The Putrescine Importer PuuP of *Escherichia coli* K-12[▽]

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The Puu pathway is a putrescine utilization pathway involving gamma-glutamyl intermediates. The genes encoding the enzymes of the Puu pathway form a gene cluster, the *puu* gene cluster, and *puuP* is one of the genes in this cluster. In *Escherichia coli*, three putrescine importers, PotFGHI, PotABCD, and PotE, were discovered in the 1990s and have been studied; however, PuuP had not been discovered previously. This paper shows that PuuP is a novel putrescine importer whose kinetic parameters are equivalent to those of the polyamine importers discovered previously. A *puuP*⁺ strain absorbed up to 5 mM putrescine from the medium, but a Δ *puuP* strain did not. *E. coli* strain MA261 has been used in previous studies of polyamine transporters, but PuuP had not been identified previously. It was revealed that the *puuP* gene of MA261 was inactivated by a point mutation. When *E. coli* was grown on minimal medium supplemented with putrescine as the sole carbon or nitrogen source, only PuuP among the polyamine importers was required. *puuP* was expressed strongly when putrescine was added to the medium or when the *puuR* gene, which encodes a putative repressor, was deleted. When *E. coli* was grown in M9-tryptone medium, PuuP was expressed mainly in the exponential growth phase, and PotFGHI was expressed independently of the growth phase.

Polyamines (putrescine, spermidine, and spermine) are aliphatic amines that have two or more amino groups in their chemical structure. Polyamines are distributed widely, from prokaryotic (28) to eukaryotic (17) cells, and are found at especially high concentrations in proliferating cells such as cancer cells (15) and bacteria in the exponential growth phase (5). In vivo, polyamines play important roles as growth factors. For example, polyamines, as cations, bind to nucleic acid to stabilize its structure (30) and promote the synthesis of protein and nucleic acid (4). In addition, polyamines are involved in the modulation of cellular functions by interacting with glutamate receptors such as the *N*-methyl-D-aspartate receptor (29). Recently it was described that *Proteus mirabilis*, a gram-negative bacterium and a common urinary tract pathogen of humans, used putrescine as a cell-to-cell signaling molecule to induce differentiation into swarm cells (19, 23). If the synthesis of polyamines is blocked, then cell growth is stopped or profoundly slowed (27). The supplementation of exogenous polyamines restores the growth of these cells (4).

All polyamines have putrescine as a backbone structure, and putrescine is the precursor of all polyamines. The concentration of putrescine in *Escherichia coli* cells is very high (30 mM) (4) and is regulated by synthesis, transport, and degradation.

We previously reported that the putrescine degradation pathway, the Puu pathway, involves γ -glutamylated intermediates in *E. coli* (12). All members of the Puu pathway are encoded by genes neighboring each other, which are named the *puu* gene cluster. The *puu* gene cluster includes an open reading frame encoding a putrescine transporter, PuuP, which was identified in the previous study (12).

The transport of putrescine in *E. coli* has been studied for many years. Four transporters that are able to take up putrescine into *E. coli* cell have been identified (Fig. 1). PotFGHI (18) was previously identified as an ATP-dependent putrescine transporter that is a member of the ATP-binding cassette (ABC) transporter family. PotABCD (3) is a spermidine transporter and is also a member of the ABC transporter family, and PotABCD can take up putrescine with lower affinity. PotE is active in both the excretion and uptake of putrescine (8, 9). Excretion is based on a putrescine-ornithine antiporter activity (8), and uptake is dependent on the membrane potential (9). PuuP is the most recently discovered putrescine transporter, which depends on proton motive force (12). It is thought that the presence of PuuP has a large influence on putrescine homeostasis in *E. coli*, because deletion of the *puuP* gene abolished the utilizability of putrescine as a sole nitrogen source and the complementation of *puuP* gene on the plasmid restored this utilizability (12). However, the properties and regulation of PuuP remain to be investigated. Furthermore, the significance of the multiplicity of genes encoding putrescine transporters should be studied to understand polyamine homeostasis in *E. coli*.

This report examines the properties of PuuP, regulation of *puuP* expression, and significance of PuuP among the putrescine transporters in putrescine homeostasis.

