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Uracil Uptake in *Escherichia coli* K-12: Isolation of *uraA* Mutants and Cloning of the Gene

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Mutants defective in utilization of uracil at low concentrations have been isolated and characterized. The mutations in question (*uraA*) map close to the *upp* gene encoding uracil phosphoribosyltransferase. By complementation analysis, a plasmid that complements the *uraA* mutation has been isolated. The *uraA* gene was shown to be the second gene in a bicistronic operon with *upp* as the promoter proximal gene. The nucleotide sequence of the gene was determined, and the gene encodes a hydrophobic membrane protein with a calculated M_r of 45,030. The UraA protein has been identified in sodium dodecyl sulfate-polyacrylamide gels in the membrane fraction of minicells harboring the *uraA* plasmids.

Preformed pyrimidine nucleosides and nucleobases are taken up and metabolized in *Escherichia coli* by the pyrimidine salvage pathways (Fig. 1). The enzymes and proteins responsible for the transport and interconversion of pyrimidine compounds have been characterized to some extent. It has been shown that *E. coli* can take up both nucleosides and nucleobases (23). Two active transport systems, NupC and NupG (24), are responsible for the transport of nucleosides. NupC transports all nucleosides (except guanosine and deoxyguanosine), whereas NupG transports all nucleosides (including guanosine and deoxguanosine). The genes encoding these nucleoside transporters have been cloned and sequenced elsewhere (12). Whether systems other than NupC and NupG are involved in nucleoside transport is presently under study.

In respect to nucleobase transport, the cytosine transport system has been characterized to some extent. The cytosine molecules are transported by an active transport system, which is designated CodB; after deamination, which is catalyzed by the cytosine deaminase, the further metabolism of the cytosine is dependent on an active uracil phosphoribosyltransferase (UPRTase). *codB*, the gene encoding CodB, has been sequenced (8). All other nucleobases except thymine are metabolized by a phosphoribosyltransferase after they have entered the cell.

Selections for 5-fluorouracil (FU)-resistant mutants generally result in *upp* mutants that are deficient in UPRTase activity (27). The *upp* mutants cannot use uracil as a pyrimidine source (2). Nevertheless, FU-resistant mutants may be sensitized to low concentrations of FU when a purine ribonucleoside is added to the growth medium (4, 29, 32). The toxicity is based on the intracellular formation of 5-fluorouridine through the sequential action of purine nucleoside phosphorylase and uridine phosphorylase (23). This observation suggested that the uracil transport system was composed of more than the UPRTase. There are several reports of additional factors involved in nucleobase transport in microorganisms. Benson and coworkers reported the isolation of a purine-requiring mutant of *Salmonella typhimurium* with an altered guanine uptake but a normal level of purine phosphoribosyltransferase activities (3). Burton reported a mutant defective in adenine transport, which had normal levels of adenine phosphoribosyltransferase (5).

In preliminary experiments, we have also identified a pyrimidine-requiring mutant which is deficient in the uptake of uracil but has a normal level of UPRTase activity (23). This mutation maps very close to the *upp* gene (1, 31).

During cloning of the *upp* gene (1), we also cloned the *uraA* gene. We report here a characterization of the *uraA* gene and the subsequent cloning and sequencing of the gene. In addition, by using gene fusions, we show that the *uraA* gene is the second gene in a bicistronic operon, where it is expressed from the promoter upstream of *upp*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Bacteriophage Mucts62 and Tn*10* mutagenesis were performed as described elsewhere (13, 16). P1 transduction was performed as described elsewhere (22). Bacteria were grown at 37°C in L broth (22) or in AB medium with glucose as the carbon source (6). Bacteria harboring plasmids were grown in the presence of ampicillin (100 μg ml^{-1}) or tetracycline (10 μg ml^{-1}). 5-UMP (100 μg ml^{-1}), uracil (10 μg ml^{-1}), or cytosine (5 μg ml^{-1}) was added to the growth medium of pyrimidine auxotrophs.

