γ-Glutamylputrescine Synthetase in the Putrescine Utilization Pathway of Escherichia coli K-12*

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Glutamate-putrescine ligase (γ-glutamylputrescine synthetase, PuuA, EC 6.3.1.11) catalyzes the γ-glutamylation of putrescine, the first step in a novel putrescine utilization pathway involving γ-glutamylated intermediates, the Puu pathway, in Escherichia coli. In this report, the character and physiological importance of PuuA are described. Purified non-tagged PuuA catalyzed the ATP-dependent γ-glutamylation of putrescine. The Km values for glutamate, ATP, and putrescine are 2.07, 2.35, and 44.6 mM, respectively. There are two putrescine utilization pathways in E. coli: the Puu pathway and the pathway without γ-glutamylation. Gene deletion experiments of puuA, however, indicated that the Puu pathway was more critical in utilizing putrescine as a sole carbon or nitrogen source. The transcription of puuA was induced by putrescine and in a puuR-deleted strain. The amino acid sequences of PuuA and glutamine synthetase (GS) show high similarity. The molecular weights of the monomers of the two enzymes are quite similar, and PuuA exists as a dodecamer as does GS. Moreover the two amino acid residues of the two enzymes are conserved in PuuA, and it was experimentally shown that the corresponding amino acid residues in PuuA were involved in the metal-catalyzed oxidation similarly to GS. It is suggested that the intracellular concentration of putrescine is optimized by PuuA transcriptionally and posttranslationally and that excess putrescine is converted to a nutrient source by the Puu pathway.

γ-Glutamyl linkage is an amide linkage between the γ-position carboxyl group of glutamate and an amino group of various compounds. Compounds that have a γ-glutamyl linkage are called γ-glutamyl compounds, which are widely found in both prokaryotic and eukaryotic cells. For example, the peptidoglycan of Escherichia coli has a γ-glutamyl linkage between D-glutamate and meso-2,6-diaminopimelic acid (1), the virulence of Bacillus anthracis is dependent on a capsule made of poly-γ-glutamic acid (2), theanine (γ-glutamyethylamide) is a major “umami” component of Japanese green tea (3), and glutathione (γ-glutamylcysteinylglycine) is a very important antioxidant (4) in living cells.

Putrescine is one of the polyamines that are found in a wide range of organisms from bacteria to plants and animals, and are critical for cell proliferation, differentiation, and transformation; and are involved in DNA, RNA, and protein synthesis as well as in stabilizing membrane and cytoskeletal structures (5, 6). An increased concentration of polyamine is observed in cancer cells (7), there is a significantly elevated concentration (more than millimolar) of putrescine in plants under various stress conditions, and its concentration is very high (estimated to be over 30 mM) in E. coli cells (8).

We previously reported (9) that putrescine imported from medium by PuuB, a putrescine importer, is degraded to succinic semialdehyde, a precursor of succinate, via γ-glutamyl intermediates by products of the puu gene cluster (Fig. 1). In this metabolic pathway, which we named the Puu pathway (Fig. 2), putrescine is first γ-glutamylated to γ-Glu-Put by PuuA (glutamate-putrescine ligase, γ-Glu-Put synthetase, EC 6.3.1.11). Secondarily γ-Glu-Put is oxidized to γ-Glu-γ-butyraldehyde by PuuB (γ-Glu-Put oxidase) and further oxidized to γ-glutamyl-γ-aminobutyrate (γ-Glu-GABA) by PuuC (γ-Glu-γ-butyraldehyde dehydrogenase). Then γ-Glu-GABA is hydrolyzed to glutamate and GABA by PuuD (γ-Glu-GABA hydrolase) (10), and then GABA is deaminated to succinic semialdehyde by PuuE (GABA:α-ketoglutarate aminotransferase). PuuA catalyzes the first reaction of this Puu pathway as shown below (Scheme 1).

**SHEME 1**

Glutamate + Putrescine + ATP → γ-Glutamylputrescine + H2O + ADP + P1

Putrescine has been reported to be degraded without γ-glutamylation (11–13)(not γ-glutamylated) to γ-aminobutyraldehyde by YgiG (putrescine:α-ketoglutarate aminotransferase)

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Puua was initially annotated as the putative glutamine synthetase (GS) by computer analysis based on the amino acid sequence. GS (14) catalyzes the condensing reaction of glutamate and ammonia with the aid of ATP (Scheme 2).

Glutamate + Ammonia

\[ \text{Glutamine} \rightarrow \text{GABA} + \text{H}_2\text{O} + \text{ATP} \]

\[ \text{P}_1 \]

SCHEME 2

Schemes 1 and 2 are very similar because both reactions are \( \gamma \)-glutamylation, the condensing reaction yielding an amide linkage between the \( \gamma \)-carboxyl group of glutamate and the amino group of putrescine or ammonia using ATP.

