

Specificity and Topology of the *Escherichia coli* Xanthosine Permease, a Representative of the NHS Subfamily of the Major Facilitator Superfamily

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The specificity of XapB permease was compared with that of the known nucleoside transporters NupG and NupC. XapB-mediated xanthosine uptake is abolished by 2,4-dinitrophenol and exhibits saturation kinetics with an apparent K_m of 136 μ M. A 12-transmembrane-segment model was confirmed by translational fusions to alkaline phosphatase and the α fragment of β -galactosidase.

Escherichia coli can utilize nucleosides as the sole carbon source. Two nucleoside permeases, NupC and NupG, are known and have been characterized in some detail (15). The NupG protein can facilitate uptake of all tested purine and pyrimidine nucleosides, while NupC has specificity towards the pyrimidine nucleosides, adenosine, and their deoxy derivatives. They are both powered by the proton motive force. Specificity towards xanthosine has not been examined in detail, but xanthosine has been found to inhibit the uptake of adenosine in the wild type and in a *nupC* mutant (11). Moreover, a *nupC nupG* double mutant was unable to grow on xanthosine as the sole carbon source, whereas a *nupC* or *nupG* mutant grew well (8). This indicates that both permeases can transport xanthosine.

The presence of several genes (*xapAB* and *xapR*) dedicated to the metabolism of the purine nucleoside xanthosine was indicated by Buxton et al. (4, 8). They suggested that together with the catabolizing enzyme xanthosine phosphorylase (*xapA*), a new uptake system could exist. When the operon was sequenced (20), it became clear that this function at least in part could be assigned to the presumed product of the *xapB* gene. Supporting its role as a nucleoside transporter, XapB showed 58% identity to NupG and was found to be enriched in the membrane fraction in minicell experiments (20). The hydrophathy profile of the protein indicates that it is a membrane protein with 12 transmembrane segments. Finally, xanthosine phosphorylase is induced to less than 10% of the wild-type level by the inducer xanthosine in a *xapB* mutant, emphasizing the importance of XapB (20).

Our goal in the present work was to characterize XapB and compare it with NupG and NupC. We cloned the corresponding genes into the low-copy-number plasmid pSU18 (10 copies/cell) (2). The resulting plasmids, pGD227, pGD253, and pGD257, express *xapB*, *nupG*, and *nupC*, respectively. Since expression of all three permeases is under complex control and is induced by nucleosides, we cloned the genes without their

normal promoters and expressed them from the plasmid-borne *lac* promoter under derepressed conditions (*lacI*).

Purine nucleoside phosphorylase and xanthosine phosphorylase catalyze the phosphorylytic breakdown of nucleosides, releasing ribose-1-phosphate (ribose-1-P) and the nucleobase moiety. Ribose-1-P is converted to ribose-5-P by phosphoribomutase and can be used as an energy source. We exploited this by examining the requirement for nucleoside permeases to grow on nucleosides as the sole carbon source (Table 1). The XapB, NupG, and NupC permeases cloned in pSU18 were transformed into the transport-negative *E. coli* strain SØ6687 (*araD139 ΔlacU169 strA thi codAB ΔnupC ΔnupG ΔxapB::Kan^r*). Since growth on xanthosine requires induction of xanthosine phosphorylase, which is a slow process, we also expressed *xapAB* for comparison. Fast-growing mutants which outgrew their parents in liquid cultures appeared frequently; therefore, growth was monitored on plates, where mutants are easily distinguished. As shown in Table 1, expression of *xapB* results in growth on all nucleosides tested except guanosine. When both *xapA* and *xapB* are expressed, the same growth pattern is seen except that cells seem to grow better on purine nucleosides in the presence of XapA. XapA expressed alone did not result in growth on any of the nucleosides (data not shown). Expression of *nupG* results in growth on all nucleosides except adenosine and xanthosine, although microcolonies appear on xanthosine after 5 days of incubation. When *nupC* is expressed, the cells grow well on all tested nucleosides except xanthosine and guanosine.