Plasmid construction. The plasmids used in this study are listed in Table 2. Plasmid pBM6 has been previously described (1). For construction of plasmid pSA17, pBM6 was partially digested with *Nru*I and religated, thus resulting in a *purMN*$^+$ *upp uraA*$^+$ plasmid in which the promoter region of *upp* was retained. For construction of pDF17 or pDF18, a 2-kb *Mlu*I deletion of the *purMN* operon was made in the pSA17 or the pBM6 plasmid. For construction of pSA19, a 2-kb *Cla*I-*Eco*RI fragment from pBM6 with filled-in 3′-recessed ends was subcloned into the *Eco*RI site (with filled-in 3′-recessed ends) of the expression vector pUHE23-2, which contains the A1 phage T7 promoter combined with two *lac* operator sites (9). For construction of the gene fusion plasmids, a 1.8-kb *Mlu*I-*Bgl*II fragment from pBM6 containing the *upp* promoter was subcloned into the transcriptional fusion plasmid pJN17 (26), resulting in pPA24. In order to remove the *upp* promoter, an *Eco*RV deletion was performed on pPA24, thus removing 400 bp and resulting in pPA28.

DNA methods. All DNA manipulations were carried out by standard methods (20). Restriction endonucleases and other DNA-modifying enzymes were purchased from Boehringer Mannheim or New England Biolabs, Inc. Subclones for DNA sequencing were constructed in pBluescript phagemids (21) (Stratagene Cloning Systems). Sequencing was performed by the dideoxy-chain termination technique with either the Sequenase version 2 (U.S. Biochemical) or, for resolving compressions, the Taqtrack (Promega) sequencing system. Both strands of the *uraA* region were sequenced. Specific oligonucleotides used as primers were obtained from either the Danish Biotechnological Institute or the Center for Microbiology, The Technical University of Denmark.

Uptake and assays. Complementation of *uraA* mutations was always done in

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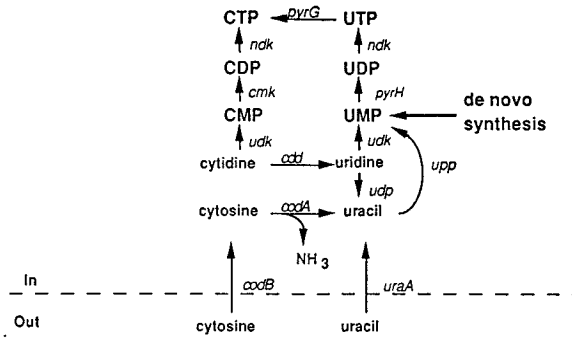

FIG. 1. Pyrimidine salvage pathways. The individual enzymes are identified by their gene symbols as follows: *cdd*, cytidine deaminase; *cmk*, CMP kinase; *codA*, cytosine deaminase; *codB*, cytosine permease; *ndk*, nucleoside diphosphokinase; *pyrG*, CTP synthetase; *pyrH*, UMP kinase; *udk*, uridine kinase; *udp*, uridine phosphorylase; *upp*, uracil phosphoribosyltransferase; and *uraA*, uracil permease.

a two-step manner: (i) test of growth rate on uracil as a pyrimidine source and (ii) uptake analysis. Determination of uracil uptake was done by measuring [2-^{14}C]uracil (New England Nuclear) uptake in exponentially growing cells at room temperature. Cells were removed from a growing culture, filtered, and washed in AB medium and resuspended in the growth medium lacking pyrimidines. The optical density at 436 nm was measured before addition of [2-^{14}C]uracil (50 Ci/mol) or [2-^{14}C]cytosine (50 Ci/mol). Cell suspension (200 μl) was filtered through 0.45-μm-pore-size nitrocellulose filters and was subsequently washed with 2.0 ml of AB medium. The radioactivity in the dried filters was determined by liquid scintillation counting.

Galactokinase (7) and UPRTase (31) assays were performed as described elsewhere. Protein concentrations were determined by using a Protein Determination kit from Bio-Rad.

Plasmid copy number. The specific activity of β-lactamase in crude cellular extracts was used as a relative estimate of plasmid copy number (28).

