JOURNAL OF BACTERIOLOGY, Feb. 2011, p. 989–993 Vol. 193, No. 4
0021-9193/11/$12.00 doi:10.1128/JB.01178-10

NOTES

Escherichia coli Dihydropyrimidine Dehydrogenase Is a Novel NAD-Dependent Heterotetramer Essential for the Production of 5,6-Dihydrouracil▽

Ryota Hidese,[1] Hisaaki Mihara,[2] Tatsuo Kurihara,[1] and Nobuyoshi Esaki[1]*

Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan,[1] and Department of Biotechnology, Institute of Science and Engineering, College of Life Sciences, Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan[2]

Received 1 October 2010/Accepted 3 December 2010

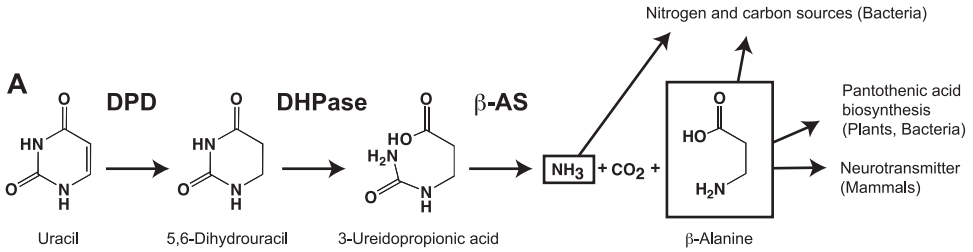
The reductive pyrimidine catabolic pathway is absent in *Escherichia coli*. However, the bacterium contains an enzyme homologous to mammalian dihydropyrimidine dehydrogenase. Here, we show that *E. coli* dihydropyrimidine dehydrogenase is the first member of a novel NADH-dependent subclass of iron-sulfur flavoenzymes catalyzing the conversion of uracil to 5,6-dihydrouracil *in vivo*.

The reductive pyrimidine catabolic pathway is found in most eukaryotes. It degrades uracil or thymine into β-amino acids through dihydropyrimidine and *N*-carbamoyl-β-amino acid intermediates (Fig. 1A). These reactions are catalyzed by dihydropyrimidine dehydrogenase (DPD), dihydropyrimidinase, and β-alanine synthase in both mammals (16) and plants (25). β-Alanine, a product of the reductive pathway, not only is a substrate for the synthesis of pantothenic acid in plants and several bacteria (20, 25) but also acts as a neurotransmitter in mammals (19). Mammalian DPD, the first and rate-limiting enzyme of the reductive pyrimidine catabolic pathway, catalyzes the NADPH-dependent reduction of uracil and thymine to 5,6-dihydro derivatives (16). The mammalian enzyme is composed of two identical subunits of approximately 111 kDa. Each subunit carries one FMN, one FAD cofactor, and four [4Fe/4S] clusters and contains separate binding sites for the electron-donating cosubstrate NADPH and the electron-accepting pyrimidines (16). Bacterial enzymes for the reductive degradation of pyrimidine were originally discovered and identified 50 years ago in a uracil-fermenting bacterium, *Clostridium uracilicum* (1–5). In this bacterium, the reductive pyrimidine catabolic pathway is important because it enables uracil and thymine to be used as nitrogen and carbon sources for growth (Fig. 1A). Since then, the presence of DPD activity has been demonstrated in several bacterial strains, including *Alcaligenes eutrophus* (4, 15), *Pseudomonas* sp. (9, 22–24), and *Escherichia coli* B (21). However, little is known about the molecular characteristics of DPD in bacteria.

