Functional Identification of the Hypoxanthine/Guanine Transporters YjcD and YgfQ and the Adenine Transporters PurP and YicO of Escherichia coli K-12*

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The evolutionarily broad family nucleobase-cation symporter-2 (NCS2) encompasses transporters that are conserved in binding site architecture but diverse in substrate selectivity. Putative purine transporters of this family fall into one of two homology clusters: COG2233, represented by well studied xanthine and/or uric acid permeases, and COG2252, consisting of transporters for adenine, guanine, and/or hypoxanthine that remain unknown with respect to structure-function relationships. We analyzed the COG2252 genes of Escherichia coli K-12 with homology modeling, functional overexpression, and mutagenesis and showed that they encode high affinity permeases for the uptake of adenine (PurP and YicO) or guanine and hypoxanthine (YjcD and YgfQ). The two pairs of paralogs differ clearly in their substrate and ligand preferences. Of 25 putative inhibitors tested, PurP and YicO recognize with low affinity 6-benzoyladenine, 2,6-diaminopurine, and purine, whereas YjcD and YgfQ recognize 1-methylguanine, 8-azaguanine, 6-thioguanine, and 6-mercaptopurine and do not recognize any of the PurP ligands. Furthermore, the permeases PurP and YjcD were subjected to site-directed mutagenesis at highly conserved sites of transmembrane segments 1, 3, 8, 9, and 10, which have been studied also in COG2233 homologs. Residues irreplaceable for uptake activity or crucial for substrate selectivity were found at positions occupied by similar role amino acids in the Escherichia coli xanthine- and uric acid-transporting homologs (XanQ and UacT, respectively) and predicted to be at or around the binding site. Our results support the contention that the distantly related transporters of COG2233 and COG2252 use topologically similar side chain determinants to dictate their function and the distinct purine selectivity profiles.

The external supply of purine nucleobases can be vital to cells that either rely on salvage pathways for nucleotide synthesis or use purines as nitrogen or carbon sources through catabolism. Entry of these solutes is regulated by a range of different membrane transport systems depending on the type of organism and cell. Based on genomic and phylogenetic evidence in conjunction with the relatively few study paradigms available, most microbial cells use distinct transporter and active site motifs for purine uptake relative to humans or other mammals (1–3). As a characteristic example, the putative purine transporters encoded in bacterial or fungal genomes fall into one of only two structural folds corresponding to the nucleobase-cation symporter families NCS1 (1) and NCS2 (2). The NCS1 transporters have no structural homologs in animals, whereas NCS2 transporters are present in all major taxa of organisms, including mammals, but their human homologs are strikingly different in selectivity and not related with nucleobase uptake (3, 4). Apart from the evolutionary implications, understanding the structure-function-selectivity relationships of such differences would offer a means to design novel, selective purine-related antimicrobials for optimized therapies (4, 5).

Of the relatively few bacterial or fungal purine nucleobase transporters that have been characterized functionally in detail, the majority belong to the evolutionarily diverse family NCS2 (see Fig. 1). The purine transporters of this family fall into one of two homology clusters: COG2233, represented by well studied permeases for xanthine and/or uric acid (2-oxy purines), and COG2252, consisting of transporters for adenine, guanine, and/or hypoxanthine (2-non-oxy purines) that remain largely unknown with respect to structure-function relationships (Fig. 1). The well studied purine permeases of COG2233 include the xanthine permease XanQ of Escherichia coli (6), the uric acid permease UacT of E. coli (7), and the xanthine/uric acid permease UapA of Aspergillus nidulans (8). Systematic insight from mutagenesis studies of these homologs and structural...
modeling on the template of the single structurally known member of the family (the uracil permease UraA) (2) have indicated that their key binding site determinants are similar even though the overall sequence identity is low, ranging from 22 to 28%. On the other hand, the COG2233 homologs retain characteristic sequence motifs that are different in transporters of the poorly studied COG2252 cluster of the family (Fig. 2).

In this work, we provide a first insight on the structure–function relationships of COG2252 members of family NCS2 using the homologs of \( E. \) coli \( K-12 \) as study paradigms. With respect to the nucleobase uptake-related coding potential, the \( E. \) coli \( K-12 \) genome includes 10 members of family NCS2 and two members of NCS1. The NCS1 members CodB and YbbW (AILP) are predicted as a cytosine permease and allantoin permease, respectively, from genomic and/or genetic evidence (9, 10). The COG2252 members that belong to COG2233 have been identified functionally as uracil (UraA) (11), xanthine (XanQ and XanP) (12), uracil and xanthine (RutG), or uric acid (UacT) permeases (7). The NCS2 members of cluster COG2252 (YgfQ, YjcD, YicO, and PurP) are related in sequence with the fungal and plant AzgA-like adenine-guanine-hypoxanthine transporters (13, 14), whereas PurP is annotated as a high affinity adenine transporter based on genetic (15, 16) and systems biology evidence (17). Here, we cloned and overexpressed the four COG2252 genes of \( E. \) coli and showed that PurP and YicO are high affinity transporters specific for adenine, whereas YjcD and YgfQ are high affinity transporters for hypoxanthine and guanine. Then we subjected PurP and YjcD to site-directed mutagenesis at positions of residues that are conserved and functionally important in homologs of the COG2233 cluster (7, 18–23) but differ in the coding sequences of COG2252 genes. Our data provide support to the contention that the distantly related purine transporters of the two homology clusters use distinct but topologically equivalent side chains to dictate the binding site function and selectivity.

