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Molecular Identification of Pseudouridine-metabolizing Enzymes*

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Pseudouridine, a non-classical nucleoside present in human urine as a degradation product of RNAs, is one of the few molecules that has a glycosidic C-C bond. Through a data base mining approach involving transcriptomic data, we have molecularly identified two enzymes that are involved in the metabolism of pseudouridine in uropathogenic *Escherichia coli*, the principal agent of urinary tract infections in humans. The first enzyme, coded by the gene *yeiC*, specifically phosphorylates pseudouridine to pseudouridine 5′-phosphate. Accordingly, *yeiC*$^-$ mutants are unable to metabolize pseudouridine, in contrast to wild-type *E. coli* UTI89. The second enzyme, encoded by the gene *yeiN* belonging to the same operon as *yeiC*, catalyzes the conversion of pseudouridine 5′-phosphate to uracil and ribose 5-phosphate in a divalent cation-dependent manner. Remarkably, the glycosidic C-C bond of pseudouridine is cleaved in the course of this reaction, indicating that YeiN is the first molecularly identified enzyme able to hydrolyze a glycosidic C-C bond. Though this reaction is easily reversible, the association of YeiN with pseudouridine kinase indicates that it serves physiologically to metabolize pseudouridine 5′-phosphate rather than to form it. YeiN is homologous to *Thermotoga maritima* IndA, a protein with a new fold, which we now show to act also as a pseudouridine-5′-phosphate glycosidase. Data base mining indicates that most eukaryotes possess homologues of pseudouridine kinase and pseudouridine-5′-phosphate glycosidase and that these are most often associated in a single bifunctional protein. The gene encoding this bifunctional protein is absent from the genomes of man and other mammals, indicating that the capacity for metabolizing pseudouridine has been lost late in evolution.

Pseudouridine is a nucleoside that is formed post-transcriptionally in RNAs such as tRNAs, ribosomal RNAs, and small RNAs, where it represents one of the main non-classical nucleosides (1). Mammalian ribosomal RNA contains about ≈100 pseudouridines per ribosome, and tRNAs contain an average of 3–4 pseudouridines. The binding of uracil to ribose through C5 rather than through N1 allows the presence of an additional N-bound hydrogen, which by immobilizing a water molecule stabilizes secondary structures and reinforces base stacking. Pseudouridine is formed post-transcriptionally by pseudouridine synthases, which act on maturing tRNAs or rRNAs by severing the normal glycosidic C-N bond, flipping the uracil moiety along its N3-C6 axis and forming a glycosidic C-C bond with C5 without any additional input of energy.

Pseudouridine is one of the few examples of molecules that have a glycosidic C-C bond. Other examples are the C-mannosyl residues bound to tryptophan (2), the C-glucosides bound to catechol derivatives (C-glucosylated form of microcin E492, Ref. 3), and some nucleoside analogues (formycin A, Ref. 4; pyrazofurin, Ref. 5). No enzyme that hydrolyzes a glycosidic C-C bond has yet been molecularly identified.

Pseudouridine can be used by *Escherichia coli* as a source of uracil for a strain deficient in pyrimidine synthesis (uridine auxotroph) (6). Pseudouridine was shown to be phosphorylated in *E. coli* extracts by a kinase to pseudouridine 5′-phosphate (PsiMP)[3] (7), which is hydrolyzed to ribose 5-phosphate and uracil (8) (Scheme 1). Neither of these enzymes has been identified, despite the interest that may represent the identification of an enzyme that cleaves a C-C bond. Pseudouridine metabolism is likely to be restricted to only some species. The finding that this nucleoside is present in human urine, where its concentration is increased in tumor-bearing patients (9, 10), suggests that pseudouridine is not metabolized in man.

By exploiting available data base information, we have been able to identify the two genes that encode pseudouridine kinase and pseudouridine-5′-phosphate glycosidase. The goal of this work is to report these findings.

EXPERIMENTAL PROCEDURES

Materials—Reagents, of analytical grade whenever possible, were from Sigma, Acros (Geel, Belgium), Roche Applied Sciences (Mannheim, Germany), PerkinElmer Life Sciences (Coignieres, France), or Merck (Darmstadt, Germany). Q-Sepharose, HisTrap, HisSpinTrap, Nap-5, and PD-10 column were purchased from GE Healthcare (Diegem, Belgium). Pseudouridine was from Berry and Associates (Dexter, MI).

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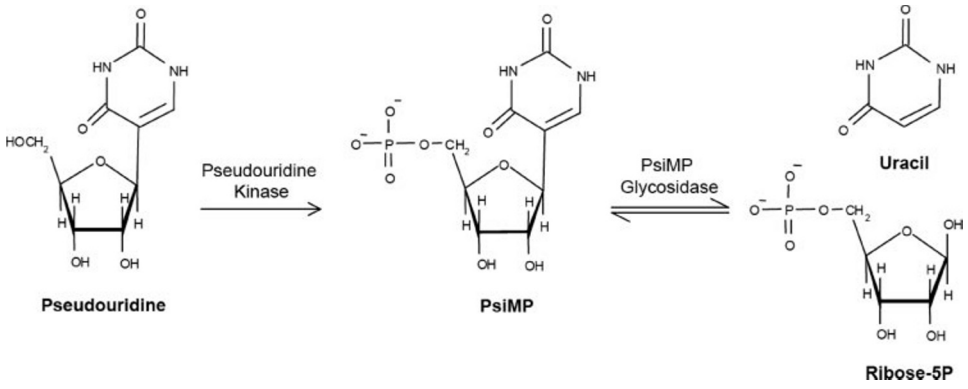
[3] The abbreviations used are: PsiMP, pseudouridine 5′-phosphate; LB, Luria-Bertani; ppm, parts per million; PCA, perchloric acid.



SCHEME 1. **Reactions catalyzed by pseudouridine kinase and pseudouridine-5′-phosphate glycosidase.**

Vivaspin-15 centrifugal concentrators were from Vivascience (Göttingen, Germany). *Thermotoga maritima* genomic DNA was from American Type Culture Collection (Manassas, VA). Dowex 1-X8 (100–200 mesh) was purchased from Acros. Enzymes and restriction enzymes were purchased from Sigma, Roche Applied Sciences, or Fermentas (St Leon-Rot, Germany). D-[1-^{14}C]Ribose was from American Radiolabeled Chemicals (St Louis, MO). Sephadex G-10 was from Pharmacia Fine Chemicals (Uppsala, Sweden).

Expression and Purification of YeiC—A 5′-primer containing the putative ATG codon (AAAT**CATATG**CGCGAAAAGGATTATGTCGTAATTATAGG) in a NdeI site (in bold) and a 3′ primer containing the putative stop codon (AA**GGATCC**TTAATTCAGACATTCTGCGTTCTCCACTAACG) flanked by a BamHI site were used to PCR-amplify genomic DNA from *E. coli* K12 with Pfu polymerase. A ~950-bp product was obtained. This fragment was restricted with NdeI and BamHI and ligated in pET-15b. This vector was used to transform *E. coli* BL21 and checked by sequencing. The resulting bacteria were grown in Luria-Bertani (LB) medium containing 100 mg/liter ampicillin. The culture was grown at 37 °C until A_{600} reached 0.5–0.6. It was then cooled on ice for 20 min, and the inducer isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.4 mM. After overnight incubation at 37 °C, the cells were collected by centrifugation, resuspended in buffer A (20 mM Hepes pH 7.1, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml antipain, 1 mg/ml lysozyme), and submitted to three cycles of freezing and thawing. The bacterial extract was incubated on ice for 1 h with 0.5 mg/ml DNaseI in the presence of 10 mM $MgSO_4$, and centrifuged for 30 min at 10,000 × *g*. The resulting supernatant was recentrifuged for 30 min at 15,000 × *g*, filtered on a 0.22-μm filter, diluted 2-fold with buffer B (25 mM Hepes pH 7.1, 5 μg/ml leupeptin, 5 μg/ml antipain, 300 mM NaCl, and 20 mM imidazole), and loaded on a HisSpinTrap column. The column was washed twice with 600 μl of binding buffer, and the His-tagged protein was eluted with 400 μl of buffer C (25 mM Hepes pH 7.1, 5 μg/ml leupeptin, 5 μg/ml antipain, 300 mM NaCl, and 500 mM imidazole). The purified protein was then analyzed by SDS/PAGE, desalted on a Nap5 column equilibrated with buffer D (25 mM Hepes pH 7.1, 5 μg/ml leupeptin, 5 μg/ml antipain, 25 mM KCl), supplemented with 10% (w/v) glycerol, and stored at −70 °C.