Minicell analysis. Plasmid-bearing derivatives of the minicell-producing strain BD1854 were grown overnight in AB minimal medium with glucose as the carbon source. Protein synthesis and fractionation of minicell-synthesized proteins were carried out as described elsewhere (12). Membrane fractions were isolated from six times as many cells as the total cellular fractions. Polyacrylamide gel electrophoresis (PAGE) was performed on sodium dodecyl sulfate (SDS)-polyacrylamide slab gels (12.5%) (18). Before electrophoresis, the samples were incubated in standard SDS-sample buffer at 37°C for 1 h. Samples of 10 μl were applied to the gel, and protein bands were visualized by staining with Coomassie brilliant blue and by autoradiography.

Nucleotide sequence. The nucleotide sequence data appear in the EMBL Nucleotide Sequence Data Library under the accession number X73586.

RESULTS

Selection and characterization of *uraA* mutants. Early attempts to isolate mutants defective in uracil uptake resulted in *upp* mutants with no or low UPRTase activity. In order to avoid this problem, two approaches were pursued.

In the first approach, a pyrimidine-requiring parental strain (SØ106) was used, with cytosine serving as the pyrimidine source. Cytosine can be metabolized only by deamination to uracil. Strain SØ106 was lysogenized with phage Mucts62. Lysogens were plated on glucose minimal medium containing cytosine with filter pads containing from 2.5 to 10 μg of FU. Colonies appearing in the cleared zone around the filter pads were tested for lysogeny and were analyzed for UPRTase activity and uracil and cytosine uptake. Three of 50 colonies tested were found to have normal UPRTase activities and normal uptake of cytosine but were defective in uracil uptake.

The lysogens were cured for the prophage Mucts62 by plating at 42°C. One of the resulting strains, designated BM610, was analyzed for uracil and cytosine uptake and was later used for complementation studies.

In the second approach, a wild-type strain was tested for resistance to low concentrations of FU. The pyrimidine prototrophic strain CSH62 is resistant to 0.02 μg of FU ml^{-1} and was subsequently spread onto plates containing 0.03 μg of FU ml^{-1} following Tn*10* mutagenesis as described in Materials and Methods. The colonies appearing were analyzed for UPRTase activity and for uracil and cytosine uptake. In this way, a mutant carrying a Tn*10* inserted within *uraA* was isolated as a mutant with normal UPRTase activity and cytosine uptake but defective uracil uptake. The *uraA*::Tn*10* allele was moved into strain SØ106 by P1 transduction, generating SØ1345. Genetic analysis revealed that *uraA*::Tn*10* and Δ*uraA2* are allelic (data not shown). Mapping revealed that the *uraA*::Tn*10* is 88% cotransducible with *purM* (SØ1537) at 54 min on the *E. coli* linkage map.

Strain SØ1345 was analyzed for uracil and cytosine uptake and was compared with the parent strain. As seen in Fig. 2, the mutant strain was unable to take up uracil, whereas cytosine transport was unaffected. The mutants have no defect in the uptake of the purine nucleobases (data not shown).

At a low concentration of uracil (9 μM), the pyrimidine-requiring *uraA* mutants have lower growth rates than the parent. At 90 μM uracil, the mutant strains grow like the parent strain, and the mutant strains take up uracil to the same extent as the parent strain and incorporate uracil into nucleic acids at the same rate.

Cloning of the *uraA* gene and operon expression of the *upp* and *uraA* genes. A *upp*-complementing plasmid has been isolated from a ColEl plasmid DNA library (31). This plasmid also showed a *uraA*-complementing phenotype. However, it was previously assumed that the UPRTase was the only component of the uracil transport system; it was therefore necessary to separate the two genes.