In this study, we report that Puua is an important enzyme that catalyzes the first step of the Puu pathway and is regulated by a complicated regulation system. We also discuss the similarities between GS and Puua.

MATERIALS AND METHODS

Strain and Plasmid Construction

The strains, plasmids, and oligonucleotides used in this study are listed in Table 1. The strains were derivatives of \( E. \text{coli} \) K-12. Strain SH639 (15) has a deletion of the ggt gene. P1 transduction, DNA manipulation, and transformation were performed by the standard methods (16, 17). A DNA fragment of Kohara phage 257 (18) containing the puu gene cluster was used to make plasmids. The cloned regions of DNA on plasmids and the deleted regions of DNA are summarized in Fig. 1. In strain SK212, disruption of the puuR gene was carried out as follows. The 4.5-kb EcoRV-EcoRV fragment, including puuD, was isolated from Kohara phage 257 and cloned between the XmnI site and the blunt-ended ScaI site of pACYC184 to obtain plasmid 2. The 220-bp region between EcoRI and XmnI sites was replaced with the 1.2-kb HincII kanamycin resistance cassette of pUC4K. The plasmid was linearized using Sall. Homologous recombination was performed using this linearized plasmid to delete residues 65–137 of a putative 185-residue PuuR protein (Fig. 1).
| Strain, plasmid, or oligonucleotide | Characteristic or sequence | Source or Ref. |
|------------------------------------|--------------------------|----------------|
| Strain                            |                          |                |
| KP7500                            | W3110 but F lacZAM15 galK2 gafT22 λ lambda in (rrnD-rrnE)1 | T. Miki        |
| JD2571                            |                          | T. Miki        |
| JD24090                           |                          | T. Miki        |
| MG165                             | F prototrophic           | C. A. Gross    |
| SK121                             | F ΔgJ-2                  | 15             |
| SK21                              | ΔψψR:kan^+               | This study     |
| SK231                             | pkD6/F ΔψψCRE::(FRT-kan^-) -FRT | This study     |
| SK237                             | Sh6.39 but ΔψψDR::CRE::FRT | This study     |
| SK293                             | pBR322/SK287             | This study     |
| SK308                             | pBelobac1.1/SH639        | This study     |
| SK310                             | Sh6.39 but Δψψac::2-FRT | This study     |
| SK326                             | pSK324/YG110             | This study     |
| SK327                             | pSK325/YG110             | This study     |
| SK350                             | Sh6.39 but Δψψc::mini-Tn10kan | This study     |
| SK351                             | Sh6.39 but Δψψac::2-FRT Δψψc::mini-Tn10kan | This study     |
| SK352                             | Sh6.39 but Δψψc::mini-Tn10kan | This study     |
| SK353                             | Sh6.39 but Δψψac::2-FRT Δψψc::mini-Tn10kan | This study     |
| SK50                              | rkΔm3ΔCA::(FRT-cat^-)FRT | This study     |
| SK58                              | Δψψac::(FRT-cat^-)FRT ΔψψCRE::(FRT-kan^-) -FRT | This study     |
| SK62                              | pBR322/SO58              | This study     |
| SK63                              | pkHG8/SO58               | This study     |
| SK97                              | pSO97/YG110              | This study     |
| SK106                             | pSO82/SK310              | This study     |
| SK115                             | pBelobac1.1/SK310        | This study     |
| TK251                             | pkD66/MG1655             | Laboratory stock |
| KG110                             | F λds6gal (t6^- m6^-) ΔψψR::kan^+ (DE3) | 18             |
| Plasmid                           |                          |                |
| pACYC184                          | pl5A replicon cat^- tet^- | New England Biolabs |
| pBelobac11                        | Mini-F replicon cat^-    | New England Biolabs |
| pBR322                            | ColE1 replicon mpr^- bla^- tet^- | New England Biolabs |
| pKD3                              | oriB bla^- FRT-cat^- -FRT | 19             |
| pKD12                             | oriB bla^- FRT-cat^- -FRT | 19             |
| pKD46                             | oriR101 replicon repA101"araC^- gan^-bat^-exo^-"(araR)_bla^- | This study     |
| pKHG3                             | pl5A replicon mpr^- tet^- puuA^- puuD^- puuR^- | This study     |
| pKHG8                             | ColE1 replicon mpr^- bla^- puuA^- | This study     |
| pSK169                            | ColE1 replicon bla^- puuC^- puuB^- puuE^- | This study     |
| pSK324                            | ColE1 replicon bla^- puuACpkuupu (T7p) | This study     |
| pSK325                            | ColE1 replicon bla^- puuACpkuupu (T7p) | This study     |
| pSK92                             | Mini-F replicon cat^- puuA^- | This study     |
| pSO97                             | ColE1 replicon bla^- puuA^- (T7p) | This study     |
| pSO105                            | ColE1 replicon bla^- puuACpkuupu (T7p) | This study     |
| pUC19                             | ColE1 replicon bla^- | New England Biolabs |
| Oligonucleotide                   |                          | GE Healthcare  |
| pKD3-2                            | 5'-CTATGAGATATCCCTCTTTA-3' |                |
| pKD3-1                            | 5'-GCTGAGCTGCGGCTACACCC-3' |                |
| pKD3-4                            | 5'-CTCTGGAGGCAGCTCAACCC-3' |                |
| puuA-1                            | 5'-CTTGTGGTGAGGCTCAACCC-3' |                |
| puuA-2                            | 5'-CTCTGGAGGCAGCTCAACCC-3' |                |
| puuA-3                            | 5'-CTCTGGAGGCAGCTCAACCC-3' |                |
| puuA-4                            | 5'-CTCTGGAGGCAGCTCAACCC-3' |                |
| puuA-11                           | 5'-CTCTGGAGGCAGCTCAACCC-3' |                |
| puuA-RT1                          | 5'-GAAGCCGAGAACACACACC-3' |                |
| puuA-RT2                          | 5'-GAAGCCGAGAACACACACC-3' |                |
| gapA-RT1                          | 5'-TGAAGCCTGCGGCTAAT-3' |                |
| gapA-RT2                          | 5'-TGAAGCCTGCGGCTAAT-3' |                |
γ-Glutamylputrescine Synthetase of E. coli K-12