Expression and Purification of YeiN—The preparation of the expression vector was performed as described above. The PCR primers were: AAAT**CATATG**TCTGAATTAAAAATTTCCCCTGAATTATTAC and AAGGATCCTTAACCCGCGAGACGCTGATATTC. This vector was used to transform *E. coli* BL21 and checked by sequencing. Expression and preparation of the extract were carried out as described for YeiC. The His-tagged protein was purified on HisTrap HP column (1 ml), as previously described (11). The fraction containing YeiN, as determined by SDS-PAGE analysis, was concentrated (using Vivaspin-15), desalted on a PD-10 column equilibrated with buffer D supplemented with 10% glycerol, and stored at −70 °C.

Expression and Purification of IndA from T. maritima—A 5′-primer containing an NdeI site (in bold) flanked by a sequence coding for a purification tag (MGSDKIHHHHHH, underlined) and the putative ATG codon (in italics) (GACCGC**CAT*ATG***GGCAGCGATAAAATCCATCATCATCATCATCACATG</u>ATCATAGAAAGCAGGATAGAAA) and a 3′ primer containing the putative stop codon (GACCGC**GGATCC**TCACGAGCGTTTGAGTTTTACC) flanked by a BamHI site were used to PCR-amplify genomic DNA from *T. maritima* with Pwo polymerase. A ~900-bp product was obtained. This fragment was restricted with NdeI and BamHI, and ligated in pET-3a. This vector was used to transform BL21 and checked by sequencing. Expression, preparation of the extract, and purification were performed as described for YeiC, except that the expression was carried out at 18 °C.

Measurement of Enzymatic Activities—Activities were assayed spectrophotometrically (340 nm) at 30 °C. Unless otherwise stated, the assay medium (reaction mixture of 600 μl) contained 25 mM Hepes pH 7.1, 25 mM KCl, 1 mM $MgCl_2$, and 1 mM dithiothreitol.

The pseudouridine kinase activity of *E. coli* YeiC was assayed through the production of ADP with a pyruvate kinase/lactate dehydrogenase-coupled assay. The assay buffer contained 0.5 mM ATP-Mg, 0.25 mM phosphoenolpyruvate, 0.15 mM NADH and different nucleoside concentrations, as well as 10 μg of rabbit muscle pyruvate kinase and 10 μg of rabbit muscle lactate dehydrogenase.

The pseudouridine-5′-phosphate glycosidase activity of *E. coli* YeiN was assayed through the production of ribose 5-phosphate with a ribose-5-phosphate isomerase/ribulose-5-phosphate reductase-coupled assay. The assay buffer contained 50 μM CTP-Mg, 0.15 mM NADPH, 1.7 units/ml ribose-5-phosphate isomerase from spinach, 0.3 units/ml *Haemophilus influenzae* ribulose-5-phosphate reductase (12), and the indicated concentrations of metal ions and nucleotide 5′-monophosphates.

The pseudouridine-5′-phosphate glycosidase activity of *T. maritima* IndA was measured in a two-step assay. The enzyme (7.5 μg/ml) was first incubated for 0, 5, or 10 min at

different temperatures (50–90 °C) in an assay medium containing 25 mM Hepes pH 7.1, 0.5 mM $CoCl_2$, and 1 mM PsiMP (final volume: 400 μl). The tubes were transferred on ice to stop the reaction and the formed ribose 5-phosphate was measured spectrophotometrically with spinach ribose 5-phosphate isomerase (1.7 units/ml) and *H. influenzae* ribulose 5-phosphate reductase (0.3 units/ml), in the presence of 50 μM CTP-Mg, 0.15 mM NADPH, and 1 mM $MgCl_2$ (final volume: 600 μl).

Reversibility of the Reaction Catalyzed by YeiN—HPLC assays were used to study the forward and backward reactions of YeiN. The reactions were assayed at 30 °C, in a medium containing 25 mM Hepes pH 7.1, 0.5 mM $CoCl_2$ and 50 μg/ml YeiN, as well as 0.5 mM PsiMP (forward reaction) or 1 mM uracil and 0–1 mM ribose 5-phosphate (backward reaction). The reaction was stopped after 0–120 min by mixing a portion of the incubation medium with 0.25 vol. of cold 10% (w/v) perchloric acid. After neutralization, the samples (50 μl) were analyzed by chromatography on a Partisphere Sax column (4.6 × 125 mm; from Whatman) using a gradient of 0.01–0.5 M $NH_4H_2PO_4$ (pH 3.7) at a flow rate of 2 ml/min. A_{254} was measured, the reference wavelength being 350 nm. Concentrations were calculated by comparing the integrated peak surfaces with those obtained with titrated solutions of PsiMP and uracil.

Synthesis of [^{14}C]Pseudouridine—For the synthesis of [^{14}C]ribose 5-phosphate, 5 μCi of D-[1-^{14}C]ribose was incubated for 1 h at 30 °C with 5 μg (25 milliunits) of ribokinase in a solution (final volume: 100 μl) containing 25 mM Hepes pH 7.1, 25 mM KCl, 5 mM $MgCl_2$, 10 μM ribose, and 1 mM ATP-Mg. The incubation was stopped by the addition of 25 μl of ice-cold 10% (w/v) $HClO_4$. After neutralization with K_2CO_3, the salts were removed by centrifugation, and the supernatant was diluted with water. The sample (1 ml) was applied onto a 1-ml Dowex 1-X8 column, which was washed with 3 ml of water. [^{14}C]Ribose 5-phosphate was eluted with 6 × 0.5 ml of 0.5 M NaCl. The elution fractions containing radioactivity were pooled.

For the synthesis of [^{14}C]pseudouridine, 5 mM uracil and 2.5 × 10^6 cpm [^{14}C]ribose 5-phosphate were incubated for 2 h at 30 °C in a medium containing 25 mM Hepes pH 7.1, 50 μM $CoCl_2$, 1 mM $MgCl_2$, 50 μM ribose 5-phosphate, and 50 μg/ml YeiN (final volume: 1 ml). The sample was then treated with 1.4 units (1 μg) alkaline phosphatase for 30 min at 37 °C, and the reaction was stopped by addition of 250 μl of ice-cold 10% (w/v) $HClO_4$. The neutralized perchloric extract was diluted to a volume of 2 ml and phosphorylated with YeiC (2.5 μg/ml) for 30 min at 30 °C in the presence of 25 mM Hepes pH 7.1, 25 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EGTA, and 1 mM ATP-Mg. The reaction was stopped by heating for 5 min at 80 °C. The sample was diluted three times with water and applied on a Q-Sepharose column (3 ml). After washing with 3 ml of water, [^{14}C]PsiMP was eluted with a gradient (100–300 mM) of NaCl. The elution fractions containing radioactivity were pooled (8 ml) and incubated with 1.4 units of alkaline phosphatase for 30 min at 37 °C in the presence of 25 mM Hepes pH 7.1, 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$. The reaction was stopped by heating 5 min at 80 °C, and the preparation was centrifuged to remove denatured proteins.

E. coli UTI89 yeiC$^-$—The *E. coli* UTI89 *yeiC*$^-$ mutants were constructed by transferring the Δ*yeiC::kan* allele from the Keio collection (13) into the UTI89 wild-type strain by P1 transduction.

