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A Putrescine-Inducible Pathway Comprising PuuE-YneI in Which γ-Aminobutyrate Is Degraded into Succinate in *Escherichia coli* K-12▽

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γ-Aminobutyrate (GABA) is metabolized to succinic semialdehyde by GABA aminotransferase (GABA-AT), and the succinic semialdehyde is subsequently oxidized to succinate by succinic semialdehyde dehydrogenase (SSADH). In *Escherichia coli*, there are duplicate GABA-ATs (GabT and PuuE) and duplicate SSADHs (GabD and YneI). While GabT and GabD have been well studied previously, the characterization and expression analysis of PuuE and YneI are yet to be investigated. By analyzing the amino acid profiles in cells of Δ*puuE* and/or Δ*gabT* mutants, this study demonstrated that PuuE plays an important role in GABA metabolism in *E. coli* cells. The similarity of the amino acid sequences of PuuE and GabT is 67.4%, and it was biochemically demonstrated that the catalytic center of GabT is conserved as an amino acid residue important for the enzymatic activity in PuuE as Lys-247. However, the regulation of expression of PuuE is significantly different from that of GabT. PuuE is induced by the addition of putrescine to the medium and is repressed by succinate and low aeration conditions; in contrast, GabT is almost constitutive. Similarly, YneI is induced by putrescine, while GabD is not. For *E. coli*, PuuE is important for utilization of putrescine as a sole nitrogen source and both PuuE and YneI are important for utilization of putrescine as a sole carbon source. The results demonstrate that the PuuE-YneI pathway was a putrescine-inducible GABA degradation pathway for utilizing putrescine as a nutrient source.

Polyamines (putrescine, spermidine, and spermine) are aliphatic amines that have two or more amino groups in their chemical structures. Polyamines are distributed widely from prokaryotic (21) to eukaryotic (14) cells and are particularly found at high concentrations in proliferating cells, such as cancer cells (3) and bacteria in the exponential phase (4). *Escherichia coli* contains putrescine and spermidine. Spermine, however, is absent because the *E. coli* genome does not encode spermine synthase (22).

We previously reported a putrescine utilization pathway, the Puu (*pu*trescine *u*tilization) pathway, that involves γ-glutamylated intermediates of *E. coli* (6) (Fig. 1A). In the Puu pathway, putrescine, imported by PuuP (9), is γ-glutamylated by PuuA (8) at the expense of an ATP molecule. The resulting γ-glutamylputrescine is oxidized by PuuB to γ-glutamyl-γ-aminobutyraldehyde, which is then dehydrogenated by PuuC into γ-glutamyl-γ-aminobutyrate. In the next step, the γ-glutamyl group is hydrolyzed by PuuD (7), generating γ-aminobutyrate (GABA). The amino group of the resulting GABA is transferred by GABA aminotransferase (GABA-AT) to α-ketoglutarate (α-KG) to yield glutamate and succinic semialdehyde, which is oxidized by succinic semialdehyde dehydrogenase (SSADH) to yield succinate (Fig. 1A).

For *E. coli*, it has been previously reported (18) that GABA-AT and SSADH were encoded by *gabT* and *gabD*, respectively. However, the levels of GABA-AT and SSADH activity remained even in the Δ*gabT* and Δ*gabD* strains, respectively (18). In addition, it has been reported that these activities in Δ*gabT* and Δ*gabD* were induced when putrescine in the media acted as a nitrogen source, but the relevant genes were not identified (18). In the first description of the Puu pathway, it was simply reported that PuuE was a second GABA-AT and that both PuuE and GabT were important for utilizing putrescine as the sole nitrogen source (6). However, the role of PuuE in GABA metabolism and the regulation of expression of *puuE* remained to be investigated. Recently, YneI, whose gene is located far from *puu* gene cluster, was predicted to be the second SSADH by computer annotation and was proven biochemically to have SSADH activity (2). The role of YneI in the putrescine degradation pathway remained unclear.

In this article, we demonstrate that PuuE and YneI constitute a putrescine-inducible pathway that degrades GABA to succinate. In addition, to understand why *E. coli* has these duplicate enzymes, we compare the two sets of enzymes that degrade GABA to succinate via succinic semialdehyde, GabT-GabD and PuuE-YneI, from the viewpoint of regulation of expression.

MATERIALS AND METHODS

Strain and plasmid construction. P1 transduction, DNA manipulation, and transformation were performed according to standard methods (12, 15). To construct KEI01 (Table 1), pKJ150, including the DNA sequence of *kan*$^+$-*ymjA*$^+$-*puuP*$^+$*A*$^+$*D*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$-*pspF*$^+$-*cat*$^+$, was linearized using BamHI and PpuMI. The resulting 18-kb fragment was used to transform KJ153 by electroporation. Kanamycin-resistant KEI01 was obtained. In order to facilitate the homol-

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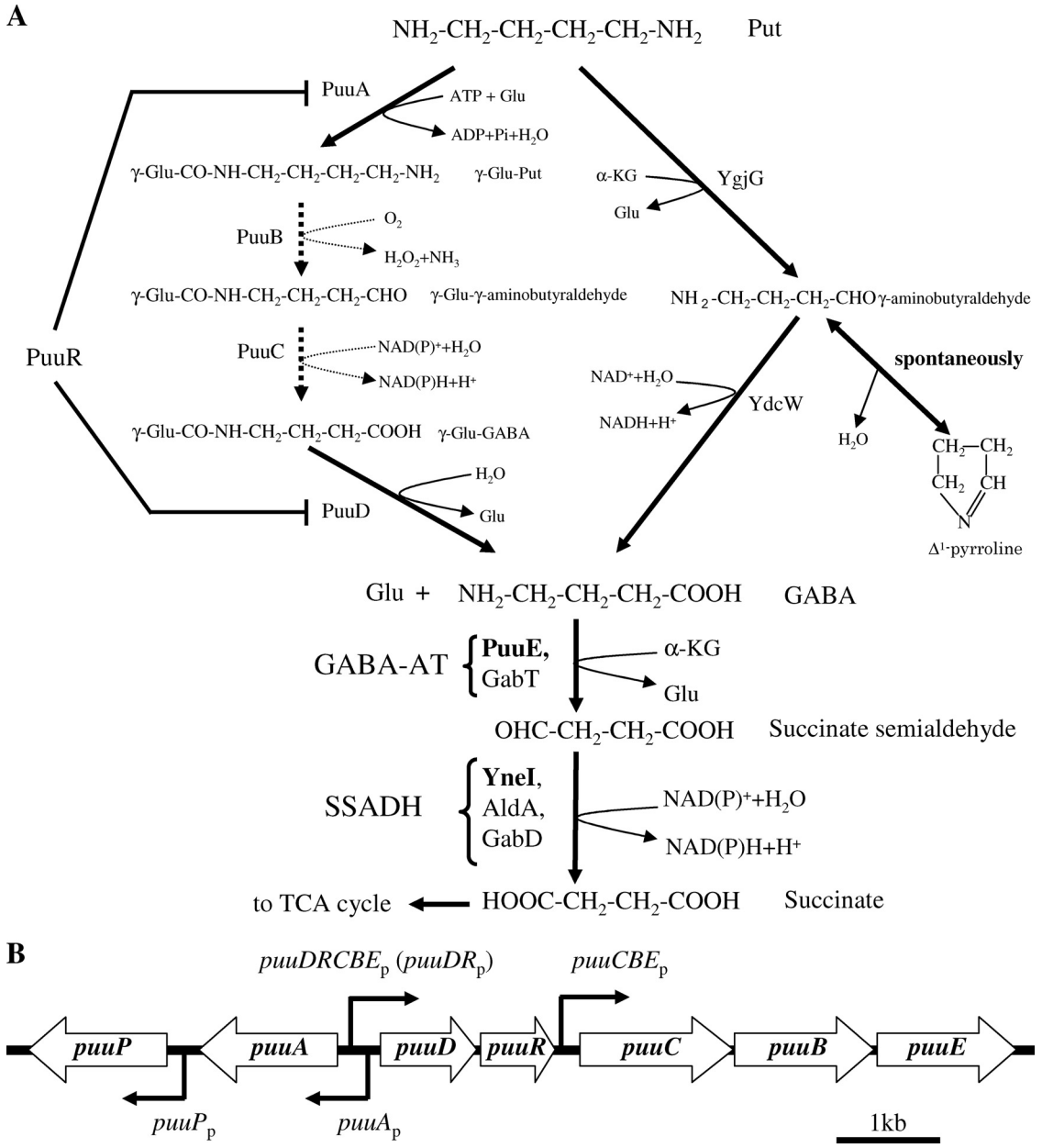