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FIG. 1. Transporters of putrescine of *E. coli* that are discussed in this paper. PuuP and PotFGHI are putrescine importers described previously. PotABCD comprises an uptake system that is preferential for spermidine but also takes up putrescine, albeit with lower affinity. YdcSTUV is annotated as a putative putrescine transporter in several databases, but this activity has never been confirmed experimentally. PotE is putrescine-ornithine antiporter that was reported to take up putrescine depending on the culture conditions.

MATERIALS AND METHODS

Strain and plasmid construction. The *ggt* gene of SH639 (Table 1) was disrupted as described previously (25). P1 transduction, DNA manipulation, and transformation were performed by standard methods (16, 20) In SK212, disruption of the *puuP* gene was performed as follows. pSK207, including the DNA sequence of $\Delta puuR::kan^+$, was linearized using *S*all. The resulting 6.4-kb fragment was used to transform strain JC7673 by electroporation, and kanamycin-resistant SK210 was obtained. $\Delta puuR::kan^+$ was transduced into SH639 to obtain SK212. In SO22, disruption of the *puuP* gene was carried out as follows. pSO13, including the DNA sequence of $\Delta puuP::tet^+$, was linearized using *X*baI and *S*acI. The resulting 4.9-kb fragment was used to transform strain JC7673 by electroporation, and tetracycline-resistant SO22 was obtained. To construct YT20, the *potABCD* genes of KJ101 were disrupted by a method described previously by Datsenko and Wanner (2) using the PCR product amplified from pKD3 with the primers $\Delta potABCD$ -1 and $\Delta potABCD$ -2. As a result of homologous recombination, the region from the start codon of *potA* to the termination codon of *potD* was replaced by *Flp* recombination target (FRT)-*cat*⁺-FRT in YT20. In the construction of SK337, the *potFGHI* genes of KJ101 were disrupted as described previously (2) using the PCR product amplified from pKD13 with the primers $\Delta potFGHI$ -1 and $\Delta potFGHI$ -2. As a result of homologous recombination, in SK337 the region from the start codon of *potF* to the termination codon of *potI* was replaced by FRT-*kan*⁺-FRT. In the construction of SK359, the *ycdSTUV* genes of KJ101 were disrupted by the same method (2) using the PCR product amplified from pKD13 with the primers $\Delta ycdSTUV$ -1 and $\Delta ycdSTUV$ -2. Homologous recombination replaced the region from the start codon of *ycdS* to the termination codon of *ycdV* with FRT-*kan*⁺-FRT. To construct YT1, the *potE* gene of KJ101 was disrupted by the same method (2) using the PCR product amplified from pKD13 with the primers $\Delta potE$ -1 and $\Delta potE$ -2. After homologous recombination, the FRT-*kan*⁺-FRT sequence replaced the region from 45 bp downstream of the start codon of *potE* to 47 bp upstream of the termination codon of *potE* (whole length, 1.3 kb). To construct the multiple mutants, P1 transductions using P1(SO22, $\Delta puuP::tet^+$), P1(SK337, $\Delta potFGHI::FRT-kan^+-FRT$), P1(SK359, $\Delta ycdSTUV::FRT-kan^+-FRT$), P1(YT1, $\Delta potE::FRT-kan^+-FRT$), or P1(YT20, $\Delta potABCD::FRT-cat^+-FRT$) were performed. In the elimination of the *kan*⁺ and *cat*⁺ genes from FRT-*kan*⁺-FRT and FRT-*cat*⁺-FRT, respectively, the recombination reaction by *Flp* flippase carried by pCP20 was used. Colony PCRs were performed with primers which annealed upstream and downstream of the genomic regions containing mutations, and the sizes of DNA

amplified were measured to confirm that the intended insertion or deletion mutations were obtained.

Media and reagents. In all experiments, unless otherwise noted, strains were grown at 37°C with shaking at 140 rpm in 60 ml of medium in a 300-ml Erlenmeyer flask. M9-tryptone (14) was used in the analysis of extracellular polyamine concentration and in bacterial cultures for real-time reverse transcription-PCR (RT-PCR) analysis. The compositions of W-Glc-Put (including 0.2% putrescine as the sole nitrogen source) and W-Put-AS (including 0.4% putrescine as the sole carbon source) were described previously (14). The composition of medium B was described previously (6). In the growth experiments on M9-Glc-Put and M9-Put-AS, the strains were precultured on an LB plate at 37°C and then streaked on a nutrient-limited plate and incubated at 20°C.