Consumption of [^{14}C]Pseudouridine by E. coli UTI89 Wild Type and yeiC$^-$ *Mutants—E. coli* UTI89 wild-type and mutant strains were precultured overnight in LB medium (containing 100 μg/liter kanamycin for the mutants). The precultures were used to seed LB medium at an A_{600} of 0.2. When A_{600} reached 0.5, 1-ml samples were taken and further incubated with [^{14}C]pseudouridine (50,000 cpm) and 100 μM pseudouridine for 4 h at 37 °C. The cells were centrifuged for 1 min at 13,000 rpm and perchloric acid extracts were prepared from the extracellular medium and the cell pellet with 250 μl and 100 μl of ice-cold 10% (w/v) $HClO_4$, respectively. After a 10-min centrifugation at 13,000 rpm, the supernatants were neutralized with K_2CO_3, and the pellets were resuspended in Soluene-350. Samples of these extracts were counted to determine the acid-soluble and -insoluble radioactivity. The soluble part of the extracellular medium was treated or not with pseudouridine kinase (YeiC) in an incubation medium containing 25 mM Hepes pH 7.1, 25 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM ATP-Mg, or with alkaline phosphatase in the presence of 25 mM Hepes pH 7.1 and 25 mM KCl. The samples were applied onto 1-ml Dowex 1-X8 columns equilibrated with 20 mM Hepes pH 7.1. The columns were washed with 2× 1 ml of 20 mM Hepes pH 7.1, and the retained radioactivity was eluted with a stepwise 0–1 M NaCl gradient. The fractions were counted for radioactivity.

Preparation of Pseudouridine 5′-Phosphate—For the synthesis with YeiN, 500 μmol of uracil and 250 μmol of ribose 5-phosphate were incubated in the presence of 25 mM Hepes pH 7.1, 500 μM $CoCl_2$, and 2.5 mg of YeiN for 1 h at 30 °C. The sample was heated for 5 min at 80 °C and centrifuged. The resulting supernatant was diluted 3-fold and loaded onto a 20-ml Q-Sepharose column. The column was washed with 60 ml of water and PsiMP was eluted with a linear NaCl gradient (0–1 M in 150 ml). Fractions containing PsiMP (assayed with YeiN) were pooled, concentrated to a volume of 2 ml, and loaded on a Sephadex G10 fine column (0.9 × 50 cm) equilibrated with water to separate the phosphorylated compound from salts. Fractions containing PsiMP were pooled and freeze-dried for NMR analysis.

For synthesis with YeiC, 100 μmol of pseudouridine were incubated in the presence of 25 mM Hepes pH 7.1, 12.5 mM ATP-Mg, 25 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, and 200 μg of YeiC over 30 min at 30 °C. The sample was heated for 5 min at 80 °C and centrifuged. The phosphorylation product was purified as described above.

NMR Analysis of Pseudouridine 5′-Phosphate—Samples (400 μl) containing 10 or 25 μmol of PsiMP in water were adjusted to pH 6.0, mixed with 50 μl of D_2O (for locking on the deuterium signal), and placed in 5-mm outer diameter tubes. All spectra were recorded at a constant temperature of 0 °C. ^{31}P NMR spectra were acquired by 1D NMR methods (14) on a Bruker AVANCE 500 spectrometer at 202.5 MHz (11.7 Tesla) equipped with a Bruker 5-mm BBI (broad band inverse) probe. Typical Fourier transform spectral parameters were: 45° (6 μs) flip angle, 50 kHz spectral width, 8 K data points, 0.6-s repetition time. 1000 transients were accumulated. Chemical shift

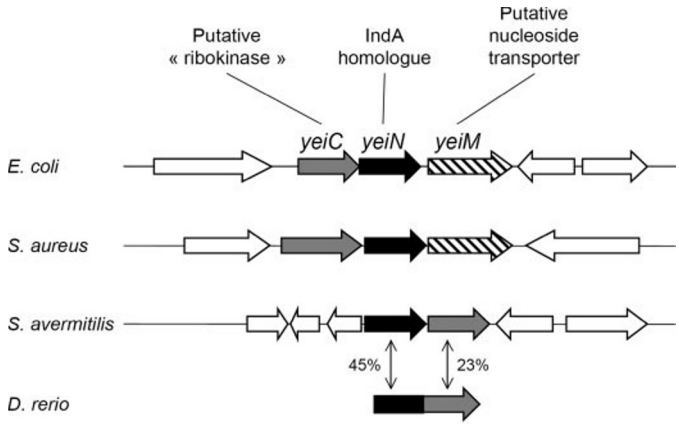


FIGURE 1. **Genomic environment of *yeiC* and *yeiN* in different bacteria and existence of a homologous bifunctional protein in eukaryotes.** In the genome of various strains of *E. coli* (K12, UTI89), *yeiC* (*gray arrows*) forms a putative operon with *yeiN* (*black arrows*), a homologue of *Erwinia carotovora indA*, and with *yeiM* (*hatched arrow*), a putative nucleoside transporter. A similar operon is found in *Shigella flexnerii* (not shown) and in *S. aureus*. The association of *yeiC* and *yeiN* without a putative nucleoside transporter is found in many other bacterial genomes, including those of *Streptomyces avermitilis* and *Rhizobium leguminosarum*. Putative bifunctional proteins comprising a N-terminal YeiN domain fused with a C-terminal YeiC domain are found in many eukaryotes including vertebrates, insects, fungi, protozoa, though not in mammals.

values were quoted in parts per million (ppm) and were referenced to the isotropic chemical shift of H_3PO_4 (0 ppm). ^{1}H 1D NMR spectra were obtained with the WATERGATE solvent-suppression pulse sequence (15) on a Bruker AVANCE 500 spectrometer equipped with a Bruker 5 mm BBI (broad band inverse) probe at 500.2 MHz (11.7 Tesla). Typical Fourier transform spectral parameters were: 90° (7 μs) flip angle, 10 kHz spectral width, 5 K data points, 6-s repetition time. 512 transients were accumulated. Chemical shift values were referenced to the isotropic chemical shift of sodium 3-(trimethylsilyl)-propionate-2, 2, 3, 3-d4 (TSP, 0 ppm).

RESULTS

Data Base Analysis—Microarray studies indicate that *yeiC*, which encodes a putative enzyme of the ribokinase/adenosine kinase/pfkB family, is the most overexpressed gene (about 6-fold) when a uropathogenic strain of *E. coli* is grown on human urine (16). This suggests that YeiC catalyzes the phosphorylation of a sugar derivative present in urine.

Functionally related genes are often associated in operons in bacterial genomes. Genome analysis may therefore provide clues to the function of a putative protein. Analysis of the environment of *yeiC* homologues in other bacterial genomes disclosed two significant associations (Fig. 1). A first one was with a gene encoding a putative nucleoside transporter, which was found in the genomes of *E. coli* and *Staphylococcus aureus*. This suggested that YeiC serves to phosphorylate a nucleoside.

The second significant association was with a gene coding for a protein of unknown function, designated YeiN in *E. coli* and IndA in many other bacterial genomes. Alignments of YeiC and YeiN with some of their homologues are shown in Fig. 2. *indA* is a gene, which, on the basis of mutational insertion (17) has been proposed to participate in the synthesis of the pigment indigoidine in *Erwinia chrysanthemi* (see "Discussion"). More recent

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A
E.coli          MREKDYVVIIGSANIDVAGY-SHESLNYADSNPGKIKFTPGGVGRNIAQN 49
S.aureus        MKNSQNIVCIGAANIDKQLY-LKSELVNKSSNPVSSISVSGGVARNLAYN 49
R.leguminosarum --MKKKILVLGGAHIDRRGR-ICGETAPGASNPGTWFEEPGGGGFNAARN 47
D.rerio         QKMDSKTIVIGGINVDFIAKGTTKKLLFGQTNPGSVCQSFGGVGRNIADC 395
                   ..  : :*. ::*       .    :** .    ** . * *

E.coli          LALLGN-KAWLLSAVGSDFYGQSLLTQTNQSGVYVDKCLIVPGENTSSYL 98
S.aureus        IGKLSNKKTILLTLLGNDKEADYILNEAN-KFIDLKHIKIIENMNTGMFT 98
R.leguminosarum LARLGF-QVTMISPRGGDPMGETVGEAADFAGIDDRPFVFLDR-KTPSYT 95
D.rerio         LSRLGH-KPLFISAIGKDSHSDAVLNYCK--HMDTSAVARLQDQRTATYC 442
                :. *.  :  ::: * *  .: :    .   :       :  .*  :

E.coli          SLLDNTGEMLVAINDMNISNAITAEYLAQH--GEFIQRAKVIVADCNISE 146
S.aureus        SVIDYNGEMICGFADMDIYNLINNKYLNEK--IEVIKNADCVVVDSNIPK 146
R.leguminosarum AIIEKDGNLVIALADMDLYRFFVPRRLSIRWVREAFAAHDFVLFDANLPE 145
D.rerio         AVITESGELSLGLGDMDIHQQIQEQYVSQF--VDQLSSASLIVLDGNIPV 490
                :::   *::  .: **:: . :  . :      : :   . :: * *:.