**EXPERIMENTAL PROCEDURES**

**Materials**—[2,8-\(^3\)H]Adenine (31.8 Ci mmol\(^{-1}\)), [2,8-\(^3\)H]hypoxanthine (27.7 Ci mmol\(^{-1}\)), [8-\(^3\)H]guanine (21.2 Ci mmol\(^{-1}\)), [8-\(^3\)H]xanthine (28 Ci mmol\(^{-1}\)), [5,6-\(^3\)H]uracil (44.9 Ci mmol\(^{-1}\)), and [8-\(^{14}\)C]uric acid (57.8 mCi mmol\(^{-1}\)) were purchased from Moravek Biochemicals. Non-radioactive nucleobases were from Sigma. Oligodeoxynucleotides were synthesized from BioSpring GmbH. High fidelity DNA polymerase was from Kapa Biosystems. Restriction endonucleases used were from Takara. Horseradish peroxidase (HRP)-conjugated avidin was from Amersham Biosciences. All other materials were reagent grade and obtained from commercial sources.

\[^{3}\] M. Botou, P. Lazou, K. Papakostas, and S. Frillingos, manuscript in preparation.
**Bacterial Strains and Plasmids**—E. coli K-12 was transformed according to Inoue et al. (24). TOP10F’ (Invitrogen) was used for initial propagation of recombinant plasmids. T184 (25) or single gene knock-out strains (Keio collection; provided from the E. coli Genetic Stock Center) (26) were used for expression of pT7-5-borne purP, yicO, yicD, uraA, xanQ, xanP, uacT, rutG, ybbY, codB, or ybbW from the lacZ promoter/operator.

**DNA Manipulations**—Construction of expression plasmids and biotin acceptor domain (BAD)-tagged versions of COG2252 homologs was essentially as described previously for XanQ and XanP (12). The coding sequences of genes were amplified by PCR on the template of genomic DNA prepared from E. coli T184 and transferred to plasmid vector pT7-5 by restriction fragment replacement; BAD-tagged versions were prepared using two-stage (overlap extension) PCR (27) on the templates of pT7-5/purP (or other NCS2 or NCS1 gene as indicated) and pT7-5/xanQ-BAD. For construction of mutants, two-stage PCR was performed on the template of PurP-BAD or YjcD-BAD. The entire coding sequence of all constructs was verified by double strand DNA sequencing (MWG-Biotech).

**Growth of Bacteria**—E. coli Keio strain JW3692 (pT7-5/purP) or JW4025 (pT7-5/yjcD) harboring the given plasmids was grown aerobically at 37 °C in Luria-Bertani medium containing kanamycin (0.025 mg/ml) and ampicillin (0.1 mg/ml). E. coli T184 harboring the given plasmids was grown at the same conditions except that streptomycin (0.01 mg/ml) was used instead of kanamycin. Fully grown cultures were diluted 10-fold, allowed to grow to midlogarithmic phase, induced with isopropyl 1-thio-B-galactopyranoside (0.5 mM) for an additional 1.5 h at 37 °C, harvested, and washed with appropriate buffers (see below).

**Transport Assays and Kinetic Analysis**—E. coli cells were washed twice in potassium phosphate buffer (0.1 M), pH 7.5; normalized to an A420 of 10 (35 μg of total protein/50 μl) in the same buffer; and assayed for active transport of radiolabeled

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**FIGURE 2. Sequence alignment of COG2252 members of E. coli with other NCS2 homologs.** The full-length sequences of the homologs shown in Fig. 1 and of YbbY were aligned with ClustalW, and the part of this alignment referring to the 10 E. coli NCS2 members is presented. Each COG2252 was also analyzed with homology structure prediction using HHpred and threaded on the template of the x-ray structure of UraA. The structure-based alignment of the COG2252 homologs did not differ significantly from the result of the ClustalW alignment except in TM3, TM10, and the C-term of the gate domain (α12–α14). In these regions, the structure-based alignment was used; the shifts of the initial ClustalW alignment are denoted with forward slashes. Secondary structural elements (α-helices and β-strands) of the UraA template (2) are indicated above the sequence alignment. Highly conserved amino acids are indicated in red, and amino acids that are invariant (or invariant with one exception) in the functionally known COG2252, COG2233, or purine-transporting COG2233 members are shaded in orange. Strictly invariant COG2252 or COG2233 residues are indicated with red asterisks on top or on bottom of the alignment, respectively. The boxed sequence regions represent highly conserved motifs of the COG2233 cluster (E-value < e^-39) and of the COG2252 cluster (E-value < e^-29) revealed with the program MEME.
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substrates. *E. coli* JW3692 was assayed for active transport of [3H]adenine (0.04–40 μM) and [3H]hypoxanthine (0.1–100 μM), JW4025 was assayed for active transport of [3H]guanine (0.1–40 μM), and T184 was assayed for active transport of [3H]xanthine (1–100 μM), [3H]uracil (1–100 μM), and [14C]uric acid (0.01–0.4 mM) by rapid filtration at 25 °C at pH 7.5 as described (12). For kinetic uptake measurements, initial rates were assayed in JW3692 or JW4025 cells at 5–15 s, and data were fitted to the Michaelis-Menten equation using Prism4 to determine $K_m$ and $V_{max}$ values. For ligand competition experiments with PurP, YicO, YgfQ, YjcD, and selected mutants, uptake of [3H]adenine or [3H]hypoxanthine was assayed in JW3692 cells in the absence or presence of unlabeled analogs at the indicated concentration range. Data were fitted to the equation $y = B + (T - B)/(1 + 10^{(\log IC_{50} - \log x)})$ for sigmoidal dose response (variable slope) where $x$ is the concentration variable, $y$ (activity) values range from $T$ (top) to $B$ (bottom), and $h$ is the Hill coefficient using Prism4 to obtain IC$_{50}$ values; in all cases, $h$ was close to −1, consistent with the presence of one binding site. $K_i$ values were calculated from the Cheng-Prusoff equation, $K_i = IC_{50}/[1 + (L/K_m)]$ where $L$ is the permeant concentration and $K_m$ is the value obtained for this permeant, assuming a simple model of competitive inhibition with the binding site of the transporter (17).