The plasmid pBM6, which has been previously described (1), was shown to complement both *upp* and *uraA* mutations (Fig. 3). In order to exclude the possibility that a high level of UPRTase was sufficient for complementation of a *uraA* mutation, it was necessary to eliminate the plasmid-directed

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Reference or source
BD1854	*minA minB thi his rpsL lac mtl man mal xyl tonA*	Laboratory collection
BM604	*thi galE* Δ(*attB-bio*) *deoA103 deoC argA lysA cytR upp udp pyrF30*	1
BM610	*lacZ rpsL thi pyrF30* Δ*uraA2*	This work
CSH62	*thi*	Cold Spring Harbor Laboratory
NM522	Δ(*lac-proAB*) *hsdD5 supE thi* F *proAB lacI*^{q}*Z*Δ*MI5*	Laboratory collection
SØ106	*lacZ rpsL thi pyrF30*	1
SØ268	*cdd pyrE60 thi-1 argE3 his-4 proA2 thr-1 leu-6 mtl-1 xyl-5 ara-14 galK2 lacY1 str-31 supE44*	26
SØ1345	*lacZ rpsL thi uraA*::Tn*10 pyrF30*	This work
SØ1537	*galE purM48 metB1 relA1*	1
SØ6305	*lacZ rpsL thi upp*::Tn*5 pyrF30*	1

TABLE 2. Plasmids used in this work

Plasmid	Description
pBM6	Ap^r Tc^r; contains a 5.5-kb fragment which carries the *purMN* operon and the *upp-uraA* operon (1)
pBM61	Tc^r; *Pst*I deletion of pBM6 containing the *uraA* gene, which is not expressed
pBM91	Ap^r; contains a 3-kb fragment which carries the *purMN* operon (34)
pSA4	Ap^r; *Sal*I deletion of pBM6 containing the *purMN* operon and the *upp* gene
pSA17	Ap^r Tc^r; internal *Nru*I deletion in the *upp* gene; *purMN uraA*
pSA18	Ap^r Tc^r; contains a 3.9-kb fragment which carries the *purMN* operon and the *upp-uraA* operon
pUHE23-2	Ap^r Cm^r (9)
pSA19	Ap^r; pUHE23-2 with a 2-kb fragment containing the *uraA* gene
pDF18	Ap^r Tc^r; same as pBM6, with a deletion of the *purMN* operon
pJN17	Ap^r (26)
pPA24	Ap^r *upp*$^+$ *galK*$^+$
pPA28	Ap^r

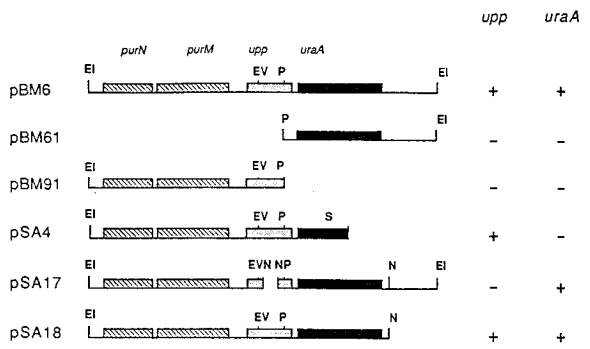

FIG. 3. Complementation analysis of *upp* (SØ6305) and *uraA* (BM610). EI, *Eco*RI; EV, *Eco*RV, N, *Nru*I; P, *Pst*I; S, *Sal*I; +, complementation; −, no complementation.

UPRTase synthesis by deleting part of the *upp* gene but retaining the *uraA* expression. *Pst*I deletion plasmids pBM61 and pBM91 (Fig. 3) resulted in the loss of ability to complement either the *upp* or the *uraA* mutation. In order to analyze whether the *uraA* gene was transcribed from the promoter upstream from the *upp* gene, an internal deletion of the *upp* gene was made (pSA17). This plasmid complements the *uraA* mutation, suggesting that *upp* and *uraA* may constitute an operon.

Figure 4 shows the uracil uptake of the *uraA* mutant containing three different plasmids. The results reveal that expression of the *upp* gene even on a high-copy-number plasmid does not result in any uracil uptake when the plasmids lack the *uraA* gene [SØ1345(pSA4)] and also that *upp* is the limiting component of the uptake system of uracil [compare SØ1345 (pSA18) and SØ1345(pSA17)].