SK310, the puuA gene was disrupted according to the method of Datsenko and Wanner (19) using oligonucleotides puuA-1 and puuA-2. This strain deleted the predicted ATP binding motif of PuuA (Figs. 1 and 8). In S058, disruptions of puuADR and puuCBE were performed by the modified method of Datsenko and Wanner (19) as follows. To disrupt puuCBE, the DNA fragment of Kohara clone 257 (18), containing the puuCBE gene region, was digested with NarI and HincII and cloned into pUC19 digested with NarI and HincII to obtain pSK169. The FRT-kan+/-FRT fragment was amplified by PCR using oligonucleotides pKD13-1 and pKD13-4 as primers and plasmid pKD13 as a template with KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The PCR product was ligated with the 3.9-kb DraIII (blunt-ended with a Blunting kit (Takara, Kyoto, Japan)) and EcoRV fragment of pSK169, which deleted all of the puuB and most of the puuC and puuE genes (Fig. 2). The obtained plasmid was cleaved with HindIII and AatII, and the 3.0-kb fragment was used to transform TK251 by electroporation at 30 °C. Kanamycin-resistant transformant SK231 was obtained. To disrupt puuADR, the FRT-cat+/-FRT fragment was amplified by PCR using oligonucleotides pKD13-1 and pKD13-2 as primers and plasmid pKD13 as a template with KOD-plus DNA polymerase. The PCR product was ligated with the 4.7-kb XmnI and HpaI fragment of pKHG3, which deleted all of the puuAD and most of the puuR genes. The obtained plasmid was cleaved with SacI and Nrlu, and the 2.8-kb fragment was used to transform SK231 by electroporation. Chloramphenicol-resistant transformant S056 was obtained. Then S058 was made by P1 transduction of the ΔpuuADR::(FRT-cat+/-FRT) ΔpuuCBE::(FRT-kan+/-FRT) alleles from S056 to SH639. To construct pKHG8, a 2.0-kbp Smal and Hpal fragment of pKHG3 was ligated with pBR322 digested with EcoRV and Nrlu. pSO105, which carries puuA42852N, was constructed with the QuickChange technique (Stratagene) using oligonucleotides puuA-3 and puuA-4 as primers and pSO97 as a template but using KOD-plus DNA polymerase (Toyobo). pSK324, which carries puuA42852N, was similarly constructed using oligonucleotides puuA-5 and puuA-6.

