

Transthyretin-related proteins function to facilitate the hydrolysis of 5-hydroxyisourate, the end product of the uricase reaction

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Abstract Purine catabolic pathway in *Bacillus subtilis* is consisted of more than 14 genes. Among these genes, *pucL* and *pucM* are required for uricase activity. While PucL is known to encode the uricase itself, the function of PucM is still unclear although this protein is also indispensable for uric acid decomposition. Here, we provide evidence that PucM, a transthyretin-related protein, functions to facilitate the hydrolysis of 5-hydroxyisourate, the end product of the uricase reaction. Based on these results, we propose that transthyretin-related proteins present in diverse organisms are not functionally related to transthyretin but actually function as a hydroxyisourate hydrolase.

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1. Introduction

Purines are major components of nucleic acids and nucleotides and are continuously formed and degraded in biosphere [1]. In *Bacillus subtilis*, the natural purine bases serve as an alternative nitrogen source when the preferred nitrogen sources (e.g., glutamate plus ammonia or glutamine) are exhausted. Mutational analyses of the purine-utilizing system in *B. subtilis*, complemented with functional studies, led to the identification of the 14 *puc* genes and the *gde* gene consisting of the purine degradation pathway [2]. All 14 *puc* genes except *pucI* are located in six transcription units (Fig. 1A), which

are expressed when cells are grown with glutamate as the nitrogen source, but not when grown on glutamate plus ammonia (nitrogen catabolic control system). The urate oxidase (E.C. 1.7.3.3) activity has been assigned to the *pucL* and *pucM* genes (Fig. 1B). PucL shows a certain sequence identity, starting from Met171, to several known uricases (urate oxidase) and is therefore believed to encode the uricase protein itself [3]. By contrast, PucM, whose inactivation also results in an uricase-defective phenotype, shows certain sequence similarity to the eukaryotic transthyretin (TTR) proteins, implying that the role of this gene in the purine degradation pathway is possibly distinct from that of PucL [2]. In *Escherichia coli*, there exists a PucM orthologue protein YedX whose sequence similarity to PucM is very high. Like PucM, this protein also contains the conserved TRP signature sequence (YRGS) at its C-terminal end [4]. In several prokaryotes the gene encoding TRP is located within the operon for proteins playing roles in purine degradation, such as xanthine dehydrogenase, uricase, allantoinase, and ureidoglycolate hydrolase [4]. Thus far, a family of transthyretin-related proteins (TRPs) has been identified from 47 different species [4]. Notably, the four amino acid sequence motif (YRGS) at the C-terminus of the proteins clearly separates members of the TRP family not only from TTR but also from other sequences listed as TTR-like in databases (Fig. 1C).

To date the exact role of *pucM* gene product in uric acid oxidation in *B. subtilis* has not been clarified. It is partly due to the assumption that urate oxidase can directly convert urate to allantoin [5]. However, a number of studies demonstrated that allantoin is not the exact product of the urate oxidase reaction. Actually, urate oxidase catalyzes the conversion of urate to 5-hydroxyisourate (HIU), which is then spontaneously converted to allantoin under in vitro conditions [6,7]. The half-life of HIU at neutral pH is estimated to be about 30 min in vitro and nonenzymatic decomposition of HIU generates a racemic mixture of allantoin. However, allantoinase is specific for (*S*)-allantoin and the racemization of allantoin at neutral pH takes almost 10 h, suggesting that nonenzymatic conversion would be far too slow to support the pathway [8,9]. These observations led to the discovery of a novel enzyme, designated as HIU hydrolase (HIUHase), which facilitates the stereo-specific conversion of HIU to (*S*)-allantoin in soybean [10]. Here, we

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Abbreviations: HIU, 5-hydroxyisourate; HIUHase, 5-hydroxyisourate hydrolase; LC-MS, liquid chromatography-mass spectrometry; OHCU, 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazole; TRP, transthyretin-related protein; TTR, transthyretin