[1,4-¹⁴C]putrescine dihydrochloride (107 mCi/mmol) was purchased from GE Healthcare, Buckinghamshire, England.

Transport assay. The transport assay was performed as described previously (11) using [¹⁴C]putrescine dihydrochloride, except that M9-tryptone medium was used instead of M63 medium. In the kinetic analysis of PuuP, YT75 was grown in M9-tryptone and harvested in the exponential phase (*A*₆₀₀ = 0.5). The cells were incubated with 0.1, 0.5, 2, 5, or 10 μM [¹⁴C]putrescine dihydrochloride.

Analysis of polyamine concentration. The polyamines in the samples were measured by high-pressure liquid chromatography as described previously (14).

Real-time RT-PCR analysis. Real-time RT-PCR analysis was performed as described previously (14). *puuP*-specific primers *puuP*-RT1 and *puuP*-RT2 were designed using PRIMER 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to amplify a 95-nucleotide fragments. The number of transcripts in a sample was determined by comparing the number of cycles required for the reaction to reach a common threshold, with a plot of threshold cycle values against standards (pSO19). The relative expression levels of *puuP* compared to the controls were calculated using OPTICON (Bio-Rad). The relative amounts of transcripts in the samples were further standardized by amplification of the *gapA* gene as an internal control using primers *gapA*-RT1 and *gapA*-RT2.

RESULTS

Uptake of putrescine from the medium by PuuP. To analyze the uptake of extracellular putrescine by PuuP during growth in the M9-tryptone, the putrescine concentration in the culture