Media and Growth of Bacteria

In all experiments, except when studying the influence of the overexpression of native PuuA on protein purification, strains were grown at 37 °C with reciprocal shaking at 140 rpm in 60 ml of medium in a 300-ml Erlenmeyer flask. M9-tryptone (M9 minimal medium 16) except that 1% Bacto tryptone was used instead of 0.2% glucose was used in the analysis of the intracellular amino acid and polyamine profiles. In the study to determine whether E. coli can grow using putrescine as the sole source of nitrogen, W-Glu-Put medium (W salts minimal medium 20) containing 0.4% glucose as the sole carbon source and 0.2% putrescine as the sole nitrogen source) was used. To determine whether E. coli can grow using putrescine as the sole source of carbon, M9-put-AS medium (M9 minimal medium 16) except that 0.4% putrescine and 0.4% ammonium sulfate were used instead of 0.2% glucose and 0.2% ammonium chloride, respectively) was used. In growth experiments in carbon- and nitrogen-limited medium, strains were precultured on an LB plate at 37 °C, streaked on a nutrient-limited plate, and incubated at 20 °C. To overexpress mutagenized PuuA, strains were grown in 60 ml of LB broth containing 100 μg/ml ampicillin at 37 °C with shaking at 140 rpm in a 300-ml Erlenmeyer flask. To overexpress native PuuA for protein purification, strains were grown in 200 ml of LB broth containing 100 μg/ml ampicillin at 37 °C with shaking at 140 rpm in a 1-liter Erlenmeyer flask. γ-Glu-Put was enzymatically synthesized and purified as described previously (9).

Analysis of Amino Acids and Polymamines

Amino acids and polymamines in the samples were measured using an HPLC system (model LC-20AD; Shimadzu, Kyoto, Japan) equipped with a Shim-pack Amino-Na column (Shimadzu) with gradient elution at 60 °C at a flow rate of 0.6 ml/min or using an HPLC system (model LC-20AD; Shimadzu) equipped with a TSKgel Polyamimepak (Tosoh, Tokyo, Japan) with gradient elution at 40 °C at a flow rate of 0.4 ml/min. The running program for the HPLC system using the Shim-pack Amino-Na column was described previously (9). In the analysis using TSKgel Polyamimpepak, two buffers, buffer A (18.6 mM trisodium citrate dehydrate, 400 mM sodium chloride, 20.8 mM HCl, 4% methanol, 0.016% octanoic acid, 0.0156% Brij-35) and buffer B (93 mM trisodium citrate dehydrate, 2 mM sodium chloride, 104 mM HCl, 20% methanol, 0.008% octanoic acid, 0.078% Brij-35), were used. The column was originally equilibrated with buffer A. After the sample was injected, the concentration of buffer B was kept at 0% for 5 min. Then it was increased to 100% and maintained until 30 min. Then the column was regenerated by 0.2 N NaOH from 30 to 35 min. After the regeneration step, the column was equilibrated again by buffer A from 35 to 45 min. o-Pthalaldehyde was used as the detection reagent (21) as described previously (3), and fluorescence was detected with a fluorescence detector (model RF-10AXL; Shimadzu) at an absorbance of 470 nm with excitation at 340 nm. Standard compounds were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma-Aldrich except γ-Glu-Put, which was synthesized as described previously (9). In our HPLC system equipped with the Polyamimpepak, γ-Glu-Put, putrescine, cadaverine, and spermidine were eluted at 10.5, 17.5, 22.3, and 28.1 min, respectively. In the preparation of whole-cell samples, 1 ml of A600 = 1 culture was centrifuged, and the pellet was washed with 1 ml of M9-glucose minimal medium (16). The washed pellet was resuspended in 0.2 ml of 5% trichloroacetic acid (v/v; Nacalai Tesque) and boiled in a boiling water bath for 15 min to break the cell. The suspension was centrifuged, the supernatant was applied to HPLC after filtration using Millex-LH (Millipore, Billerica, MA), and the precipitated protein was dissolved in 1 ml of 0.1 N NaOH. The protein concentration of the solution was measured by the Lowry method (22), and the polyamine concentration of the cell was calculated as nmol/mg of protein.

Assays for PuuA Activity

HPLC Method—γ-Glu-Put synthetase activity was determined by measuring the decrease of glutamate. A reaction mixture containing 10 mM monosodium glutamate, 10 mM putrescine dihydrochloride, 7.5 mM ATP, 30 mM MgCl2, and 100 mM imidazole-HCl buffer (pH 8.0) was incubated at 37 °C. After
PuuA was purified from a cell-free extract prepared from a 200-ml culture of SO97 by 0–40% ammonium sulfate precipitation and column chromatography using a HiTrap Blue column (column volume, 5 ml; GE Healthcare). During purification, the protein was basically dissolved in buffer C (20 mM imidazole–HCl (pH 9.0), 10 mM monosodium glutamate, 100 mM putrescine dihydrochloride, 7.5 mM ATP, 25 mM MgCl$_2$, 10 mM KCl, 1 mM phosphoenolpyruvate, 0.14 mM NADH, 5 units/ml pyruvate kinase (Oriental Yeast, Tokyo, Japan), 12.6 units/ml lactate dehydrogenase (Oriental Yeast), and 1 µg/ml PuuA was incubated at 25 °C. The change in absorbance at 340 nm due to oxidation of NADH was followed using a UV-visible spectrophotometer (model UV-1600PC; Shimadzu).