In the course of our investigation of iron-sulfur proteins that depend on the *iscS* gene for iron-sulfur cluster maturation in *E. coli*, we found two putative iron-sulfur proteins, PreT and PreA, which exhibited ca. 30% identity to the N- and C-terminal halves, respectively, of mammalian DPD (11). It was revealed that the *preT* and *preA* genes (formerly *yeiT* and *yeiA*, respectively) form an operon structure on the genome. Bioinformatic analysis revealed that genes with sequence identities to *preT* and *preA* are also present as a putative operon in a number of bacteria, including *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, *Bacillus clausii*, and *Clostridium tetani*. Interestingly, the corresponding homologous genes, *pydX* and *pydA*, of *P. aeruginosa* are located in a gene cluster whose function is computationally predicted to be pyrimidine-reductive degradation (13) (Fig. 1B). In contrast, the *preT-preA* genes do not associate with any other putative genes for the reductive pyrimidine catabolic pathway (Fig. 1B). Moreover, a previous study suggests that *E. coli* K-12 does not possess the reductive pyrimidine catabolic pathway (18), and no gene for a homolog of β-alanine synthase is seen in the genome sequence. Although *E. coli* HyuA and AllB exhibit 35% and 26% identities, respectively, to human dihydropyrimidinase, they do not exhibit catalytic activity toward dihydropyrimidine (7). Furthermore, a new pathway for pyrimidine degradation was recently found in *E. coli* and termed the Rut pathway, which is completely different from the reductive catabolic pathway (8, 10). Thus, these findings raise questions about the molecular and physiological function of PreT-PreA in *E. coli.* In this study, we purified the complex of PreT and PreA (EcDPD) from *E. coli* K-12 and characterized the enzymological properties of the as-isolated enzyme. In addition, we show that *preTA* is essential for the *in vivo* production of 5,6-dihydrouracil (DHU) from uracil and that DHU which accumulates during the exponential growth is converted to uracil by EcDPD after the early stationary phase. Furthermore, multiple sequence alignment analysis revealed that EcDPD is the first member of a novel subclass in the DPD family proteins.

Methods. *E. coli* K-12 W3110 (a wild-type strain) and JW2133 (a Δ*preT* strain) were obtained from the National

* Corresponding author. Mailing address: Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan. Phone: 81-774-38-3240. Fax: 81-774-38-3248. E-mail: esakin@SCL.kyoto-u.ac.jp.
▽ Published ahead of print on 17 December 2010.



B

Mammals	DPD	DHPase	β-AS
Pseudomonas aeruginosa	PydX-PydA	PydB	PydC
Escherichia coli K12	PreT-PreA	—	—

FIG. 1. Reductive pyrimidine catabolic pathway (A) and the bacterial enzymes predicted to correspond to the mammalian enzymes involved in the reductive pyrimidine catabolic pathway (B). (A) Reductive pyrimidine degradation is accomplished via a sequential catalysis by dihydropyrimidine dehydrogenase (DPD), dihydropyrimidinase (DHPase), and β-alanine synthase (β-AS). The final product of the pathway, β-alanine, functions as a neurotransmitter in mammals, a substrate for pantothenic acid biosynthesis in plants and bacteria, and a carbon and nitrogen source in many bacteria. Thymine is also degraded by the same pathway. (B) The mammalian enzymes for the reductive pyrimidine catabolic pathway are shown with the corresponding predicted proteins from *Pseudomonas aeruginosa* and *Escherichia coli* K-12.

BioResource Project (NIG, Japan). [2-^{14}C]DHU was purchased from Moravek Biochemicals (Brea, CA). EcDPD was purified from the *E. coli* W3110 extract though DEAE-Toyopearl (Tosoh, Tokyo, Japan), Butyl-Toyopearl (Tosoh), and Superdex 200 (GE Healthcare, Uppsala, Sweden) columns. The pyrimidine-reductive activity of the enzyme was determined at 30°C in 100 mM potassium phosphate buffer (pH 6.0) containing 1 mM uracil or thymine and 150 μM NADH. The dihydropyrimidine oxidation activity of the enzyme was determined at 30°C in 100 mM CAPS[3-(cyclohexylamino)-1-propanesulfonic acid]-NaOH (pH 11.0) containing 100 μM NAD^+ and 1 mM DHU. One unit of enzyme activity was defined as the amount of enzyme that consumed 1 μmol of a substrate per min. For DHU analysis, *E. coli* cells cultivated in LB medium at 37°C were harvested at the early stationary phase (optical density at 600 nm [OD_{600}] = 2.5) and disrupted by sonication. Extraction of pyrimidine and dihydropyrimidines from the cell extracts and the following high-pressure liquid chromatography (HPLC) analysis were performed according to a previous paper (14). The mass spectrometric measurements were performed using an API 3000 triple-quadrupole instrument (PE Sciex, Toronto, Canada). A homology search was performed using the BLAST program at DDBJ (http://www.ddbj.nig.ac.jp/). Multiple alignments were obtained with the CLC sequence viewer (http://www.clcbio.co.jp/). Phylogenetic trees were constructed by the UPGMA (unweighted pair group method with arithmetic average) procedure using the CLC sequence viewer (bootstrap scores for 1,000 iterations).