**Results**

Functional Identification of PurP, YicO, YjcD, and YgfQ—The COG2252 genes *ygfQ*, *yjcD*, *yicO*, and *purP* were mobilized from the *E. coli* K-12 genome; transferred to transcriptional control of the lacZ promoter/operator in plasmid vector pTT7-5; and induced for overexpression in *E. coli* host cells. Several K-12 strains were used as *E. coli* host in these experiments to establish optimal conditions for assaying the uptake of each purine nucleobase. At first, aerobically grown T184 was used at conditions of negligible endogenous activity of xanthine, hypoxanthine, or uric acid uptake (12). However, T184 cells displayed a high background of adenine and guanine uptake. This may be due in part to endogenous expression of PurP, which is known to be linked with the ability of *E. coli* to utilize adenine as a nitrogen source (15, 16). We analyzed the RNA expression profile of the endogenous NCS2 and NCS1 genes and found that aerobically grown T184 cells express both purP and yicD transcripts, which may account for the high adenine/guanine background (data not shown). Then we tested a range of single gene knock-out strains (Keio collection) and established that JW3692 (purP knock-out) can be used as a host for adenine uptake assays and JW4025 (yicD knock-out) can be used for guanine uptake assays, whereas other strains, including JW2850 (ΔxanQ), JW5470 (ΔyicD), JW5636 (ΔpurP), JW5467 (ΔyicO), and JW0327 (ΔguoB), display significant background for both adenine and guanine uptake. With respect to hypoxanthine uptake, several strains were tested, including T184 (12), JW4025, JW5467, and JW3692, and found to be suitable as a host, but JW3692 was preferred for direct comparisons between adenine and hypoxanthine uptake in the same cell system.

Using the appropriate host strain in each case, we found that all COG2252 constructs can be expressed in the *E. coli* membrane at high levels and display highly significant purine uptake activities with distinct substrate profiles (Fig. 3). PurP and YicO transport [3H]adenine but not guanine, hypoxanthine, xanthine, uric acid, or uracil, and YjcD and YgfQ transport [3H]guanine and [3H]hypoxanthine but not adenine, xanthine, uric acid, or uracil. No other NCS2 or NCS1 homolog of *E. coli* can transport adenine, guanine, or hypoxanthine to a significant extent as tested in parallel assays (Fig. 3 and data not shown). Kinetic analysis revealed that PurP transports adenine with very high affinity ($K_m$ 1.0 μM), YicO is also a high affinity transporter for adenine ($K_m$ 6.5 μM), and YjcD and YgfQ are high affinity transporters for guanine ($K_m$ 1.6 and 1.8 μM, respectively).
respectively) and hypoxanthine ($K_m$ 11.2 and 22.5 µM, respectively) (Table 1). Analysis of the purine selectivity profiles with competitive inhibition experiments showed that the adenine transporters PurP and YicO can also recognize hypoxanthine albeit with very low affinity ($K_i$ 370 and 330 µM, respectively) but do not recognize guanine, xanthine, uric acid, uracil, cytosine, or thymine, whereas the hypoxanthine uptake activity of YjcD and YgfQ is inhibited with high affinity by guanine ($K_i$ 4 and 3 µM, respectively) but not at all by adenine, xanthine, uric acid, or any of the pyrimidine nucleobases (Fig. 4). The specificity profiles were investigated further by assaying transport of the appropriate substrate in the presence or absence of a series of analogs (Fig. 4 and Table 2). PurP and YicO cannot recognize guanine, 6-mercaptopurine, or 3-methyladenine and are competed with low affinity by 7-methyladenine or 9-methyladenine ($K_i$ 370 and 330 µM, respectively), 2,6-diaminopurine ($K_i$ 9 and 71 µM, respectively), or purine ($K_i$ 3 and 21 µM, respectively). YjcD and YgfQ recognize a broader range of guanine and purine analogs, including 1-methylguanine ($K_i$ 33 and 105 µM, respectively), 5-thioguanine ($K_i$ 4 and 4 µM, respectively), 8-azaguanine ($K_i$ 44 and 154 µM, respectively), and 6-mercaptopurine ($K_i$ 18 and 57 µM, respectively). Overall, there is a clear distinction in specificity between the two adenine transporters and the two guanine/hypoxanthine transporters (no analog is recognized with high affinity by both transporter types), whereas PurP and YjcD display higher affinity than their iso-functional paralogs with respect to substrate or analogs tested (Table 2).

Delineation of Mutagenesis Targets for Structure-Function Analysis of PurP and YjcD—The four transporters characterized here are structurally homologous to the well studied NCS2 proteins UraA (2), XanQ (6), UacT (7), and UapA (8) but belong to a separate cluster of orthologs for which studies on the role of individual side chains are not yet available. To initiate a rationally designed mutagenesis study in homologs of this cluster, we selected as replacement targets residues that are functionally important in the well studied NCS2 proteins and fall in characteristic sequence motifs of the family. Highly conserved sequence motifs of the functionally known COG2233 homologs fall in TM1, TM3, TM5, TM8, TM9, TM10, and TM12 (Fig. 2). Amino acid residues delineated as important from the previous mutagenesis studies of purine-transporting COG2233 members are at the motifs of TM1, TM3, TM8, TM9, and TM10. These functionally linked positions are either invariably conserved or falling to distinct conservation patterns (Fig. 5). They are occupied by residues that are irreplaceable in XanQ (Glu-272, Asp-304, Gln-324, and Asn-325), UapA (Glu-356, Asp-388, Gln-408, and Asn-409), and UacT (His-37, Glu-270, Asp-298, Gln-318, and Asn-319); crucial for the substrate selectivity profile in XanQ (Asn-93, Asp-276, and Ala-323), UacT (Thr-100 and Ser-317), and UapA (Ser-154); or subject to constraints with respect to the allowed site-directed replacements in XanQ (His-31, Asp-276, and Asn-326), UacT (His-86 and Asp-360), and UapA (Met-274 and Val-320). The above...
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### Table 1