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

Strain, phage, plasmid, or oligonucleotide	Characteristics or sequence	Source or reference
Strains		
JC7673	F ⁻ <i>thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 supE344 recB21 recC22 sbcB15</i>	A. J. Clark
KJ101	SH639/pKD46	This study
MA261	<i>speB speC gly leu thr thi</i>	1
SH639	F ⁻ Δ ggt-2	24, 26
SK210	JC7673 but Δ puuR::kan ⁺	This study
SK212	SH639 but Δ puuR::kan ⁺	This study
SK337	SH639 but Δ potFGHI::FRT-kan ⁺ -FRT	This study; SH639 $\times$ P1(SK210)
SK359	SH639 but Δ ycdSTUV::FRT-kan ⁺ -FRT	This study
SK365	SH639 but Δ potABCD::FRT Δ potFGHI::FRT Δ puuP::tet ⁺ Δ ycdSTUV::FRT	This study
SK421	SH639 but Δ potABCD::FRT Δ potE::FRT Δ potFGHI::FRT Δ ycdSTUV::FRT	This study
SK422	SH639 but Δ potABCD::FRT Δ potE::FRT Δ puuP::tet ⁺ Δ ycdSTUV::FRT	This study
SK423	SH639 but Δ potE::FRT Δ potFGHI::FRT Δ puuP::tet ⁺ Δ ycdSTUV::FRT	This study
SK424	SH639 but Δ potABCD::FRT Δ potE::FRT Δ potFGHI::FRT Δ puuP::tet ⁺	This study
SK425	SH639 but Δ potABCD::FRT Δ potE::FRT Δ potFGHI::FRT Δ puuP::tet ⁺ Δ ycdSTUV::FRT	This study
SO22	JC7673 but Δ puuP::tet ⁺	This study
SO23	SH639 but Δ puuP::tet ⁺	This study; SH639 $\times$ P1(SO22)
SO24	SO23/pSO19	12
YT1	SH639 but Δ potE::FRT-kan ⁺ -FRT	This study
YT4	MA261 but Δ puuP::tet ⁺	This study; MA261 $\times$ P1(SO23)
YT20	SH639 but Δ potABCD::FRT-cat ⁺ -FRT	This study
YT75	pYT71/SK425	This study
Phage Kohara 257		10
Plasmids		
pACYC184	p15A replicon, cat ⁺ tet ⁺	New England Biolabs (Ipswich, MA)
pBR322	ColE1 replicon, rop ⁺ bla ⁺ tet ⁺	New England Biolabs
pCP20	pSC101 replicon, (ts) bla ⁺ cat ⁺ Flp (λ Rp) cI857	2
pKD3	oriR γ bla ⁺ FRT-cat ⁺ -FRT	2
pKD13	oriR γ bla ⁺ FRT-kan ⁺ -FRT	2
pKD46	oriR101 replicon, repA101 ^{ts} araC ⁺ gam ⁺ -bet ⁺ -exo ⁺ (araBp) bla ⁺	2
pKHG3	p15A replicon, tet ⁺ puuA ⁺ puuD ⁺ puuR ⁺ ; 4.5-kb EcoRV fragment of Kohara 257- λ DNA was ligated with 3.2-kb ScaI and XmnI fragment of pACYC184	This study
pMW219	pSC101 replicon, kan ⁺	New England Biolabs
pSK207	p15A replicon, tet ⁺ puuA ⁺ puuD ⁺ Δ puuR::kan ⁺ ; 1.2-kb HincII fragment (tet ⁺) of pUC4K was ligated with 6.6-kb AflII and XmnI fragment of pKHG3, which deleted a 0.22-kb central region of 0.56-kb puuP	
pSO6	pSC101 replicon, kan ⁺ ymjA ⁺ puuP ⁺ ; 3.9-kb AatI fragment of Kohara 257- λ DNA was ligated with pMW219 cleaved with SmaI	This study
pSO8	pSC101 replicon, kan ⁺ ymjA ⁺ puuP ⁺ ; 4.7-kb AatI fragment (incompletely digested) of Kohara 257- λ DNA was ligated with pMW219 cleaved with SmaI	This study
pSO10	pSC101 replicon, kan ⁺ puuP ⁺ ; to eliminate the ymjA gene from pSO6, 2.3-kb BsmI (blunt-ended) fragment of pSO6 was ligated with pMW219 cleaved with SmaI	This study
pSO13	pSC101 replicon, kan ⁺ ymjA ⁺ Δ puuP::tet ⁺ ; 2.7-kb MscI and SspI fragment (tet ⁺) of pBR322 was ligated with 7.2-kb HpaI fragment of pSO8, which deleted a 1.3-kb N-terminal region of 1.4-kb puuP	This study
pSO19	ColE1 replicon, rop ⁺ bla ⁺ tet ⁺ puuP ⁺ ; 1.9-kb EcoRI fragment of pSO10 was ligated with pBR322 cleaved with EcoRI	This study
pUC4K	ColE1 replicon, bla ⁺ kan ⁺	Pharmacia
pYT71	p15A replicon, cat ⁺ puuP ⁺ ; 1.9-kb EcoRI fragment of pSO19 was blunt ended and ligated with pACYC184 cleaved with EcoRV	This study
Oligonucleotides		
Δ potABCD-1	5'-CATTAGCCACATCCTTGCTAACTAAAAACGGGCGGTAATACCACCGCCC GCTTGCTGAAGTGTAGGCTGGAGCTGCTTC-3'	
Δ potABCD-2	5'-AAGAGTCACCAAGGTGGTTAAACCACAAACCCGCATCGGTAAGCCATCCG TTGCGTTTACCATATGAATATCCTCCTTAG-3'	
Δ potE-1	5'-ATGAGTCAGGCTAAATCGAACAAAATGGGCGTCTGTTAGTTAACCGTGTA GGCTGGAGCTGCTTC-3'	
Δ potE-2	5'-TTAACCGTGTTTATTTTTCAGTTCAAAGCGTGGTGATACCAGACCATTCGG GGGATCCGTCGACC-3'	
Δ potFGHI-1	5'-ACAACCTTTTGTTCGTTTGTAAACGAACCTTCAGAAGGAAAGAGATATTC GGGATCCGTCGACC-3'	
Δ potFGHI-2	5'-GACATGCGCGGCATTATGTAGCCAGGTTGGCAAATTTAGTGTCTTGTAG GCTGGAGCTGCTTCG-3'	
Δ ycdSTUV-1	5'-CAAGGAGATATTTTACGGCACGGCGGCTGAACAATTAATTACGACAGG AGTAAGACCTTGTTGATAGGCTGGAGCTGCTTC-3'	
Δ ycdSTUV-2	5'-GCTAACCAAGTTCTCCGTTAATCAGTAACCTATGTTGCATAGCATATTCCTG TATCAGTGGATTCCGGGGATCCGTCGACC-3'	
puuP-RT1	5'-ATAAAGATAACGCCACCGTAGACC-3'	
puuP-RT2	5'-CGATTGTCTGTTTCTCCTTCCTC-3'	
gapA-RT1	5'-TGAAAGGCTTCTGGGCTACACT-3'	
gapA-RT2	5'-CGAAGTTGTCGTTTACAGAGCGATACC-3'

