sup>−4</sup> M) for the binding site in LamB (13–15) is very similar to the K<sub>n</sub> of adenosine (4 × 10<sup>−4</sup> M) that we report here for the binding site in Txs. The presence of a binding site is of physiological importance. A calculation of the flux of maltotriose through both the substrate-specific LamB channel and a general diffusion porin showed that at a maltotriose concentration of 10<sup>−4</sup> M the LamB channel is much more efficient than the porin (15), although at a higher substrate concentration of 10<sup>−3</sup> M the flux through the porin exceeds that through LamB.

Nucleosides occur in nature mainly as degradation products of nucleic acids. Their uptake is important for cell growth because they can serve as carbon and nitrogen sources and as precursors in nucleic acid synthesis (27). They are relatively small molecules (average molecular weight is around 250), and, at high concentrations, they can permeate the outer membrane by diffusion through the porins (30, 49). For reasons discussed in detail by Nikiado and Vaara (2), at low substrate concentration, the permeation of nucleosides across the outer membrane is rate-limiting for their overall uptake, because E. coli has two high affinity nucleoside transport systems, NupC and NupG, with apparent K<sub>c</sub> values in the range of 0.3–0.6 μM (22–25, 27). Consequently, the presence of a nucleoside-specific Txs pore should offer a significant growth advantage in very dilute environments.

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