To further establish the operon structure of the *upp* and *uraA* genes, we then constructed a *uraA*::*galK* operon fusion in pJN17 and performed a promoter deletion analysis as well as an analysis of the regulation of the galaktokinase synthesis. By growing the cells with 5′-UMP as the pyrimidine source, the cells are partly starved for pyrimidines, which causes increased *upp* expression (1). Extracts from plasmid-harboring strains were analyzed for *galK* expression and pyrimidine regulation (Fig. 5). Removal of the *upp* promoter resulted in loss of *uraA*::*galK* expression. The increased expression of *uraA*::*galK* expression upon growth with 5′-UMP as the sole pyrimidine source suggested that the regulation of *uraA* expression is analogous to that of *upp*.

Determination of the *uraA* nucleotide sequence. The complementation analysis showed that the *uraA* gene is positioned downstream from the *upp* gene. Therefore, we initiated subcloning of small fragments from the region into phagemid vectors and determined the nucleotide sequence on both strands. At 100 bp downstream from the *upp* stop codon, an

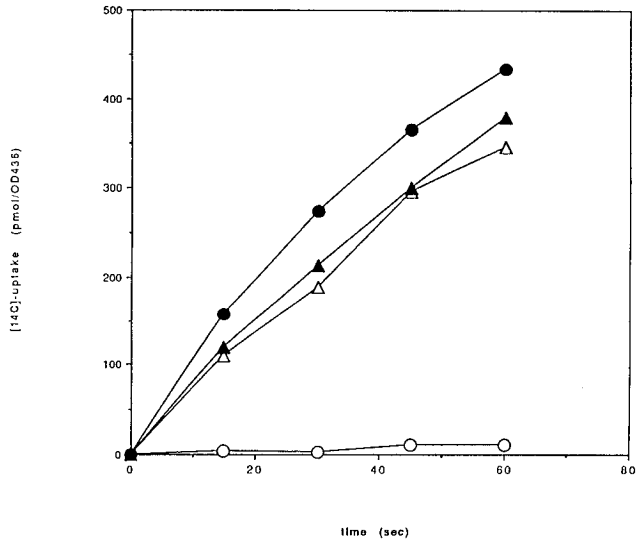

FIG. 2. Comparison of uracil and cytosine uptake by the *uraA* mutant and parent cells. Cells were grown with cytosine as the pyrimidine source. Mutant cells (SØ1345) (circles) and parent cells (SØ106) (triangles) are indicated. Values for uracil uptake (final concentration in assay mixture, 0.7 μM) (open symbols) and cytosine uptake (final concentration in assay mixture, 0.3 μM) (closed symbols) are shown. An optical density at 436 nm (OD_{436}) of 1 ≈3 × 10^8 cells.

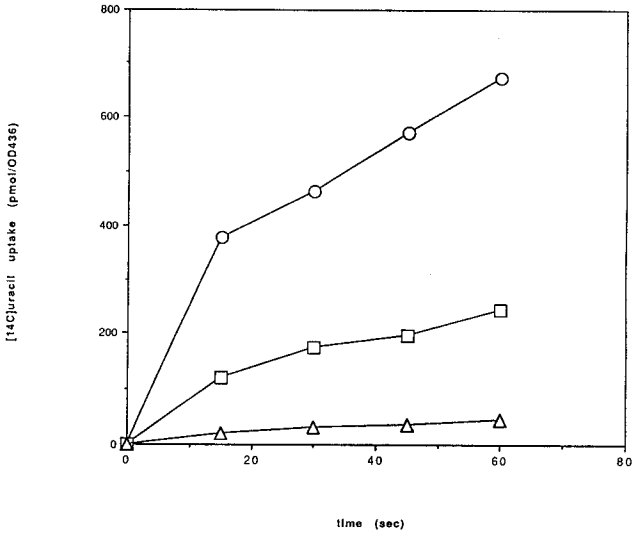

FIG. 4. Uracil uptake by plasmid-harboring cells of SØ1345 (*uraA*). Cells were grown with uracil as the pyrimidine source. Squares, SØ1345(pSA17) (*upp uraA*$^+$); triangles, SØ1345(pSA4) (*upp*$^+$); circles, SØ1345(pSA18) (*upp*$^+$ *uraA*$^+$). Final uracil concentration in the assay mixture, 0.7 μM). An optical density at 436 nm (OD_{436}) of 1 ≈3 × 10^8 cells.