FIG. 1. (A) Degradation pathway of putrescine to succinate. PuuA (8) and PuuB, PuuC, and PuuD (7) comprise the first-half of the Puu pathway that degrades putrescine to GABA via γ-glutamylated intermediates. YgjG (16) and YdcW (17) comprise the pathway for degrading putrescine to GABA without γ-glutamylation. It has been reported that PuuR is a transcriptional repressor of *puuA* (8), *puuD* (7), and *puuP* (9). Abbreviations: Put, putrescine; Glu, glutamate; γ-Glu-Put, γ-glutamylputrescine; γ-Glu-γ-aminobutyraldehyde, γ-glutamyl-γ-aminobutyraldehyde; γ-Glu-GABA, γ-glutamyl-γ-aminobutyrate; GABA, γ-aminobutyrate; α-KG, α-ketoglutarate; GABA-AT, GABA-aminotransferase; SSADH, succinic semialdehyde dehydrogenase. (B) Organization of the *puu* gene cluster. $puuP_p$ is a putative promoter. The positions of the other promoters have been described previously (13); however, our unpublished data suggest that the transcription from $puuCBE_p$ is relatively weak and that transcription from $puuDR_p$ continues to yield *puuDRCBE* mRNA. TCA, tricarboxylic acid.

ogous recombination and introduction of cat^+ gene, KEI01 was transformed with pKD46, a temperature-sensitive suicide plasmid carrying the Red recombinase system. The resulting strain, KEI02 was transformed with the above-mentioned 18-kb fragment carrying kan^+-*ymjA*$^+$-*puuP*$^+$*A*$^+$*D*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$-*pspF*$^+$-cat^+ by electroporation. Kanamycin- and chloramphenicol-resistant strain KEI04 was obtained. To construct KEI05, the *yneI* gene of KJ101 was disrupted according to the method described previously (1) using the PCR product amplified from pKD13 with the primers yneI-up(c) and yneI-down. The *yneI* gene was replaced by FRT (FLP recombination target)-kan^+-FRT in KEI05 through homologous recombination. pKD46 was cured through single-colony isolation by streaking at 37°C. (In the construction of KEI07, KEI09, KEI10, KEI12, and KJ102, the same procedure was performed to eliminate pKD46.) To construct KEI07, the *gabD* gene of KJ101 was disrupted according to the method described previously (1) using the PCR product amplified from pKD3 with the primers gabD-up2 and gabD-down(c). The *gabD* gene of KJ101 was replaced by FRT-cat^+-FRT in KEI07 through homologous recombination. To construct KEI09, pKJ150, including the DNA sequence kan^+-*ymjA*$^+$-*puuP*$^+$*A*$^+$*D*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$-*pspF*$^+$-cat^+, was linearized using BamHI and PpuMI. The resulting 18-kb fragment was used to transform TK251 by electroporation, and kanamycin- and chloramphenicol-resistant KEI09 was obtained. To construct KEI10, the *aldA* gene of KJ101 was

TABLE 1. Strains, plasmids, and oligonucleotides used in this study

Strain, phage, plasmid, or oligonucleotide	Characteristic(s) or sequence	Source or reference
Strains		
KEI01	SH639, except Δ*gabT*::FRT *kan*$^+$-*ymjA*$^+$-*puuP*$^+$*A*$^+$*D*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$	This study
KEI02	pKD46/KEI01	This study
KEI04	SH639, except Δ*gabT*::FRT *kan*$^+$-*ymjA*$^+$-*puuP*$^+$*A*$^+$*D*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$-*pspF*$^+$-*cat*$^+$; the control of KJ155	This study
KEI05	SK639, except Δ*yneI*::FRT-*kan*$^+$-FRT	This study
KEI06	SH639, except Δ*yneI*::FRT; *kan*$^+$ of Δ*yneI*::FRT-*kan*$^+$-FRT allele in KEI05 was eliminated	This study
KEI07	SH639, except Δ*gabD*::FRT-*cat*$^+$-FRT	This study
KEI08	SH639, except Δ*gabD*::FRT; *cat*$^+$ of Δ*gabD*::FRT-*cat*$^+$-FRT allele in KEI07 was eliminated	This study
KEI09	MG1655, except *kan*$^+$-*ymjA*$^+$-*puuP*$^+$*A*$^+$*D*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$-*pspF*$^+$-*cat*$^+$; the control of KEI12	This study
KEI10	SH639, except Δ*aldA*::FRT-*kan*$^+$-FRT	This study
KEI11	SH639, except Δ*aldA*::FRT; *kan*$^+$ of Δ*aldA*::FRT-*kan*$^+$-FRT allele in KEI10 was eliminated	This study
KEI12	MG1655, except *kan*$^+$-*ymjA*$^+$-*puuP*$^+$*A*$^+$*D*$^+$*R*(*3Ala*)*C*$^+$*B*$^+$*E*$^+$-*pspF*$^+$-*cat*$^+$	This study
KEI14	SH639, except Δ*yneI*::FRT Δ*gabD*::FRT-*cat*$^+$-FRT	This study; KEI06 × P1 (KEI07)
KEI15	SH639, except Δ*aldA*::FRT-*kan*$^+$-FRT Δ*gabD*::FRT	This study; KEI08 × P1 (KEI10)
KEI16	SH639, except Δ*aldA*::FRT Δ*yneI*::FRT-*kan*$^+$-FRT	This study; KEI11 × P1 (KEI05)
KEI17	SH639, except Δ*aldA*::FRT Δ*yneI*::FRT; *kan*$^+$ of Δ*yneI*::FRT-*kan*$^+$-FRT allele in KEI16 was eliminated	This study
KEI18	SH639, except Δ*gabD*::FRT Δ*yneI*::FRT; *cat*$^+$ of Δ*gabD*::FRT-*cat*$^+$-FRT allele in KEI14 was eliminated	This study
KEI19	SH639, except Δ*aldA*::FRT Δ*gabD*::FRT; *kan*$^+$ of Δ*aldA*::FRT-*kan*$^+$-FRT allele in KEI15 was eliminated	This study
KEI26	pKEI21/MG1655	This study
KJ101	pKD46/SH639	This study
KJ102	SH639, except Δ*gabT*::FRT-*kan*$^+$-FRT	This study
KJ107	SH639, except Δ*gabT*::FRT; *kan*$^+$ of Δ*gabT*::FRT-*kan*$^+$-FRT allele in KJ102 was eliminated	This study
KJ109	SH639, except Δ*gabT*::FRT Δ*puuE*::*kan*$^+$	6
KJ117	pQE-80L/KJ109	This study
KJ121	pKJ120/KJ109	This study
KJ123	pKJ122/KJ109	This study
KJ124	SH639, except Δ*puuDR*::FRT Δ*puuCBE*::FRT Δ*gabT*::FRT-*kan*$^+$-FRT	This study; SK247 × P1 (KJ102)
KJ127	pBelobac11/KJ102	This study
KJ128	pBelobac11/KJ124	This study
KJ130	pKJ126/KJ124	This study
KJ131	pSK299/KJ124	This study
KJ153	pKD46/KJ107	This study
KJ155	SH639, except Δ*gabT*::FRT *kan*$^+$-*ymjA*$^+$-*puuP*$^+$*A*$^+$*D*$^+$*R*(*3Ala*)*C*$^+$*B*$^+$*E*$^+$-*pspF*$^+$-*cat*$^+$	This study
MG1655	F$^-$ prototrophic	C. A. Gross
SH639	F$^-$ Δ*ggt-2*	19, 20
SK187	SH639, except Δ*puuE*::*kan*$^+$	6
SK247	SH639, except Δ*puuDR*::FRT Δ*puuCBE*::FRT	6
TK251	pKD46/MG1655	Laboratory stock
Phages		
Kohara 257		5
Kohara 305		5
Plasmids		
pACYC184	p15A replicon, *cat*$^+$ *tet*$^+$	New England Biolabs (Ipswich, MA)
pACYC-*aldH-ordL-goaG*	p15A replicon, *cat*$^+$ *puuC*$^+$*B*$^+$*E*$^+$	6
pBelobac11	Mini-F replicon, *cat*$^+$	New England Biolabs
pBR322	ColE1 replicon, *rop*$^+$ *bla*$^+$ *tet*$^+$	New England Biolabs
pCP20	pSC101 replicon, (Ts) *bla*$^+$ *cat*$^+$ Flp (λRp) *c*I857	1
pKD3	*oriR*γ *bla*$^+$ FRT-*cat*$^+$-FRT	1
pKD13	*oriR*γ *bla*$^+$ FRT-*kan*$^+$-FRT	1
pKD46	*oriR101* replicon, *repA101*(Ts) *araC*$^+$ *gam*$^+$-*bet*$^+$-*exo*$^+$ (*araBp*) *bla*$^+$	1
pKEI03	ColE1 replicon *bla*$^+$ *yneI*$^+$; the DNA fragment of Kohara clone 305 was digested with HincII, and the resulting 2.5-kb fragment was ligated to pUC19 cleaved by HincII	This study
pKEI21	ColE1 reolicon *bla*$^+$ *lacI*^{q} $T5_p$-$(His)_6$-*yneI*	This study
pKJ111	ColE1 replicon *bla*$^+$ *puuE*$^+$	This study
pKJ112	p15A replicon, *cat*$^+$ *puuE*$^+$; pKJ111 and pACYC-*aldH-ordL-goaG* were digested with SmaI and BsiWI, and the resulting 0.5-kb fragment of pKJ111 and 5.2-kb fragment of pACYC-*aldH-ordL-goaG* were ligated	This study
pKJ115	ColE1 replicon, *bla*$^+$ *lacI*^{q} $T5_p$-$(His)_6$-*puuE*$^+$	This study
pKJ120	ColE1 replicon, *bla*$^+$ *lacI*^{q} *puuE*$^+$ ($T5_p$)	This study
pKJ122	ColE1 replicon, *bla*$^+$ *lacI*^{q} *puuE*(*K267A*) ($T5_p$)	This study
pKJ126	Mini-F replicon, *cat*$^+$ *puuD*$^+$*R*$^+$*C*$^+$*B*$^+$; pSK299 was digested with HincII and ClaI and blunt ended, and the resulting 5.7-kb fragment was ligated to pBelobac11 cleaved by HpaI	This study
pKJ133	ColE1 replicon, *bla*$^+$ *puuD*$^+$*R*(*3Ala*)	This study
pKJ134	p15A replicon, *tet*$^+$ *ymjA*$^+$-*puuP*$^+$*A*$^+$*D*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$-*pspF*$^+$*A*$^+$; the DNA fragment of Kohara clone 257 was digested with ScaI, and the resulting 12.0-kb fragment was ligated to pACYC184 cleaved by ScaI	This study
pKJ148	p15A replicon, *tet*$^+$ *kan*$^+$-*ymjA*$^+$-*puuP*$^+$*A*$^+$*D*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$-*pspF*$^+$*A*$^+$; pUC4K was digested with HincII, and the resulting 1.3-kb fragment including the *kan*$^+$ gene was ligated to pKJ134 cleaved by PstI and blunt ended	This study
pKJ150	p15A replicon, *tet*$^+$ *kan*$^+$-*ymjA*$^+$-*puuP*$^+$*A*$^+$*D*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$-*pspF*$^+$-*cat*$^+$-*pspA*$^+$; pACYC184 was digested with HincII and SacII and blunt ended, and the resulting 1.9-kb fragment including the *cat*$^+$ gene was ligated to pKJ148 cleaved with BglII and blunt ended	This study