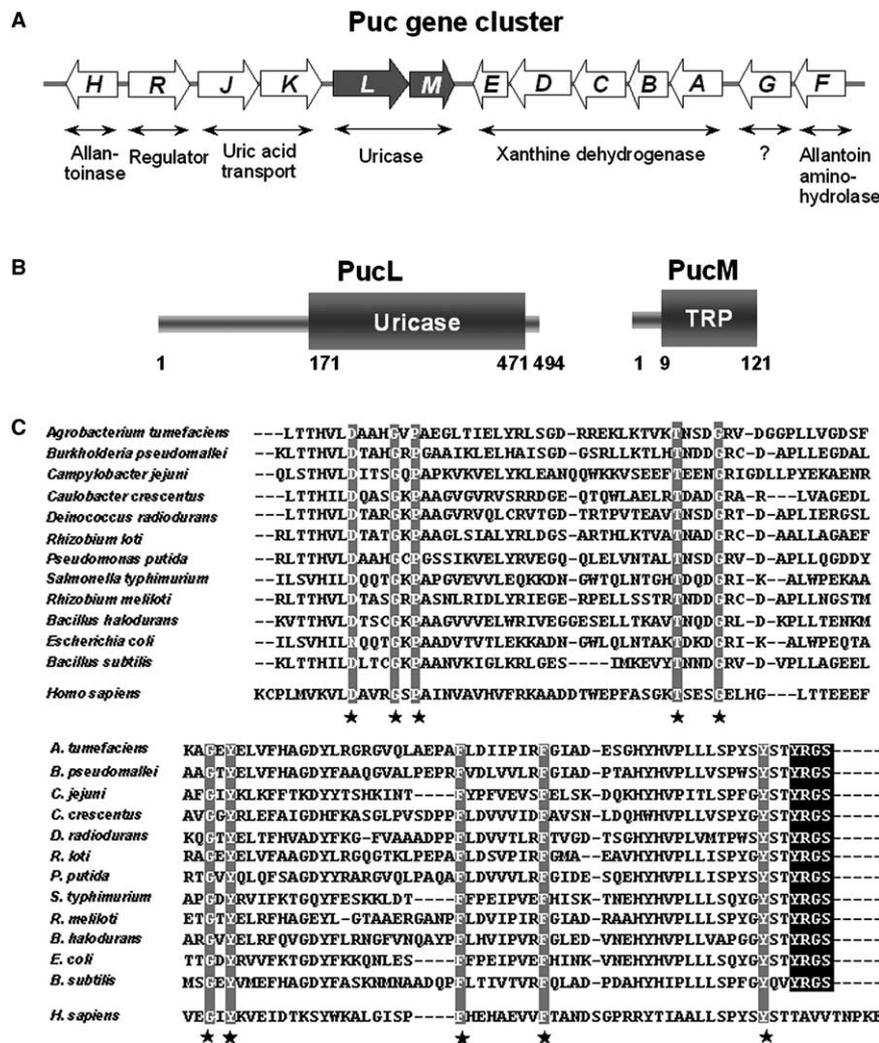


Fig. 1. The organization of *Bacillus* genes for purine catabolism and the sequence similarity among transthyretin-related proteins. (A) The organization of *Bacillus puc* gene cluster. (B) Schematic representation of PucL (uricase) and PucM proteins. The amino acid numbers are shown to indicate position of each domain in the respective proteins. (C) Multiple sequence alignment of transthyretin-related proteins (TRPs) present in micro-organisms and human transthyretin (TTR). The sequences are derived from GenBank database and the consensus sequences are highlighted and also indicated by an asterisk.

provide evidence that PucM as well as other TRPs (e.g., YedX) facilitate the hydrolysis of HIU and therefore can be re-designated as HIU hydrolases.

