The RihA, RihB, and RihC Ribonucleoside Hydrolases of Escherichia coli

SUBSTRATE SPECIFICITY, GENE EXPRESSION, AND REGULATION*

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Pyrimidine-requiring cdd mutants of Escherichia coli deficient in cytidine deaminase utilize cytidine as a pyrimidine source by an alternative pathway. This has been presumed to involve phosphorylation of cytidine to CMP by cytidine/uridine kinase and subsequent hydrolysis of CMP to cytosine and ribose 5-phosphate by a putative CMP hydrolase. Here we show that cytidine, in cdd strains, is converted directly to cytosine and ribose by ribonucleoside hydrolase encoded by the previously uncharacterized gene ybeK, which we have renamed rihA. The RihA enzyme is homologous to the products of two unlinked genes, yeik and yaaF, which have been renamed rihB and rihC, respectively. The RihB enzyme was shown to be a pyrimidine-specific ribonucleoside hydrolase like RihA, whereas RihC hydrolyzed both pyrimidine and purine ribonucleosides. The physiological function of the ribonucleoside hydrolases in wild-type E. coli strains is enigmatic, as their activities are paralleled by the phosphorylorytic activities of the nucleoside phosphorylases, and a triple mutant lacking all three hydrolytic activities grew normally. Furthermore, enzyme assays and lacZ gene fusion analysis indicated that rihB was essentially silent unless activated by mutation, whereas rihA and rihC were poorly expressed in glucose medium due to catabolite repression.

In Escherichia coli and Salmonella enterica serovar typhimurium, exogenous ribonucleosides are predominantly metabolized by nucleoside phosphorylases, which phosphorylorytically cleave the N-glycosidic bond, yielding ribose 1-phosphate and the corresponding nucleobase (Fig. 1) (for reviews, see Refs. 1 and 2). Uridine phosphorylase encoded by the udp gene is specific for uridine, whereas purine-nucleoside phosphorylase encoded by the xapA gene is capable of cleaving all the purine ribonucleosides, except xanthosine, which is metabolized by xanthosine phosphorylase, the xapA product. Nucleoside phosphorylases capable of cleaving cytidine are not known; however, cytidine is efficiently converted to uridine by cytidine deaminase, the cdd gene product.

Some ribonucleosides may also be phosphorylated directly to the corresponding nucleoside 5’-monophosphates by nucleoside kinases, encoded by the udk and gsk genes (Fig. 1). However, these enzymes generally constitute a minor pathway of nucleoside salvage because they are tightly feedback-inhibited by nucleotides and compete poorly for their nucleoside substrates against the nucleoside phosphorylases and cytidine deaminase (3–5). A kinase specific for adenosine has not been found in E. coli, but adenosine is predominantly converted to inosine by adenosine deaminase (6).

Although the general picture of nucleoside metabolism as outlined here has not changed considerably over the last 20 years, there has been some indications that additional unknown enzymes might be involved in the assimilation of nucleosides in E. coli. Thus, it is well known that pyrimidine-requiring mutants that are deficient in both the udk and udp genes still have the ability to utilize uridine as a pyrimidine source by some unknown pathway (6). It has also been known for many years that pyrimidine-requiring cdd mutants of E. coli deficient in cytidine deaminase can grow slowly in glucose minimal medium with cytidine as a pyrimidine source (7). In this case, an alternative pathway for conversion of cytidine to uracil nucleotides has been inferred to involve phosphorylation of cytidine to CMP by cytidine/uridine kinase, followed by hydrolysis of CMP to cytosine and ribose 5-phosphate by a putative CMP hydrolase. Cytosine could then be further metabolized to uracil nucleotides by cytosine deaminase and uracil phosphoribosyltransferase encoded by the codA and upp genes, respectively. The evidence for the operation of this alternative pathway of cytidine salvage rested on the finding that growth of pyr cdd mutants on cytidine was eliminated by additional mutational blocks in the udk, codA, or upp genes (7).

In this work, we attempted to identify the gene for the putative CMP hydrolase by subjecting a pyrF cdd strain to transposon mutagenesis and screening for mutants that had lost the ability to utilize cytidine as a pyrimidine source. Contrary to our expectations, all the obtained mutants contained normal levels of CMP hydrolyase activity, but they were deficient in a cytidine hydrolyase activity encoded by the previously uncharacterized ybeK gene, which we have renamed rihA. Thus, the major pathway for conversion of cytidine to uracil nucleotides in a cdd mutant appears to be initiated by direct hydrolysis of cytidine to cytosine and ribose catalyzed by the RihA hydrolase. Interestingly, this enzyme was also found to be an efficient uridine hydrolase and a major contributor to the unknown pathway of uridine salvage operating in the absence of uridine kinase and uridine phosphorylase.

Further analyses revealed that the E. coli genome contains two additional ribonucleoside hydrolyase genes, yeik and yaaF, which have been renamed rihB and rihC, respectively. To investigate the possible physiological function of the three nucleoside hydrolases, we have determined their substrate specificity and have characterized mutant strains in which each of the

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structural genes has been disrupted singly or in combination, as well as strains that overproduce the hydrolytic activities from multicopy plasmids. Finally, we have mapped the corresponding promoters of these genes and present initial studies of their regulation using lacZ gene fusions.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Media**—The bacterial strains used in this study are all derivatives of *E. coli* K12 and are listed in Table I. Generalized transductions with lysates of bacteriophage P1vir were performed as described (8). Minimal medium plates contained AB minimal medium (15) solidified with 0.2% glucose or glycerol as a carbon source, 1 mg/ml thiamin, and 15 mg/ml nucleobases or 30 mg/ml nucleosides when required. Rich medium was Luria broth (LB medium).