E.coli          EALAWILDNAAN--VPVFVDPVSAWKCVK--VRDRLNQIHTLKPNRLEAE 192
S.aureus        ESIQYLIQLCGKSNIPIGIIPASIKKVRN--ILKNLAKIDYLIPNKKEAE 194
R.leguminosarum ETIAAIVAKARSLAKPVAAIAISPAKVVR--LRPCIGGIDYLFLNEAEAA 193
D.rerio         STINYVCRIAKKHAVPVWFEPTDADKACKPFLSESWKALAFTSPNLTELC 540
                .::  :   . .   *:  . .  * .  :      :     *  *

E.coli          TLSG--------IALSGREDVAKVAAWFH---QHGLNRLVLSMGGDGVYY 231
S.aureus        AFWN--------VSLNTKSDYKKIKKISK---TSEIKNIVITNGNDDIFY 233
R.leguminosarum ALTG--------ERP---EEAAGWPPLLN---EIGIRNAVVTRGRRELVA 229
D.rerio         TMNHTLGLPTPAELPRSLEDVLGCAPALSRPLLEHLHCVVVTLGSLGVLV 590
                ::              .:              :.  *:: *   :

E.coli          SDISGESGWSAP--------------------IKTNVINVTGAGDAMMAG 261
S.aureus        TNGELSIFKNVQ--------------------QSSNIINDTGAGDVFSAS 263
R.leguminosarum LCEGRAVTLQPP--------------------IADTVADVTGAGDSLAAG 259
D.rerio         CGEHEAGTVNLQPRKQKRKARLCAVHYPALTLSSEETVNVSGAGDSFAGA 640
                          .                          : :**** : ..

E.coli          LASCWVDGMPFAESVRFAQGCSSMALSCEYTNNPDLS---IANVISLVEN 308
S.aureus        VFHYWLENYEVDN----------------YLEMALFN---ACSTVSTIYN 294
R.leguminosarum TLAALISGLPLEEAVRHGTAAATLTVQSQHAVNENLTPDLLNEALALVPK 309
D.rerio         MMVGILQGLHTDSCVQMGLLAARRSLLSPHPIDPSLTADAIRPEKSHFQQ 690
                     :..    .              :    :.       : . :

E.coli          AECLN----- 313
S.aureus        TSMIN----- 299
R.leguminosarum VRILH----- 314
D.rerio         TKPAFMWMED 700
                .

B
E.coli          ----------------MSELKISPELLQISPEVQDALKNKKPVVALESTI 34
S.aureus        ----------------MANLQK---YIEYSREVQQALENNQPIVALESTI 31
R.leguminosarum ------------MTLMKPRLSR---------EMAEAIAAGSPVVALESTI 29
D.rerio         MLWRFSTFLRRCFTTTHSRHCKQDSLFTIHPDVKEALAHHRPVVALESTI 50
T.maritima      ------------------------------MIIESRIEKGKPVVGMETTV 20
                                             : . :    *:*.:*:*:

E.coli          ISHGMPFPQNAQTAIEVEETIRKQGAVPATIAIIGGVMKVGLSKEEIELL 84
S.aureus        ISHGMPYPQNVEMATTVEQIIRNNGAIPATIAIIDGKIKIGLESEDLEIL 81
R.leguminosarum ITHGMPYPANLETALGVETVIRENGAIPATIAVVKGELRVGLEHDELEEL 79
D.rerio         ITHGMPYPHNLSTAKEVEAIVRAEGSIPATIGILEGRIHVGLSSDELDFL 100
T.maritima      FVHGLPRKEAIELFRRAKEISREKGFQLAVIGILKGKIVAGMSEEELEAM 70
                : **:*     .    .:   * :*   *.*.:: * :  *:. :::: :

E.coli          GREGHNVTKVSRRDLPFVVAAGKNGATTVASTMIIAALAGIKVFATGGIG 134
S.aureus        ATS-KDVAKVSRRDLAEVVAMKCVGATTVATTMICAAMAGIQFFVTGGIG 130
R.leguminosarum AQS-KGIVKASGRDLAVAMIRGQSAGTTVSATMLMADLAGIDVFATGGVG 128
D.rerio         AQS-KTALKVSRRDLPYVISKGLSGGTTVSGTMIAANKAGIPVFVTGGIG 149
T.maritima      MRE--GADKVGTREIPIVVAEGKNAATTVSATIFLSRRIGIEVVVTGGTG 118
                  .     *.. *::. .:      ..***: *:: :   ** ...*** *

E.coli          GVHRGAEHTFDISADLQELANTNVTVVCAGAKSILDLGLTTEYLETFGVP 184
S.aureus        GVHKGAEHTMDISADLEELSKTNVTVICAGAKSILDLPKTMEYLETKGVP 180
R.leguminosarum GVHRGAEQTFDISADLTELGRTKTAVVCAGVKSILDIAKTLEYLETQRVP 178
D.rerio         GVHRDGENSLDVSADLTELGRTPIAVVSAGVKSILDIGRTLEYLETQGVC 199
T.maritima      GVHPG---RVDVSQDLTEMSSSRAVLVSSGIKSILDVEATFEMLETLEIP 165
                *** .    .*:* ** *:. :  .::.:* *****:  * * ***  :

E.coli          LIGYQ-TKALPAFFCRTSPFDVSIRLDSASEIARAMVVKWQSGLNGGLVV 233
S.aureus        VIGYQ-TNELPAFFTRESGVKLTSSVETPERLADIHLTKQQLNLEGGIVV 229
R.leguminosarum VIAYG-TEDFPAFFTRRSGFKADHRLDTPEEIAKAMWLHHQLGTGTGLLI 227
D.rerio         VATYGDSKSFPAFFSRQSGFTSPYNISSPQEAADLIASTFSLGLKSGLLL 249
T.maritima      LVGFR-TNEFPLFFSRKSGRRVP-RIENVEEVLKIYESMKEMELEKTLMV 213
                :  :  :: :* ** * *       :.. ..        .     :::

E.coli          ANPIPEQFAMPEHTINAAIDQAVAEAEAQGVIGKESTPFLLARVAELTGG 283
S.aureus        ANPIPYEHALSKAYIEAIINEAVVEAENQGIKGKDATPFLLGKIVEKTNG 279
R.leguminosarum ANPIPEASALAPDFIDGTIADAVRDADERGIDRKELTPFLLARINELTKG 277
D.rerio         AVPIPEEHAATGQQIEDAIQTAVTEASSKGVTGRDVTPFILQRVSELTKG 299
T.maritima      LNPVPEEYEIPHDEIERLLEKIELEVE-----GKEVTPFLLKKLVEMTNG 258
                  *:*     .   *:  :    :..      :: ***:* :: * * *

E.coli          DSLKSNIQLVFNNAILASEIAKEYQRLAG---------------------- 312
S.aureus        KSLAANIKLVENNAALGAKIAVAVNKLL---------------------- 307
R.leguminosarum ESLKANIELVKNNARLAARIAVAYAPLKKARN---------------------- 309
D.rerio         KSLQANIALIRNNARVGSQIAHALSKLNENKEGHVRGNIKTQKTMDQKMD 349
T.maritima      RTLKANLALLEENVKLAGEIAVKLKRS---------------------- 285
                 :* :*: *: :*. :...**
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FIGURE 2. **Sequence alignment of YeiC (*A*) and YeiN (*B*) homologues.** The *E. coli* sequences are aligned with those of their homologues in *S. aureus*, *R. leguminosarum*, and the fish *D. rerio*. YeiN is also aligned with its *T. maritima* homologue, which has been crystallized (19). (*): strictly conserved residues; (:): conserved substitutions; (.): semiconserved substitutions.