Continued on following page

TABLE 1—*Continued*

Strain, phage, plasmid, or oligonucleotide	Characteristic(s) or sequence	Source or reference
pKJ151	ColE1 replicon, *rop*$^+$ *bla*$^+$ *puuD*$^+$*R*(*3Ala*)*C*$^+$*B*$^+$*E*$^+$; pKJ133 and pSK291 were digested with SmaI, and the resulting 1.3-kb fragment of pKJ133 and 6.7-kb fragment of pSK291 were ligated	This study
pKJ152	p15 replicon, *tet*$^+$ *kan*$^+$-*ymjA*$^+$*puuP*$^+$*A*$^+$*D*$^+$*R*(*3Ala*)*C*$^+$*B*$^+$*E*$^+$ *pspF*$^+$-*cat*$^+$-*pspA*$^+$; pKJ150 and pKJ151 were digested with AflII, and the resulting 2.7-kb fragment of pKJ151 was ligated to the 16.7-kb fragment of pKJ150	This study
pQE-80L	ColE1 replicon, *bla*$^+$ *lacI*^{q} $T5_p$-$(His)_6$	Qiagen
pSK103	ColE1 replicon, *bla*$^+$ *puuD*$^+$*R*$^+$; the DNA fragment of Kohara clone 257 was digested with PvuII, and the resulting 3.0-kb fragment containing the *puuDR* gene was ligated into pUC19 cleaved by SmaI	This study
pSK109	ColE1 replicon, *bla*$^+$ *rop*$^+$ *puuD*$^+$; pSK103 was digested with EcoRI, and the resulting 1.6-kb fragment was ligated into pBR322 cleaved by EcoRI	This study
pSK134	ColE1 replicon, *bla*$^+$ *rop*$^+$ *puuD*$^+$; pSK134 is pSK109 introduced into JM110 to demethylate its BsaBI site	This study
pSK137	ColE1 replicon, *bla*$^+$ *rop*$^+$ *puuD*$^+$; pSK134 was digested with EcoRV and BsaBI, and the resulting 4.5-kb fragment was self-ligated	This study
pSK169	ColE1 replicon, *bla*$^+$ *puuR*$^+$*C*$^+$*B*$^+$*E*$^+$	6
pSK291	ColE1 replicon, *bla*$^+$ *rop*$^+$ *puuD*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$; pSK137 and pSK169 were digested with NarI and HindIII, respectively, and the resulting 4.3-kb fragment of pSK137 containing the region from 7840 to 9300 (*puuD′*) of Kohara clone 257 was ligated to the 5.1-kb fragment of pSK169 containing the region from 9300 to 14350 (*′puuDR*$^+$*C*$^+$*B*$^+$*E*$^+$) of Kohara clone 257	This study
pSK299	Mini-F replicon, *cat*$^+$ *puuD*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$; pSK291 was digested with HincII, and the resulting 7.0-kb fragment including *puuD*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$ was ligated to pBelobac11 cleaved by HpaI	This study
pUC19	ColE1 replicon, *bla*$^+$	New England Biolabs
pUC4K	ColE1 replicon, *bla*$^+$ *kan*$^+$	New England Biolabs
Oligonucleotides		
aldA-up	5′-CAATGTATTCACCGAAAACAAACATATAAATCACAGGAGTCGCCCGTGTAGGCTGGAGC TGCTTC-3′	
aldA-down(c)	5′-CTGACGCGCACAGGCGGAGGAAAAAACCTCCGCCTCTTTCACTCAATTCCGGGGATCCG TCGACC-3′	
for_delta_gabT1	5′-ATGAACAGCAATAAAGAGTTAATGCAGCGCCGCCAGTCAGGCGATTGTGTAGGCTGGA GCTGCTTC-3′	
for_delta_gabT2	5′-CTACTGCTTCGCCTCATCAAAACACTGGCTGATGATCTCCAGACCATTCCGGGGATCCGT CGACC-3′	
gabD-up2	5′-TACACGCCGCATTTAATCAATAACCTTTGAAAACAGGATGTAGCGGTGTAGGCTGGAGC TGCTTC-3′	
gabD-down(c)	5′-CGGCGCTGCATTAACTCTTTATTGCTGTTCATTCGCATTCTCCAGCATATGAATATCCTCC TTAG-3′	
PCR_amp_goaG_1	5′-GTGGGATCCATGAGCAACAATGAATTCCATCAG-3′	
PCR_amp_goaG_2	5′-GTTAAGCTTTTAATCGCTCAGCGCATCCTGCAAAA-3′	
QC_aldH-ordL_lo_1	5′-GAAACAGGAGTCATAATGAGCAACAATGAATTCCATC-3′	
QC_aldH-ordL_lo_2	5′-GATGGAATTCATTGTTGCTCATTATGACTCCTGTTTC-3′	
QC_His_BamH1_lo_1	5′-GGAGAAATTAACTATGAGCAACAATGAATTCCATCAGCGTCG-3′	
QC_His_BamH1_lo_2	5′-CGACGCTGATGGAATTCATTGTTGCTCATAGTTAATTTCTCC-3′	
QC_puuE_K267A_1	5′-GACGATGGCGGCCAGCCTCGCGGGCGGG-3′	
QC_puuE_K267A_2	5′-CCCGCCCGCGAGGCTGGCCGCCATCGTC-3′	
QC_puuR_3Ala_1	5′-CGCCGAACTCTCCGGGCTGGCGGCCGCGGCTATCAGTACGATAG-3′	
QC_puuR_3Ala_2	5′-CTATCGTACTGATAGCCGCGGCCGCCAGCCCGGAGAGTTCGGCG-3′	
yneI_1	5′-GCGGTACCATGACTCATAAAGGAGATACCCCGATGACC-3′	
yneI_2	5′-GCAAGCTTTCAGATCCGGTCTTTCCACACCGTCTGGAT-3′	
yneI_up(c)	5′-TATTATTCATTTTAAAGCAAGAGTAAATCTGCGTATCTTCATACCATTCCGGGGATCCGT CGACC-3′	
yneI_down	5′-AGGTCTGAAAAGACCTGCGAGTATATCAGAGCTGAATATGTCGCGTGTAGGCTGGAGCT GCTTCG-3′	