**Isolation of a rihA:cam Mutant, CN1932**—The pyrF cdd strain CN1930 (Table I) was subjected to transposon mutagenesis with mini-Tn10 cam from ANK1316 as described by Kleckner et al. (16). A phage P1 lysate prepared on a pool of ~20,000 kanamycin resistant transposants was subsequently used to transduce CN1879 (ade-1, aux-1, thi-1, leu-1, cam-1; Tn10:kan4) as a source of transposon inserts in the vicinity of the cam gene. Among 20,000 kanamycin resistant transposants, 1078 were obtained. Of these, 114 were obtained in the presence of 20 μg/ml chloramphenicol in the selection medium. DNA sequencing revealed that the chloramphenicol resistance gene was inserted at positions 8643–8651 in the rihA gene sequence (GenBank™/EBI accession number AE000169) in the opposite direction of rihA.

**Isolation of a rihB:cam Mutant, CN2264**—The genome of CN2264 (cdd-Tn10 ribB, Iss0-activated) (Table I) was mutagenized with mini-Tn10 cam as described above. A phage P1 lysate prepared on a pool of ~20,000 chloramphenicol resistant transposants was subsequently used to transduce CN2300 (cdd ribB, Iss0-activated) (Table I) to tetracycline resistance. By replica plating onto LB medium + 20 μg/ml chloramphenicol, we identified a total of 327 tetracycline resistant transductants, which had received a cam insertion by co-transduction with cdd-Tn10. Among these transductants, we found three clones that had lost the ability to utilize cytidine as a pyrimidine source. For each of these strains, we sequenced the region of the cam gene that had been disrupted.

**Isolation of a rihC:cam Mutant, CN2403**—The genome of CN1930 (Table I) was subjected to transposon mutagenesis with mini-Tn10 cam as described above. A phage P1 lysate prepared on a pool of ~20,000 chloramphenicol resistant transposants was subsequently used to transduce CN2316 (car-403: Tn10) (Table I) to pyrimidine prototrophy. By replica plating onto glucose minimal plates with chloramphenicol, we isolated a total of 220 clones presumed to have the chloramphenicol resistance gene inserted in the vicinity of the car gene. By PCR screening of these clones using the primer pairs cam-down + ribC-BamHI or cam-down + ribC-EcoRI (Table II), we identified one clone (CN2403) that gave a PCR product of ~400 base pairs with cam-down + ribC-BamHI. Sequencing of this product revealed that the cam gene was inserted in the *ribC* reading frame in the same orientation as rihC, at nucleotides 7271–7279 (GenBank™/EBI accession number AE000113).

**Isolation of a deoD gsk::kan Mutant, CN1980**—The gsk-3 mutant CN1932 (Table I) was mutagenized with mini-Tn10 kan from ANK1316 as described by Kleckner et al. (16). A phage P1 lysate prepared on a pool of ~20,000 kanamycin resistant transposants was used to transduce CN1879 (ade-1, aux-1, thi-1, leu-1, cam-1; Tn10:kan4) with mini-Tn10 gsk from AE000169) in the opposite direction of gsk. Among 20,000 kanamycin resistant transposants, 1078 were obtained. Of these, 114 were obtained in the presence of 20 μg/ml chloramphenicol in the selection medium. DNA sequencing revealed that the chloramphenicol resistance gene was inserted at positions 8643–8651 in the gsk gene sequence (GenBank™/EBI accession number AE000169) in the opposite direction of gsk.

**Verification of Genomic Mutations by Colony PCR Amplification**—An aliquot of a bacterial colony was suspended in 25 μl of a standard PCR reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.1 mM deoxynucleotides, 0.5 μM oligonucleotide primers, and 0.25 units of Taq polymerase (PerkinElmer Life Sciences). PCR amplifications were performed for 40 cycles (94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1.5 min). Following by 72 °C for 7 min. The amplified products were separated on agarose gels and eluted overnight in 50 μl of water at room temperature. Direct sequencing of the eluted fragment was performed using ThermoSequenase (U. S. Biochemical Corp.) and a 32P-labeled primer.

**Plasmid Constructions**—DNA manipulations, transformations, and restriction analyses were performed according to standard procedures (17). PCR amplifications were performed on 1 μg of genomic DNA using *Pfu* polymerase (Strategene) according to the manufacturer’s recommendations. The DNA oligonucleotides used as primers in PCRs are listed in Table II. Plasmids containing cloned PCR fragments were verified by DNA sequencing.