2. Materials and methods

2.1. Cloning of uricase, pucM, yedX, and hTTR

The 975-bp DNA fragment encoding PucL (uricase) (forward primer: 5'-CGCGGATCC ATG AAA AGA ACC ATG TC, reverse primer: 5'-ACGCGT CGA CAT TCA GGC TTT CAG GCT CC) and the 366-bp DNA fragment encoding PucM (forward primer: 5'-CGCGGATCC ATG TCG GAG CCT GAA AGC, reverse primer: 5'-ACGCGT CGA CAT TTA ACT CCC CCT ATA CAC C) were amplified by PCR from the genome of *Bacillus subtilis* 168. The 370-bp DNA fragment encoding YedX (forward primer: 5'-CGGGATCC ATG GCA CAA CAA AAC ATT C, reverse primer: 5'-ACGCGT CGA CTT AAC TGC CAC GAT AGG TTG) was amplified by PCR from the genomic DNA of *E. coli*. Human TTR gene (forward primer: 5'-CGC GGATCC GGC CCT ACG GGC ACC GGT G, reverse primer: 5'-ACGCGT CGA CAT TCA TTC CTT GGG ATT GGT G) was also amplified from the human liver cDNA library by

PCR using a pair of the primers covering the coding sequence of the mature protein (without the predicted signal sequence). The resulting PCR products (containing the *Bam*HI and *Sal*I sites; underlined) were subsequently cloned into the expression vector pGEX-6p-1 (Pharmacia) for the production of GST-tagged recombinant proteins in *E. coli*.

2.2. Protein expression and purification

Plasmids carrying GST-tagged uricase, PucM, hTTR, and YedX were introduced into *E. coli* BL21-DE3 and the transformed bacteria were grown at 30 °C. Purification of the GST-fusion proteins was performed by using Glutathione-Sepharose 4B (Amersham Pharmacia Biotech) in a batch absorption mode. GST moiety was removed from GST-fusion proteins by using Precision Protease™ as described in the manufacturer's manual. SDS-PAGE analysis of purified proteins revealed that all proteins were highly pure (~98%; Fig. 2).

2.3. Enzyme assays

Urate oxidase activity was assayed by monitoring the changes in absorbance at 290 nm, which is caused by the enzymatic oxidation of uric acid [8]. The assay mixture (final volume of 1.0 ml) consisted of 50 mM potassium phosphate buffer (pH 7.5), 50 μM urate, and 0.06 μM purified enzyme. The assays were carried out aerobically at 30 °C unless otherwise specified.

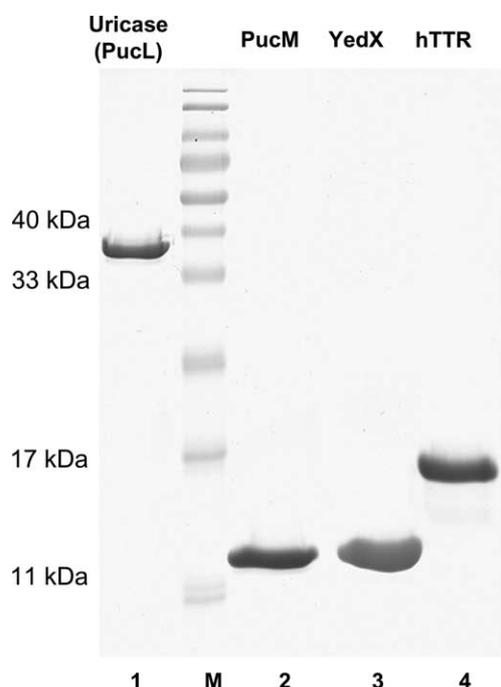


Fig. 2. SDS-PAGE analysis of purified recombinant proteins. Recombinant proteins purified by using GSH-agarose beads (PucL, 3 μ g; other proteins, 5 μ g) were separated in a 15% SDS-PAGE gel. Lane 1, *B. subtilis* Uricase (PucL); M, Molecular-weight standards (PageRuler™ Prestained Protein Ladder); Lane 2, *B. subtilis* PucM; Lane 3, *E. coli* YedX; Lane 4, Human TTR.