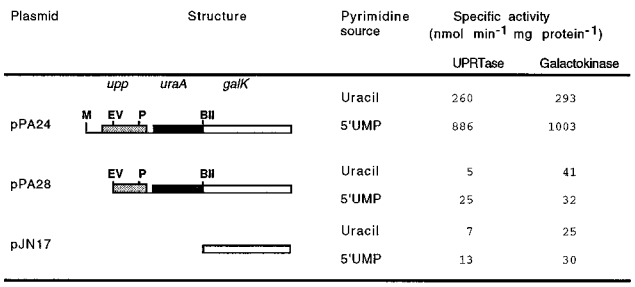

FIG. 5. Effect of *upp* promoter deletion on *uraA* gene expression. The depicted plasmids were constructed in plasmid pJN17. SØ268 cells were grown with the pyrimidine sources indicated. Cells were harvested, and enzyme activities were determined. Specific activities are corrected for copy number. M, *Mlu*I; EV, *Eco*RV; P, *Pst*I; BII, *Bgl*II.

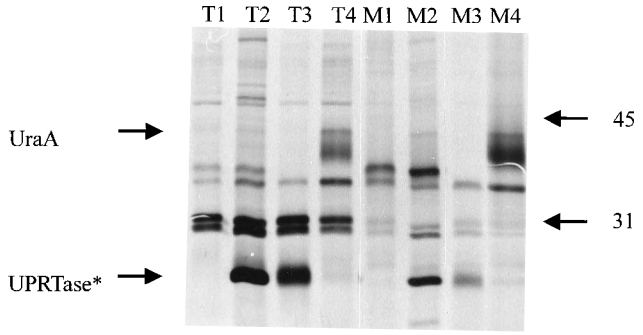

FIG. 6. Identification and subcellular location of the uracil permease. Plasmid-encoded polypeptides were labeled in minicells with [^{35}S]methionine; this was followed by cell fractionation, SDS-PAGE, and autoradiography as described in Materials and Methods. The positions of UraA and UPRTase polypeptides and the positions and sizes (in kilodaltons) of polypeptide standards are indicated. Lanes: M1 to M4, membrane fractions; T1 to T4, total cellular proteins; T1 and M1, pBR322; T2 and M2, pDF18 (*upp*$^+$ *uraA*$^+$); T3 and M3, pUHE23-2; T4 and M4, pSA19 (*uraA*$^+$). The bands migrating as 22-kDa polypeptides in lanes T3 and M3, which is similar in size to UPRTase, derive from chloramphenicol acetyltransferase.

open reading frame encoding a polypeptide of 429 amino acid residues was found.

The open reading frame encoding 429 amino acid residues starting at bp 24 is preceded by a Shine-Dalgarno sequence (GAGGA) positioned at 12 to 16 bp. The open reading frame encodes a polypeptide with an abundance of nonpolar amino acids (59.7%). This is suggestive of a membrane protein.

There are no sequences resembling a promoter upstream from the *uraA* translation start codon. Trailing the open reading frame, there is a region of dyad symmetry beginning at position 1320 which may form a stem-loop structure. However, there is no run of T residues immediately after this region. There is no open reading frame downstream from the *uraA* gene within 300 bp. However, there is a weak pyrimidine-independent promoter activity on the 300-bp *Nru*I-*Eco*RI fragment which has been identified by insertion of the fragment in a *galK* promoter fusion vector (data not shown).