For construction of pRibA, a 4.7-kilobase region containing *ribA* and neighboring genes (from nucleotide 8399 (GenBank™/EBI accession number AE000169) to nucleotide 12797 (GenBank™/EBI accession number AE000170)) (Fig. 2) was subcloned from genomic DNA and inserted between the EcoRI and BglII sites of the medium-copy vector pET17b (Novagen). For construction of pRibC, the *ribC* gene and flanking regions (nucleotides 7235–8485; GenBank™/EBI accession number AE000305) (Fig. 2) were PCR-amplified from genomic DNA with the *ribB-EcoRI* and *ribH-BamHI* primers and inserted between the EcoRI and BamHI sites of the high-copy vector pGEM3 (Promega). For construction of pRibH, the *ribH* gene and flanking regions (nucleotides 6444–7588; GenBank™/EBI accession number AE000113) (Fig. 2) were PCR-amplified from genomic DNA with the *ribC-EcoRI* and *ribC-
Table I
Bacterial strains

| Strain* | Genotype* | Ref/source/construction† |
|---------|-----------|--------------------------|
| CN1524 (CSH26) | [CSH26] | 8 |
| CN1565 (SK701) | [CSH26] | 8 |
| CN1728 (C928) | [CSH26] Δg20- lac-lac his F88 rpsL super suppressor thp thi F88 pro* cod* lac+ | S. Kushner |
| CN1685 | [CSH26] pyrF: Tn5 | CN1524 × P1(CN1565), KanR |
| CN1879 (SO1736) | [SO003] purE deo adk 2 ubb 2419: Tn10 | 9 |
| CN1892 (HO692) | [SO003] deo adk: Tn5 gsk-3 | 10 |
| CN1893 (SO415) | [SO003] upp-11 udk-2 | 11 |
| CN1928 | [CSH26] pyrF: Tn5 cdd: Tn10 | CN1685 × P1(SO1452), TetR |
| CN1930 | [CSH26] pyrF: Tn5 cdd: Tn10 F10 pro* cod* lac+ | CN1928 × P1(CN1728), Pro+ |
| CN1932 | [CSH26] gsk-3 | 5 |
| CN1980 | [SO003] purE deo gsk: kan | See “Experimental Procedures” |
| CN2045 | [CSH26] udk-2 | his+ cam rihC derivatives of CN1930, rapid growth on glucose + cytidine |
| CN2056 | [CSH26] udk-2 udp: Tn5 | CN2045 × P1(CN1892), KanR |
| CN2258 | [CN1930] rihA: cam | See “Experimental Procedures” |
| CN2264 | [CN1930]car-403 | CN1930, rapid growth on glucose + cytidine |
| CN2290 | [CN1930] udk-2 | CN1930, rapid growth on glucose + cytidine |
| CN2076 (SO5069) | purR6: Tn10 pdxH15(Am) relA1 spo11 thi1-1 | 12 |
| CN2300 | [CN1930] cdd: rihB (IS50-activated) | CN2264 × P1(CN1524), CdrR d |
| CN2316 (CLT246) | [VH1000] car-403 - Tn10 | 13 |
| CN2324 | [CN1930] rihB: cam | CN1930, rapid growth on glucose + cytidine |
| CN2349 | F α rihB lac-3 thi recA-56 | 14 |
| CN2389 | [CSH26] car-403: Tn10 udk-2 udp: Tn5 | CN2056 × P1(CN2316), TetR |
| CN2390 | [CSH26] car-403: Tn10 udk udp: Tn5 rihA: cam | CN2339 × P1(CN2258), CamR |
| CN2403 | [VH1000] rihC: cam | See “Experimental Procedures” |
| CN2428 | [CSH26] pyrF: Tn5 rihA: cam | CN1980 × P1(CN2402), CamR |
| CN2545 | [SO003] purE deo gsk: kan rihC: cam | CN2390 × P1(CN2545), PyrR (HxR) d |
| CN2547 | [CSH26] udk-2 udp: Tn5 rihA: cam rihC: cam deoD | 11 |
| CN2550 (SO430) | [SO003] cdd udk add | CN2547 × P1(CN2076), TetR (PdxR) |
| CN2553 | [SO003] cdd udk udp: Tn5 rihA: cam rihC: cam deoD purR6: Tn10 pdxH15(Am) | CN2553 × P1(CN2550), PdxR (TetR AR) |
| CN2572 | [CSH26] udk-2 udp: Tn5 rihA: cam rihC: cam deoD add | CN2572 × P1(CN3242), TetR d |
| SO1452 | araD139 Δlac-lac-lacI189 pdl: lac: Tn10 | Laboratory collection |

* Original strain designations are given in parentheses.

† Unselected markers are shown in parentheses. Resistance to tetracycline (TetR), kanamycin (KanR), or chloramphenicol (CamR) was scored on LB medium containing 15 or 20 µg/ml antibiotics. The udk-2 allele confers resistance to 5-fluorouridine at 10 µg/ml on glucose minimal plates supplemented with 20 µg/ml uracil (FURR) phenotype. The cdd-2 allele was selected by its ability to allow utilization of deoxyctydine as a pyrimidine source (CDR phenotype). The deoD mutation prevents utilization of 1 mg/ml inosine as a carbon source (HxR phenotype). The pdxR mutation gives rise to a requirement for 1 µg/ml pyridoxal hydrochloride in minimal medium (PdxR phenotype). The adeD mutation was found to confer sensitivity to adenine at 30 µg/ml on glucose minimal plates supplemented with 1 mM adenine and 40 µg/ml histidine (ARR phenotype).

‡ The presence of the mutant rih alleles was verified by PCR analysis.

§ The adeD mutation in this strain arose spontaneously and was not recognized in the original description.