**Purification of PuuA**

PuuA was purified from a cell-free extract prepared from a 200-ml culture of SO97 by 0–40% ammonium sulfate precipitation and column chromatography using a HiTrap Blue column (column volume, 5 ml; GE Healthcare). During purification, the protein was basically dissolved in buffer C (20 mM imidazole–HCl (pH 9.0), 10 mM monosodium glutamate, 100 mM putrescine dihydrochloride, 7.5 mM ATP, 25 mM MgCl$_2$, 10 mM KCl, 1 mM phosphoenolpyruvate, 0.14 mM NADH, 5 units/ml pyruvate kinase (Oriental Yeast, Tokyo, Japan), 12.6 units/ml lactate dehydrogenase (Oriental Yeast), and 1 µg/ml PuuA was incubated at 25 °C. The change in absorbance at 340 nm due to oxidation of NADH was followed using a UV-visible spectrophotometer (model UV-1600PC; Shimadzu).

**Real Time RT-PCR Analysis**

Total RNA was extracted and purified using an RNA Mini kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. One microgram of total RNA was treated with DNase I (final concentration, 0.1 units/µl; amplification grade; Invitrogen). After adding EDTA (final concentration, 2.27 mM) to inactivate DNase I, cDNA was synthesized by an iScript cDNA Synthesis kit (Bio-Rad) from 1 µg of starting total RNA primed with the random primers included in the kit according to the manufacturer’s instructions. *Thi* RNase H (final concentration, 0.52 units/µl; Toyobo) was added to the reaction mixture to remove RNA. *puuA*-specific primers, *puuA*-RT1 and *puuA*-RT2, were designed to amplify 95-nucleotide fragments using Primer3 software. The real time PCR mixture (brought to a final volume of 10 µl with deionized water) contained 5 µl of iQ™ SYBR Green Supermix (Bio-Rad), 3 pmol of each of the two primers, and a 2-µl cDNA sample in a 96-well optical reaction plate mounted in a DNA Engine Opticon Continuous Fluorescence Detection System (Bio-Rad). The thermal cycling conditions were as follows: 95 °C for 15 min and 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. To ensure the absence of nonspecific PCR products, melting curve analysis and agarose gel electrophoresis were performed after each run. The number of transcripts in a sample was determined by comparing the number of cycles (C) required for the reaction to reach a common threshold (t) with a plot of Ct values against the standard pKHG8. The relative expression levels of *puuA* compared with controls were calculated using Opticon (Bio-Rad). The relative amount of transcripts between samples was further standardized by amplification of the *gapA* gene as an internal control using primers *gapA*-RT1 and *gapA*-RT2.

**Sequence Alignment of Proteins**

Sequence alignment between PuuA and GS from *E. coli* K-12 was performed by the “needle” program of EMBOSS-Align (24). The gap penalty was 10.0, and the extend penalty was 0.5.

**RESULTS**

*PuuA Converts Putrescine to γ-Glutamylputrescine in Vivo*—As described previously (9), SK247 (*puuDRCBE*) deletes PuuB (γ-Glu-Put oxidase), which catalyzes the next step of PuuA in the Puu pathway (Fig. 1), and this strain accumulated γ-Glu-Put (Fig. 4A). The concentrations of putrescine in SK247 and...
SH639 (parental strain) were 150 and 341 nmol/mg, respectively. SO62 (pBR322/ΔpuuA) did not accumulate γ-Glu-Put, whereas strains with puuA on a plasmid (SO63) or genomic DNA (SK293) on the same genetic background accumulated γ-Glu-Put (Fig. 4B). The concentrations of putrescine in SO62, SO63, and SK293 were 450, 122, and 210 nmol/mg, respectively. These results indicated that γ-Glu-Put is generated by the reaction catalyzed by PuuA in vivo.

PuuA Reaction In Vitro—Purified PuuA catalyzed the γ-glutamyltransferase activity of putrescine (Scheme 1). This reaction, requiring Mg$^{2+}$ and Mn$^{2+}$, can be substituted for Mg$^{2+}$. When any one of the substrates and a cofactor (putrescine, glutamate, ATP, and the metal ion) were omitted from the reaction mixture, γ-Glu-Put was not formed.

PuuA Activity for Other Amines and Ammonia—PuuA could use several diamines, spermidine, and spermine instead of putrescine (Fig. 5). The relative activity of diamines depended on the length of the methylene chain; PuuA exhibited the highest activity for putrescine, which has four carbon atoms, and activity dropped when the methylene chain of diamine was longer or shorter than C$_4$ (Fig. 5). PuuA activity for spermine or spermidine was <10% of that for putrescine. PuuA could not catalyze the γ-glutamyltransferase activity of ornithine or GABA. PuuA was originally annotated as GS; however, it could not form glutamine from ammonia and glutamate.