disrupted according to the method described previously (1), using the PCR product amplified from pKD13 with the primers aldA-up and aldA-down(c). The *aldA* gene of KJ101 was replaced by FRT-*kan*$^+$-FRT in KEI10 through homologous recombination. To construct KEI12, *puuR*(*3Ala*) was introduced into the *puu* gene cluster (Fig. 1B) as follows. pKJ152, including the gene sequence *kan*$^+$-*ymjA*$^+$-*puuP*$^+$*A*$^+$*D*$^+$*R*(*3Ala*)*C*$^+$*B*$^+$*E*$^+$-*pspF*$^+$-*cat*$^+$, was linearized using BamHI and PpuMI. The resulted 18-kb fragment was used to transform TK251 by electroporation, yielding kanamycin- and chloramphenicol-resistant KEI12. To confirm that the mutation was introduced appropriately, the genomic region around *puuR*(*3Ala*) was amplified by PCR using KOD-plus DNA polymerase, and the amplified DNA fragment was sequenced. To construct KJ102, the *gabT* gene of KJ101 was disrupted according methods described previously (1) using the PCR product amplified from pKD13 with the primers for_delta_gabT1 and for_delta_gabT2. The *gabT* gene of KJ101 was replaced by FRT-*kan*$^+$-FRT in KJ102 through homologous recombination. To construct the multiple mutants, P1 transductions were performed respectively. To eliminate the *kan*$^+$ and *cat*$^+$ genes from FRT-*kan*$^+$-FRT and FRT-*cat*$^+$-FRT, respectively, a recombination reaction using Flp flippase carried by pCP20 was used. In KJ155, *puuR*(*3Ala*) was introduced into *puu* gene cluster using the same method as in the construction of KEI12, except that the recipient strain was KJ107. pKEI21 was constructed as follows. The *yneI* gene was amplified by PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan), pKEI03 as a template, and the primers yneI_1 and yneI_2, following the manufacturer's instructions. yneI_1 and yneI_2 were designed to add a KpnI restriction site at 5′ end of *yneI* and a HindIII restriction site at the 3′ end of *yneI*, respectively. The amplified fragment was ligated to pQE-80L digested by KpnI and HindIII. pKJ111 was constructed as follows. The region including the *puuCB* gene was looped out from pSK169 (8) with QC_aldH-ordL_lo_1 and QC_aldH-ordL_lo_2 as the mutagenic primers, using the QuikChange method, except that KOD-plus DNA polymerase was used. pKJ115 was constructed as follows. The *puuE* gene was amplified by PCR using KOD-plus DNA polymerase, pSK169 (8) as a template, and the primers PCR_amp_goaG_1 and PCR_amp_goaG_2, following the manufacturer's instructions. PCR_amp_goaG_1 and PCR_amp_goaG_2 were designed to add a BamHI restriction site at the 5′ end of *puuE* and a HindIII restriction site to the 3′ end of *puuE*, respectively. The amplified fragment was ligated to pQE-80L digested by BamHI and HindIII. The DNA region amplified by the PCR was sequenced to confirm that no sequence changes had occurred. pKJ120 was constructed as follows. The region including the RGS-His epitope, BamHI site, and the start codon originally included in pQE-80L was looped out from pKJ115 using QC_His_BamH1_lo_1 and QC_His_BamH1_lo_2 as the muta-

genic primers, using the QuikChange method, except that KOD-plus DNA polymerase was used. The resulting plasmid was sequenced to confirm that the intended mutation was introduced and that no unintended mutations had been introduced. The mutation *puuE*(*K267A*) carried on pKJ122 was introduced to the *puuE* gene using the QuikChange method, except that KOD-plus DNA polymerase (Toyobo) was used, in this introduction of the mutation, and the template pKJ112 and the mutagenic primers QC_puuE_K267A_1 and QC_puuE_K267A_2 were used. The 0.8-kb region of *puuE* between the BanIII site and the DraIII site, including the A267K mutation, was sequenced to confirm that unintended nucleotide changes had not been introduced, and the plasmid sequenced was digested by BanIII and DraIII. The resulting 0.8-kb fragment including *puuE*(*K267A*) was substituted into the same region of pKJ120 to obtain pKJ122. To confirm the substitution, the region around the mutation was sequenced again. The mutation of *puuR*(*3Ala*) carried on pKJ133 was introduced to the *puuR* gene using the QuikChange method, except that KOD-plus DNA polymerase was used, in this introduction of mutation, and pSK103 as a template and the mutagenic primers QC_puuR_3Ala_1 and QC_puuR_3Ala_2 were used. The 3.0-kb region including the *puuR* gene cloned originally from the DNA of Kohara clone 257 was sequenced to confirm that unintended nucleotide changes had not been introduced.

Media and reagents. In all experiments, unless otherwise noted, strains were grown in a 300-ml Erlenmeyer flask at 37°C, with shaking at 140 rpm in 60 ml of M9-tryptone (M9 minimal medium [12], except that 1% Bacto tryptone was used instead of 0.2% glucose). W salts minimal medium containing glucose and putrescine (W-Glc-Put) and M9 medium containing putrescine and ammonium sulfate (M9-Put-AS) (8) were used for the growth experiments in nitrogen source-limited medium and carbon source-limited medium, respectively. In the growth experiments on M9-Glc-Put and M9-Put-AS, the strains were precultured on LB plates at 37°C and then streaked on nutrient-limited plates and incubated on M9-Glc-Put at 37°C and on M9-Put-AS at 20°C. To overexpress *puuE* for protein purification, KJ121 was grown in 500 ml LB broth containing 100 μg/ml ampicillin at 37°C with shaking at 140 rpm in a 2-liter Erlenmeyer flask. To induce *puuE*, 2 h after inoculation, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.5 mM. Three hours after induction with IPTG, the cells were harvested. To overexpress His_6-*yneI* for protein purification, KEI26 was grown in 100 ml LB broth containing ampicillin (100 μg/ml) at 37°C with shaking at 140 rpm in a 500-ml Erlenmeyer flask. To induce *yneI*, 2.5 h after inoculation, IPTG was added to the culture to a final concentration of 0.5 mM. Five hours after induction with IPTG, the cells were harvested.

Enzyme assays. The GABA-AT activity shown in Fig. 2 and used to calculate kinetic parameters was assayed as described previously (6). For other GABA-AT assays, a simplified enzymatic method described previously was used (18). In the experiment to confirm generation of succinate in the reaction catalyzed by the SSADH activity of YneI, a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.8), 2 mM succinic semialdehyde, and 2 mM NADP was incubated with the enzyme solution at 30°C. After the reaction was terminated by boiling at 100°C for 3 min, the concentration of succinate in the reaction mixture was determined using the Roche enzymatic kit for succinate, catalogue no. 176281 (Roche, Basel, Switzerland). For other SSADH assays, a simplified enzymatic method described previously was used (18).