FIG. 2. Putrescine concentrations in culture supernatants with and without PuuP. Open circles, putrescine concentration in the culture supernatant of SH639 (*puuP*⁺); closed circles, putrescine concentration in the culture supernatant of SO23 (SH639 but Δ *puuP*); open squares, growth of SH639; closed squares, growth of SO23. (A) Change in putrescine concentration in the culture supernatants of *puuP*⁺ and Δ *puuP* strains. (B) Change in putrescine concentration in the culture supernatant of *puuP*⁺ and Δ *puuP* strains grown in M9-tryptone medium supplemented with 1 mM putrescine.

supernatant was measured at different growth phases (Fig. 2). In the *puuP*⁺ strain, the putrescine concentration in the culture supernatant increased to 30 μ M at 3.75 h, dropped to 0 μ M at 4.5 h, and did not increase again before the end of the incubation at 28 h after inoculation (Fig. 2A). On the other hand, in the Δ *puuP* strain, the putrescine concentration increased to around 60 μ M at 4 h, was maintained at between 50 and 60 μ M until 6.75 h after inoculation, and then gradually decreased to 2 μ M at 28 h (Fig. 2A). It is thought that the increase of putrescine in the early growth phase was caused by the export of putrescine to the medium by *E. coli* and that the rapid drop in the concentration of extracellular putrescine in the *puuP*⁺ strain from 3.75 to 4.5 h (the exponential phase) after inoculation was caused by the vigorous putrescine uptake from the medium by PuuP expressed at this stage. Furthermore, it is thought that a transporter(s) other than PuuP functioned after 7 h after inoculation (the stationary phase) to gradually decrease the putrescine concentration in the medium.

To assess what quantity of putrescine is taken up from the medium by PuuP, putrescine was added to the medium at a concentration of 1 mM (Fig. 2B). A rapid fall in the concentration of putrescine from 0.82 to 0 mM was observed between 4 and 7 h after inoculation only in the case of *puuP*⁺ cells. The Δ *puuP* strain imported less than 0.1 mM until 24 h after inoculation (stationary phase), although putrescine was added to the medium at a concentration of 1 mM (Fig. 2B). The PuuP-dependent decrease in the putrescine concentration in the

culture supernatant was up to 5 mM putrescine in the medium (data not shown).

PuuP-dependent uptake of putrescine in *E. coli* cells. In previous work (12), putrescine uptake was measured by counting ¹⁴C label in cells incubated with ¹⁴C-labeled putrescine. To confirm that putrescine was absorbed without degradation outside the cell, SH639 (*puuP*⁺), SO23 (Δ *puuP*), and SO24 (pBR322-*puuP*⁺/ Δ *puuP*) were grown for 5 h in M9-tryptone and in M9-tryptone supplemented with 5 mM putrescine, and the putrescine concentration in the cells was measured by high-pressure liquid chromatography. The intracellular putrescine concentrations in SH639, SO23, and SO24 grown in M9-tryptone supplemented with 5 mM putrescine were 33, 8, and 89% higher than those in strains grown in M9-tryptone, respectively. This result suggests that PuuP takes up intact putrescine without external degradation.

Comparison of the kinetic parameters of PuuP with those of PotFGHI and PotABCD. In previous work (7), the kinetic values for putrescine uptake by PotFGHI and PotABCD were determined using bacteria harboring pACYC184 with *potABCD* or *potFGHI* grown in medium B. To compare the kinetic parameters of PuuP with those values reported in the previous work (7), the K_m and V_{max} values for putrescine uptake by PuuP were measured using YT75 (pACYC184-*puuP*/SK425) grown in M9-tryptone. When the kinetic parameters were determined, bacteria were grown in the media in which each transporter was induced well, because PotFGHI activity of the bacteria grown in M9-tryptone was very weak and PuuP activity of SK421 grown in medium B was weaker than that in M9-tryptone (data not shown). The K_m and the V_{max} of PuuP were 3.7 μ M and 19.9 nmol/min/mg of protein, respectively. This result showed that the putrescine uptake capability of PuuP was equivalent to that of PotFGHI and PotABCD (Table 2), although the substrate affinity of PuuP was lower than that of PotFGHI.