Next, we tested the uptake of ¹⁴C-labeled nucleosides in transport-negative strain GD1333 (*araD139 ΔlacU169 strA thi cod ΔnupC ΔnupG ΔxapABR::Kan^r cytR deoD zjj::Tn10*) containing pGD227, pGD253, pGD257, or pSU18. As shown in Fig. 1, XapB can transport all tested nucleosides except guanosine, although the uptake of uridine and cytidine is only slightly above the background level. A similar specificity is seen with NupG, with the main difference being that NupG can transport all nucleosides except xanthosine. NupC is the most efficient permease for the uptake of pyrimidine nucleosides, while inosine and adenosine uptake is only slightly above the control level and xanthosine and guanosine cannot be taken up at all. It should be noted, however, that we do not know the

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TABLE 1. Growth on nucleosides as sole carbon sources^a

Nucleoside	Growth				
	Control	XapB	XapAB	NupC	NupG
Xanthosine	–	++	+++	–	(+)
Guanosine	–	–	–	–	+++
Inosine	–	+++	++++	++	+++
Adenosine	–	++	+++	+++	–
Uridine	–	+	+	+	+
Cytidine	–	+++	+++	+++	+++
Thymidine	–	+++	+++	++	+++

^a SØ6687 harboring the indicated transporters on plasmids was grown with nucleosides as the sole carbon source on AB minimal plates (5). +++++, excellent growth after 3 days at 37°C; +++, good growth; ++, poor growth; +, barely visible growth; –, no growth; (+), microcolonies appeared after 5 days.

concentration of each permease, and only differences in nucleoside specificity can be tested. Thus, at a low nucleoside concentration (2 μM), xanthosine can only be transported by XapB and guanosine can only be transported by NupG. With all purine nucleosides except xanthosine, a small amount of uptake is seen with the control plasmid. A similar background growth is seen in growth experiments when a *cytR* mutant is used as the host (data not shown). Since the strain used for the uptake experiments is *cytR*, a likely explanation for the background is that another purine-specific, low-affinity, *cytR*-regulated permease exists. Such a permease has recently been identified by Bente Mygind (personal communication). The presence of another *cytR*-regulated permease can also explain the appearance of fast-growing mutants in the growth experiments, since *cytR* mutants easily appear under selection pressure (7).

It has previously been reported that the initial nucleoside uptake rate, mediated by NupG and NupC, equals the exchange rate (17). This also applies to XapB, since similar time curves were seen for xanthosine uptake in cultures grown with and without unlabeled xanthosine added prior to the addition of labeled xanthosine (data not shown). The initial uptake rate (or the exchange rate) of xanthosine was measured by taking samples 10, 20, 30, and 40 s after addition of radiolabeled xanthosine. The initial uptake rate is constant for up to approximately 40 s. By varying the substrate concentration, we determined the saturation kinetic parameters of XapB. Because of the very low solubility of xanthosine, we took advantage of the finding that the uptake rate equals the exchange rate. Thus, when the initial uptake rate was measured at high xanthosine concentrations, unlabeled xanthosine was added prior to the assay, and only trace amounts of labeled xanthosine were added to start the assay. The data were fitted to the Michaelis-Menten equation using the Ultrafit 3.0 software (BioSoft), and the K_m was found to be $136 \pm 26 \mu\text{M}$. This value is much higher than the values of 0.3 to 1 μM determined for NupC- and NupG-mediated cytidine transport (12, 15, 16). It is, however, in the same range as many eukaryotic nucleoside transporters, e.g., the NupC homologue in rat of 37 μM (10). Moreover, the high K_m is in good agreement with the finding that full induction of *xapAB* requires an extracellular concentration of xanthosine above 1 mM (G. Dandanell, unpublished data). The low solubility of xanthosine is a technical problem only in the uptake assays and not in the induction and growth

experiments. It could be argued that the outer membrane limits the uptake of xanthosine in strains in which *xapB* is overexpressed. Nucleosides enter the outer membrane through OmpC, OmpF, and Tsx. Although Tsx is a nucleoside-specific porin, it is only necessary at a very low nucleoside concentration (<0.1 μM) (15). When we compared xanthosine uptake in wild-type cells with uptake in a *cytR* mutant, which has sevenfold-higher expression of *tsx* (3), we found no difference in xanthosine uptake, strongly indicating that diffusion through the outer membrane is not limiting in our experiments (data not shown).

To find out if the xanthosine permease is energized by the proton motive force, we examined the effect of the uncoupler 2,4-dinitrophenol on xanthosine uptake. When we added 2,4-dinitrophenol to a final concentration of 1 mM, it completely abolished uptake, whether added 10 min prior to addition of ¹⁴C-labeled xanthosine or 60 s after some uptake had taken place (data not shown).