The *uraA* gene product and the subcellular localization. In order to detect the UraA protein, plasmids harboring *uraA* were transformed into the minicell-producing strain BD1854. The minicells were analyzed for plasmid-encoded polypeptides. From the deduced amino acid sequence, the UraA protein was believed to be an integral membrane protein; therefore, membrane fractions were isolated and analyzed.

Early attempts to identify the UraA protein showed no obvious candidates for the UraA protein either in the total protein samples or in the samples from the membrane fractions. The *uraA* gene was therefore subcloned into the expression vector pUHE23-2, in which it was expressed from a strong promoter (9). The resulting plasmid was designated pSA19 (for a description of the construction, see Materials and Methods). When extracts from minicells harboring pSA19 were analyzed, a new strong band appeared on the autoradiograph that migrated as a polypeptide with an approximate molecular mass of 42 kDa, which did not appear in samples from vector-harboring minicells (compare lanes M3 and M4 and lanes T3 and T4 in Fig. 6). This rather diffuse band is highly enriched in the membrane fraction. From the amino acid sequence, UraA is expected to have a molecular mass of 45 kDa.

The identification of the UraA protein produced from the expression vector made it possible to identify a faint band visible only in the membrane fraction of extracts from the pDF18 plasmid, in which the *uraA* gene is expressed from its own promoter (Fig. 6, lanes T2 and M2).

DISCUSSION

In this study, we have identified a gene, *uraA*, that encodes a uracil transporter by isolating mutants that are deficient in uracil uptake at low concentrations. Furthermore, the gene was cloned and sequenced, and we have shown that the *uraA* gene is the second gene in an operon with the *upp* gene as the promoter-proximal gene. Between the *upp* gene and the *uraA* gene, there is a structure which resembles a *rho*-independent terminator (1). The function of this possible terminator is currently under investigation.

The polypeptide encoded by the *uraA* structural gene was identified in minicell experiments as a membrane-associated protein with an apparent molecular mass of approximately 42 kDa in SDS-polyacrylamide gels. It is surprising that the apparent molecular mass of 42 kDa is so close to the value predicted from the amino acid sequence of 45,030 Da. Typically, membrane proteins migrate considerably faster in SDS-polyacrylamide gels than predicted from the amino acid sequence, presumably because of the binding of excess SDS to the hydrophobic regions of these proteins (33). When the UraA protein is overproduced, it is seen as a smear to lower M_r values which might be explained as a consequence of the binding of various amounts of SDS. Membrane proteins often appear diffuse in SDS-polyacrylamide gels.

It is also notable that the UraA protein gives rise only to a very faint band when the *uraA* gene is expressed from its normal promoter, as opposed to the UPRTase protein, which gives rise to a very strong band. This might indicate that the UPRTase protein and the UraA protein are synthesized in different amounts.

In the present work, we demonstrate that the UraA protein is necessary for uracil uptake at low exogenous uracil concentrations, even under conditions with high UPRTase activity. Therefore, we suggest that uracil enters the cytoplasm by facilitated diffusion across the cytoplasmic membrane where the UraA protein is a membrane-bound facilitator. Internal uracil is trapped as UMP by the action of UPRTase or as uridine by the action of uridine phosphorylase in *upp* mutants. This system is analogous to the glycerol transport system, which is one of two other reported facilitated transport system in *E. coli* (35). The glycerol transport system is composed of two proteins, the facilitator (GlpF) and the glycerol kinase (GlpK), which is the trapping enzyme (37). The genes encoding these two proteins also constitute an operon (25).