Analysis of GABA concentration. GABA concentrations in the samples were measured using the high-performance liquid chromatography (HPLC) system described previously (6).

Analysis of transcription of *yneI* and *puuE*. Total mRNAs from KEI12 [*puuR*(*3Ala*)] and KEI09 (*puuR*$^+$) were analyzed using the DNA-Chip system (Nandemo Array, Gene Frontier, Kashiwa, Japan).

RESULTS

In addition to GabT, PuuE catalyzes the GABA aminotransferase reaction *in vivo*. To show that PuuE has GABA-AT activity in addition to that of GabT, the Δ*gabT* (KJ102) and Δ*puuE* (SK187) single mutants and the Δ*gabT* Δ*puuE* (KJ109) double mutant were constructed. When the GABA-AT activity of SH639 (*gabT*$^+$ *puuE*$^+$) was set to 100%, the activities of KJ102 (Δ*gabT puuE*$^+$) and SK187 (*gabT*$^+$ Δ*puuE*) were 68 and 35%, respectively (Fig. 2A). In addition, the double mutant KJ109 (Δ*gabT* Δ*puuE*) had almost no GABA-AT activity (Fig. 2A), as described previously (6). The intracellular amino acid profiles of SH639 (*gabT*$^+$ *puuE*$^+$) and KJ102 (Δ*gabT puuE*$^+$) were almost identical (Fig. 2B). However, SK187 (*gabT*$^+$ Δ*puuE*) accumulated GABA, and further accumulation of GABA was observed in KJ109 (Δ*gabT* Δ*puuE*) (Fig. 2B). These results suggest that PuuE, as well as GabT, has GABA-AT activity and metabolized GABA via succinic semialdehyde in *E. coli* cells.

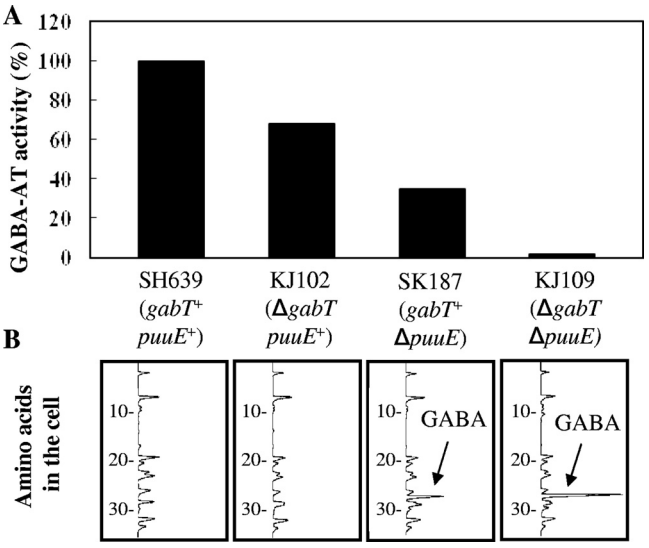
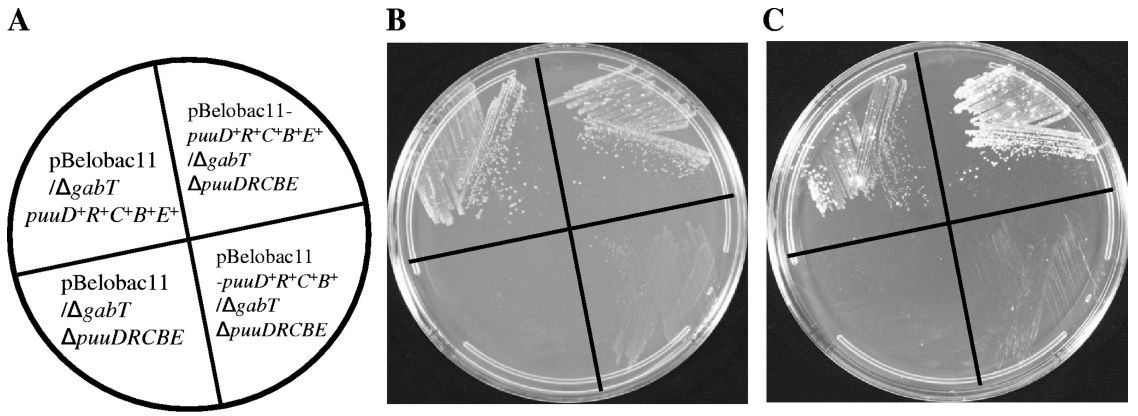


FIG. 2. (A) GABA-AT activity of Δ*gabT* and/or Δ*puuE* mutants. The *gabT*$^+$ *puuE*$^+$ (SH639), Δ*gabT puuE*$^+$ (KJ102), *gabT*$^+$ Δ*puuE* (SK187), and Δ*gabT* Δ*puuE* (KJ109) strains were inoculated to the same initial cell density (A_{600} of 0.03) into 60 ml M9-tryptone medium supplemented with 5 mM putrescine in a 300-ml Erlenmeyer flask and were grown at 37°C with reciprocal shaking at 140 rpm. Seven hours after inoculation, the cell extract was prepared. Values are shown as relative activity compared with the activity of SH639 (*gabT*$^+$ *puuE*$^+$), considered to be 100%. (B) The intracellular amino acid profile of Δ*gabT* and/or Δ*puuE* mutants. The same strains used in panel A were grown under the same conditions as in panel A. Five hours after inoculation, cells were harvested and the polyamine profile and the concentrations in the cells were analyzed by a method described previously (6). Numbers on the left-hand side of each chart indicate the elution time in minutes.

Physiological role of PuuE. To confirm the importance of PuuE in utilizing putrescine as a nutrient source in *E. coli*, a complementation experiment was performed. KJ127(pBelobac11/Δ*gabT puuD*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$), KJ128(pBelobac11/Δ*gabT* Δ*puuDRCBE*), KJ130(pBelobac11-*puuD*$^+$*R*$^+$*C*$^+$*B*$^+$/Δ*gabT* Δ*puuDRCBE*), and KJ131(pBelobac11-*puuD*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$/Δ*gabT* Δ*puuDRCBE*) were grown on plates with putrescine as the sole source of nitrogen or carbon. The strains, which have *puuE*$^+$ on genomic DNA (KJ127) or on the single-copy plasmid, pBelobac11 (KJ131) were able to grow on a plate with putrescine as the sole source of nitrogen (Fig. 3B) or carbon (Fig. 3C), respectively. The Δ*puuDRCBE* strain (KJ128) was not able to grow and the *puuD*$^+$*R*$^+$*C*$^+$*B*$^+$ Δ*puuE* (KJ130) strain grew slightly on a plate with putrescine as the sole source of nitrogen (Fig. 3B) or carbon (Fig. 3C), respectively. When incubated at 37°C, none of the strains was able to grow on the W-Put-AS plates (data not shown), for reasons not understood. The results indicate that the GABA-AT activity of PuuE is important for utilization of putrescine as the sole nitrogen or carbon source in *E. coli*.



	W-Glc-Put	M9-Put-AS
Salt	W	M9
Carbon source	0.4 % glucose	0.4 % $PutCl_2$
Nitrogen source	0.2 % $PutCl_2$	0.4 % $(NH_4)_2SO_4$

FIG. 3. Importance of PuuE in utilizing putrescine as a nutrient source. KJ127(pBelobac11/Δ*gabT puuD*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$), KJ128(pBelobac11/Δ*gabT* Δ*puuDRCBE*), KJ130(pBelobac11-*puuD*$^+$*R*$^+$*C*$^+$*B*$^+$/Δ*gabT* Δ*puuDRCBE*), and KJ131(pBelobac11-*puuD*$^+$*R*$^+$*C*$^+$*B*$^+$*E*$^+$/Δ*gabT* Δ*puuDRCBE*) were precultured on LB plates at 37°C, and single colonies were streaked on W-Glc-Put at 37°C and M9-Put-AS at 20°C. The compositions of the plates are shown in the table at the bottom of the figure. The amounts of glucose (0.4%), putrescine dihydrochloride (0.4% and 0.2%), and $(NH_4)_2SO_4$ (0.4%) are equivalent to 22.2 mM, 24.8 and 12.4 mM, and 30.3 mM, respectively. (A) Summary of the genotypes of the strains that were streaked; (B) complementation experiment with *puuE* on W-Glc-Put plate; (C) complementation experiment with *puuE* on the M9-Put-AS plate.