| Permease       | $K_m$ (μM) | $V_{max}$ (nmol min$^{-1}$ mg$^{-1}$ protein) | $V_{max}/K_m$ (μl min$^{-1}$ mg$^{-1}$) |
|----------------|------------|---------------------------------------------|----------------------------------------|
| **Kinetics for adenine uptake (JW3692)** |            |                                             |                                        |
| PurP(WT)       | 1.0 ± 0.2  | 1.7 ± 0.1                                   | 1700                                   |
| YicO(WT)       | 6.5 ± 0.3  | 11.0 ± 0.2                                  | 1692                                   |
| PurP mutants   |            |                                             |                                        |
| T38A           | 1.4 ± 0.2  | 2.0 ± 0.1                                   | 1429                                   |
| A91G           | 1.1 ± 0.2  | 1.4 ± 0.1                                   | 1273                                   |
| A91S           | 1.4 ± 0.3  | 11.7 ± 0.8                                  | 8357                                   |
| T271A          | 1.6 ± 0.8  | 1.9 ± 0.3                                   | 1187                                   |
| T271S          | 4.2 ± 0.6  | 3.7 ± 0.2                                   | 881                                    |
| D298N          | 3.7 ± 0.8  | 6.4 ± 0.6                                   | 2000                                   |
| I317A          | 0.3 ± 0.1  | 2.0 ± 0.3                                   | 6667                                   |
| S319A          | 0.9 ± 0.4  | 5.8 ± 0.8                                   | 6445                                   |
| S319N          | 0.9 ± 0.3  | 3.7 ± 0.3                                   | 4111                                   |
| **Kinetics for guanine uptake (JW4025)** |            |                                             |                                        |
| YicD(WT)       | 1.6 ± 0.2  | 4.0 ± 0.3                                   | 2500                                   |
| YgfQ(WT)       | 1.8 ± 0.1  | 3.4 ± 0.1                                   | 1889                                   |
| YjcD mutants   |            |                                             |                                        |
| T35A           | 42.3 ± 6.7 | 20.9 ± 1.9                                  | 494                                    |
| A88G           | 50.1 ± 12.0| 72.5 ± 10.6                                 | 1447                                   |
| A88S           | 48.2 ± 4.0 | 30.3 ± 1.5                                  | 629                                    |
| D302N or I321E | ND         | ND                                          |                                        |
| **Kinetics for hypoxanthine uptake (JW3692)** |            |                                             |                                        |
| YjcD(WT)       | 11.2 ± 0.4 | 2.7 ± 0.1                                   | 241                                    |
| YgfQ(WT)       | 23.2 ± 3.7 | 13.8 ± 1.1                                  | 595                                    |
| YjcD mutants   |            |                                             |                                        |
| T35A           | 30.3 ± 4.4 | 3.0 ± 0.2                                   | 99                                     |
| A88G           | 4.2 ± 1.5  | 4.5 ± 0.5                                   | 1071                                   |
| A88S           | 18.4 ± 3.6 | 2.8 ± 0.2                                   | 152                                    |
| D302N          | 12.4 ± 2.3 | 4.0 ± 0.3                                   | 322                                    |
| I321E          | 53.5 ± 8.1 | 10.0 ± 0.9                                  | 190                                    |
| D271A or N     | ND         | ND                                          |                                        |
| T275S or E322D | ND         | ND                                          |                                        |

side chains are generally not conserved in the COG2252 homologs. Although different, the COG2252 side chains at the corresponding positions are strongly conserved or invariant (Fig. 5) and fall at highly conserved sequence regions as well (Fig. 2).

The relevant amino acid side chains of PurP and YjcD (Thr-38/35, Ala-91/88, Asp-267/271, Thr-271/275, Asp-298/302, Ile-317/321, Glu-318/322, and Ser-319/323) were subjected to site-directed replacements with Ala, same character or similar character amino acids, and/or amino acids occupying the corresponding positions of COG2233 members (see below). Homology modeling of PurP and YjcD on the structural template of UraA (2) showed that the selected side chains fall at the putative binding site region or at the periphery (Fig. 6). Notably, the COG2252 homologs appear to form a more relaxed helical structure at the beginning of the α-helix of TM10 (Fig. 6) so that the 3-amino acid helical turn of Ile-317/321, Glu-318/322, and Ser-319/323 corresponds to a 4-amino acid helical turn in the topology of XanQ (Ala-323, Gln-324, Asn-325, and Asn-326) or other COG2233 transporters. The significance of this observation is discussed in the Discussion section.

**Site-directed Mutagenesis of PurP**—Based on the above considerations, we engineered 19 PurP mutants, namely T38A, T38H, A91S, A91G, D267E, D267A, D267N, T271D, T271A, T271S, T271N, D298N, D298E, I317A, I317E, E318D, E318Q, S319A, and S319N. After verification of the sequence, each PurP mutant was transferred and expressed in E. coli JW3692 and assayed for its expression levels in the membrane and its ability to catalyze active transport of adenine (Fig. 7). Of the 19 mutants, nine (T38A, A91S, A91G, T271A, T271S, D298E, I317A, S319A, and S319N) were highly active (40–120% of the rate achieved with wild type and 50–140% of the steady-state accumulation level), four (D267E, T271N, I317E, and E318D) displayed very low or negligible uptake rates (2–8% of wild type) and accumulated to significant but very low levels (18–25% of wild type) and the other active mutants (Table 1).

The specificity profile of each active mutant was determined and compared with wild-type PurP in adenine uptake inhibition assays (Table 2). It was found that mutants A91S, T271S, and D298E displayed significantly lower affinities than wild type (higher $K_m$ values) for both the high affinity ligands 9-N6-benzoyladenine, purine, and 2,6-diaminopurine and the low affinity ligand 9-methyladenine, whereas mutant T271A displayed significantly lower affinity than wild type for N6-benzoyladenine and purine but nearly wild-type affinity for the other analogs. Less significant differences were observed with the other
mutants, including lower affinity of T38A, I317A, and S319N for purine and lower affinity of T38A, S319A, and S319N for 2,6-diaminopurine. No significant deviations from wild type were found with the other purines or analogs tested (Table 2).