The function of the *puuP* gene of MA261 is impaired. Although PuuP had an extremely large influence on the uptake of extracellular putrescine (Fig. 2B), PuuP was not found during studies of other putrescine importers. In the study of other putrescine importers, *potFGHI*, *potABCD*, and *potE* were expressed from plasmid vectors introduced into derivatives of a polyamine-requiring strain, MA261 (*speB speC*) (7). *SpeB* and *SpeC* comprise pathways of putrescine synthesis, and MA261 is defective in putrescine synthesis because of *speB* and *speC* mutations. The growth of MA261 is stimulated by the addition of putrescine to medium B because extracellular putrescine is imported into the cells by a putrescine importer(s). In the present findings, a large amount of putrescine taken up from the medium by *E. coli* was dependent on PuuP. Therefore, it

TABLE 2. Kinetic parameters of polyamine importers^a

Importer	K_m (mM)	V_{max} (nmol/min/ mg-protein)	Medium
PuuP	3.7	19.9	M9-tryptone
PotFGHI	0.5	22.1	Medium B
PotABCD	1.5	19.8	Medium B
PotE	1.8	3.4	Medium B

^a Data for PotFGHI, PotABCD, and PotE are from reference 7.

FIG. 3. Impairment of the *puuP* gene of MA261. Putrescine concentrations in the culture supernatants of MA261 (*speB speC*) and YT4 (MA261 but Δ *puuP*) are shown. M9-tryptone medium was supplemented with 1 mM putrescine. Open circles, putrescine concentration in the culture supernatant of MA261 (*speB speC*); closed circles, putrescine concentration in the culture supernatant of YT4 (MA261 but Δ *puuP*); open squares, growth of MA261; closed squares, growth of YT4.

was expected that the PuuP-dependent import of putrescine would stimulate the growth of MA261, and YT4 (MA261 but Δ *puuP*) was constructed. However, growth stimulation depending on PuuP in MA261 was not observed in M9-tryptone medium supplemented with 1 mM putrescine (Fig. 3). Surprisingly, the decrease of putrescine in the culture supernatant that was observed in the genetic background of SH639 (Fig. 2A) was not observed in MA261 (Fig. 3). To elucidate the reason for this discrepancy, the nucleic acid sequence of *puuP* of MA261 was determined. The open reading frame and the 384-bp upstream region of *puuP* including the *puuP* promoter were sequenced, and a mutation of adenine-329 to guanine was found. This mutation resulted in a replacement of Tyr-110 by Cys in PuuP. A transport assay was performed to confirm that the Y110C mutation resulted in a deficiency of putrescine uptake activity by PuuP. The [14 C]putrescine uptake activity of the PuuP Y110C mutant was reduced to one-eighth of that of wild-type PuuP (data not shown). These results suggested that *puuP* of MA261 was mutated at some step during strain construction and that this mutation impaired the putrescine uptake activity of PuuP. It is suggested that one of the reasons that PuuP had not been discovered during the study of other polyamine importers was because of the Y110C mutation in PuuP of MA261.

Growth phase-dependent regulation of two putrescine importers, PuuP and PotFGHI, in M9-tryptone medium. There are four putrescine importers (PotFGHI, PotE, PotABCD, and PuuP) that were described previously and one putative putrescine importer (YdcSTUV) in *E. coli*. To clarify which importer functions in specific growth phases in *E. coli*, five strains that have only one putrescine importer gene and SK425, in which all genes encoding known or annotated putrescine importers were deleted, were constructed. Transport assays with strains that were harvested in the exponential phase ($A_{600} = 0.5$) and in the stationary phase ($A_{600} = 3$ to 3.5) were performed (Fig. 4A). In the exponential phase (Fig. 4A), SK421, which had only *puuP*⁺ as a putrescine importer, exhibited the highest [14 C]putrescine transport activity. However, in the stationary phase (Fig. 4A), the transport activity of SK421 was decreased significantly. In the stationary phase, the [14 C]putrescine transport activity of SK422 (only *potF*⁺*G*⁺*H*⁺*I*⁺)