Enzyme characteristics. We found that, unlike the dimeric NADPH-dependent mammalian DPDs, as-isolated EcDPD was a heterotetrameric iron-sulfur flavoenzyme composed of two PreT and two PreA subunits and that it catalyzed the reduction of uracil and thymine to DHU and 5,6-dihydrothymine (DHT), respectively, by using NADH as a specific cosubstrate. In addition to the reduction of pyrimidines, EcDPD catalyzed the reverse reaction (i.e., NAD^+-dependent oxidation of 5,6-dihydropyrimidine to pyrimidine). The enzyme also showed catalytic activity for 5-fluorouracil (0.67 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) but not for orotate. The enzyme showed the highest k_{cat} and k_{cat}/K_m values for uracil (Table 1). An amino acid sequence comparison of PreT with the N-terminal half of pig DPD indicated that Lys365, which is essential for binding the phosphate group of NADPH (6), is replaced by Glu286 in PreT. The negatively charged Glu286 may interfere with the binding of NADP(H) to the active site of EcDPD.

Analysis of pyrimidines and dihydropyrimidines in *E. coli* extracts. Although the genotype of the JW2133 strain is Δ*preT*, expression of neither PreT nor PreA was detected by Western blotting (data not shown), suggesting that the deletion of *preT* had a polar effect on the expression of the downstream *preA* gene. Therefore, the JW2133 strain was employed as a *preTA*-deficient (Δ*preT-preA*) strain in this study. The JW2133 strain grew similarly to the wild-type W3110 strain in LB medium. We analyzed the extracts of the wild-type and JW2133 strains by HPLC (Shimadzu, Kyoto, Japan) to investigate the occurrence of DHU. Four main peaks were detected in the extract from the W3110 strain (Fig. 2). The three peaks at 20.3, 33.1, and 42.5 min in the W3110 extract corresponded to DHU, uracil, and thymine, respectively. The ESI-MS (electrospray ionization-mass spectrometry) analysis also identified the compounds eluted at the retention times of 20.3, 33.1, and 42.5 min as $[DHU + H]^+$ (MW, 115), $[uracil + H]^+$ (MW, 113), and

TABLE 1. Kinetic parameters of EcDPD

Substrate	K_m (μM)	V_{max} ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
Uracil[a]	38	0.43	0.64	0.17×10^{-1}
Thymine[a]	87	0.26	0.39	0.45×10^{-2}
DHU[b]	160	0.44	0.65	0.41×10^{-2}
DHT[b]	130	0.18	0.28	0.22×10^{-2}

[a] Determined at pH 6.0.
[b] Determined at pH 11.0.

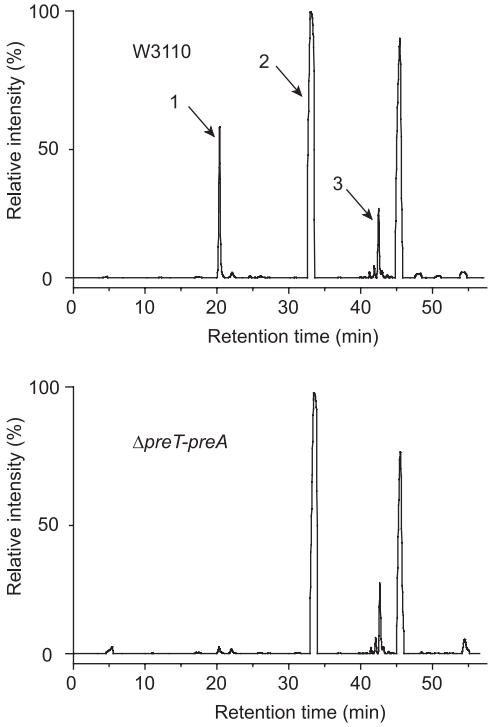


FIG. 2. HPLC analysis of extracts of *E. coli*. Pyrimidines and reduced pyrimidines extracted from the wild-type strain (W3110) and the EcDPD-deficient strain (Δ*preT-preA*) were separated by HPLC and detected at 220 nm. Peaks 1, 2, and 3 are those corresponding to DHU, uracil, and thymine, respectively. The *y* axes represent the relative absorbance intensities at 220 nm.