HIU hydrolase activity was assayed for its ability to catalyze the hydrolysis of HIU [8]. HIU was generated in situ (in 1-cm path-length cuvette) by the addition of 0.06 μ M recombinant uricase to a 1-ml of 50 mM potassium phosphate buffer (pH 7.5) containing 50 μ M urate. Sufficient amount of uricase was used to convert the urate to HIU within 10 min. When the urate oxidase reaction almost reached its end-point, as determined by monitoring the absorbance at 292 nm, PucM, YedX or hTTR (0.06 μ M each) was added to the reaction mixture. The disappearance of HIU was subsequently monitored at 312 nm.

2.4. LC-MS analysis of the reaction products

Urate (purity >99%) was prepared at a concentration of 100 ng/mL in HPLC-grade water. Electrospray ionization mass spectrometry was performed using a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron Co., USA) equipped with a Finnigan electrospray source. The system was operated by using Xcalibur software (version 1.3 SP2, Thermo Electron). HPLC separations were performed on the Finnigan Surveyor™ Modular HPLC Systems (Thermo Electron Co., USA), using a TSK-GEL Amide-80 column (5 μ m, 2.0 \times 150 mm, Tosoh Co., Japan). 20 μ l of enzyme reaction mixture (incubated for 10 min after adding PucM) was loaded and eluted isocratically with 60% acetonitrile in 10 mM ammonium acetate (pH 8.0) for 10 min at a flow rate of 0.2 ml/min.

Mass analysis was performed with an electrospray ionization (ESI) source in the negative ion mode. The spray needle voltage was 5 kV, ion transfer capillary temperature at 200 °C, nitrogen sheath gas flow rate at 60 arbitrary units, and auxiliary gas flow rate setting at 5 arbitrary units. The ion trap containing helium damping gas was introduced according to the manufacturer's recommendations. Full-scan mass spectra were obtained in the range m/z 50–300, with 3 microscans and a maximum ion injection time of 300 ms. For identification of the enzyme reaction products and intermediates, such as HIU, 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazole (OHCU), and allantoin (Fig. 6), the instrument was operated in the selected ion monitoring (SIM) mode and tandem mass spectrometry (MS/MS). All experiments were controlled by the menu-driven software provided with the system and performed under automatic gain control conditions.

3. Results

3.1. Uricase activity is accelerated by PucM but not by human transthyretin

Since it was uncertain how PucM influences uric acid catabolism, we first tested whether PucM protein affects the activity of uricase (PucL). By monitoring the decay of urate at 290 nm, we measured the enzymatic activity of purified uricase (0.06 μ M) in the absence or presence of PucM. Although uricase was capable of decomposing uric acid without PucM for 10 min in a linear mode, its activity was markedly accelerated upon the addition of an equal molar amount of PucM (Fig. 3). In contrast to PucM, human transthyretin (hTTR) did not exert any effect on the uricase activity (data not shown). The finding that uric acid decomposition is greatly enhanced only by PucM suggested that PucM is functionally different from hTTR even though they share some sequence similarity. Although PucM was shown to facilitate the decomposition of uric acid, it was still unclear whether PucM functions as an additional enzyme promoting the removal of the end product of uricase reaction or as an activator of uricase. Interestingly, other studies have revealed that uric acid is first decomposed into HIU and then eventually hydrolyzed to allantoin [6,7]. These findings, together with our results, led us to speculate that *Bacillus pucM* mutant grows slowly because it cannot quickly convert HIU into allantoin, which may require PucM function.

3.2. PucM and other transthyretin-related proteins but not transthyretin exhibit HIU hydrolase activity

To figure out if PucM indeed facilitates the decay of HIU, we measured the effect of PucM on the decomposition of HIU by monitoring the absorbance at 312 nm. In our assay conditions, within 10-min incubation of urate with purified uricase, the production of HIU reached the maximum level and then decreased slowly afterward (Fig. 4A). As expected, addition of purified PucM (0.06 μ M) greatly accelerated the disappearance of HIU in the reaction mixture. The effect of PucM on HIU decay was quite dramatic; the half-life of HIU in the presence of PucM was less than a minute while that in the absence of PucM was more than 30 min (Fig. 4B). Next, to determine if YedX, *E. coli* TRP, is also functionally similar to PucM, we measured the HIU decomposing activity of YedX. Indeed, YedX exhibited HIU hydrolyzing activity and its effect was comparable to that of PucM (Fig. 4B). To rule out the possibility that this effect was due to the presence of a transthyretin-like domain in these proteins, we tested if hTTR is also capable of HIU hydrolysis. Similar to its inability to increase uric acid decomposition, hTTR again failed to promote the hydrolysis of HIU in the assay condition (Fig. 4B). Collectively, these results led us to conclude that the TRPs, but not TTR itself, are in fact an enzyme facilitating the hydrolysis of HIU.