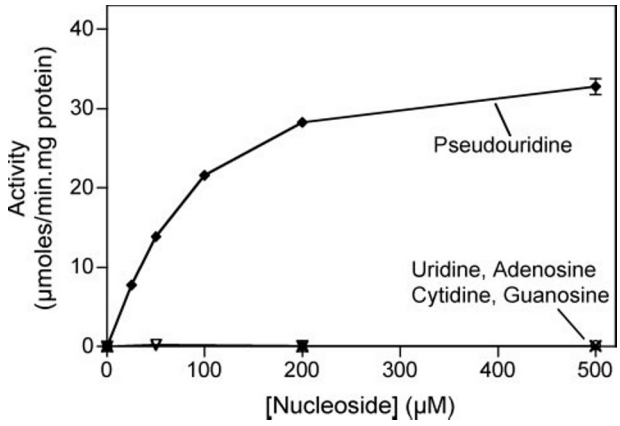

FIGURE 3. **Pseudouridine kinase activity of YeiC.** The enzymatic activity was measured spectrophotometrically through the production of ADP in the presence of 0.5 mM ATP and of the indicated concentrations of pseudouridine (◆), uridine (△), adenosine (×), guanosine (▽), or cytidine (○). Results are the means of three values ± S.E.

results indicate, however, that another protein, IndC, has the full capacity of synthesizing indigoidin (18), questioning the role of IndA in this process. Interestingly, the IndA homologue of *T. maritima* has been crystallized and shown to have a new fold (19). The association of *yeiC* with *yeiN/indA* was found not only in bacterial genomes, but also in eukaryotic sequences. Blast searches indeed indicated the existence in eukaryotes of putative bifunctional proteins comprising a YeiN/IndA N-terminal domain and a YeiC C-terminal domain. Such proteins were found in fish (*Danio rerio*), birds (*Gallus gallus*), invertebrates (*Drosophila melanogaster*, *Strongylocentrotus purpuratus*, *Caenorhabditis elegans*), and fungi (*Aspergillus*, *Schizosaccharomyces pombe*), but not in man and other mammals, nor in *Saccharomyces cerevisiae*.

The association of a putative nucleoside kinase, highly expressed when bacteria are grown in urine, with an enzyme with a novel structure suggested that the product of the reaction underwent an unusual reaction. These considerations led us to hypothesize that these enzymes served to metabolize pseudouridine, one of the most abundant nucleosides present in urine (20). Both *E. coli* YeiC and YeiN (IndA) were produced as fusion proteins with a His tag at the N terminus. They were overexpressed in *E. coli* and purified to homogeneity by metal affinity chromatography.

YeiC Is a Pseudouridine Kinase—The ability of YeiC to phosphorylate pseudouridine and other substrates was determined by measuring enzymatically the formation of ADP in the presence of different nucleosides and ATP. As shown in Fig. 3, the enzyme converted ATP to ADP in the presence of pseudouridine but not in the presence of uridine, adenosine, guanosine, or cytidine, indicating that it was specific for pseudouridine. The enzyme was dependent on the presence of Mg^{2+}. Its K_m for pseudouridine was 120 μM in the presence of 0.5 mM ATP-Mg, and its K_m was 330 μM for ATP-Mg in the presence of 200 μM pseudouridine (Fig. 3).

HPLC analysis of the products indicated that YeiC converted pseudouridine to a more polar compound, which was retained on the Partisphere Sax column (anion exchanger) and eluted at 5 min. This compound had a similar UV absorption spectrum as pseudouridine, with a peak at 264 nm. It was purified by chromatography on an anion-exchanger column and characterized. Mass spectrometry analysis indicated that it had the expected mass (m/z value of 323 for the monodeprotonated species) for a phosphorylated derivative of pseudouridine. ^{31}P and ^{1}H 1D NMR spectra of pseudouridine phosphate were acquired (Fig. 4*A*) as well as those of pseudouridine and uridine 5′-monophosphate for the sake of comparison (not shown). ^{31}P 1D NMR analysis confirmed that the product was indeed phosphorylated (Fig. 4*B*). The ^{1}H 1D NMR spectrum of the product (Fig. 4*C*) was distinctly different from that of UMP (not shown). A major difference was the absence of the resonance corresponding to H5 and appearing as a doublet at 5.95 ppm in the UMP spectrum. Compared with pseudouridine, the chemical shifts of the H5′ and H5″ protons of the product of YeiC were displaced downfield by ~0.27 ppm (135 Hz) compared with pseudouridine and they appeared as octuplets instead of quadruplets, indicating additional coupling with ^{31}P. This indicated that phosphorylation had occurred on carbon atom C5′ of the ribose moiety (Fig. 4*D*). We also noted that the H6 chemical shift of pseudouridine 5′-monophosphate was about 7.87 ppm, whereas its value is ≈7.68 ppm in pseudouridine. This shift change can be attributed to the *anti* conformation adopted by pseudouridine 5′-monophosphate. This pseudouridine 5′-monophosphate H6 chemical shift variation of about 0.2 ppm (100 Hz) is in agreement with previous comparative studies on conformations of uridine, pseudouridine, and their derivatives (21, 22) .

YeiN Is a Pseudouridine 5′-Phosphate Glycosidase—We tested the ability of YeiN to convert PsiMP to ribose 5-phosphate and uracil. Ribose 5-phosphate formation was followed using ribose-5-phosphate isomerase and *H. influenzae* ribulose-5-phosphate reductase (12). In the presence of Mn^{2+}, YeiN was found to catalyze the formation of ribose 5-phosphate from PsiMP, though not from any of the four classical ribonucleotide monophosphates (AMP, UMP, GMP, CMP) (Fig. 5*A*). No consumption of NADPH was observed when ribose-5-phosphate isomerase was omitted from the reaction mixture, indicating that ribose 5-phosphate was formed and not ribulose 5-phosphate. The K_m for PsiMP was 60 μM in the presence of 0.5 mM Mn^{2+}. The reaction could also be studied through the formation of uracil by HPLC. A compound with the same elution time as uracil (0.7 min) was formed when the enzyme was incubated with PsiMP. We also determined the metal ion dependence of YeiN. As shown in Fig. 5*B*, the highest activities were recorded with Fe^{2+}, Co^{2+}, and Mn^{2+}, whereas Zn^{2+} and, to a lesser extent, Ni^{2+} behaved as inhibitors.