Taken together, the PurP mutagenesis data revealed that Asp-267 (TM8) and Glu-318 (TM10) are irreplaceable for function (all conservative side-chain changes at these positions inactive); Thr-38 (TM1), Thr-271 (TM8), and Asp-298 (TM9) are replaceable but subject to constraints with respect to the allowed changes (at least one conservative side-chain change inactivate); replacements of Ala-91 (TM3), Thr-271 (TM8), and Asp-298 (TM9) result in decreased affinity for substrate and/or ligands; and replacement of Ile-317 (TM10) affects the specificity profile because I317A allows higher affinity transport of adenine but lower affinity recognition of purine.

**Site-directed Mutagenesis of YjcD**—In the same vein, we engineered 19 mutants in the YjcD background, namely T35A, T35H, A88S, A88G, D271E, D271A, D271N, T275D, T275A, T275S, T275N, D302N, D302E, I321A, I321E, E322D, E322Q, S323A, and S323N. After verification of the sequence, each YjcD mutant was transferred in *E. coli* JW3692 and assayed for its expression levels in the membrane and its ability to catalyze active transport of hypoxanthine (Fig. 8). Of the 19 mutants, five (T35A, A88G, A88S, D302N, and I321E) were highly active (35–120% of the rate achieved with wild type and 40–90% of the steady-state level), four (D271A, D271N, T275S, and E322D) displayed very low uptake activity (rates 15–20% and steady-state levels 18–25% of wild type), and 10 (T35H, D271E, T275D, T275A, T275N, D302E, E322Q, S323A, S323N, and I321A) were practically inactive (rates 0–8% and steady-state levels 0–12% of wild type). All 19 mutants showed high protein levels in the membrane (Fig. 8). On kinetic analysis of the hypoxanthine uptake, T35A, T271A, T271N, I321E, and D322D displayed 3-, 5.5-, 2-, 5-, and 4-fold lower affinity (higher *Km*) relative to wild type, respectively, whereas A88G showed 2.7-fold higher affinity (lower *Km*) and A88S and D302N did not differ significantly from wild type (Table 1).

The specificity profile of each active mutant was examined using hypoxanthine uptake inhibition assays (Table 2). It was found that mutants T35A, I321E, A88G, and A88S deviate from the wild-type profile with T35A displaying lower affinity for guanine, 1-methylguanine, 6-thio, or 8-aza derivatives; A88S displaying lower affinity for guanine, 1-methylguanine, and 8-aza-guanine; A88G showing lower affinity for guanine and 1-methylguanine but higher affinity for 6-thioguanine and 6-mercaptopurine; and I321E displaying higher affinity for guanine but lower affinity for 1-methylguanine, 8-azaguanine, and 8-bromoxanthine (Table 2). The low affinity of mutants T35A, A88G, and A88S for guanine as revealed in the hypoxanthine inhibition assays is corroborated by the high *Km* values for guanine uptake found for these mutants on guanine uptake kinetic analysis (Table 1). It is clear from our data that the replacement of Ala-88 with Gly reverses selectivity of YjcD from a guanine-prefering profile (wild-type *Km* is 1.8 μM for guanine, 1-methylguanine, and 8-azaguanine) to that of an adenine-prefering profile (wild-type *Km* is 1.5 μM for adenine and 2.8 μM for guanine).

**FIGURE 4. Specificity profiles of PurP, YicO, YjcD, and YgfQ.** *E. coli* JW3692 expressing PurP, YicO, YjcD, or YgfQ was assayed for initial rates of [3H]adenine (0.1 μM) uptake (histogram bars on the left) or [14C]hypoxanthine (1 μM) uptake (histogram bars on the right) at 5–15 s at 25 °C in the absence or presence of the indicated unlabeled nucleobases or analogs at 0.6 mM (for adenine antagonism) or 1 mM (for hypoxanthine antagonism). The uptake rates retained are presented as percentages of the rate measured in the absence of competitors with standard deviations (error bars) from three independent determinations shown. The inhibitory effect of competitors yielding ≥50% inhibition of the uptake activity was subjected to kinetic analysis to determine *K* values (Table 2). The chemical structures of high affinity substrates and analogs recognized by PurP (*K* < 1 μM) and YicO (*K* < 71 μM) are shown on the left. The chemical structures of high affinity substrates and analogs recognized by YjcD (*K* < 41 μM) and YgfQ (*K* < 148 μM) are shown on the right. A, adenine; G, guanine; HX, hypoxanthine; X, xanthine; UA, uric acid; U, uracil; T, thymine; G, cytosine; Alp, allopurinol; Pu, purine; N6bA, N6-benzoyladename; diNP, 2,6-diaminopurine; 7MA, 7-methyladenine; 7MG, 7-methylguanine; 8azG, 8-azaguanine; 8azX, 8-azaxanthine; 8BrX, 8-bromoxanthine; 6SX, 6-thioxanthine; 2SX, 2-thioxanthine.
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TABLE 2
Specificity profiles of PurP, YicO, YjcD, YgfQ, and mutants

| Permease | Competitive use | Km (µM) | IC50 (µM) |
|----------|-----------------|---------|-----------|
| PurP(wt) | HX              | 96.2    | 2.1       |
| YicO(wt) | HX              | 96.2    | 2.1       |
| YjcD     | HX, HX         | 96.2    | 2.1       |
| YtgQ     | HX, HX         | 96.2    | 2.1       |

We have shown in this study that the genome of E. coli K-12 encodes two homologous but functionally distinct pairs of high affinity transport proteins for the uptake of adenine versus guanine/hypoxanthine, namely the two adenine-specific permeases PurP and YicO and the two guanine/hypoxanthine permeases YjcD and YgfQ. The four proteins belong to cluster COG2252 of the evolutionarily broad family NCS2 and are closely related to each other, ranging in sequence identity from 81% (between YjcD and YgfQ) to 34% (between YjcD and PurP). Previous reports on the functional profile of these proteins are described in Table 2.