FIG. 4. Growth phase-dependent regulation of two putrescine importers, PuuP and PotFGHI, in M9-tryptone medium. (A) Putrescine import activities of various putrescine importers (PuuP, PotFGHI, PotE, and PotABCD) and of a putative putrescine importer (YdcSTUV) at the exponential and stationary growth phases. SK421 (only *puuP*⁺, shown as PuuP), SK422 (only *potF*⁺*G*⁺*H*⁺*I*⁺, shown as PotFGHI), SK365 (only *potE*⁺, shown as PotE), SK423 (only *potA*⁺*B*⁺*C*⁺*D*⁺, shown as PotABCD), SK424 (only *ycdS*⁺*T*⁺*U*⁺*V*⁺, shown as YdcSTUV), and SK425 (all genes encoding known or annotated putrescine importers were deleted, shown as Δ all) were grown in M9-tryptone medium and harvested in the exponential growth phase ($A_{600} = 0.5$, black bars) or in the stationary phase ($A_{600} = 3$ to 3.5, white bars). Cells were incubated with 10 μ M [14 C]putrescine. The linearity between the uptake of [14 C]putrescine and the incubation time was confirmed. (B) Putrescine concentrations in the culture supernatants of strains SK421 (only *puuP*⁺, closed circles), SK422 (only *potF*⁺*G*⁺*H*⁺*I*⁺, open squares), and SK425 (all genes encoding known or annotated putrescine importers were deleted, closed triangles).

I⁺) was the highest among these strains, although the transport activity was low (Fig. 4A). To evaluate the influence of the activity of PuuP and PotFGHI on the environment, the concentrations of putrescine in the culture supernatant of SK421 (only *puuP*⁺), SK422 (only *potF*⁺*G*⁺*H*⁺*I*⁺), and SK425 (all genes encoding known or annotated as putrescine importers were deleted) were measured (Fig. 4B). The concentration of putrescine in the culture supernatant of SK421 (only *puuP*⁺) decreased from 25 μ M to almost 0 μ M in the exponential phase but increased gradually to 15 μ M in the stationary phase because SK421 lacks *potFGHI*, which are expressed in the stationary phase. On the other hand, the putrescine concentration in the culture supernatant of SK422 (only *potF*⁺*G*⁺*H*⁺*I*⁺) increased to 60 μ M because SK422 lacks *puuP*,

FIG. 5. Utilizability of putrescine as a sole source of nitrogen or carbon depending on PuuP. (A) Left panel, arrangement of strains on the plates. SK421 (only *puuP*⁺), SK422 (only *potF*⁺*G*⁺*H*⁺*I*⁺), SK365 (only *potE*⁺), SK423 (only *potA*⁺*B*⁺*C*⁺*D*⁺), SK424 (only *ycdS*⁺*T*⁺*U*⁺*V*⁺), and SK425 (all genes encoding known or annotated putrescine importers were deleted) were streaked (see also Fig. 4A) on W-Glc-Put (including putrescine as a sole nitrogen source, center panel) or M9-Put-AS (including putrescine as a sole carbon source, right panel). (B) Compositions of W-Glc-Put and M9-Put-AS.

which is expressed in the exponential phase, and then decreased to 50 μ M. This result was consistent with the [¹⁴C] putrescine uptake activity of SK421 (only *puuP*⁺) being high in the exponential phase and the [¹⁴C]putrescine uptake activity of SK422 (only *potF*⁺*G*⁺*H*⁺*I*⁺) being relatively high in the stationary phase (Fig. 4B). The accumulation of putrescine in the culture supernatant of SK425 (all genes encoding known or annotated as putrescine importers were deleted) was highest in these three strains (Fig. 4B). The concentration of putrescine in the culture supernatant of SK425 was 90 μ M at 30 h after inoculation (Fig. 4B).

Relationship between putrescine transporters and the ability to utilize putrescine. To compare the importance of the transporters in the uptake of putrescine, the strains that had only one remaining putrescine importer were grown on plates (Fig. 5A) with putrescine as a sole carbon or nitrogen source (Fig. 5B). Only SK421, which has only PuuP as a putrescine transporter, could grow on putrescine as a sole source of nitrogen or carbon. This result clearly indicates that only PuuP out of five putrescine importers which are known or annotated is essential for utilization of putrescine as a sole nitrogen or carbon source and can support the growth of *E. coli*.