The hydrophathy profile of XapB indicates that xanthosine permease has 12 transmembrane segments (TMs) (20). We used the TopPred2 program (21) and the TMHMM program (18) to predict the topology of XapB (the TMHMM model is shown in Fig. 2). Both programs predict that XapB has 12 TMs, and to confirm this model, we made *xapB* fusions to a dual *phoA-lacZ*(α) reporter, a method developed by Alexeyev and Winkler (1). The combination of alkaline phosphatase (*phoA*) and β -galactosidase (*lacZ*) to study membrane topology has been controversial. However, by fusing all three proteins into a single polypeptide and measuring the ratio of alkaline phosphatase and β -galactosidase activity, differences in expression levels and protein stability of different fusions can largely be ignored. Fusion points just preceding a TM segment were designed so that the *phoA-lacZ*(α) part would not be buried in the lipid bilayer and putative topogenic signals in the loop would be preserved. In addition, four fusions predicted to be positioned in a TM were constructed. First, dual reporter vector pMN8h was constructed by inserting the *phoA* gene lacking the N-terminal export signal sequence into pSU19 (a *Bam*HI-*Ban*II *phoA* fragment was isolated from pUI310 [9]). Next a *Ban*II (Klenow)-*Bsr*BI fragment was deleted, creating an in-frame fusion between *phoA* (lacking the eight C-terminal amino acids) and the α -fragment of β -galactosidase with a 6-amino-acid linker (SSNSLA) between. All *xapB-phoA-lacZ*(α) fusions were constructed by amplifying the *xapB* fragment by PCR and ligating the PCR product into pMN8h. These plasmids were transformed into TG1 (19), which express the ω -subunit of β -galactosidase, and the activity of alkaline phosphatase and β -galactosidase was determined in exponentially growing cells (TG1 is not *phoA*; however, the background alkaline phosphatase activity level was negligible in all our experiments). As shown in Fig. 2, all fusions predicted to be positioned in periplasmic loops give high alkaline phosphatase/ β -galactosidase ratios, whereas all fusions predicted to be in cytoplasmic loops give high β -galactosidase/alkaline phosphatase ratios. The four fusions predicted to be in the membrane all give very low ratios (less than 3). These data correlate with the model shown in Fig. 2. The only exceptions are K64 in the first cytoplasmic loop and M94 in the second periplasmic loop. The normalized alkaline phosphatase/ β -galactosidase ratio is high for K64 because the absolute alkaline phosphatase