3.3. LC-MS/MS analysis of reaction products also demonstrates that HIU is decomposed by PucM

To further confirm that PucM mediates the hydrolysis of HIU, we analyzed the products generated from the uricase reaction – in the absence or presence of PucM – with an LC/MS spectrometer. When urate (m/z 167, $[M-H]^-$) was incubated with uricase alone, the mass peak for HIU (m/z 183, $[M-H]^-$) appeared at the expected position (Fig. 5A and

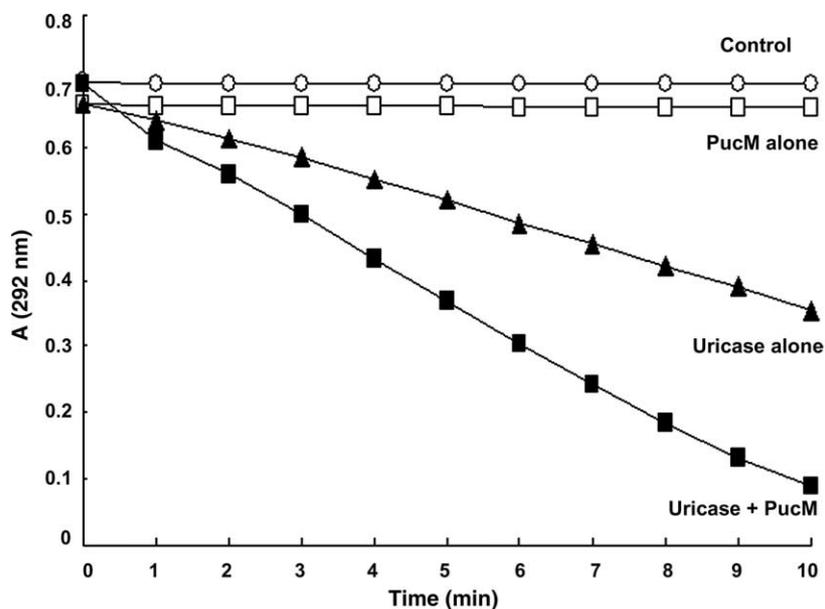


Fig. 3. Acceleration of uric acid decomposition by purified PucM. Uricase reaction was monitored by decrease of absorbance at 290 nm. Before the addition of uric acid, an equal amount of PucM (0.06 μ M) was pre-incubated with uricase for 5 min and then urate was added in the solution to start the reaction.

B). In addition, small mass peaks for OHCU (m/z 201, $[M-H]^-$; the intermediate between HIU and allantoin, Fig. 6) and allantoin (m/z 157, $[M-H]^-$; the final product of

uric acid decomposition, Fig. 6) appeared indicating that a fraction of HIU is hydrolyzed spontaneously in the absence of PucM (Fig. 5B). When PucM was added to the reaction