Enrichment of PuuE and His_6-YneI. PuuE was enriched from a cell extract of KJ121 by 20 to 40% ammonium sulfate fractionation. PuuE was enriched to 93% homogeneity, as judged by measuring the density of the protein band by SDS-PAGE using Image J software (NIH, Bethesda, MD) (Fig. 4A, lane 4). His_6-YneI was enriched from a cell extract of KEI26 by a HiTrap chelating HP 1-ml column (GE Healthcare, Buckinghamshire, England) following the manufacturer's instructions. His_6-YneI was eluted from the column when the concentration of imidazole was 100 mM and was enriched to 94% homogeneity (Fig. 4C).

The property of PuuE. Kinetic parameters were calculated using enriched nontagged PuuE. In the forward reaction, which is the transfer reaction of the amino group from GABA to α-KG forming glutamate and succinic semialdehyde, the optimum pH, K_m for GABA, K_m for α-KG, and k_{cat} were 9.0, 1.57 mM, 5.1 mM, and 39.5 s^{-1}, respectively. In the reverse reaction, which is the transfer reaction of the amino group from Glu to succinic semialdehyde forming GABA and α-KG, the optimum pH, K_m for succinic semialdehyde, K_m for Glu, and k_{cat} were 8.0, 0.0577 mM, 18.0 mM, and 8.56 s^{-1}, respectively. PuuE was pyridoxal-phosphate dependent.

The important residue of PuuE for enzymatic activity. The identity and similarity of the amino acid sequences of PuuE and GabT were 54.2% and 67.4%, respectively. The sequence alignment between PuuE and GabT (data not shown) suggested that Lys-267 of PuuE (corresponding to Lys-268 in GabT, which is an important residue for GabT activity [10]) is an important residue for the activity. Site-directed mutagenesis of PuuE(K267A) was performed to confirm that Lys-267 is an important residue of PuuE. Wild-type *puuE* [*puuE*(WT)] and *puuE*(*K267A*) were similarly overexpressed using the expression vector, pQE-80L (Fig. 4A); however, PuuE(K267A) had no GABA-AT activity (Fig. 4B). This result confirms that Lys-267 of PuuE is an important residue of this enzyme.

Synchronous expression of *puuE* with the other genes of the *puu* gene cluster. To analyze the change in expression of *puuE* with intracellular or extracellular putrescine concentration, the GABA-AT activities of cell extracts of KJ102 (Δ*gabT puuE*$^+$) at different growth phases were measured. The PuuD (7) and PuuE activity was decreased along with the depletion of putrescine of culture supernatant (Fig. 5A) and the decrease of putrescine in the cell (Fig. 5B). In a previous study (9), it was observed that PuuP caused a rapid decrease of putrescine in the culture supernatant in the early stationary phase (Fig. 5A). These results imply that the expression of *puuE* is regulated in the same manner as other members of the *puu* gene cluster and that the products of the genes of *puu* gene cluster utilize excess putrescine no longer required in the early stationary phase as a nutrient source.

Regulation of the expression of *puuE*. In a previous study, the expression of *puuD* was induced by the addition of putrescine and repressed by the addition of succinate to the medium (7). Furthermore, the expression of *puuD* was repressed when *E. coli* was grown under low-aeration conditions (7). Induction by putrescine was also observed in the regulation of *puuA* (8). Because the expression of *puuE* was regulated in similar manner to *puuD* (Fig. 5), one member of the *puu* gene cluster, the effects of the addition of putrescine, succinate, and GABA, the substrate of the GABA-AT reaction, to the medium on the expression of *puuE* were analyzed and compared to that of *gabT*. Similarly, the effects of aeration

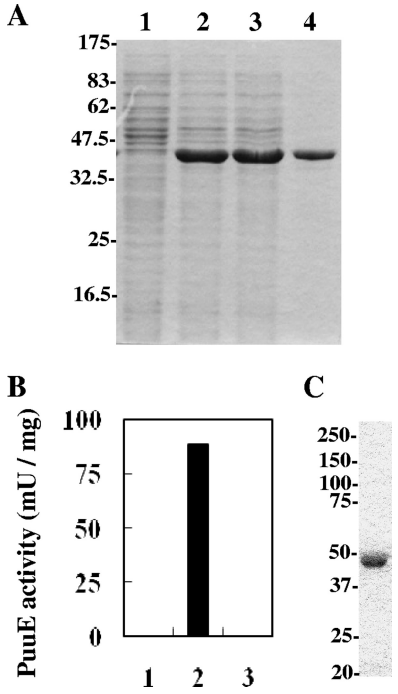


FIG. 4. K267A mutation introduced into the predicted catalytic residue of PuuE. Strains KJ117(pQE-80L/Δ*puuE* Δ*gabT*), KJ121(pQE-80L-*puuE*$^+$/Δ*puuE* Δ*gabT*), and KJ123[pQE-80L-*puuE*(*K267A*)/Δ*puuE* Δ*gabT*] were inoculated to the same initial cell density (A_{600} of 0.03) into 60 ml M9-tryptone in a 300-ml Erlenmeyer flasks and were grown at 37°C with reciprocal shaking at 140 rpm. Two hours after inoculation, IPTG was added to a final concentration of 0.5 mM. Three hours after induction, cells were harvested. (A) Expression of *puuE*(WT) and *puuE*(*K267A*) from the overexpression vector. A cell extract was prepared, and 20 μg protein was applied to SDS-PAGE: lane 1, KJ117(pQE-80L/Δ*puuE* Δ*gabT*); lane 2, KJ121(pQE-80L-*puuE*$^+$/Δ*puuE* Δ*gabT*); lane 3, KJ123[pQE-80L-*puuE*(*K267A*)/Δ*puuE* Δ*gabT*]; lane 4, enriched PuuE (5 μg). (B) GABA-AT activity of PuuE(K267A). The GABA-AT activities of cell extracts of the strains were measured: lane 1, KJ117(pQE-80L/Δ*puuE* Δ*gabT*); lane 2, KJ121(pQE-80L-*puuE*$^+$/Δ*puuE* Δ*gabT*); and lane 3, KJ123[pQE-80L-*puuE*(*K267A*)/Δ*puuE* Δ*gabT*]. (C) Enriched His_6-YneI (5 μg).

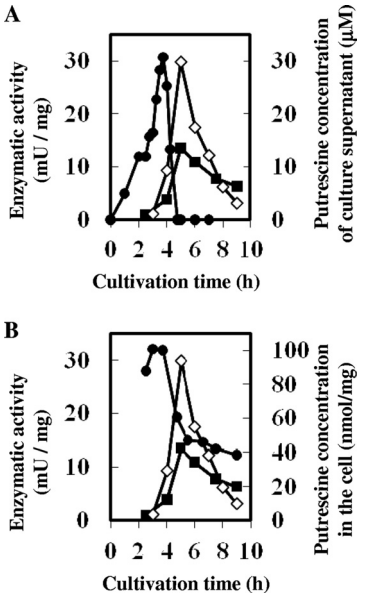


FIG. 5. Regulation of *puuE* by putrescine concentration in culture supernatant or in the cell. (A) Comparison of PuuE activity, PuuD activity, and putrescine concentration in culture supernatant. Closed circles, putrescine concentration of culture supernatant of SH639; open diamonds, GABA-AT activity of KJ102 (SH639 but Δ*gabT*); closed squares, PuuD activity of SH639. (B) Comparison of PuuE activity, PuuD activity, and putrescine concentration in the cell. Closed circles, putrescine concentration in the cell of SH639; open diamonds, GABA-AT activity of KJ102 (SH639 but Δ*gabT*), which is the same data set as described for panel A; closed squares, PuuD activity of SH639, which is the same data set as described for panel.

on the expression of *puuE* and *gabT* were examined. The expression of *gabT* was not influenced by putrescine (Fig. 6B) and GABA (Fig. 6C) compared with that of the control (Fig. 6A). In addition, the expression of *gabT* was not significantly influenced by succinate (Fig. 6D) or low aeration (Fig. 6E) compared with the control (Fig. 6A). On the other hand, PuuE activity was strongly induced by the addition of putrescine (Fig. 6B) and completely repressed by the addition of succinate (Fig. 6D) and under low aeration (Fig. 6E). GABA did not influence the expression of *puuE*, as was the case for *gabT* (Fig. 6C). The expression of *gabT* was nearly constitutive, while that of *puuE* was strongly influenced by the culture conditions.