**Bam**HI primers and inserted between the EcoRI and BamHI sites of the medium-copy vector pBR322 (19).

For construction of low-copy rih lacZ gene fusions, the promoters and N-terminal coding regions of rihA, rihB, and rihC (nucleotides 9481–9933, 8254–8485, and 6444–6677, respectively) were PCR-amplified from genomic DNA with the rih EcoRI + rih HindIII, rihB EcoRI + rihB HindIII, and rihC EcoRI + rihC HindIII primer pairs, respectively. These PCR products were cloned into the low-copy lacZ vector pCN2423 between a unique EcoRI site upstream of the lac promoter and a unique HindIII site at codon 5 of the lacZ gene. Plasmid pCN2423 has the same overall structure as the previously described medium-copy vector pCPN5 (20), except that it contains the pSC101 replicon derived from pLG339 (21). High-copy gene fusions of the rih genes to the lacZ gene were constructed by subcloning of the promoter containing fragments from the low-copy plasmids between the unique EcoRI and HindIII sites of the high-copy vector pUC8 (22).

Assays of Nucleoside Hydrolase Activities—Unless otherwise noted, cells were grown in glycerol minimal medium, with uracil as a pyrimidine source when required. 30 ml of bacterial culture was harvested on ice at A660 = 0.5. Cells were collected by centrifugation, washed, and resuspended in 50 ml Tris-HCl (pH 7.5) to A660 = 20. Cells were disrupted by sonic treatment, and the extract was cleared by centrifugation at 20,000 × g for 3 min in a refrigerated microcentrifuge. Assays were performed at 37 °C by mixing appropriately diluted extract with 1C-labeled nucleoside substrate at a final concentration of 1 mM in a total volume of 75 µl of 40 mM Tris-HCl (pH 7.5). At time intervals, 15-µl samples were taken out, boiled for 2 min, and cooled on ice. After a 5-min centrifugation at 20,000 × g, 5 µl of supernatant was applied to a thin-layer chromatography plate for separation of substrate and reaction products, which were subsequently quantitated by counting in an Instant Imager (Packard Instrument Co.). The enzymatic activities were calculated from the initial slope of a plot of the amount of radioactivity remaining as a function of time. The reaction products were separated from the substrate by chromatography on polyethyleneimine-cellulose plates developed in water. The 1C-labeled nucleosides were from several different suppliers; similar products can be obtained from Moravek Biochemicals Inc.

Assays of CMP Hydrolase Activity—Assays of CMP hydrolase activity were performed as described for the nucleoside hydrolase assays, except that [U-14C]CMP was used as substrate, and reaction products were separated from the substrate by chromatography on polyethyleneimine-cellulose plates developed in 1 M acetic acid to 2 cm above the origin and then in 1 M acetic acid and 3 M LiCl (9:1) to 15 cm above the origin. Measurements of β-Galactosidase Synthesis—Differential rates of β-galactosidase synthesis were measured at 37 °C as described previously (20), except that induction with isopropyl-β-D-thiogalactopyranoside (IPTG) was performed at a concentration of 1 mM and for 16 hours at 37 °C.
TABLE II  
DNA oligonucleotides used for PCR amplifications or primer extension analyses

| Name          | Sequence (5’→3’)* |
|---------------|-------------------|
| 1224 cam-down | CGCCAGGGTTTCCCCAGTCAGAC |
| IS50-EcoRIb   | cggaaacctGTTACATGTTAGGAGTCAC |
| rihA-EcoRI    | cggaaacctGTATGGATCCTATTGGGATGCA |
| rihB-HindIII  | cggaaacctGAAGATTGGGATTACTGTCACAGCT |
| rihB-EcoRI    | cggaaacctTCATCATGACCCGGATCACAATC |
| rihB-BamHI    | cggaaacctGTTGTTGCAGAATGGTGGCACGTTTGCAGC |
| rihC-BamHI    | cggaaacctCAGAACACTTCTCATGACATCC |
| rihC-EcoRI    | cggaaacctTCGTCAATGCCGGGGTCAGTATC |

* Nucleotides complementary to their genomic target sequences are shown in uppercase letters, whereas sequence tags containing restriction sites for subsequent cloning of PCR products are in lowercase letters.

This primer was used together with rihB-HindIII for specific detection of the IS50 insertion upstream of rihB.

![Fig. 2. Genetic organization of the rihA (a), rihB (b), and rihC (c) regions at 15, 48, and 0.6 min of the genomic map, respectively. The maps are based on the annotation of the E. coli genome sequence (18). Horizontal lines indicate the extent of regions cloned in plasmids. The promoters of the rih genes are symbolized by black arrowheads.](http://www.jbc.org/)

RESULTS

Isolation of rihA:cam Mutants Deficient in Cytidine Salvage—With the aim of identifying the putative CMP hydrolase gene of E. coli, we subjected a pyrF cdd mutant strain, CN1930 (Table I), to transposon mutagenesis with mini-Tn10 cam (16). Following penicillin enrichment, we obtained, by replica plat-
mulated as the major reaction product in extracts of pRihA transformants, even if they were CodA⁻ (data not shown), presumably because cytosine deaminase could not keep pace with the increased cytidine hydrolase activity. Unlike cytosine deaminase, the RihA cytidine hydrolase appeared to be strictly specific for ribonucleosides. Even the pRihA transformant with 40-fold increased cytidine hydrolase activity showed no enzymatic activity with deoxycytidine as substrate, and it was completely unable to grow with deoxycytidine as a pyrimidine source.