Previous results have indicated that the reaction catalyzed by pseudouridine-5′-phosphate glycosidase (called pseudouridylate synthase in some works) is easily reversible (23). This could indeed be confirmed. Incubation of YeiN with uracil and ribose 5-phosphate followed by HPLC separation led to the appearance of a compound that had the same elution time and UV spectrum as PsiMP. This product was purified by anion-exchange chromatography and gel filtration. Its analysis by NMR indicated that it had the same ^{1}H and ^{31}P spectrum as the product of YeiC, *i.e.* pseudouridine 5′-phosphate. In particular, no duplication of the shift corresponding to H1′ was observed (not

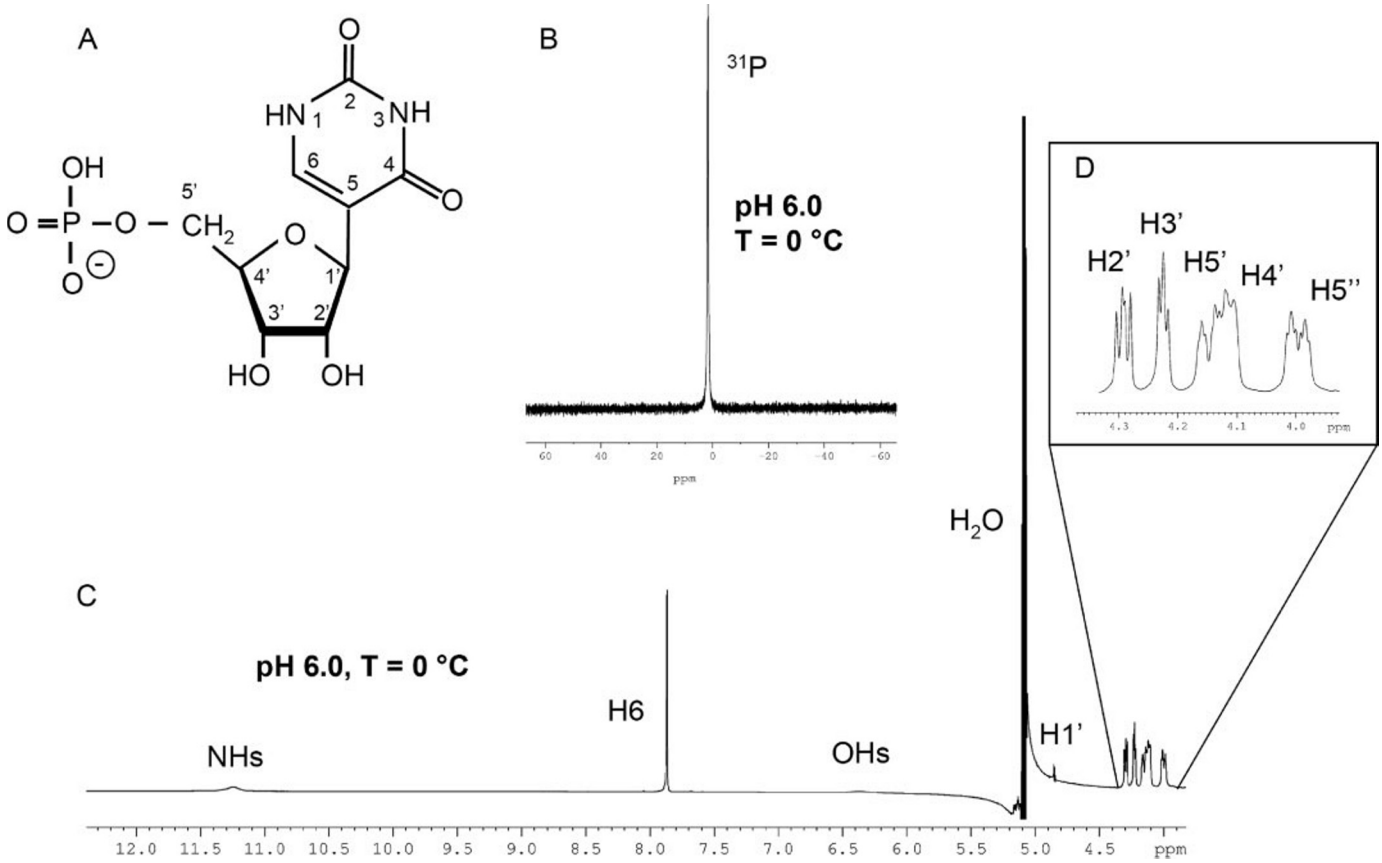

FIGURE 4. **NMR spectrum of the phosphorylation product of pseudouridine.** The product of pseudouridine phosphorylation by YeiC was purified and submitted to NMR analysis. *A*, structure of pseudouridine 5′-monophosphate. *B*, ^{31}P 1D NMR spectrum of the YeiC product. *C*, ^{1}H 1D NMR spectrum of the YeiC product. *D*, enlargement of the ribose proton region of the ^{1}H 1D NMR spectrum.

shown), indicating that only one anomer was present in the preparation.

The equilibrium constant of the glycosidase reaction was determined by allowing the hydrolytic reaction or the condensation reaction to proceed until the concentration of the reaction product(s) reached a plateau (Fig. 6). A value of 2.3×10^{-4} M could be calculated for the ratio [Ribose 5-P][Uracil]/[PsiMP] in both cases. From this, a $\Delta G^{\circ\prime}$ value of + 4.9 kcal/mol was calculated for the hydrolysis of the glycosidic bond of PsiMP.

The synthetic reaction was also utilized to prepare radiolabeled PsiMP from [^{14}C]ribose 5-phosphate and uracil. Treatment of this compound with alkaline phosphatase led to the formation of radiolabeled pseudouridine, which was used in the metabolic studies described below.

T. maritima IndA Is Also a Pseudouridine-5′-phosphate Glycosidase—IndA, the *T. maritima* homologue of YeiN, which has been crystallized, only possesses 30% sequence identity with YeiN and, unlike the latter, it is not associated with a putative ribokinase. It was therefore of interest to determine if it was also a pseudouridine-5′-phosphate glycosidase. As thermophile enzymes are sometimes poorly active at room temperature, incubations were performed at temperatures ranging from 50 to 90 °C. The *T. maritima* YeiN homologue indeed acted as a pseudouridine-5′-phosphate glycosidase, as indicated by its ability to form ribose 5-phosphate from PsiMP (Fig. 7) and to convert ribose 5-phosphate and uracil to PsiMP (not shown). The enzyme was, however, extremely temperature-dependent, because no activity could be observed at or below 50 °C.

Metabolic Utilization of Pseudouridine by E. coli—Incubation of a uropathogenic strain of *E. coli* (UTI89) with pseudouridine radiolabeled on its ribose portion for 4 h, followed by centrifugation, indicated that ≈40% of the radioactivity had been incorporated in the pellet whereas 40% was present in the medium (Fig. 8). The radioactive material present in the bacterial pellet was insoluble in the presence of perchloric acid (PCA), indicating that part of the radioactivity had been incorporated in nucleic acids and/or proteins. In contrast, the radioactive material present in the supernatant was soluble in the presence of PCA. When neutralized PCA extracts from the supernatant were applied on an anion-exchanger (Dowex 1-X8), the radioactivity was partially eluted in the flow-through fractions and partially retained on the resin. The proportion of retained radioactive material was unaffected by prior treatment with alkaline phosphatase, indicating that the anionic metabolites were not phosphate esters (and therefore differed from PsiMP). The non-retained radioactive compound(s) did not correspond to pseudouridine, as indicated by the fact that it did not bind to anion-exchanger after incubation with pseudouridine kinase and ATP. Taken together, these findings showed that the radiolabeled compound(s) differed from pseudouridine and PsiMP, indicating that *E. coli* UTI89 had completely consumed radiolabeled pseudouridine. When similar experiments were performed with *yeiC*$^{-}$ mutants, all the radioactivity