[thymine + H]$^+$ (MW, 127), respectively. In contrast, only three main peaks were detected in the JW2133 extract (Fig. 2). The peak at the retention time of 20 min was nearly absent in the JW2133 strain, unlike that of the wild-type strain. This phenotype was fully complemented with a plasmid expressing *preTA* (data not shown). These data suggest that *preTA* is important for the *in vivo* production of DHU and that EcDPD physiologically converts uracil into DHU. Although a chemical structure for the compound eluted at about 45.5 min could not be identified by LC-MS analysis, there was no difference in the peak intensities between the wild-type and *preTA*-deficient strains.

We investigated the time course of DHU production during the growth of the W3110 strain in LB media. The maximum production of DHU was observed at the early stationary phase (Fig. 3A), and the amount of DHU produced afterwards gradually decreased. We also measured the amount of DHU in the cultivation medium during the growth of the W3110 strain, but none was detected, suggesting that DHU produced in the cell is not excreted into the medium. This prompted the question of why DHU levels decreased in the wild-type cells after the early stationary phase. Therefore, we further examined the metabolic fate of DHU by radio-HPLC (Shimadzu) using [2-^{14}C]DHU. [2-^{14}C]DHU added to the cultivation medium was incorporated into the W3110 cells, and three main radiolabeled peaks were detected at 44, 62, and 68 min (Fig. 3B). The radiolabeled compounds with retention times of 44, 62, and 68 min were identified as DHU, uracil, and thymine, respectively, based on comparison with the retention times of