Properties of PuuA—The optimal pH of PuuA of the γ-Glu-Put synthetase reaction is 9. The optimal Mg$^{2+}$ concentration of PuuA is 25 mM. The $V_{max}$ of PuuA is 6.71 μmol/min/mg of protein, and the calculated $K_{cat}$ for PuuA monomer is 5.94 s$^{-1}$. The $K_m$ is 2.07 mM for glutamate, 2.35 mM for ATP, and 44.6 mM for putrescine. PuuA was inactivated by EDTA treatment, and activity was not recovered by the addition of Mg$^{2+}$ or Mn$^{2+}$. By gel filtration of native PuuA, the molecular weight of native PuuA was calculated to be 630,000. Because the molecular weight of PuuA monomer was 53,000 by SDS-PAGE analysis, it was suggested that PuuA forms a dodecamer as does GS.

Comparison of the Puu Pathway with the YgjG-YdcW Pathway—E. coli metabolizes putrescine to GABA by two pathways (Fig. 2): the Puu pathway (Fig. 2, left pathway) (9) and the metabolic pathway, first reported in the 1980s (Fig. 2, right pathway) (11), that is composed of YgjG (13) and YdcW (12). In the latter pathway, putrescine is metabolized to GABA without γ-glutamyltransferase activity. In contrast, in the Puu pathway, putrescine is γ-glutamylated once and metabolized to γ-Glu-GABA via γ-glutamyl-γ-aminobutyraldehyde, and then the γ-glutamyl linkage of γ-Glu-GABA is hydrolyzed by PuuD to form GABA and Glu. To determine which pathway is more important in the utilization of putrescine as a sole nutrient source, several gene deletion mutants involved in puuA, ygjG, and ydcW were constructed, and the strains were incubated at 20 °C on a W-Glc-Put plate containing putrescine as the sole source of nitrogen and an M9-Put-AS plate containing putrescine as the sole
 Importance of PuuA in utilizing putrescine as a nutrient source. The strains ΔygjG, SK352; ΔydcW, SK350; puuAΔygjGΔydcW, SH639; ΔpuuA, SK310; ΔydcWΔpuuA, SK351; ΔygjGΔpuuA, SK353; pBelobac11/puuA*, SK308; pBelobac11/ΔpuuA, SO116; and pBelobac11-ΔpuuA/ΔpuuA, SO115 were precultured on an LB plate at 37 °C, and single colonies were streaked, respectively, on W-Glc-Put and M9-Put-AS plates and incubated at 20 °C. The composition of the plates is shown in the table under the figure, and the genotypes of the streaked strains are indicated to the left of the plates. A, influence of deleting the gene involved in two degradation pathways of putrescine on W-Glc-Put plate containing putrescine as the sole source of nitrogen. B, influence of deleting the gene involved in two degradation pathways of putrescine on M9-Put-AS plate containing putrescine as the sole source of carbon. C, complementation experiment of puuA on W-Glc-Put plate. D, complementation experiment of puuA on M9-Put-AS plate.

FIGURE 7. Expression pattern of puuA. The transcriptional level of puuA was analyzed by real time RT-PCR. A, transcriptional level of puuA of SH639 in M9-tryptone medium supplemented with putrescine. The transcriptional level of puuA was quantitated and compared with the puuA expression in M9-tryptone (control), taken as 1. B, influence of the deletion of puuR. The transcriptional level of puuA with ΔpuuR was compared with puuA* (SH639), taken as 1.
PuuA activities of the cell-free extract of strains, which overexpressed mutagenized PuuAs, were measured. PuuA activity was impaired to 9% of wild-type PuuA (measured using the cell-free extract of SO97) by the H282N mutation (SO106), to 3% by R257Q (SK326), and to 0.05% by the double mutation of H282N and R357Q (SK327). These results are consistent with previous results that GSH269N exhibits 3% and GSH269N/R344Q exhibits only 0.1% activity of GS\textsuperscript{WT} (26). All mutated PuuAs were very rapidly inactivated (after >3 h of incubation in buffer C at 4 °C).

**DISCUSSION**

In our previous report (9), MalE-fused PuuA was used to show that PuuA catalyzes the \(\gamma\)-glutamylation of putrescine in vitro and in vivo because we could not express and purify non-tagged PuuA efficiently. Accordingly the physiological role and kinetic parameters of PuuA could not be evaluated meaningfully. Also regulation of the expression of \(puuA\) was not fully reported except that the regulation of \(puuA\) occurring in response to O\(_2\) was mediated by ArcA and FNR (27). Furthermore experimental comparison of two putrescine degradation pathways in \(E.\ coli\) (9, 10, 12, 13) (Fig. 2) has not been performed, and the similarity between GS and PuuA has not been discussed. The following discussion will address these issues.