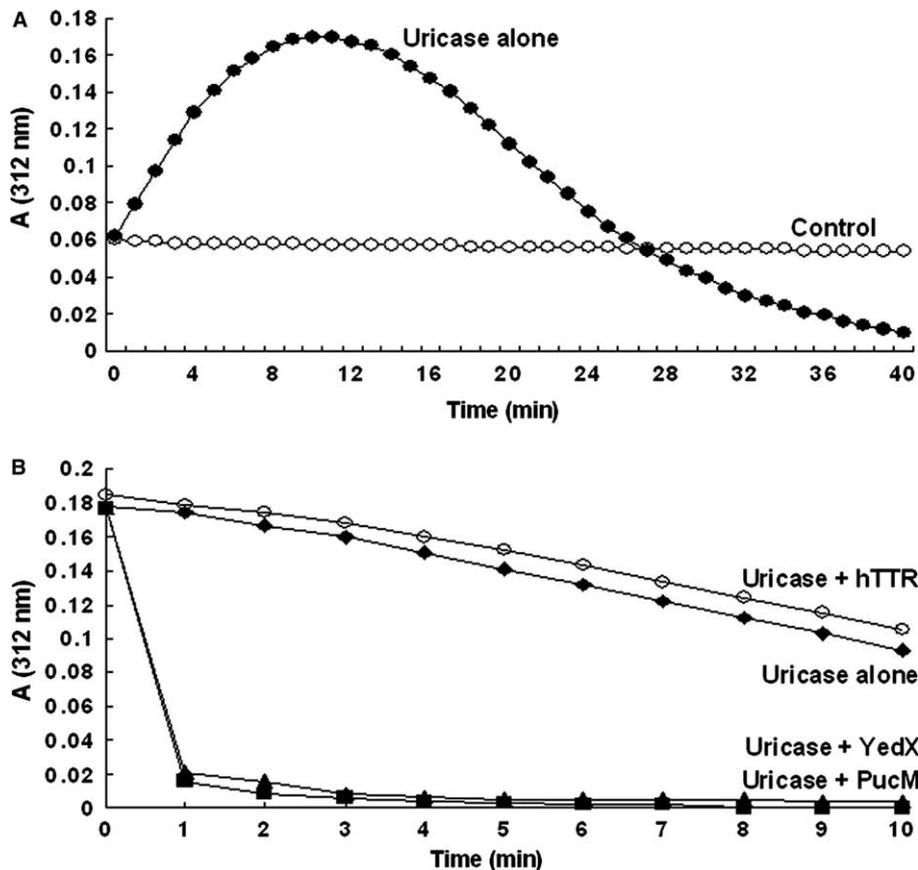


Fig. 4. HIU hydrolase activities of PucM, YedX and hTTR. The recombinant uricase was pre-incubated with urate for 10 min to generate HIU from urate. After addition of PucM, YedX, or hTTR in the reaction mixture, HIUase activity of these proteins was monitored by measuring the absorbance at 312 nm.