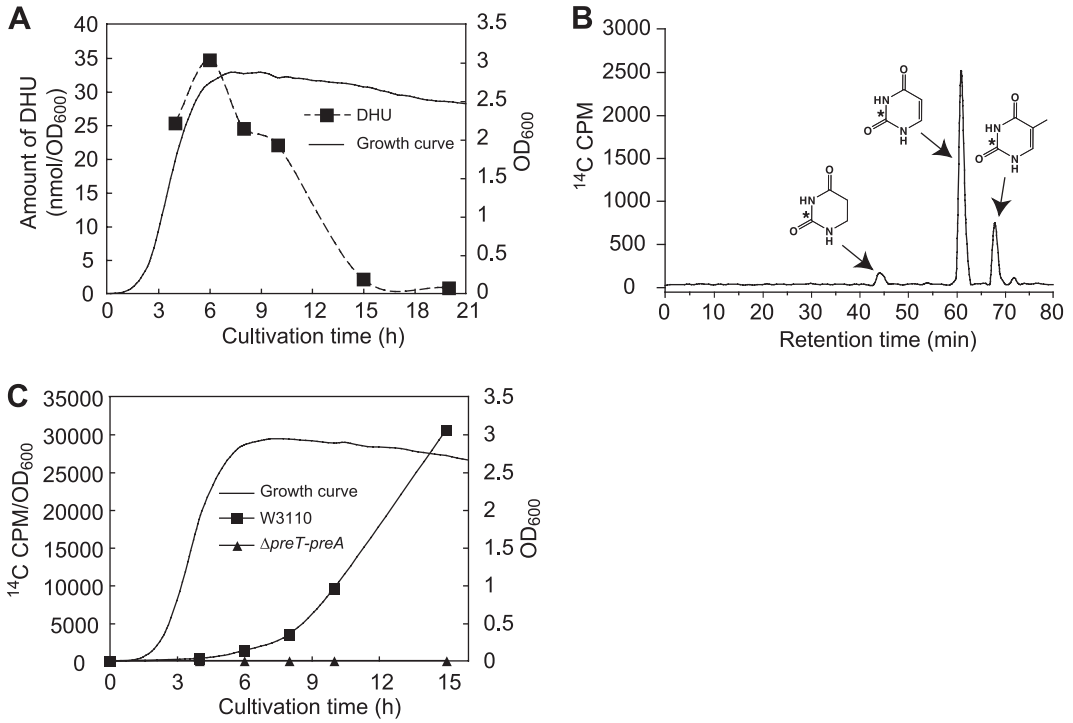


FIG. 3. Time course of DHU production in *E. coli* W3110 (A), analysis of metabolites derived from [2-^{14}C]DHU in *E. coli* W3110 (B), and the uptake of [2-^{14}C]DHU by W3110 and Δ*preT-preA* strains (C). (A) DHU in the cell extracts was determined at various time points during the growth of the wild-type W3110 strain in LB medium. (B) The radiolabeled extracts were prepared from the wild-type strain grown in LB medium containing 0.5 μM [2-^{14}C]DHU and were analyzed by radio-HPLC. (C) The radioactivity in W3110 and Δ*preT-preA* cells grown in LB medium containing 0.5 μM [2-^{14}C]DHU was measured by a liquid scintillation counter (Beckman Coulter, Brea, CA).

authentic compounds. Intriguingly, the radioactivity in the W3110 cells rapidly increased in early stationary phase, corresponding to the time course pattern of DHU production (Fig. 3C). The radiolabeled uracil and thymine formed from [2-^{14}C]DHU were incorporated into nucleic acids (i.e., DNA and RNA). In contrast, the radioactivity in the JW2133 cells was significantly lower than that in the wild-type strain (Fig. 3B). Because the radioactivity in the JW2133 cells slightly increased (up to 60 cpm/OD_{600} unit), [2-^{14}C]DHU was probably incorporated into the mutant strain. These results suggest that DHU formed from uracil by EcDPD in the cell during the early stationary phase is converted back to uracil and further metabolized to form thymine, with the pyrimidines thus formed eventually being incorporated into nucleic acids. Therefore, DHU may serve as a backup supply for the pyrimidine pool during the stationary phase of growth of *E. coli*. In fact, the amount of uracil was highest (66 nmol/OD_{600} unit) at the exponential phase, rapidly decreased to 17 nmol/OD_{600} unit at the early stationary phase, and then was maintained at a constant level during the stationary phase (data not shown).

Sequence analysis. On the basis of our experimental and published results and analysis of homologous sequences in bacterial genomes, DPDs can be classified into four types (Fig. 4). DPDs from mammals belong to type I; they are encoded as a single polypeptide and prefer NADPH as a cosubstrate. This subclass is phylogenetically distinct from bacterial DPDs. Type II comprises bacterial NADPH-dependent DPDs such as *P. aeruginosa* PydX-PydA, which are composed of two different polypeptides. The type II DPD proteins are widely distributed in various bacteria, including *P. putida* and *Brevibacillus agri*, and the coding genes are generally clustered with genes for the reductive pyrimidine catabolic pathway. Type III includes putative DPDs found in *Clostridium tetani* and *Solibacter usitatus*, which contain an NADPH-binding motif, but most or all of the genes homologous to other reductive pyrimidine catabolic pathway genes are missing in these bacterial genomes. The bacterial NADH-dependent DPDs, including EcDPD, consist of two different polypeptides and are classified as type IV. Type IV DPD is also found in *Salmonella* spp. and *Shigella* spp., which do not have a putative gene cluster for the reductive pyrimidine catabolic pathway. There is a relatively close sequence similarity between type III and type IV DPDs, but they are separated by the nicotinamide cofactor-binding motif.

The *rut* gene cluster involved in the Rut pathway consists of seven tandem genes, and their expression is negatively regulated by RutR, which is the master regulator of the genes involved in the synthesis and degradation of pyrimidines in *E. coli* (12, 17). Intriguingly, a gene encoding a RutR homolog is clustered with the genes involved in the reductive pyrimidine catabolic pathway in bacteria with a type II DPD gene (13). However, neither a RutR-homologous gene nor a RutR-binding consensus sequence is present around type III and IV DPD genes, suggesting that the gene regulation of type III and IV DPDs is independent of the Rut pathway. It is also interesting that *Salmonella* spp. have the RutR regulator but do not have the Rut pathway enzymes (10).

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (B) 19370040 (to N.E.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by a Grant-in-Aid for Encouragement of Young Scientists 21780094 (to H.M.).