**Importance of PuuA Reaction in \(E.\ coli\) Cells**

Because polyamines, including putrescine, are highly reactive molecules, they play important roles in cell growth and proliferation (5, 6, 28). Abnormal accumulation of polyamine, however, can lead to inhibition of cell growth and protein synthesis (29); therefore, polyamine concentration should be regulated strictly and promptly to maintain optimum cell growth. It was reported previously that \(N\)-acetylpolyamine had less effect on growth and protein synthesis than non-acetylated polyamine (30), and a pathway to synthesize \(N\)-acetylpolyamine to decrease the toxicity of excess polyamine exists in both prokaryotes and eukaryotes (6, 31). Because the \(\gamma\)-glutamylation of putrescine by PuuA is similar to the acetylation of polyamines, PuuA is predicted to play a role in detoxifying excess polyamine. A noteworthy property of PuuA is the extraordinarily high \(K_m\) value for putrescine (44.6 mM). This high \(K_m\) value is consistent with the high \(K_m\) value (9.2 mM) for putrescine of His-tagged YgjG (\(\gamma\)-ketoglutarate aminotransferase) of \(E.\ coli\) (13). The intracellular concentration of free putrescine in \(E.\ coli\) was reported previously to be significantly high (12 mM) (32). Around this concentration, the reaction

**FIGURE 8. Alignment of PuuA and GS of \(E.\ coli\) K-12.** Identical and similar amino acid residues are indicated by black and gray shading, respectively. Under the alignment, the amino acid residues involved in the n1, n2, ammonium ion, glutamate, and ATP binding sites in GS are indicated. Above the alignment, two mutated amino acid residues in PuuA and a putative ATP binding motif deduced in PROSITE are indicated. Gaps are indicated by dashes. Alignment was performed by the needle program of EMBASSY-Align, and shading was performed using the BOXSHADE program.
velocity of PuuA is not saturated and is almost proportional to putrescine concentration because of its high $K_m$ value. This indicates that PuuA works efficiently when putrescine concentration in cells is so high that intracellular putrescine has a detrimental effect on $E. coli$. As a result, $E. coli$ cells can regulate the intracellular concentration of putrescine by PuuA without changing the expression level of $puuA$. In contrast, several $puuA$ overexpression strains exhibited severe growth defects (data not shown) probably because putrescine concentration decreases abnormally as a result of an excess amount of PuuA in the cell. It is predicted that the PuuA level, which influences the concentration of putrescine, must be strictly regulated.

Expression of $puuA$—As shown in Fig. 8, the transcription level of $puuA$ was up-regulated by the addition of putrescine to the medium and overexpressed by the deletion of $puuR$. These results are consistent with the expression pattern of $puuD$ (10). The observed expression regulation of $puuA$ and the fact that the predicted $puuA$ and $puuD$ promoters are overlapped and in reverse directions (Fig. 1) suggest that all $puu$ genes, including $puuA$ and $puuD$, are synchronously regulated by PuuR and/or other regulators. For example, it was reported recently that ArcA and FNR repressed the expression of $puuA$ when $E. coli$ was grown under anaerobic conditions and that there are putative binding motifs of ArcA and FNR upstream of the open reading frame of $puuA$ (27). These results indicated that excess putrescine is appropriately catabolized by products of the $puu$ gene cluster that are regulated by ArcA, FNR, and PuuR to decrease the toxicity of putrescine and to utilize putrescine as both nitrogen and carbon sources.