Role of Cytidine/URidine Kinase in the Salvage of Cytidine by a cdd Mutant—The genetic evidence for the existence of a CMP hydrolase rested on the finding that growth of pyr cdd strains on cytidine was prevented by mutational inactivation of the udk gene (7). This was very puzzling since our results indicated that cytidine was converted directly to cytosine by the RihA hydrolase. To reinvestigate this problem, we introduced a cdd mutant into the pyrF cdd strains CN1930 and CN2269, the latter being an uracil kinase mutant derivative of CN2258 (Fig. 5 c), in agreement with the previous study (7). However, the negative effect of both the udk and rihA mutations was more than eliminated by introduction of pRihA, to the extent that cytidine salvage was no longer growth-limiting. Thus, cytidine/uridine kinase per se was not required for salvage of cytidine in a cdd mutant background, provided the RihA activity was sufficiently high. It should also be noted that even the parental strain (CN1930) was substantially growth-limited by the rate of cytidine salvage, unless transformed with pRihA.

The negative effect of the udk mutation on cytidine salvage in the pyrF cdd background may be rationalized by considering that there are two different pathways for salvage of cytidine in a pyr cdd mutant. The cytidine hydrolase pathway only needs to satisfy the requirement for uracil nucleotides, whereas cytosine nucleotides can be synthesized directly from cytidine via the uracil-kinase reaction (Fig. 1). In the udk mutant, however, the entire cellular pyrimidine requirement would have to be channeled via the cytidine hydrolase reaction, which was already limiting for the supply of uracil nucleotides in the parental strain (CN1930).

With glycerol as a carbon source, on the other hand, the endogenous RihA activity was not limiting for growth on cytidine either for CN1930 or for the udk derivative CN2269 (Fig. 5 b). The rihA mutant, however, was still severely compromised for growth on cytidine. These results emphasized that cytidine/uridine kinase was not necessary for salvage of cytidine in a pyr cdd mutant and further suggested that rihA might be derepressed during growth on glycerol. Accordingly, CN1930 and CN2269 contained 3-fold higher cytidine hydrolase activity when cells were grown on glycerol rather than on glucose (Fig.
was further aggravated by the rihA disruption, but was completely eliminated by introduction of pRihA. In glycerol medium, CN2389 was not limited by the rate of uridine hydrolysis, presumably because of the de-repression of the rihA gene (Fig. 5c). In contrast, the rihA derivative CN2390 remained pyrimidine-restricted, although the residual uridine hydrolyase activity in this strain (Fig. 6a) allowed for a fairly high growth rate compared with glucose + uridine medium (Fig. 6b). These results suggested glucose-mediated repression of the residual activity, which can probably be ascribed to the RihC nucleoside hydrolase described below.

Selection of Mutants with an Activated rihB Gene and Isolation of rihB::cam Disruption Mutants—Mutants of CN1930 with increased cytidine hydrolyase activity were readily obtained by selection for rapid growth in glucose minimal medium with cytidine as a pyrimidine source. One group of mutants were found to contain an amplification of the rihA region and were not characterized further. Another group of three independent mutants, however, seemed to contain a mutation that activated another cytidine hydrolyase gene since growth of these mutants on cytidine was not eliminated by introduction of the rihA::cam disruption. The total cytidine hydrolyase activity in one of these mutants (CN2264) was increased 3-fold, which allowed for nearly unrestricted growth on glucose + cytidine (at 80% of the growth rate with cytosine as a pyrimidine source).

The mutation responsible for the increased cytidine hydrolyase activity in CN2264 was coarsely mapped by conventional methods to be near the cdd gene at 48 min of the genetic map (data not shown). To identify the particular gene affected by the mutation, we subjected the 48 min region of the genome in CN2264 to random insertion mutagenesis with the mini-Tn10 cam transposon and isolated three mutants that had lost the ability to grow rapidly on cytidine. All three strains were found to have a cam insertion at either of two different positions within the yeiK gene, which we have renamed rihB (see “Experimental Procedures”). Since rihB was one of two genes that showed strong homology to rihA in a BLAST search of the E. coli genome, we inferred that the increased cytidine hydrolyase activity of the original up-mutant (CN2264) was caused by activation of the rihB locus.

The mutation responsible for activation of the rihB gene in CN2264 was discovered fortuitously during cloning of the cam insert from one of the rihB::cam disruption mutants (CN2324). The restriction pattern of the resulting plasmid deviated from what we expected from the genomic DNA sequence, and DNA sequencing revealed that it was because an IS50 insertion had integrated 23 base pairs upstream of the rihB gene with the transposase gene in the opposite orientation of the rihB gene with the transposase gene in the opposite orientation of rihB. Subsequent PCR analyses revealed that this IS50 insertion was present in the original up-mutant (CN2264) as well as in the two other independent mutants selected for rapid growth on cytidine. In contrast, no insertion was present in the parental strain (CN1930). These findings strongly indicated that the IS50 element activated the rihB gene in the fast-growing mutants, presumably because IS50 contains an outwardly directed weak constitutive promoter (25).