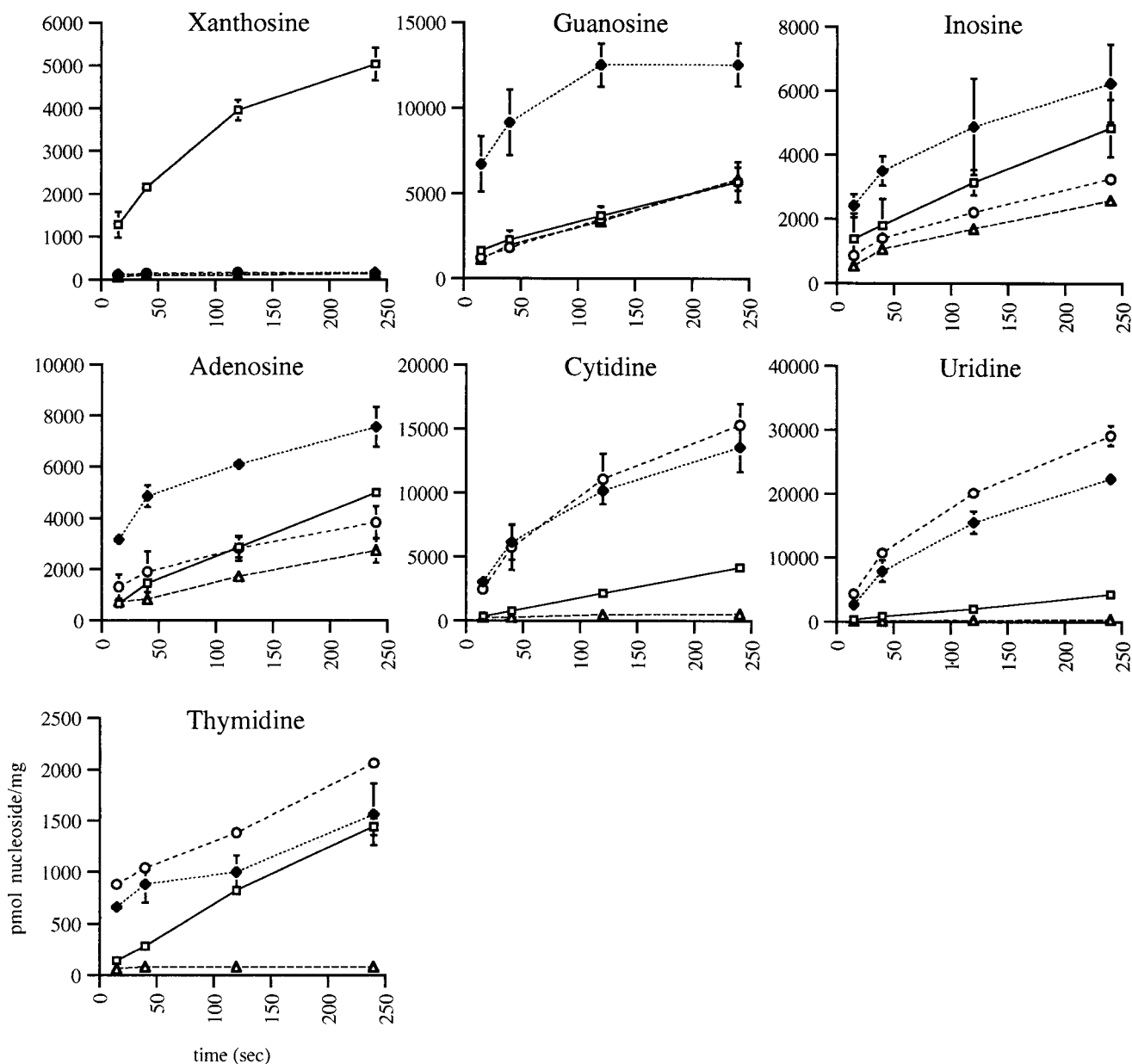


FIG. 1. Uptake of ^{14}C -radiolabeled nucleosides by GD1333. The time course of the activity of recombinant XapB (open square), NupG (solid diamond), NupC (open circle), and the control (open triangle) towards the ribonucleosides xanthosine, guanosine, inosine, adenosine, uridine, and cytidine and the deoxy ribonucleoside thymidine was assayed. Error bars indicate the standard deviation. Cells were grown exponentially in AB, B1, and glucose minimal medium. Radioactively labeled nucleoside was added (2.5 to 5 mCi/mmol) to a final concentration of 2 μM . Samples were withdrawn at different times and filtered through a 0.45- μm nitrocellulose membrane (Schleicher & Schuell). The filter was washed and dried, and radioactivity was counted in a liquid scintillation counter (17).

activity is high. Unexpectedly high activities have previously been observed for *phoA* fusions positioned N-terminally in cytoplasmic loops (6). The absolute activities of M94 are very low (data not shown), and the fusion protein could be poorly expressed or unstable. Another possibility is that the reporters might be buried in the lipid bilayer, since the second periplasmic loop is predicted to be very short (3 residues).

Concluding remarks. Both the growth experiments and the uptake assays clearly show that XapB is a permease that can transport both purine and pyrimidine nucleosides. The inter-

changing pattern of high and low PhoA-LacZ activity ratios in our *xapB-phoA-lacZ*(α) fusions points to a 12-TM topology, with N and C termini on the cytoplasmic side, as predicted by both TMHMM and TopPredII. Cells expressing only *xapB* grow well on (and can concentrate) all the nucleosides tested except guanosine (Table 1 and Fig. 1). Our growth experiments with recombinant NupC and NupG support the previous finding, with the chromosomal genes, that NupC can transport all nucleosides except guanosine and inosine, whereas NupG can transport all nucleosides (15). However, two exceptions are