Two Putrescine Degradation Pathways to Yield GABA—There are two putrescine degradation pathways in $E. coli$: the Puu pathway (9) in which putrescine is first $\gamma$-glutamylated and metabolized to GABA and the YgiG-YdcW pathway (11–13) in which putrescine is metabolized to GABA without $\gamma$-glutamyl- lation (Fig. 2). It was reported that the $\Delta ydcW$ strain on the MG1655 background could grow on M9 minimal medium supplemented with 0.4% putrescine as a sole carbon source and 0.2% ammonium sulfate as a sole nitrogen source or supplemented with 0.4% glucose as a sole carbon source and 0.2% putrescine as a sole nitrogen source (12). This result indicated that another pathway besides the YgiG-YdcW pathway plays an important role in utilizing putrescine as a nutrient source. As shown in Fig. 6, the $\Delta puuA$ strain did not grow, whereas $\Delta ygiG$ and $\Delta ydcW$ strains grew on media supplemented with putrescine as a sole nitrogen or carbon source (Fig. 6, A and B). The result strongly indicates that the more important pathway for putrescine catabolism is the Puu pathway. In the Puu pathway, putrescine is first $\gamma$-glutamylated and then metabolized further. Because the $\gamma$-glutamyl- lation of putrescine by PuuA requires ATP hydrosis, the YgiG-YdcW pathway, which does not require ATP, seems more advantageous. Nevertheless our data indicate that the Puu pathway functions more effectively even in nutrient-limited media in which $E. coli$ must save energy. If putrescine is metabolized by the YgiG-YdcW pathway, $E. coli$ could apparently save energy; however, $\gamma$-aminobutyraldehyde, an intermediate of the YgiG-YdcW pathway, is unstable and spontaneously cyclized to $\Delta^1$-pyrroline. $\gamma$-Glutamyl- lation of putrescine is suggested to prevent $\gamma$-aminobutyraldehyde cyclization (9). The result in Fig. 6 shows that the stabilization effect of the intermediate by $\gamma$-glutamyl- lation is definitely effective and exceeds the loss of energy by $\gamma$-glutamyl- lation. $\gamma$-Aminobutyraldehyde cyclizes to form a five-membered ring, whereas $\gamma$-glutamyl-$\gamma$-aminobutyraldehyde would form a 10-membered ring if cyclization were to take place at both ends of the molecule. It was reported previously (33) that a 10-membered ring is $10^{-8}$ times less likely to be formed than a five-membered ring. From the above consideration and results, it is very reasonable that the $\gamma$-glutamyl- lation of putrescine by PuuA, forming an amide bond between the amino group of putrescine and the carboxyl group of glutamate, prevents the cyclization reaction because the formation of a 10-membered ring of $\gamma$-glutamyl-$\gamma$-aminobutyraldehyde is difficult.

The reason for the mucoid growth of SO115 (pBelobac11-$puuA$/puuA$\Delta$puuA) incubated at $37^\circ$C on the W-Glc-Put plate is not known. It is possible that the $puuA$ gene on this plasmid is expressed abnormally because the putative operator region of $puuA$ was deleted. The reason why all strains, including SH639, could not grow on the M9-Put-AS plate at $37^\circ$C but grew on the same plate at $20^\circ$C also remains to be elucidated.

Similarity of PuuA and GS of $E. coli$—PuuA exhibits significant amino acid sequence similarity to GS (Fig. 8). The reactions of GS and PuuA are very similar in terms of the formation of an amide bond between the $\gamma$-position carboxyl group of Glu and ammonia (in GS) and between the $\gamma$-position carboxyl group of Glu and the amino group of putrescine (in PuuA), respectively. Furthermore gel filtration analysis revealed that PuuA exists as a dodecamer in the native state, and its activity requires Mg$^{2+}$ or Mn$^{2+}$ just like GS (25).

There are two divalent metal binding sites in GS, termed n1 and n2. The n1 site, which consists of Glu-131, Glu-212, and Glu-220 in GS, is involved in the conformational change of GS that is responsible for enzyme activity (25). Judging from the amino acid sequence, two of three amino acid residues, Glu-131 and Glu-220 in GS, are conserved in PuuA, but the region around Glu-212 in GS is significantly different from that in PuuA (Fig. 8). Moreover the binding site of the ammonium ion, which consists of Glu-50, Tyr-179, and Glu-212 in GS, is adjacent to the n1 site, and none of these three amino acid residues are conserved in PuuA (Fig. 8) probably because the substrate of PuuA is putrescine. As a result, it is possible that the conformation around the n1 site of PuuA is significantly different from that of GS.

The other metal binding site, the n2 site, composed of Glu-129, His-269, and Glu-357 in GS, is overlapped in the ATP binding site (25). All three amino acid residues that comprise the n2 site in GS are conserved in PuuA (Fig. 8). It was reported that His-269 and Arg-344 of GS were involved in forming both the ATP binding site and the n2 site (25). The two amino acid residues of PuuA, His-282 and Arg-357, which correspond to His-269 and Arg-344 of $E. coli$ GS (Fig. 8), were chosen in a site-directed mutagenesis experiment. The significant decrease of enzymatic activity of PuuA$^{H268N}$ and PuuA$^{R359K}$ mutations suggested that these amino acid residues form the same type of ATP binding site as in GS.

The n2 site is also involved in metal-catalyzed oxidative modification in GS (26). This modification is the initial and impor-
tant step in the proteolytic degradation of GS. The oxidative modification of His-269 to Asn and Arg-344 to Gln induces the loss of activity of GS followed by increased susceptibility to proteolytic degradation (26). This mechanism is thought to be one of the regulation systems of the concentration of GS protein in the cell (34). As in GS, site-directed mutagenesis of the corresponding two amino acids of PuuA decreases its enzyme activity, and mutated PuuAs were rapidly inactivated. This suggests that the intracellular concentration of PuuA protein is also regulated posttranslationally by oxidative modification and proteolysis in response to the cellular concentration of putrescine.

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