Isolation of a rihC Mutant and a rihA rihB rihC Triple Mutant—Homology searches revealed that rihA and rihB are highly homologous to a third gene, rihC (previously called yoaF), located at 0.6 min. To study the function of this putative nucleoside hydrolase gene, we isolated a rihC::cam mutant, CN2403 (Table I), by PCR screening of a collection of mutants with cam insertions in the vicinity of the carAB operon at 0.6 min. As the rihC::cam mutation was isolated in a prototrophic background, it had no detectable phenotypic consequences.

5c). The udk mutation by itself, however, had no significant effect on the cellular cytidine hydrolase activity either in glycerol or glucose medium. Thus, the poor growth of the udk mutant on glucose + cytidine (Fig. 5a) was not caused by a lower activity of cytidine hydrolase compared with the parental strain, but rather by a greater demand for the reaction.

Uridine Hydrolase Activity of RihA—We suspected that the RihA protein, if endowed with uridine hydrolase activity, might constitute the unknown pathway of uridine salvage in udk udk double mutants. To test this possibility, we introduced the rihA::cam disruption into a pyrimidine-requiring udk udk mutant strain (CN2389) to create the udk udk rihA triple mutant (CN2390) (Table I). As shown in Fig. 6a, the cellular uridine hydrolase activity was more than halved by the rihA disruption in CN2390, whereas introduction of the pRihA plasmid caused a 40-fold increase in activity compared with the RihA+ strain CN2389. These results showed that RihA is a uridine hydrolase, and comparison with the data in Fig. 5b indicated that the enzyme was approximately equally efficient with uridine and cytidine as substrates.

Growth experiments confirmed that the uridine hydrolase activity of RihA is a major contributor to the pathway of uridine salvage operating in udk udk mutants (Fig. 6b). In glucose medium, uridine salvage by CN2389 was clearly limited by the endogenous uridine hydrolase activity, which, in this case, had to satisfy the entire pyrimidine requirement. This limitation...
To facilitate studies of the individual nucleoside hydrolases, the disrupted rihA, rihB, and rihC alleles were combined in a prototrophic strain, CN2573 (Table I), which carried additional mutations in the deoD, add, udp, cdd, and udk genes (see Fig. 1). Thus, crude extracts of CN2573 were essentially devoid of nucleoside-metabolizing enzymatic activities, which might interfere with assays of the individual nucleoside hydrolases produced from recombinant plasmids. Despite the nearly complete elimination of nucleoside catabolism in CN2573, it was fully viable and grew with a normal generation of 70 min in glycerol minimal medium. Thus, none of the nucleoside hydrolase genes were essential, either alone or in combination.

**Determination of the Substrate Specificity of the Three Nucleoside Hydrolases**—To determine the substrate specificity of the three nucleoside hydrolases, we transformed the multiple mutant CN2573 (Table I) with recombinant plasmids pRihA, pRihB, and pRihC, containing the corresponding structural genes (see “Experimental Procedures”). Measurements of hydrolytic activities with different nucleoside substrates in extracts of the transformed strains revealed that both the RihA and RihB enzymes were essentially pyrimidine-specific (Fig. 7). The RihB enzyme, however, did have a clear preference for cytidine over uridine, whereas RihA was equally efficient with either substrate, in agreement with the previous results (Figs. 3 and 6a). The RihC enzyme, on the other hand, was characterized by a remarkably broad substrate specificity for both purine and pyrimidine ribonucleosides, with decreasing activity in the order uridine > xanthosine > inosine > adenosine > cytidine > guanosine (Fig. 7). Like RihA, the RihB and RihC enzymes were specific for ribonucleosides, as the transformed strains showed no detectable activity with deoxycytidine or deoxyadenosine (Fig. 7).

**Expression and Physiological Capacity of the RihB and RihC Activities**—Plasmids pRihA and pRihC are medium-copy plasmids based on the replicon from pBR322, whereas pRihB is a high-copy number plasmid derived from the pGEM3 vector. When cloned into a medium-copy vector, the rihB gene gave rise to a much lower cytidine hydrolase activity (2.6 $\mu$mol/min/g [dry weight] in glycerol medium), approximating the activity expressed from the chromosomal rihA gene in CN1930 (Fig. 3b). This result suggested that the chromosomal RihB activity was 40-fold lower than the endogenous RihA activity based on the approximate magnitude of the gene dosage effect obtained by cloning in a medium-copy vector (Figs. 3 and 6a). Taking the gene dosage effect into account, the endogenous RihC activity in glycerol medium could be estimated from the data in Fig. 7 to be on the order of 1.3 and 0.3 $\mu$mol/min/g (dry weight) with uridine and cytidine, respectively, i.e. at least 5-fold higher than the corresponding RihB activities. Thus, the low residual hydrolytic activities for cytidine and uridine in the rihA mutants CN2258 and CN2390 (Figs. 3 and 6a) could probably be accounted for by the endogenous RihC activity.

The physiological capacity of the RihC enzyme was investigated specifically by measuring the efficiency of inosine salvage in a purine-requiring strain, CN1980 (Table I), in which the two other pathways of inosine metabolism had been blocked by mutations in the deoD and gsk genes (see Fig. 1). In glycerol medium, growth of CN1980 with inosine as a purine source was severely limited by the endogenous RihC activity (Fig. 8). This growth limitation was further aggravated by disruption of the rihC gene in CN2545, but was completely eliminated by introduction of pRihC. In glucose medium, on the other hand, both CN1980 and its rihC derivative were essentially unable to grow with inosine as a purine source, and even the pRihC transformant was markedly growth-limited by the rate of inosine hydrolysis. These results indicated that rihC was repressed in glucose medium. Moreover, they demonstrated that the endogenous RihC activity was far from sufficient to satisfy the cellular purine requirement by hydrolysis of inosine, even in glycerol medium.