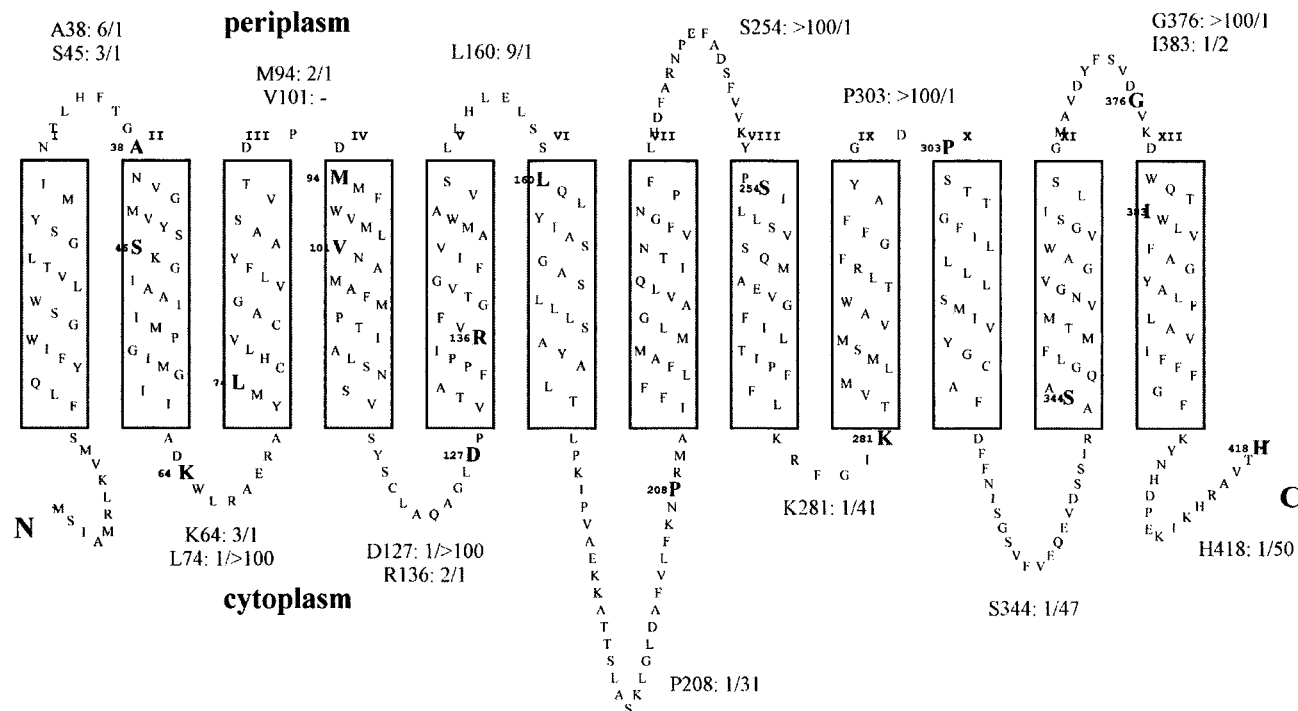


FIG. 2. Topological model of XapB. The model was constructed by the TMHMM software (18). The highlighted amino acids indicate the C-terminal amino acids of XapB and fusion points to PhoA and LacZ(α). β -Galactosidase and alkaline phosphatase activities were measured in exponentially growing cells (AB, B1, and glucose minimal medium) on whole sodium dodecyl sulfate-CHCl₃-permeabilized cells as described (13, 14) and normalized to the highest value. The alkaline phosphatase/ β -galactosidase ratios are indicated at the loops.

noted. First, NupG does not seem to support growth on adenosine despite the fact that adenosine is effectively taken up by NupG in the transport assay (Table 1 and Fig. 1). The reason is most likely that NupG transport adenosine very effectively and that the high concentration of adenosine inhibits growth. Such inhibition has also been seen in liquid cultures after addition of adenosine to exponentially growing cultures (data not shown). Second, we find that when NupC is expressed from plasmids, cells grow well on inosine (Table 1). A similar result has been observed with different plasmid constructs carrying *nupC* (B. Mygind, personal communication). This indicates that NupC does transport inosine; however, the affinity for inosine is too low to support growth in wild-type cells, in which only a single copy of *nupC* is expressed. Growth on uridine was very poor for all the constructs. We have no explanation for this finding, since they all grow well on cytidine. In *E. coli*, cytidine is deaminated to uridine by cytidine deaminase, and the cell must therefore contain all the enzymes required for the catabolism of uridine.

In our growth experiment, a strain expressing only *nupC* does not grow on xanthosine, as previously reported (8). Since the strain used here is *xapB*, it indicates that although NupC is necessary for induction of *xapAB*, it is XapB that takes up the xanthosine. These observations and our growth experiment indicate that both NupC and NupG can transport xanthosine but only with a very low affinity. We have not been able to show uptake of ¹⁴C-labeled xanthosine by NupC or NupG even though they are expressed from approximately 10 gene copies. Based on our results, we suggest that NupC and NupG are necessary for the initial induction of *xapAB* by transporting

small amounts of xanthosine into the cell. This transport, however, is not sufficient to obtain full induction of *xapAB* but is sufficient to induce *xapAB*, after which XapB can efficiently transport xanthosine into the cell. This model explains why induction of *xapAB* takes several generations.

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