**DISCUSSION**

We have shown in this study that the genome of E. coli K-12 encodes two homologous but functionally distinct pairs of high affinity transport proteins for the uptake of adenine versus guanine/hypoxanthine, namely the two adenine-specific permeases PurP and YicO and the two guanine/hypoxanthine permeases YjcD and YgfQ. The four proteins belong to cluster COG2252 of the evolutionarily broad family NCS2 and are closely related to each other, ranging in sequence identity from 81% (between YjcD and YgfQ) to 34% (between YjcD and PurP).
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not available except for PurP. The purP gene product has already been annotated in the databases as an adenine transporter based on genetic studies with E. coli mutants that were defective in the uptake and utilization of adenine (15, 16). Those genetic studies have also indicated that E. coli uses a different protein system for the uptake of guanine and hypoxanthine (34), but such a system remains unidentified to date. In a very recent report that appeared almost concurrently with the present study, Kozmin et al. (35) suggested that YjcD is a primary importer for modified purine bases, and its physiological role might, in fact, be related with the uptake of guanine and hypoxanthine. However, their suggestions were based on growth kinetics and suppression of the toxic effect of analogs in E. coli mutants and not on direct measurements of transport activities. Our current results clearly indicate that the guanine/hypoxanthine uptake system might be represented by either YjcD or YgfQ, whereas the adenine uptake system might be represented by either PurP or YicO. Analysis of the endogenous uptake activity in various single gene knock-out Keio strains implies that E. coli K-12 may use only YjcD and PurP, respectively, under the applied aerobic growth conditions. Both PurP (17, 36) and YjcD (36) have been shown to belong to the PurR regulon, which plays a critical role in the transcriptional regulation of purine metabolism in enterobacteria (17).

With respect to the functional distinction between YjcD (or YgfQ) and PurP (or YicO), the two pairs of purine transporters not only differ in their basic substrate preferences but also display no significant overlap in their purine recognition profiles (Fig. 4). Of 25 purines or analogs tested, the two adenine transporters were found to recognize only analogs modified at position 2 or 6 of the adenine ring as high affinity competitors (N6-benzoyladenine, purine, and 2,6-diaminopurine). The two guanine/hypoxanthine transporters were found to recognize a broader range of structures, including modifications at positions 1, 2, and 6 (1-methylguanine, 6-thioguanine, and 6-mercaptoguanine) and at the imidazole moiety (8-azaguanine). None of the analogs tested can serve as a high affinity ligand for both types of transporters. In view of the striking sequence similarity of the two types of transporters at critical sites (Fig. 2), it would be interesting to examine the structure-functional basis of this selectivity split. It is most interesting that the highly homologous AzgA-like transporters of the fungus A. nidulans (13) and the plant A. thaliana (14) do not display such a selectivity split but are able to use both adenine and guanine (or hypoxanthine) as substrates.

The detailed kinetic analyses of the four COG2252 transporters revealed some significant differences in affinity and specificity between the two isofunctional homologs in each pair (Tables 1 and 2). YicO displays 5–10-fold lower affinity for adenine and for any of the competing high affinity analogs relative to PurP. YgfQ displays 2–3.5-fold lower affinity for hypoxanthine and for the majority of competing analogs relative to YjcD but not for guanine and 6-thioguanine, which are recognized with almost equal affinities by both transporters. The latter observation indicates that YgfQ has a different specificity profile from YjcD in which the amino group of guanine at purine position 2 plays a more important role for the binding affinity. From the comparison of the sequences of YjcD and YgfQ (81%
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In summary of the first part of our results, we have identified the analytical functional profile of four purine transporters of E. coli that belong to homology cluster COG2252. Based on our current experimental evidence, we suggest alternative, function-based designations for these genes other than those that currently exist. The existing names do not reflect function or reflect only the initial functional observations in the case of the purine permease PurP (37). YjcD may be renamed to GhxP (guanine/hypoxanthine permease), and PurP may be designated with the alternative name AdeP (adenine-specific permease) to emphasize the functional split. The isofunctional paralogs YgfQ and YicO may be renamed to GhxQ and AdeQ, respectively.

The second part of our results refers to the identification of functionally important residues of PurP (AdeP) and YjcD (GhxP) by mutagenesis. The mutagenesis study was designed on the basis of the structural homology between COG2252 and COG2233 transporters. Despite the low sequence identity between the two clusters (ranging from 11 to 16% on ClustalW analysis), functionally important residues of the previously studied COG2233 members that are predicted to fall at conserved motifs of the binding site region correspond to distinctive but highly conserved amino acids in the COG2252 homologs (Figs. 5 and 6). The site-directed replacement analysis of these amino acids in PurP and YjcD revealed key functional similarities with topologically equivalent residues of the COG2233 transporters as summarized in Figs. 9 and 10.

Amino acid residues delineated as functionally irreplaceable in both PurP and YjcD (Asp-267/271 and Glu-318/322) correspond to functionally irreplaceable residues in the topologies of the purine-transporting COG2233 homologs UacT, XanQ, and UapA (Glu-270/272/356 and Asn-319/325/409). In addition, one of these two amino acid positions is implicated directly with substrate binding in the structurally known UraA (Glu-241) (2) and, based on docking analysis, in UapA (Glu-356) (8). The other of the two positions has been proposed to be a binding site residue in XanQ (Asn-325) based on site-directed alkylation analysis (21) and appears to play an irreplaceable role implicated directly or indirectly (8) in binding in purine-transporting homologs but not in UraA (2).