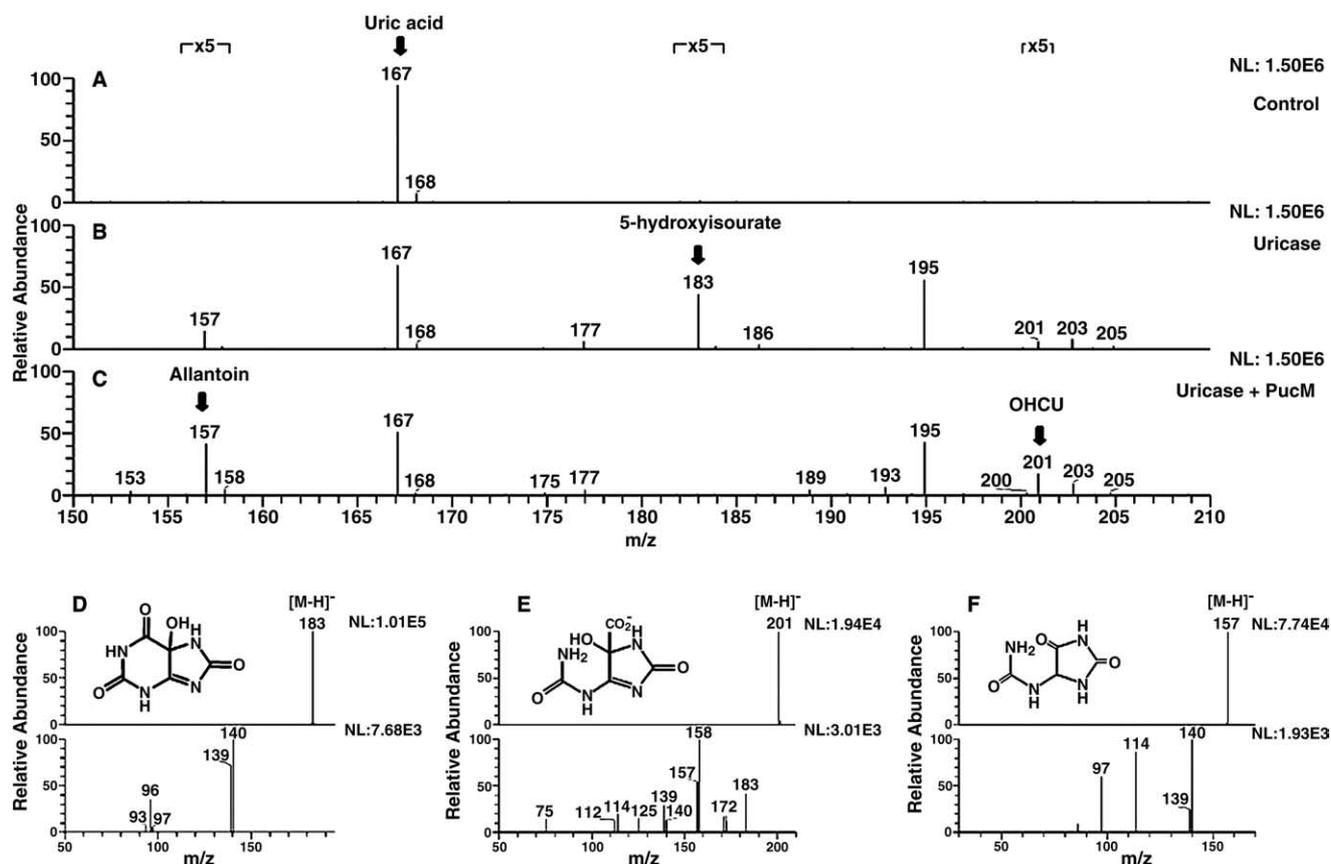


Fig. 5. LC–MS and LC–MS/MS analysis of the products derived from the reaction mixture containing uricase (PucL) and/or HIUHase (PucM). (A) Mass peaks obtained from uric acid only. (B) Mass peaks from the reaction mixture of uric acid plus PucL (uricase). Samples were immersed in a water bath at 30 °C. After 5 min, reaction mixture was withdrawn for analysis. (C) Mass peaks from the reaction mixture of uric acid, PucL and PucM. Reaction mixture was analyzed without incubation. (D) Reference mass peaks for 5-hydroxyisourate. (E) Reference mass peaks for OHCU. (F) Reference mass peaks for allantoin. All mass peaks were obtained at negative ion mode.

mixture, the mass peak for HIU rapidly disappeared and at the same time the mass peaks for OHCU and allantoin increased substantially (Fig. 5C). As a control, we performed independent LC–MS/MS analysis of allantoin, HIU, and OCHU, which were used as standards to compare the mass peaks from the reaction mixture (Fig. 5D–F). These results from LC/MS analysis clearly supported our conclusion that PucM is indeed an HIU hydrolase.

4. Discussion

TRPs are a group of proteins exhibiting sequence similarity to TTR and exist in a wide range of species. Until recently, TRPs had been considered an ancestor of TTR. However, the physiological role of TRPs remains undefined even though these two groups of proteins are structurally similar. Interestingly, a thyroxine binding assay with *E. coli* TRP and hTTR demonstrated that the bacterial TRP did not bind to thyroxine [4]. In fact, the considerable sequence similarity at the binding site within the TRP family, which may be designed for a yet unknown ligand, different from thyroid hormones, hints that this family of proteins may carry out a distinct function [2,4]. In *Bacillus subtilis*, PucM is expressed as part of an operon (*pucKJLM*) including urate transport proteins and a putative uricase (PucL). Interestingly, *pucM* mutant exhibited an