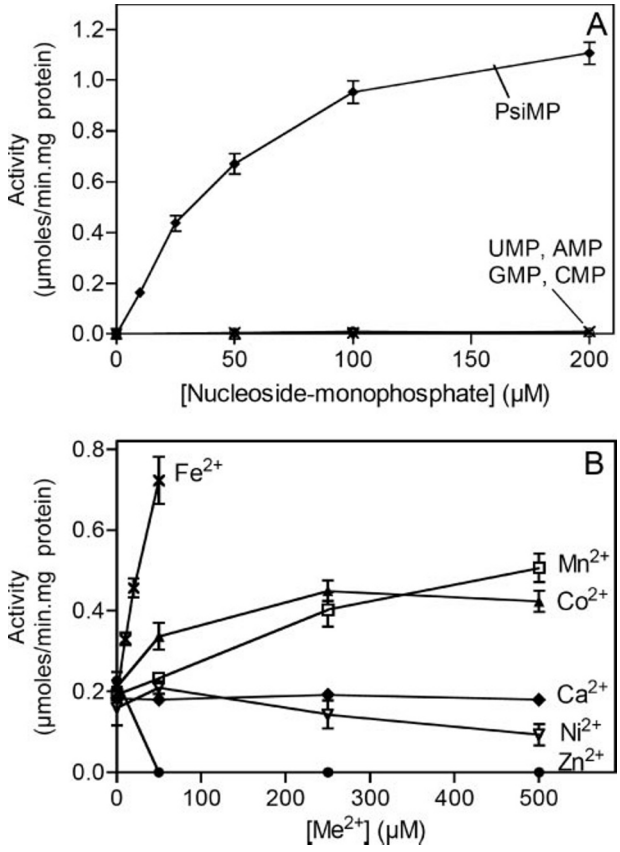

FIGURE 5. **Pseudouridine-5′-phosphate glycosidase activity of YeiN: substrate saturation curve (*A*) and metal ion dependence (*B*).** The activity was measured spectrophotometrically through the formation of ribose 5-phosphate. In *A*, the enzyme was tested in the presence of 1 mM $MgCl_2$, 0.5 mM $MnCl_2$, and the indicated concentrations of the following ribonucleoside monophosphates: PsiMP (♦), UMP (△), CMP (○), AMP (▽), and GMP (×). In *B*, the activity was tested in the presence of 1 mM $MgCl_2$ (needed for the coupling enzymes), 50 μM PsiMP, and the indicated concentrations of divalent cations.

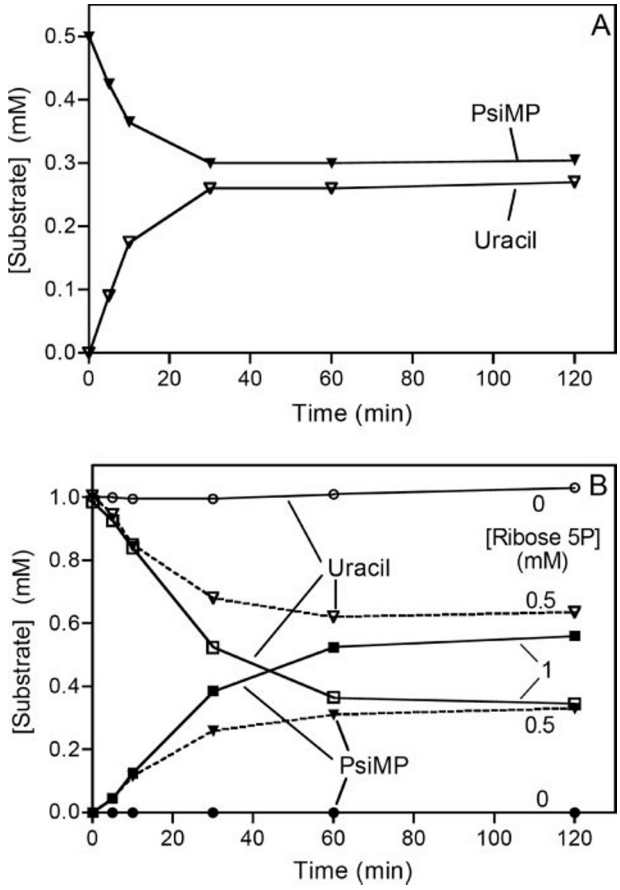

FIGURE 6. **Time course of the forward and backward reaction of *E. coli* pseudouridine-5′-phosphate glycosidase.** All incubations were carried out at 30 °C for the indicated times in the presence of 0.5 mM $CoCl_2$. The substrates were 0.5 mM PsiMP in *A* and 1 mM uracil together with 0, 0.5, or 1 mM ribose 5-phosphate in *B*. The mean of two independent experiments is shown.

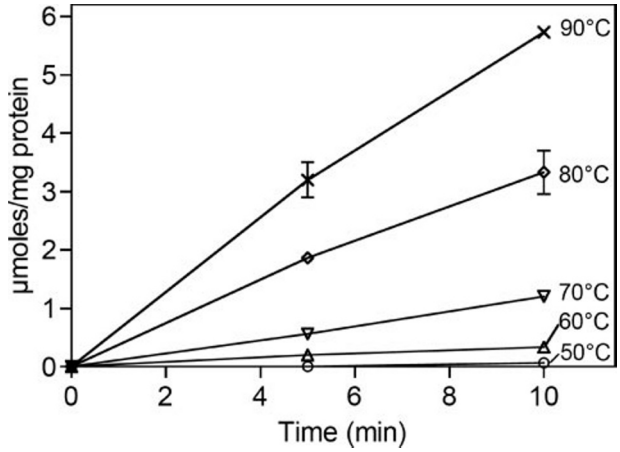

FIGURE 7. **Effect of temperature on the activity of *T. maritima* pseudouridine-5′-phosphate glycosidase.** Recombinant *T. maritima* IndA (7.5 μg/ml) was incubated at the indicated temperatures and for the indicated times in the presence of 1 mM PsiMP and 0.5 mM $CoCl_2$ The formed ribose 5-phosphate was measured spectrophotometrically at 30 °C. Means of three experiments ± S.E. are shown.

was recovered in the supernatant and corresponded to pseudouridine. These findings indicated that pseudouridine metabolism proceeds via its phosphorylation to PsiMP. The *yeiC*$^-$ mutants, however, still possessed the capacity of growing on human urine (not shown), indicating that other substrates than pseudouridine support *E. coli* growth under these conditions.

DISCUSSION

Identification of the Enzymes Involved in Pseudouridine Metabolism—Through a data base mining approach and confirmation by characterization of the relevant proteins, we have identified the genes encoding the enzymes that are responsible for pseudouridine metabolism (7, 24).

One, YeiC, is a kinase belonging to the family of ribokinase/adenosine kinase/pfkB. NMR analysis of the product of pseudouridine phosphorylation indicated it was phosphorylated on the 5th carbon of its ribose moiety, which is in agreement with the general property of this class of enzyme to phosphorylate a hydroxymethyl group bound to a furanose ring (25). Despite the close structural similarity of pseudouridine and uridine, the latter was not a substrate for pseudouridine kinase. This is likely due to the fact that the enzyme makes a hydrogen bond with the hydrogen present on N1, which is the most distinctive feature between pseudouridine and uridine. The involvement of this enzyme in the metabolism of pseudouridine is indicated by the fact that inactivation of its gene prevents *E. coli* from utilizing pseudouridine.

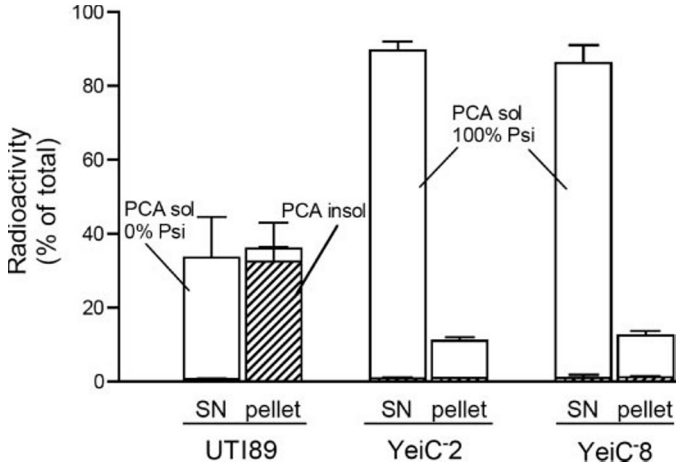

FIGURE 8. **Consumption of radiolabeled pseudouridine by *E. coli* UTI89 wild-type and *yeiC*⁻ strains.** Cells were grown in LB medium until A_{600} reached 0.5. 1-ml samples were then incubated with radiolabeled pseudouridine (50,000 cpm) for 4 h at 37 °C in LB medium. Cells were centrifuged, and perchloric extracts were prepared from the extracellular medium and the cell pellet. After further centrifugation, samples of the respective supernatant and pellets were counted to determine the PCA-soluble and insoluble radioactivity. The supernatant of the medium fraction was neutralized, treated or not with alkaline phosphatase, and chromatographed on Dowex 1-X8. The presence of radiolabeled pseudouridine was checked by phosphorylation with ATP and purified pseudouridine kinase, followed by chromatography on an anion exchanger. Two independent transformants (YeiC⁻2 and YeiC⁻8) were tested.