On the other hand, an adjacent Gln/Glu residue of the TM10 motif that appears to be irreplaceable and directly associated with binding in all COG2233 members studied thus far (2, 6, 8) identifies (and of PurP and YicO (73% identical) it is apparent that the observed specific variations in substrate preference or affinity are associated with a limited set of amino acid changes.

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FIGURE 7. Expression, uptake activities, and topology of PurP mutants. E. coli JW3692 expressing PurP with the given mutations were subjected to immunoblot analysis of membrane fractions or assayed for transport of \([^3]H\)adenine (0.1 mM, 25 °C). Upper panel, samples of membrane fractions containing 100 µg of protein were subjected to SDS-PAGE (12%) and immunoblotting using HRP-conjugated avidin. Middle panel, initial rates of adenine uptake (block histogram bars) were measured at 5–10 s, and steady-state levels of adenine accumulation (gray histogram bars) were measured at 1–10 min. Control values obtained from JW3692 harboring pT7-5 alone were subtracted from the sample measurements in all cases. Results are expressed as a percentage of the activity of wild type with standard deviations (error bars) from three independent determinations. Lower panel, topology model of PurP derived from homology threading on the template of UraA structure. The α-helical segments are indicated in rectangles with continuous (core domain) or broken line perimeters (gate domain). Residues analyzed with site-directed mutagenesis are numbered and shown in red (irreplaceable for function), purple (replacements lead to decreased substrate affinities), green (replacements lead to change of specificity), blue (constraints with respect to the allowed changes), or grayscale (remaining positions).

4 K. E. Rudd, personal communication.
is absent from the COG2252 homologs based on the structure-topology predictions (Fig. 9). This residue is invariably conserved as a Gln in the group of xanthine and/or uric acid (2-oxy purine) transporters of the family and as a Glu in uracil transporters. The relevant residue of UraA (Glu-290) is irreplaceable for binding and has been shown to bind uracil in the x-ray structure of UraA via two hydrogen bonds, one of which is with the carbonyl oxygen at position 2 of uracil (2). In the xanthine-transporting homologs XanQ (18) and UapA (37), replacement of the relevant Gln residue leads to impairment of xanthine binding probably due to disruption of an essential hydrogen bond between Gln-324/408 and the carbonyl oxygen at position 2 of xanthine as indicated recently from substrate docking analysis (8). Because the carbonyl oxygen at purine position 2 is characteristic of the structures of 2-oxy purines (xanthine and uric acid) and the xanthine-selecting COG2233 transporters do not recognize hypoxanthine (2-non-oxy xanthine), it is tempting to speculate that the lack of the essential Gln residue of TM10 in COG2252 transporters is associated with their preference for 2-non-oxy purines and their inability to recognize xanthine. It is also relevant that none of the YjcD mutants tested in this study displayed any ability to bind xanthine (Table 2), and that, inversely, none of the many mutants tested in the course of the systematic analysis of XanQ (6) display any significant ability to bind hypoxanthine. It would be interesting to investigate whether engineering of appropriate Gln insertions/deletions at the crucial α10 region might shift the transporter function from a hypoxanthine- to a xanthine-selective profile or vice versa.

Amino acid residues found in this study to be associated with selectivity mutants in YjcD (Ala-88) or in both PurP and YjcD (Ile-317/321) correspond to residues that have also been linked with selectivity mutants in the purine-transporting COG2233 homologs UacT, XanQ, and/or UapA (Thr-100/Asn-93/Ser-156 and Ser-317/Ala-323). These two amino acid positions are predicted to be at the vicinity of the purine binding site and may be associated with binding through hydrogen bonding of their side chain with a purine-binding residue (Asn93-Glu272 in XanQ) (23), hydrogen bonding of a main-chain atom with substrate (Ala-407 in UapA) (8), or a more remote indirect interaction depending on the side-chain occupancy. Interestingly, the selectivity-associated replacements of COG2233 transporters at these positions affect primarily the imidazole moiety of the ring (Fig. 9) and allow selectivity shifts between xanthine-transporting or uric acid-transporting homologs and more promiscuous, dual substrate transporters. Thus, the relevant
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mutants of the uric acid permease UacT allow highly efficient transport of xanthine (T100A and S317A) (7), mutants of the xanthine-specific XanQ allow transport of uric acid (N93S and N93A) (23) or high affinity recognition of the non wild-type ligand 8-methylxanthine (N93S, N93A, and A323S) (7, 23); and mutant S156A of the dual substrate xanthine and uric acid permease UapA shifts the profile in a xanthine-selective direction (38). In contrast, selectivity-associated mutants of COG2252 transporters affect primarily the amino group of guanine position 2 (YjcD mutants A88G and I321E) or the amino group of adenine position 6 (PurP mutant I317A) at the pyrimidine moiety (Fig. 9). It is notable as well that none of the selectivity mutants of YjcD or PurP delineated in this study as well as the corresponding residues of XanQ (6) and UacT (7) are shown as thick sticks and color-coded (red, irreplaceable for function; purple, replacements lead to decreased substrate affinities; green, replacements lead to change of specificity; blue, constraints with respect to the allowed changes; grayscale, remaining positions). Residues delineated as crucial for the selectivity profile in at least three of the transporters are highlighted with red ovals. Residues delineated at the periphery of the modeled structures of PurP and YjcD. Homology structural models of PurP, YjcD, XanQ, and UacT (Fig. 6) were displayed with PyMOL. Shown are α-helical parts of the core domain that are predicted to form the substrate coordination shelter (2). Important residues of YjcD or PurP delineated in this study as well as the corresponding residues of XanQ (6) and UacT (7) are shown as thick sticks and color-coded (red, irreplaceable for function; purple, replacements lead to decreased substrate affinities; green, replacements lead to change of specificity; blue, constraints with respect to the allowed changes; grayscale, remaining positions). Residues delineated as crucial for the selectivity profile in at least three of the transporters are highlighted with red ovals. The chemical structures of the major transported substrates in each case are also shown. The light green circles highlight sites of the purine ring associated with the observed selectivity changes and the corresponding selectivity-associated mutants in each case.