The deduced amino acid sequence of UraA contains 59.7%

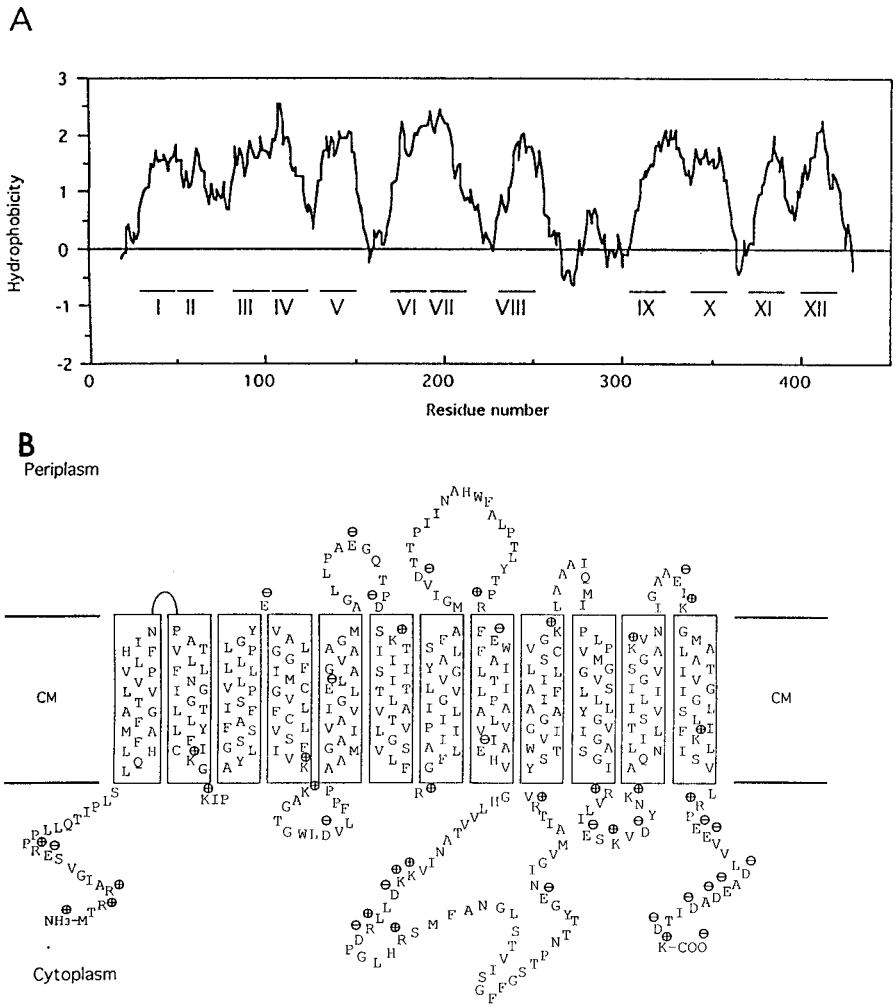

FIG. 7. Structural analysis of the UraA protein. (A) Hydrophobicity plot of the UraA protein according to Kyte and Doolittle (17) with an 11-amino-acid window. The positions of the 12 putative membrane-spanning segments are indicated by roman numerals. (B) Working model of the topology of the UraA protein prepared according to the model described by von Heijne (38). CM, cytoplasmic membrane. Pluses and minuses indicate positively and negatively charged amino acids, respectively.

nonpolar amino acid residues. The hydropathic profile of the sequence was established using an 11-amino-acid window according to Kyte and Doolittle (17). This suggests a structural arrangement of the protein with 12 hydrophobic transmembrane α-helices, which is in accordance with the consensus structure proposed by Maloney (19) (Fig. 7A). Considering that cytoplasmic domains of membrane proteins are enriched for positively charged amino acid residues (38), a working model is proposed (Fig. 7B).

We examined the deduced UraA amino acid sequence for homology with other protein sequences by using the Genetics Computer Group sequence analysis software package (10). The highest percentage of identities that was detected was to the PyrP proteins of the pyrimidine biosynthetic operons of *Bacillus subtilis* (30, 36) and *Bacillus caldolyticus* (11) (44 and 45% identical amino acid residues, respectively). In fact, the uracil transport function of these proteins has been established from their homology to the *E. coli* uracil permease (UraA) (11, 36).

There was no high degree of homology of the *E. coli* UraA to the *Saccharomyces cerevisiae* uracil permease, the only uracil transporter that has been characterized (15), in contrast to what was observed between the two UPRTases (1). This may be due to the fact that in *S. cerevisiae*, uracil is transported by an active transport mechanism (14).

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