His_6-YneI-generated succinate. In a previous study (2), the SSADH reaction of YneI was demonstrated using a simplified enzymatic method in which SSADH activity was determined by a decrease in A_{340} accompanied by a decrease in NADP; however, this method does not directly indicate the generation of succinate. In the present study, the generation of succinate by YneI was directly confirmed by a succinate-specific detection enzymatic method, in which succinyl-coenzyme A (CoA) synthetase with high substrate specificity for succinate was used. The generation of succinate by YneI was dependent on incubation time and the amount of the enzyme added to the reaction mixture (data not shown).

Putrescine-inducible SSADH: YneI. Two GABA-ATs, PuuE and GabT, catalyze the conversion of GABA to succinic semialdehyde, and *puuE* is putrescine inducible, while *gabT* is not. The product of the reaction catalyzed by GABA-AT is succinic semialdehyde, and succinic semialdehyde is degraded to succinate by SSADH (Fig. 1). It has previously been reported that GabD (18) and YneI (2) were both SSADHs. AldA is also annotated to be an SSADH. To analyze the effects of addition of putrescine or GABA to the medium on the expression of the three genes encoding SSADHs, the SSADH activities of KEI17 (*gabD*$^+$ Δ*yneI* Δ*aldA*), KEI18 (Δ*gabD* Δ*yneI aldA*$^+$), and KEI19 (Δ*gabD yneI*$^+$ Δ*aldA*) grown in M9-tryptone or M9-tryptone supplemented with 5 mM putrescine or 5 mM GABA were measured. While the SSADH activity of KEI17 (*gabD*$^+$ Δ*yneI* Δ*aldA*) was not influenced by the addition of putrescine and GABA to the medium, that of KEI19 (Δ*gabD yneI*$^+$ Δ*aldA*) grown with putrescine or GABA was 6 times or 2 times higher than that without any supplement (Fig. 7A). KEI18 (Δ*gabD* Δ*yneI aldA*$^+$) had very weak SSADH activity (Fig. 7A). These results indicate that YneI is a putrescine-inducible SSADH. The effect of addition of GABA on YneI activity was milder than that of putrescine. It is suggested that YneI and the enzymes of the Puu pathway coordinately respond to extracellular putrescine rather than extracellular

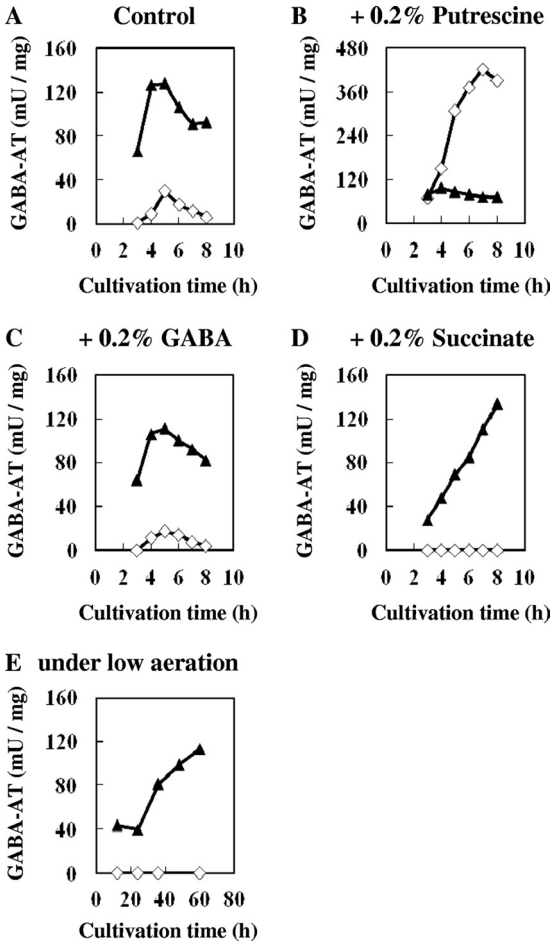


FIG. 6. Regulation of *puuE* by growth condition. KJ102 (Δ*gabT puuE*$^+$; open diamonds) and SK187 (*gabT*$^+$ Δ*puuE*; closed triangles) were inoculated to an A_{600} of 0.03 into 60 ml M9-tryptone in a 300-ml Erlenmeyer flask and were grown at 37°C with reciprocal shaking. Cells were harvested at various growth phases and cell extracts were prepared. (A) M9-tryptone without supplement and with shaking at 140 rpm; (B) M9-tryptone supplemented with 0.2% putrescine with shaking at 140 rpm. In this section, the vertical axis is shown on a different scale from that in other sections. (C) M9-tryptone supplemented with 0.2% GABA with shaking at 140 rpm. (D) M9-tryptone supplemented with 0.2% succinate with shaking at 140 rpm; (E) M9-tryptone without supplement with shaking at 60 rpm.

GABA, although we cannot eliminate the possibility that some metabolite of putrescine in the cell is the true inducer of *yneI*.

Physiological role of YneI. SSADH catalyzes the last step of the metabolic pathway that degrades putrescine to succinate. It has been reported previously (2) that YneI plays an important role in utilizing putrescine as the sole nitrogen source. However, because two amino groups of putrescine have already been utilized in the previous metabolic steps (Fig. 1A), it is more reasonable to think that SSADH plays a role in utilizing putrescine as the sole source of carbon rather than the sole source of nitrogen. To evaluate the importance of utilizing putrescine as the sole carbon source, *gabD*, *yneI*, and *aldA* single or double mutants were constructed and grown on M9-Put-AS plates with putrescine as the sole carbon source (Fig. 7B and C). The Δ*yneI* single mutant (KEI06) exhibited weak growth, and the Δ*gabD* Δ*yneI* double mutant (KEI18) did not grow on the M9-Put-AS plate. The Δ*gabD* (KEI08) and Δ*aldA* (KEI11) single mutants grew as well as the *gabD*$^+$ *yneI*$^+$ *aldA*$^+$ (SH639) strain. Because KEI19 (Δ*gabD yneI*$^+$ Δ*aldA*) showed more growth than KEI17 (*gabD*$^+$ Δ*yneI* Δ*aldA*) on the M9-Put-AS plate, *yneI* is apparently more important than *gabD* when *E. coli* utilizes putrescine as the sole carbon source.

***puuR* repressed the expression of *puuE* but had no influence on *yneI*.** PuuR is predicted by computer annotation to be a transcriptional regulator with a helix-turn-helix motif. The *puuR* gene is located in the *puu* gene cluster (Fig. 1B). In the Δ*puuR* strain, the levels of expression of *puuA* (8), *puuP* (9), and *puuD* (7) were elevated. The GABA-AT activity of the *puuE*$^+$ Δ*puuR* Δ*gabT* strain was undetected (data not shown) for unknown reasons. The 3Ala mutation was introduced into the helix-turn-helix motif of the *puuR* gene on the chromosome to construct a strain that synthesizes nonfunctional PuuR protein. The PuuE activity of KJ155 [Δ*gabT puuR*(*3Ala*)] was 15 times higher than that of KEI04 (Δ*gabT puuR*$^+$) (Fig. 8A). This result indicates that *puuE* was regulated by PuuR, as is the case for *puuA* (8), *puuD* (7), and *puuP* (9). The products of these genes constituting the Puu pathway are synchronically regulated by PuuR to utilize putrescine as a nutrient source. As described above, YneI was induced by putrescine in the medium; however, transcription of *yneI* was not enhanced by *puuR*(*3Ala*), while the transcription of *puuE* was strongly enhanced by the *puuR*(*3Ala*) mutation (Fig. 8B).