Expression of *puuP* is repressed by *puuR* at the transcription level. It was reported previously that the expression of *puuA* and *puuD* was strongly enhanced by the deletion of *puuR* (13, 14). Therefore, it was expected that the expression of *puuP* would also be enhanced by the deletion of *puuR*. Both the [¹⁴C]putrescine uptake activity and the transcription of *puuP* were strongly enhanced by deletion of the *puuR* gene (Fig. 6).

DISCUSSION

This study revealed that PuuP is an efficient putrescine importer whose kinetic parameters are not dramatically different from those of PotFGHI (18) and PotABCD (3) (Table 2). This

is clear because among all of the genes for putrescine transporters, *puuP* is indispensable for *E. coli* to grow on medium containing putrescine as a sole source of nitrogen or carbon (Fig. 5). In fact, *E. coli* took up a large amount of putrescine from the medium using PuuP (Fig. 2B). MA261, which has been used in studies of polyamine importers, has a mutation in *puuP*, which decreased the putrescine uptake activity of PuuP severely.

This study revealed that PuuP functions in the exponential growth phase and PotFGHI in the stationary growth phase in the uptake of putrescine from M9-tryptone medium (Fig. 4). Because the activity of PotFGHI was four times higher than

FIG. 6. Regulation of *puuP* by *puuR*. White bars, regulation of [¹⁴C]putrescine uptake activity of PuuP by deletion of the *puuR* gene. *puuR*⁺ (SH639) and Δ *puuR* (SK212) strains were grown in M9-tryptone medium and harvested at an *A*₆₀₀ of 0.5 (exponential phase). The cells were incubated with 10 μ M [¹⁴C]putrescine. The linearity between the uptake of [¹⁴C]putrescine and the incubation time was confirmed. Gray bars, regulation of the transcription of the *puuP* gene by deletion of the *puuR* gene. *puuR*⁺ (SH639) and Δ *puuR* (SK212) strains were grown in M9-Tryptone medium and harvested at an *A*₆₀₀ of 0.5, and extracted RNA was subjected to real-time RT-PCR analysis. The transcription level of *puuP* was quantitated and compared with the *puuP* expression in the *puuR*⁺ strain, which was taken as 1. Error bars indicate standard deviations.

that of PuuP when the strains were grown in medium B, which has been used in the study of PotFGHI (3, 7), with shaking at 100 rpm in the stationary phase (data not shown), PotFGHI appears to work more actively than PuuP under some growth conditions.

When a large amount of putrescine was added to the medium, the amount of the putrescine uptake dependent on other transporters in the stationary phase was very small (only 100 μ M), compared with up to 5 mM uptake that was dependent on PuuP (Fig. 2B). Furthermore, *puuP* was highly induced by the addition of putrescine to the medium (data not shown). It seems that only *puuP* was induced, while other putrescine importers were not induced. These results were consistent with the result that *E. coli* could grow on putrescine as a sole nitrogen or carbon source in a PuuP-dependent manner (Fig. 5). This indicates that since PuuP is induced by putrescine and takes up a large amount of putrescine in the early stage growth, it is essential for *E. coli* to form colonies by utilizing putrescine as a nutrient source.

The results described above indicate that PuuP takes up a large amount of putrescine from the medium, followed by the degradation of putrescine by the Puu pathway, and PuuP allows *E. coli* to grow on putrescine as a sole nitrogen or carbon source. Two putrescine degradation systems are known: the Puu pathway via γ -glutamylated metabolites (12) and the YdcW-YgiG pathway without γ -glutamylation (14, 21, 22). Multiple putrescine uptake and degradation systems exist in *E. coli*. No influence on the growth on putrescine as a sole carbon or nitrogen source was observed when *ydcW* and *ygiG* were deleted, while the deletion of genes encoding the Puu pathway abolished the ability to utilize putrescine as a sole carbon or nitrogen source (14), which was consistent with the result that only PuuP among the putrescine importers is effective in the utilization of putrescine as a sole nitrogen or carbon source (Fig. 5). The results indicate that when *E. coli* grows on putrescine, the Puu pathway is the mainstream putrescine degradation pathway in the exponential growth phase, and PuuP is engaged as a major importer of putrescine.

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