**Regulation of rihA and rihC by Catabolite Repression**—The repression of rihA and rihC expression in glucose medium suggested that they might be subject to catabolite repression (reviewed in Ref. 26). This possibility was investigated by the use of gene fusions of the rihA, rihB, and rihC genes to lacZ. As shown in Fig. 9, expression of both the rihA-lacZ and rihC-lacZ fusions was stimulated 3-fold by cAMP in glucose medium, albeit not quite to the level of expression seen in glycerol minimal medium. In contrast, the rihB-lacZ fusion was stimulated <20% by cAMP, indicating that the low expression of this construct in glucose medium was not caused by catabolite repression. Furthermore, the poor expression in general of the rihB-lacZ fusion supported previous indications that the low endogenous RihB activity was caused by poor expression of the rihB gene, rather than a low intrinsic activity of the enzyme.

Many genes involved in nucleoside catabolism in *E. coli*, such as *cdd* and *udp* and the *deo* operon, form a regulon that is induced by cytidine via the CytR repressor and the cAMP
Fig. 8. Efficiency of inosine salvage via the rihC reaction in a deoD gsh double mutant. The bacterial growth rate with inosine (HxR) as a purine source relative to the growth rate with hypoxanthine (Hx) is taken as an indicator of the efficiency of inosine hydrolysis to hypoxanthine. Gray bars, glucose medium; black bars, glycerol minimal medium.

DISCUSSION

Enzymology of Nucleoside Hydrolases—Nucleoside hydrolases catalyzing the irreversible hydrolysis of nucleosides to ribose and the free nucleobase are widespread in nature and have been identified in bacteria, fungi, protozoan parasites, fish, and plants, but so far not in mammals (30, 31). In most organisms, however, the physiological functions of the nucleoside hydrolases are unknown. A notable exception is the protozoan parasites, which rely on nucleoside hydrolases for salvage of purine nucleosides from the host organism, as they are unable to synthesize purines de novo (32, 33).

Based on their substrate specificity, the nucleoside hydrolases characterized so far may be divided into four different classes (30). One major class consists of relatively nonspecific hydrolases acting on both purine and pyrimidine nucleobases. A prominent member of this class is the Crithidia fasciculata inosine/uridine-prefering nucleoside hydrolase (Swiss-Prot accession number O27546), which has been extensively characterized structurally and mechanistically (34–36). The second class consists of enzymes that are strictly specific for purine nucleosides, as exemplified by the inosine/adenosine/ guanosine-prefering nucleoside hydrolase from Trypanosoma brucei (GenBank™/EBI accession number AF017231) (37). The third class consists of the uridine hydrolase encoded by the URH1 gene of Saccharomyces cerevisiae (Swiss-Prot accession number Q04179), an enzyme that is specific for uridine and ribothymidine, but shows no activity for cytidine and purine ribonucleosides (38). Finally, the fourth class is defined by a purine deoxyribonucleoside-specific hydrolase from Leishmania donovani (39). No structural or genetic information is available for this enzyme.

The RihC enzyme clearly belongs to the class of nonspecific hydrolases, whereas the RihA and RihB enzymes, being active with both cytidine and uridine, represent a new type of substrate specificity. Functionally, these enzymes are most naturally grouped in the third class with the pyrimidine-specific uridine hydrolase of S. cerevisiae, although this enzyme is inactive with cytidine. Structurally, however, the pyrimidine-specific RihA and RihB enzymes are much more homologous to the nonspecific enzymes RihC and inosine/uridine-prefering nucleoside hydrolase of C. fasciculata (Fig. 11). The greatest homology was found between RihA and RihC (homology score of 44), but all other pairwise homology scores within the group of the inosine/uridine-prefering nucleoside hydrolase and the three E. coli enzymes fell in the range of 35–38, whereas alignments of each of these proteins with Urh1 from yeast gave rise to homology scores only in the range of 25–28. On the other hand, the three E. coli enzymes and the inosine/uridine-prefering nucleoside hydrolase are more homologous to the Urh1 enzyme than they are to the strictly purine-specific inosine/ adenosine/guanosine-prefering nucleoside hydrolase (Fig. 11), which obtained homology scores of only 12–22 in pairwise alignments with each of the five other enzymes.

Thus, from a structural point of view, the pyrimidine-specific hydrolases appear to group with the nonspecific hydrolases; their inability to hydrolyze purine nucleosides may simply derive from a small number of amino acid residues that sterically restrict access of purine nucleosides to the active site. In line with this view, we found that the RihB enzyme did have a low but significant activity for the purine nucleoside xanthosine (Fig. 7). Furthermore, all three E. coli hydrolases were inactive with deoxyribonucleosides as substrates, in analogy with the inosine/uridine-prefering nucleoside hydrolase, which forms specific hydrogen bonds with the 2'-hydroxyl of the ribose ring from the conserved residues Asp-14, Asn-39, and Asp-242 in the active site (36) (see Fig. 11). The protozoan hydrolases are not active with nucleoside 5'-phosphates as substrate (35); and given the similarity to the inosine/uridine-prefering nucleoside hydrolase, it is hardly surprising that the three E. coli enzymes had no detectable CMP hydrolase activity.