Regulation of the expression of *yneI*. The expression of PuuE was completely repressed by succinate added to the medium and under low-aeration conditions (Fig. 6D and E), and other members of the Puu pathway were regulated as well. To elucidate the regulation of *yneI* and *gabD* by succinate and aeration conditions, and to determine which SSADH act together with the Puu pathway, the SSADH activities of KEI19 (Δ*gabD yneI*$^+$ Δ*aldA*) and KEI17 (*gabD*$^+$ Δ*yneI* Δ*aldA*) were measured. Succinate had little effect on repression of YneI and GabD (data not shown) in comparison with the regulation of PuuE, which was completely repressed by succinate (Fig. 6D). Under low-aeration conditions (60 rpm), the level of YneI was 20% of that under high aeration (140 rpm), but the extent of repression was less than that for PuuE (Fig. 6E). Aeration conditions had no effect on GabD (data not shown).

DISCUSSION

In the step in which GABA is converted to succinic semialdehyde, there are duplicated GABA-ATs, GabT and PuuE (Fig. 1A). However, the significance of the duplication had not previously been studied. The existence of putrescine-inducible GABA-AT was first predicted by Schneider and Reitzer (18). In this study, we identified that GabT is constitutive and PuuE is inducible.

None of the genes of the *puu* gene cluster encodes SSADH, which catalyzes the final step of the degradation pathway from putrescine to succinate (Fig. 1). The regulation of the Puu pathway enzymes, including PuuE, can be summarized as follows: induced by putrescine (Fig. 6B), repressed by succinate (Fig. 6D), overexpressed in the *puuR* mutant strain (Fig. 8), and repressed under low aeration (Fig. 6E). In the three SSADHs that have been previously studied or annotated, YneI

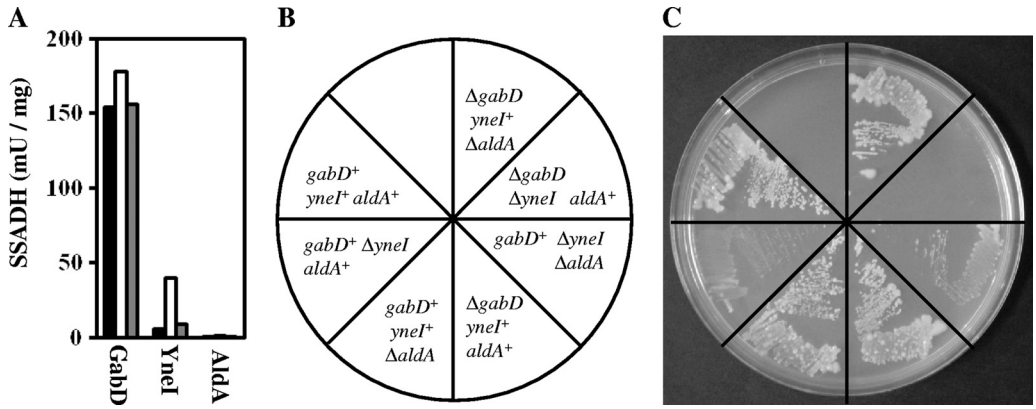


FIG. 7. YneI is a putrescine-inducible SSADH. (A) Influence of supplementation of the medium with putrescine on the expression of three SSADHs. KEI17 (*gabD*$^+$ Δ*yneI* Δ*ald*; shown as GabD), KEI19 (Δ*gabD yneI*$^+$ Δ*aldA*; shown as YneI), and KEI18 (Δ*gabD* Δ*yneI aldA*$^+$; shown as AldA) were inoculated to an A_{600} of 0.03 into 60-ml M9-tryptone supplemented with 5 mM putrescine (white bars), with 5 mM GABA (gray bars), or without any supplement (black bars) in a 300-ml Erlenmeyer flask and grown at 37°C with reciprocal shaking at 140 rpm. Cells were harvested 5 h after inoculation, and cell extract was prepared. (B and C) Importance of three SSADHs in utilizing putrescine as a carbon source. SH639 (*gabD*$^+$ *yneI*$^+$ *aldA*$^+$), KEI06 (*gabD*$^+$ Δ*yneI aldA*$^+$), KEI11 (*gabD*$^+$ *yneI*$^+$ Δ*aldA*), KEI08 (Δ*gabD yneI*$^+$ *aldA*$^+$), KEI17 (*gabD*$^+$ Δ*yneI* Δ*aldA*), KEI18 (Δ*gabD* Δ*yneI aldA*$^+$), and KEI19 (Δ*gabD yneI*$^+$ Δ*aldA*) were precultured on LB plates at 37°C, and single colonies were streaked on M9-Put-AS at 20°C.

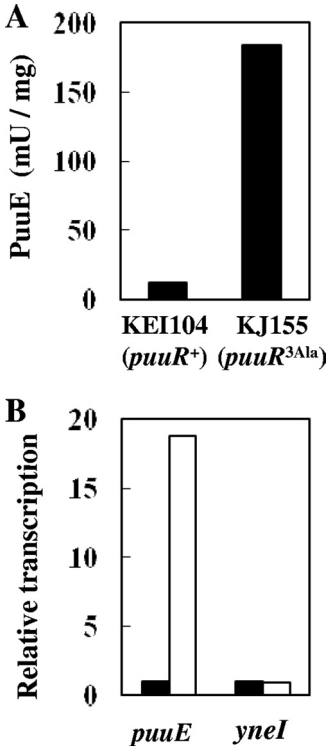


FIG. 8. Influence of the *puuR*(*3Ala*) mutation on the expression of *puuE* and *yneI*. (A) Influence of the *puuR*(*3Ala*) mutation on the expression of PuuE. KEI05 (Δ*gabT puuR*$^+$) and KJ155 [Δ*gabT puuR*(*3Ala*)] were inoculated to an A_{600} of 0.03 into 60 ml M9-tryptone in a 300-ml Erlenmeyer flask and grown at 37°C with reciprocal shaking at 140 rpm. Cells were harvested 5 h after inoculation and a cell extract was prepared to measure the PuuE activity. (B) Influence of the *puuR*(*3Ala*) mutation on the transcription of *puuE* and *yneI*. KEI09 (*puuR*$^+$; black bars) and KEI12 [*puuR*(3Ala); white bars] were inoculated to an A_{600} of 0.03 into 60 ml M9-tryptone in a 300-ml Erlenmeyer flask and grown at 37°C, with reciprocal shaking at 140 rpm. Cells were harvested 5 h after inoculation, collected by centrifugation using RNAprotect bacterial reagent (Qiagen), and frozen at −20°C. RNA extraction and transcription analysis using DNA-Chips were performed by Gene Frontier.

was regulated in a somewhat similar manner to PuuE. However, although YneI was induced by putrescine (Fig. 7A) and was repressed under low aeration, neither overexpression in the *puuR*(*3Ala*) strain (Fig. 8B) nor repression by succinate (data not shown) occurred. Therefore, there are two metabolic pathways from GABA to succinate: the constitutive GabT-GabD pathway and the putrescine-inducible PuuE-YneI pathway. Regulation of the *puuE*, *yneI*, *gabT*, *gabD*, and other *puu* genes is summarized in Table 2. The regulation of *puuE* was identical to that of the other *puu* genes; however, that of *yneI* was different to some extent because regulation by succinate and the *puuR* mutation (Fig. 8B) was not observed and mild induction by GABA (Fig. 7A) was observed (Table 2).

Although the metabolic steps catalyzed by PuuE and YneI seem to have no direct relationship with putrescine (Fig. 1A), PuuE and YneI were strongly induced by putrescine. On the other hand, PuuE was not induced by GABA, which is the direct substrate of PuuE. This result implies that putrescine is important as a nutrient source for the growth of *E. coli* in natural environments. Putrescine is often found in environments in which *E. coli* lives; for example, there is a several-hundred-micromolar concentration of putrescine in the large intestine (11), and this can be an important nutrient source.

TABLE 2. Comparison of the regulation of *puuE*, *yneI*, other *puu* genes, *gabT*, and *gabD*

Condition	Regulation of[a]:				
	puuE	*yneI*	Other *puu* genes	*gabT*	*gabD*
Putrescine	↑	↑	↑	→	→
GABA	→	↑	ND	→	→
Succinate	↓	→	↓	→	→
Low aeration	↓	↓	↓	→	→
puuR mutant	↑	→	↑	→	→

[a] ↑, expression was upregulated; →, expression was not changed; ↓, expression was downregulated; ND, not determined.

The homologs of enzymes forming the Puu pathway are only found in several Gram-negative species related to *E. coli*. These species may have an increased chance of survival by utilizing putrescine through the Puu pathway and YneI, which are immediately induced according to need, in addition to the constitutive pathway.

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