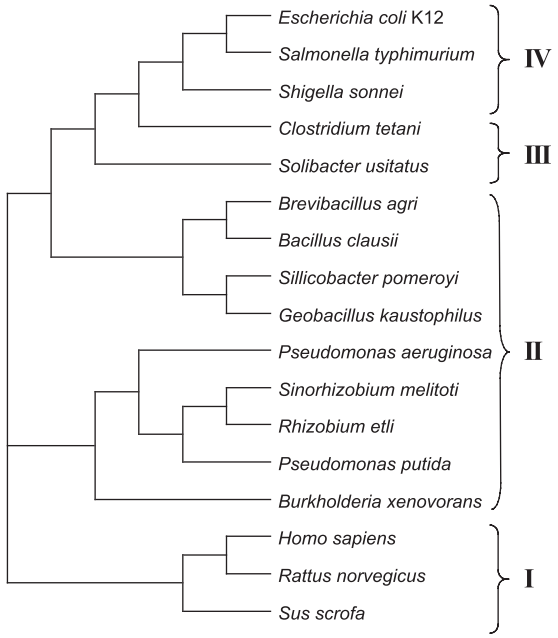


FIG. 4. Phylogenetic relationship between DPD family proteins. Subclassification into groups I, II, III, and IV is based not only on the phylogenetic relationship but also on the association with a gene cluster for the reductive pyrimidine catabolic pathway and on the nicotinamide-binding motifs. The subclassifications are as follows: type I, single polypeptide, mammalian, NADPH dependent, the reductive pyrimidine catabolic pathway; type II, heteromeric, bacterial, NADPH dependent, the reductive pyrimidine catabolic pathway; type III, heteromeric, bacterial, NADPH dependent, unknown function, no or incomplete reductive pathway; and type IV, heteromeric, bacterial, NADH dependent, unknown function, no reductive pathway. The accession numbers for the sequences are as follows: *Escherichia coli* K-12, P76440 and P25889; *Pseudomonas aeruginosa*, Q9I677 and Q9I678; *S. enterica* serovar Typhimurium, Q8ZNL8 and Q8ZNL7; *Shigella sonnei*, SSON_2202 and Q3Z059; *Clostridium tetani*, Q897C5 and Q897C4; *Solibacter usitatus*, Q01P60 and Q01P61; *Brevibacillus agri*, Q846U7 and Q846U6; *Bacillus clausii*, Q5WBE8 and Q5WBE7; *Silicibacter pomeroyi*, Q5LSJ2 and Q5LSJ1; *Geobacillus kaustophilus*, Q5L030 and Q5L029; *Sinorhizobium meliloti*, Q92N00 and Q92MZ9; *Rhizobium etli*, Q2K545 and Q2K544; *Pseudomonas putida*, Q88FQ1 and Q88FQ0; *Burkholderia xenovorans*, Q13WL3 and Q13WL4; *Rattus norvegicus*, O89000; *Homo sapiens*, Q12882; *Sus scrofa*, Q28943.

REFERENCES

1. **Campbell, L. L.** 1960. Reductive degradation of pyrimidines. 5. Enzymatic conversion of *N*-carbamyl-beta-alanine to beta-alanine, carbon dioxide, and ammonia. J. Biol. Chem. **235:**2375–2378.
2. **Campbell, L. L., Jr.** 1957. Reductive degradation of pyrimidines. I. The isolation and characterization of a uracil fermenting bacterium, *Clostridium uracilicum* nov. spec. J. Bacteriol. **73:**220–224.
3. **Campbell, L. L., Jr.** 1957. Reductive degradation of pyrimidines. II. Mechanism of uracil degradation by *Clostridium uracilicum.* J. Bacteriol. **73:**225–229.
4. **Campbell, L. L., Jr.** 1957. Reductive degradation of pyrimidines. III. Purification and properties of dihydrouracil dehydrogenase. J. Biol. Chem. **227:** 693–700.
5. **Campbell, L. L., Jr.** 1958. Reductive degradation of pyrimidines. IV. Purification and properties of dihydrouracil hydrase. J. Biol. Chem. **233:**1236–1240.
6. **Dobritzsch, D., G. Schneider, K. D. Schnackerz, and Y. Lindqvist.** 2001. Crystal structure of dihydropyrimidine dehydrogenase, a major determinant of the pharmacokinetics of the anti-cancer drug 5-fluorouracil. EMBO J. **20:**650–660.
7. **Kim, G. J., D. E. Lee, and H. S. Kim.** 2000. Functional expression and