The other enzyme, YeiN, catalyzes the hydrolysis of PsiMP to uracil and ribose 5-phosphate, as shown by HPLC and enzymatic analysis. This reaction is reversible, as indicated by the fact that the enzyme, when incubated with ribose 5-phosphate and uracil, catalyzes the synthesis of a compound with the same NMR spectrum as the product of phosphorylation of pseudouridine by pseudouridine kinase. NMR analysis showed also that the enzyme only synthesized the β-anomer of this nucleoside, which indicates its stereospecificity.

Pseudouridine-5′-phosphate glycosidase is therefore the same enzyme as the pseudouridylate synthase that catalyzes the synthesis of PsiMP from uracil and ribose 5-phosphate in extracts of *Tetrahymena pyriformis* (23, 26), *Agrobacterium-tumefaciens* (27), *E. coli* (8), and other sources (28). Accordingly, Blast searches indicate that homologues of YeiN are present in *Tetrahymena* and *Agrobacterium*. Pseudouridylate synthase was once thought to be involved in the formation of the pseudouridines present in RNAs via the formation of a hypothetical pseudouridine 5′-triphosphate. It is now known that pseudouridines are formed through isomerisation of uridines by pseudouridine synthases. Confusingly, pseudouridylate synthase (*i.e.* pseudouridine-5′-phosphate glycosidase) and pseudouridine synthases are registered in the EC data base under the same entry (EC 4.2.1.70), which is illogical because they catalyze completely different reactions. We propose to classify pseudouridine-5′-phosphate glycosidase among the hydrolases (EC 3.2.?.?), in a new subclass of glycosidases hydrolyzing C-C bonds.

Despite the reversibility of the reaction it catalyzes, pseudouridine-5′-phosphate glycosidase appears to be involved in the utilization of pseudouridine rather than its formation. The equilibrium constant of the reaction permits this, provided the concentration of ribose 5-phosphate and uracil are maintained at sufficiently low levels. Thus, at 0.1 mM ribose 5-phosphate, PsiMP and uracil, the ΔG of the reaction amounts to −0.3 kcal/mol. The role of pseudouridine-5′-phosphate glycosidase in the utilization of pseudouridine is particularly indicated by the association of this glycosidase with pseudouridine kinase in the same operon in *E. coli* and in many other bacteria, and by the fact that they form a bifunctional protein in many eukaryotes. As pseudouridine kinase catalyzes a highly exergonic reaction, obviously involved in pseudouridine utilization (see above), its companion enzyme pseudouridine-5′-phosphate glycosidase must also take part in pseudouridine degradation, at least in the organisms where the association of this enzyme with the kinase is found.

Occurrence of the Pseudouridine-metabolizing Enzyme in Nature—The presence of YeiN homologues in numerous genomes indicates that the ability of utilizing pseudouridine is shared by many bacteria and by most eukaryotes. It is remarkable that among eukaryotes, only some species have apparently lost the ability of utilizing this nucleoside. This is particularly the case for mammals. Inspection of the *G. gallus* genome indicates that the gene encoding the bifunctional protein is present between those encoding MKRN1 and DENND2A. These two genes are found next to each other in mammalian genomes, which suggests that the gene encoding the bifunctional enzyme was lost from this position during evolution of the vertebrate lineage. Consequently mammals are unable to utilize pseudouridine, which agrees with the presence of pseudouridine in human urine.

Blast searches indicate that most fungi (58 out of 68 finished or unfinished genomes) have a YeiN-YeiC homologue (sometimes appearing as two separate genes in tandem, but this may be an artifact of gene identification programs). No homologue was found in the case of *S. cerevisiae* and of the closely related yeast (29) *Ashbya gossypyi*, although well in the other closely related yeast *Kluyveromyces lactis* and in the more distant *Candida albicans*. This suggests that the capacity of metabolizing pseudouridine has been lost lately in the evolution of yeasts. This metabolic deficiency in *S. cerevisiae* is consistent with the presence of pseudouridine in beer (30).

Inactivation of the *yeiC* gene in *E. coli* UTI89 did not prevent this bacterium from growing on human urine, most probably because urine contains other substrates for *E. coli*. For instance, glucuronides and amino acids are present at concentrations that are at least 10-fold higher than that of pseudouridine (31–33), which indicates that the latter is a minor substrate. The utilization of glucuronides is consistent with the induction of enzymes involved in its metabolism (16).

Pseudouridine-5′-phosphate Glycosidase, a New Type of Glycosidase—Pseudouridine-5′-phosphate glycosidase is the first molecularly identified enzyme that catalyzes the hydrolytic cleavage of a glycosidic C-C bond. Other such enzymes most probably exist since other C-glycosides are known, though it has not yet been proven that these C-glycosides can be degraded by hydrolysis. Interestingly, the *T. maritima* homologue of YeiN has been crystallized and shown to have a new fold (19). The authors, who did not identify the enzymatic function of the protein, found that the enzyme contained in its puta-

tive catalytic site a metal ion, which they identified as Mn^{2+}, and a phosphate ester, which they tentatively identified as a glycerol 3-phosphate analog. These findings are consistent with our demonstration that the *T. maritima* YeiN homologue is also a pseudouridine-5′-phosphate glycosidase, which, as its *E. coli* homologue, is activated by divalent cations. The phosphate ester present in the catalytic site of the crystallized enzyme may correspond to ribose 5-phosphate, assuming that some of the atoms of the latter would be too mobile to provide a distinct electron density map. The finding that IndA has a completely new fold suggests that the enzyme proceeds through a novel mechanism, which may be related with the peculiar structure of the substrate. Further studies will now be made possible by the availability of the enzyme sequence.

Energetic Aspects of Pseudouridine Formation—The $\Delta G°'$ of hydrolysis of the glycosidic C-N bond in uridine is about −4 kcal/mol (calculated from the $\Delta G°'$ values of ribose 1-phosphate hydrolysis and uridine phosphorolysis (34, 35)). This value is 9 kcal/mol lower than that for the hydrolysis of the glycosidic C-C bond in PsiMP (+5 kcal/mol), consistent with empirical bond energies indicating that a C-C bond is more thermodynamically stable than a C-N bond (the difference being larger than between a C-H and a N-H bond). In addition, pseudouridine has the possibility of forming one more hydrogen bond than uridine, which can therefore stabilize pseudouridine in solution compared with uridine. Whatever the exact reason, the large difference in energy between the two bonds makes that the conversion of uridine to pseudouridine is associated with a $\Delta G°'$ of −9 kcal/mol and is therefore extremely favorable, explaining that pseudouridine synthases catalyze the conversion of uridine to pseudouridine to apparent completion without the need of additional energy (36).

Is There a Role of YeiN/IndA Homologues in the Synthesis of Indigoidine—indA has been initially found as a gene potentially involved, with *indB* and *indC*, in indigoidine synthesis in *E. chrysanthemi* (17). Recent data indicate that IndC converts glutamine to a pyridine derivative (5-amino-*3H*-pyridine-2,6-dione) and that indigoidine formation results from the spontaneous reaction of two molecules of 5-amino-*3H*-pyridine-2,6-dione (18). The role of IndA and IndB is therefore not obvious, yet IndA and IndB homologues are found next to IndC in other genomes. Because IndB is a putative phosphatase and IndA most likely acts on a phosphate ester, we speculate that they act sequentially to form a phosphate ester (possibly a ribose 5-phosphate derivative of 5-amino-*3H*-pyridine-2,6-dione, which shows some structural similarity with uracil) and then to dephosphorylate it. The resulting nucleoside could participate in the synthesis of indigoidine or have another function. In this context it is interesting to recall that plant pathogens often divert plant metabolism to form opines (37). Further work is needed to determine the exact function of IndA and IndB.

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