Role of the Putative CMP Hydrolase Pathway in Cytidine Salvage—The present results raise some serious doubts about the involvement of the CMP hydrolase activity in conversion of cytidine to uracil nucleotides in cdd mutants. In glycerol medium, the putative CMP hydrolase pathway was fully dispensable for growth on cytidine, which was efficiently metabolized via the RihA reaction in the cdd udk mutant CN2269 (Fig. 5b). The partial requirement for cytidine/uridine kinase in glucose...
medium (Fig. 5a), on the other hand, might simply be explained by its ability to provide an independent pathway for synthesis of cytosine nucleotides when flux through the RihA reaction was limited by catabolite repression. There is no genetic evidence that any of the cytidine channeled via the $udk$ reaction in glucose medium was converted to uracil nucleotides via the CMP hydrolase reaction. In glycerol medium, very little cytidine appeared to be converted to cytosine via this reaction, as shown by the poor growth of the $rihA$ mutant on glycerol + cytidine (Fig. 5b).

Physiological Function and Regulation of the Ribonucleoside Hydrolases in E. coli—The physiological role of the three ribonucleoside hydrolases in wild-type E. coli strains is enigmatic, as their activities (except for the hydrolysis of cytidine) are paralleled by the reversible nucleoside phosphorylases (Fig. 1). A similar redundancy exists for the 3'-exonucleolytic RNases, where the hydrolytic activities of RNases II and D are paralleled by the phosphorolysing enzymes polynucleotide phosphorylase and RNase PH, respectively (reviewed in Ref. 41). Unfortunately, the physiological rationale for the redundancy of
The identification of the tamate and a homolog of the DnaK chaperone, respectively, that encode a transport system for aspartate and glybeW flanked by the gltJKL that are ligands of a catalytic Ca\(^{2+}\) (35, 36), the residues that have been found to form hydrogen bonds with the ribose hydroxyls of the substrate are indicated (*), as are the residues IAG-NH this alignment. To our knowledge, the N terminus of the Urh1 protein has not been determined experimentally.

nucleobase leaving group (34). According to the sequence annotation, the Urh1 protein contains an N-terminal extension that was not included in

nucleoside-preferring nucleoside hydrolase.

environment.

some nucleoside that is only occasionally present in the

gives no clues to their physiological function. The

rihB activated coordinately with

indeed a nucleoside transporter and that the

transporter NupC. Preliminary results indicate that YeiJ is

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for increased cytidine hydrolase activity suggested that these

genes are not controlled by simple repressor proteins. So far,

for increased studies on the regulation of these genes may

appear to identify gene-specific regulatory loci for the

RNases is no less elusive than it is for the nucleoside-
catabolizing enzymes.

The finding that both rihA and rihC were subject to catabo-
lite repression might suggest a role for these genes in the provision of ribose for utilization as a carbon source. However, their low levels of expression were far from sufficient to allow utilization of nucleosides as carbon sources in strains lacking the nucleoside phosphorylase activities (data not shown). The low capacity of the nucleoside hydrolase reactions might hint that the true natural substrates of these enzymes are not the common ribonucleosides, but rather some low-level nucleoside analogs, such as modified nucleosides derived from turnover of tRNA and rRNA. Alternatively, the nucleoside hydrolases might be sufficiently induced to function in bulk metabolism of the common nucleosides under certain physiological conditions. However, we found no evidence for induction of these genes by their ordinary nucleoside substrates, so the hypothetical inducing conditions are presently unknown.

It is noteworthy that the silent rihB gene is located immedi-
ately upstream of an open reading frame, yeiJ (Fig. 2b), which encodes a protein with strong homology to the nucleoside transporter NupC. Preliminary results indicate that YeiJ is indeed a nucleoside transporter and that the yeiJ gene is activated coordinately with rihB by the IS50 insertion upstream of the rihB gene. Thus, it is likely that these genes constitute an inducible operon devoted to the transport and catabolism of some nucleoside that is only occasionally present in the environment.

Apart from this, the genetic organization of the rih genes gives no clues to their physiological function. The rihA gene is flanked by the gltsJKL operon and an open reading frame (ybeW) that encode a transport system for aspartate and glutamate and a homolog of the DnaK chaperone, respectively (Fig. 2a). The identification of the rihA promoter suggested that expression of this gene is independent of the upstream gltsJKL cluster, but it is presently unclear if ybeW might be co-transcribed with rihA. The rihC gene apparently constitutes a monocistronic operon (Fig. 2c). The downstream dapsB gene contains a promoter of its own (42), and our identification of the rihC promoter corroborated previous studies that indicated that rihC is not co-transcribed with the upstream genes of the ileS gene cluster (43).

The preferential isolation of mutants with amplifications of the rihA gene or IS50 insertions upstream of rihB in selections for increased cytidine hydrolase activity suggested that these genes are not controlled by simple repressor proteins. So far, attempts to identify gene-specific regulatory loci for the rih genes by genetic selection have been unsuccessful, but we expect that further studies on the regulation of these genes may eventually reveal their true physiological function.

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