characterization of the two cyclic amidohydrolase enzymes, allantoinase and a novel phenylhydantoinase, from *Escherichia coli*. J. Bacteriol. **182:**7021–7028.
8. **Kim, K. S., et al.** 2010. The Rut pathway for pyrimidine degradation: novel chemistry and toxicity problems. J. Bacteriol. **192:**4089–4102.
9. **Kim, S., and T. P. West.** 1991. Pyrimidine catabolism in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **61:**175–179.
10. **Loh, K. D., et al.** 2006. A previously undescribed pathway for pyrimidine catabolism. Proc. Natl. Acad. Sci. U. S. A. **103:**5114–5119.
11. **Mihara, H., R. Hidese, M. Yamane, T. Kurihara, and N. Esaki.** 2008. The *iscS* gene deficiency affects the expression of pyrimidine metabolism genes. Biochem. Biophys. Res. Commun. **372:**407–411.
12. **Nguyen Ple, M., I. Bervoets, D. Maes, and D. Charlier.** 2010. The protein-DNA contacts in RutR · carAB operator complexes. Nucleic Acids Res. **38:**6286–6300.
13. **Osterman, A.** 2006. A hidden metabolic pathway exposed. Proc. Natl. Acad. Sci. U. S. A. **103:**5637–5638.
14. **Remaud, G., M. Boisdron-Celle, C. Hameline, A. Morel, and E. Gamelin.** 2005. An accurate dihydrouracil/uracil determination using improved high performance liquid chromatography method for preventing fluoropyrimidines-related toxicity in clinical practice. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **823:**98–107.
15. **Schmitt, U., K. Jahnke, K. Rosenbaum, P. F. Cook, and K. D. Schnackerz.** 1996. Purification and characterization of dihydropyrimidine dehydrogenase from *Alcaligenes eutrophus*. Arch. Biochem. Biophys. **332:**175–182.
16. **Schnackerz, K. D., D. Dobritzsch, Y. Lindqvist, and P. F. Cook.** 2004. Dihydropyrimidine dehydrogenase: a flavoprotein with four iron-sulfur clusters. Biochim. Biophys. Acta **1701:**61–74.
17. **Shimada, T., K. Hirao, A. Kori, K. Yamamoto, and A. Ishihama.** 2007. RutR is the uracil/thymine-sensing master regulator of a set of genes for synthesis and degradation of pyrimidines. Mol. Microbiol. **66:**744–757.
18. **Simaga, S., and E. Kos.** 1981. Properties and regulation of pyrimidine catabolism in *Escherichia coli*. Int. J. Biochem. **13:**615–619.
19. **Tiedje, K. E., K. Stevens, S. Barnes, and D. F. Weaver.** 2010. Beta-alanine as a small molecule neurotransmitter. Neurochem. Int. **57:**177–188.
20. **Webb, M. E., A. G. Smith, and C. Abell.** 2004. Biosynthesis of pantothenate. Nat. Prod. Rep. **21:**695–721.
21. **West, T. P.** 1998. Isolation and characterization of an *Escherichia coli* B mutant strain defective in uracil catabolism. Can. J. Microbiol. **44:**1106–1109.
22. **West, T. P.** 1991. Pyrimidine base and ribonucleoside utilization by the *Pseudomonas alcaligenes* group. Antonie Van Leeuwenhoek **59:**263–268.
23. **West, T. P.** 2001. Pyrimidine base catabolism in *Pseudomonas putida* biotype B. Antonie Van Leeuwenhoek **80:**163–167.
24. **West, T. P.** 1994. Pyrimidine ribonucleoside catabolic enzyme activities of *Pseudomonas pickettii*. Antonie Van Leeuwenhoek **66:**307–312.
25. **Zrenner, R., et al.** 2009. A functional analysis of the pyrimidine catabolic pathway in *Arabidopsis*. New Phytol. **183:**117–132.