uricase-defective phenotype [2]. Although the possible functions of PucM in uric acid catabolism have been discussed (e.g., building of the superstructure including uricase and transporter proteins), the precise role of this TRP in purine metabolism has yet to be delineated. Our conclusion that PucM may function as HIUHase that facilitates conversion of HIU into allantoin possibly through the production of the intermediate OHCU is the first ever proposed function for TRP (Fig. 6). A wide range of species has uricase orthologues but their metabolic role varies [11]. For example, *E. coli* is not known to utilize purines, other than adenine and adenosine, as nitrogen sources. *E. coli* can produce $^{14}\text{CO}_2$ from a minimal medium containing [^{14}C] adenine, which implies allantoin production. However, neither ammonia nor carbamoyl phosphate was produced, which indicates that purine catabolism is incomplete and does not provide nitrogen during nitrogen-limited growth [12]. These findings, together with our results obtained with *E. coli* TRP, suggest that YedX is likely required for the complete purine catabolic pathway in *E. coli*.

Recently, the plant gene encoding hydroxyisourate hydrolase was identified from soybean (*Glycine max*) [13]. This gene encodes a protein that is 560 amino acids in length and contains a 31-amino acid signal sequence at N-terminus. The presence of two SKL motifs near C-terminus suggests that this HIUHase may reside in the peroxisomes. Sequence analysis revealed considerable homology between this protein and

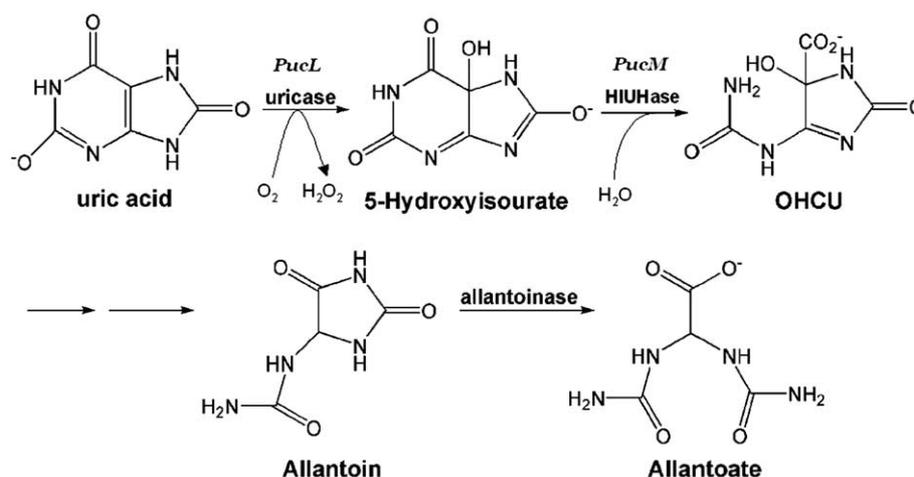


Fig. 6. Schematic representation of uric acid catabolic pathway catalyzed by uricase (PucL) and HIUHase (PucM). Decomposition of uric acid to 5-hydroxyisourate (HIU) is mediated by uricase (PucL) and the subsequent hydrolysis of HIU and production of 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazole (OHCU) is catalyzed by hydroxyisourate hydrolase (HIUHase, PucM), respectively (modified from Raychaudhuri and Tipton [1]).

members of the β -glucosidase family of enzymes. This plant HIUHase gene, however, does not show any similarity with TRP genes studied in this report (data not shown). Interestingly, there also exists a separate TRP gene in soybean (GenBank: BE824466) [5], raising a possibility that it could function as another type of HIUHase similar to PucM or YedX. Therefore, in future studies, it needs to be determined if the plant HIUHase (structurally unrelated) and/or TRP gene can carry out similar function as PucM or YedX.

Although more biochemical studies are still required to conclude that TRPs can serve as a real functional enzyme involved in uric acid metabolism, current findings nevertheless prompt us to propose that TRPs from diverse species are HIUHase, which catalyzes a previously under-recognized but important step in the purine catabolic pathway.

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