FIGURE 9. Arrangement of important residues in the modeled structures of PurP and YjcD. Homology structural models of PurP, YjcD, XanQ, and UacT (Fig. 6) were displayed with PyMOL. Shown are α-helical parts of the core domain that are predicted to form the substrate coordination shelter (2). Important residues of YjcD or PurP delineated in this study as well as the corresponding residues of XanQ (6) and UacT (7) are shown as thick sticks and color-coded (red, irreplaceable for function; purple, replacements lead to decreased substrate affinities; green, replacements lead to change of specificity; blue, constraints with respect to the allowed changes; grayscale, remaining positions). Residues delineated as crucial for the selectivity profile in at least three of the transporters are highlighted with red ovals. Residues delineated at the periphery of the modeled structures of PurP and YjcD. Homology structural models of PurP, YjcD, XanQ, and UacT (Fig. 6) were displayed with PyMOL. Shown are α-helical parts of the core domain that are predicted to form the substrate coordination shelter (2). Important residues of YjcD or PurP delineated in this study as well as the corresponding residues of XanQ (6) and UacT (7) are shown as thick sticks and color-coded (red, irreplaceable for function; purple, replacements lead to decreased substrate affinities; green, replacements lead to change of specificity; blue, constraints with respect to the allowed changes; grayscale, remaining positions). Residues delineated as crucial for the selectivity profile in at least three of the transporters are highlighted with red ovals. The chemical structures of the major transported substrates in each case are also shown. The light green circles highlight sites of the purine ring associated with the observed selectivity changes and the corresponding selectivity-associated mutants in each case.

A number of amino acid residues at the periphery of the presumed binding site of YjcD or PurP are not irreplaceable for activity or for the proper selectivity profile but are subject to constraints with respect to the allowed side-chain changes; several mutations at these positions yield low substrate affinity (YjcD T35A and PurP A91S, T271A, T271S, and D298E) or impairment of transport (YjcD T35H and D302N and PurP T38H, T271D, T271N, and D298N). Furthermore, two peripheral residues of YjcD (Thr-275 and Ser-323) were irreplaceable (Fig. 9). Similar constraints have been found with corresponding residues of the COG2233 transporters albeit to various extents of significance. For example, the invariant His of COG2233 homologs (corresponding to Thr-35/38) in TM1 was delineated as important for high affinity binding of substrate in XanQ (20), strictly irreplaceable for transport in UacT (7), and important for the proper folding and targeting to the plasma membrane in UapA (39). The less well conserved Asp/Asn/Glu of TM9 (corresponding to Asp-302/298) has been identified as irreplaceable for transport in XanQ, UacT, and UapA, but its role is obviously less critical in the COG2252 homologs (Fig. 10). The weak consensus residue Asp/His/Met of TM8 (corresponding to Thr-271) has been linked with constraints in the allowed side-chain replacements that are more stringent in XanQ (Asp-276) where a carboxyl group is needed for high uptake activity and an Asp is essential for both the selectivity and the pH profile (22). In COG2252 transporters, the corresponding residue is either subject to severe replacement constraints (PurP) or irreplaceable (YjcD). Finally, the weak consensus Asn/Ile/Val of TM10 (corresponding to Ser-323) is subject to side-chain volume constraints in XanQ (18) and UacT (7) that are probably associated with steric hindrance between TM8 and TM10 at the periphery of the binding site (7), whereas the corresponding Ser residue of COG2252 transporters is either non-essential for transport (PurP) or functionally irreplaceable (YjcD). This heterogeneity in the roles of residues...
that are peripheral to the binding site should be attributed to elaborates differences in the mechanism of the individual purine transporters, which remain to be elucidated.

Although YjcD and PurP recognize different substrates, their functionally important residues identified in this study (Fig. 9) are almost identical. In addition, our mutagenesis data did not reveal any mutations leading to change in selectivity from the one transporter type to the other. These observations imply that other, as yet unidentified COG2252 residues that differ between the two transporters might have an important contribution to the substrate recognition preferences. Given that key amino acids of the presumed binding site are identical and play a similar role in both transporters (Fig. 9), it might be assumed that residues that contribute decisively to the selectivity split are mostly at the periphery and affect the recognition of substrate through indirect side-chain interaction effects. On the other hand, the coordination of the 2-non-oxy purine bases in the binding site of COG2252 transporters might involve additional interactions that differentiate between PurP and YjcD but are not evident from the current data or play a minor role in the COG2233 homologs. To address such alternatives, further mutagenesis studies using a more thorough investigation of conserved motifs of the family (Fig. 2) in conjunction with substrate docking analyses will be needed. The ongoing Cys-scanning analysis of XanQ and other systematic studies of COG2233 transporters (6–8) will probably provide a comprehensive data set of candidate mutagenesis targets to this end.

In conclusion, we have identified several amino acid positions with important roles in the adenine or guanine/hypoxanthine transporters of E. coli and shown that functionally irreplaceable or selectivity-linked residues of these transporters correspond to residues with similar roles in the distantly related xanthine or uric acid transporters of family NCS2. This line of evidence provides support to the contention that purine transporters of the separate, weak sequence homology clusters COG2233 and COG2252 of family NCS2 may use a set of topologically similar side chains to dictate their function and selectivity preferences, reflecting an evolutionarily “deep” homology in their binding site structures.

To our knowledge, this is one of the first reports showing functional homologies of individual side chains in distantly related transport proteins that share weak similarity in linear sequence alignments. Structure-based alignments are more prompt to reveal targets for informative site-specific mutagenesis in such cases as shown both in the current study and in a recent seminal study on the structural motif homologies between the fucose permease FucP and the lactose permease LacY of the major facilitator superfamily (40).

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