A novel bacterial putrescine utilization pathway was discovered. Seven genes, the functions of whose products were not known, are involved in this novel pathway. Five of them encode enzymes that catabolize putrescine; one encodes a putrescine importer, and the other encodes a transcriptional regulator. This novel pathway involves six sequential steps as follows: 1) import of putrescine; 2) ATP-dependent γ-glutamylation of putrescine; 3) oxidation of γ-glutamylputrescine; 4) dehydrogenation of γ-glutamyl-γ-aminobutyraldehyde; 5) hydrolysis of the γ-glutamyl linkage of γ-glutamyl-γ-aminobutyrate; and 6) transamination of γ-aminobutyrate to form the final product of this pathway, succinate semialdehyde, which is the precursor of succinate.

γ-Glutamyl compounds are widely found in both prokaryotic and eukaryotic cells. For example, glutathione (γ-glutamylcysteinylglycine) is a very important antioxidant (1) for living cells.

We have studied bacterial γ-glutamyltranspeptidase (GGT) (2, 3) that catalyzes the hydrolysis of the γ-glutamyl linkage of glutathione (4). During the course of our study on Escherichia coli GGT, we unexpectedly found that the γ-glutamyl linkage of γ-glutamyl-p-nitroanilide (γ-GpNA) was cleaved by the cell-free extract of a GGT-deficient strain. The enzyme that is responsible for this reaction was purified, and the relevant gene, ycjL, was identified. Further study of ycjL and neighboring genes (ycj family) revealed that the products of the ycj gene cluster constitute a novel putrescine-utilizing pathway in which γ-Glu-Put is involved.

Putrescine, the substrate of this pathway, is one of polyamines that are found in a wide range of organisms from bacteria to plants and animals especially when these cells are proliferating or in stressful conditions (5). Also in E. coli, putrescine plays important roles in cell proliferation and normal cell growth (5). Putrescine is synthesized from ornithine by ornithine decarboxylase (SpeC) or from arginine by the sequential actions of arginine decarboxylase (SpeA) and agmatinase (SpeB) in E. coli cells (6). Putrescine is converted to spermidine, another polyamine, by the addition of an aminopropyl group derived from decarboxylated S-adenosylmethionine through the activity of spermidine synthase (SpeE) (7).

Putrescine has been reported to be converted to γ-aminobutyraldehyde by putrescine transaminase and then to be oxidized to γ-aminobutyrate (GABA) by aminobutyraldehyde dehydrogenase (8). However, neither of the genes encoding this pathway has been identified yet. GABA is converted to succinate semialdehyde by GABA aminotransferase (GabT) and then is oxidized to succinate by succinate semialdehyde dehydrogenase (GabD) (9). Recently, it was reported that the Δ gabT strain grew in minimal medium containing putrescine as a sole nitrogen source but not in minimal medium containing GABA instead of putrescine (10). This unexpected growth in the presence of putrescine likely resulted from the specific induction of a gab-independent enzyme by putrescine. It was reported that an excess amount of putrescine is exported by PotE, putrescine ornithine antiporter (11), and that putrescine outside of the cell is imported by PotFGHI, the ABC transporter (12), according to the environment in which E. coli lives.

In this paper we report on a completely new catabolic pathway for extracellular putrescine. This pathway involves the γ-glutamylation of putrescine and plays an important physiological role to utilize putrescine in nutrient starvation. All the enzymes, a transporter, and a regulator that constitute this pathway are encoded by the ycj gene cluster.2

MATERIALS AND METHODS

Media and Reagents—Strains were grown in M9-tryptone (M9 minimal medium (13) except that 1% Bacto-tryptone was used instead of 0.2% glucose) with high aeration (140 rpm) at 37 °C in the determination of enzyme activities, accumulation of amine or amino acids in cells, or overexpression of proteins. To determine the putrescine utilization of various strains, W-Put plates (W salts minimal medium (14) containing 0.4% glucose as a sole carbon source and 0.2% putrescine as a sole nitrogen source (10)) were used. [1,4-14C]Putrescine dihydrochloride (107 mCi/mmol) was purchased from Amersham Biosciences.

Bacterial Strains and Plasmids—P1 transduction and DNA manipulation and transformation were performed by the standard methods (13, 15). A DNA fragment of Kohara clone 257 (16) containing the ycj gene cluster was cloned onto high copy number plasmids. The bacterial
GGT was assayed as described previously (2). mM MgCl$_2$ and 200 mM imidazole chloride buffer (pH 7.0) was incubated at 37 °C at a flow rate of 0.6 ml/min. The gradient of phosphate monosodium citrate dehydrate, 200 mM boric acid, 0.12N NaOH (pH 7.0) linearly increased to 7% from 9 to 13 min, to 8% from 13 to 17.2 min, and then to 11%. perchloric acid, 7% ethanol (pH 3.2)) and buffer B (200 mM tri-sodium citrate dehydrate, 1% was purchased from Nacalai Tesque, Kyoto, Japan, and Sigma, except for sodium glutamate, 50 mM putrescine dihydrochloride, 15 mM ATP, 60 mM MgCl$_2$, and 200 mM imidazole chloride buffer (pH 7.0) was incubated at 37 °C. γ-Glu-Put oxidase activity was assayed by measuring the decrease in γ-Glu-Put by HPLC. The reaction mixture containing 1 mM γ-Glu-Put and 10 mM potassium phosphate buffer (pH 7.0) was incubated at 37 °C. γ-Glu-GABA hydrolysis activity was determined by measuring the glutamate released from γ-Glu-GABA. The reaction mixture containing various concentrations of γ-Glu-GABA and 50 mM imidazole-HCl buffer (pH 8.7) was incubated at 37 °C. GABA aminotransferase activity was determined by measuring the glutamate formed from GABA and α-ketoglutarate. The reaction mixture containing 10 mM GABA, 10 mM α-ketoglutarate monosodium salt, and 100 mM potassium phosphate buffer (pH 7.0) was incubated at 37 °C. Adding trichloroacetic acid (final concentration, 10%) terminated the reactions, and the filtrates were analyzed by HPLC.

**Purification of Proteins**—Following the γ-GpNA hydrolysis activity, YgJ was purified from the cell-free extract of SH639 (Δggt-2) by ammonium sulfate precipitation (30–50%) and column chromatographies using DEAE-Cellulofine A-500m (Chisso, Tokyo, Japan), phenyl-Sepharose CL-4B (Amersham Biosciences), Cellulofine GC-700m (Chisso), and MiniQ (Amersham Biosciences). During the purification, the protein was basically dissolved in buffer C (50 mM Tris-HCl (pH 8.7) and 5 mM β-mercaptoethanol). After the ammonium sulfate precipitation, the enzyme was dissolved in buffer C and dialyzed against the same buffer. The dialyzed enzyme solution was applied to DEAE-Cellulofine A-500m previously equilibrated with buffer C. Without washing the column, YgJ was eluted with 0.2 mM NaCl in buffer C from the column. In the purification step using phenyl-Sepharose CL4B, the enzyme solution was dialyzed against buffer D (0.8 mM ammonium sulfate in buffer C) and applied to the column equilibrated with buffer D. The column was washed with buffer D, and YgJ was eluted at the end of a linear gradient formed between buffer D (0.8 mM ammonium sulfate in buffer C) and

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### Table 1

| Strains or plasmid | Characteristic or sequence | Source or Ref. |
|-------------------|---------------------------|---------------|
| KJ107             | SH639ΔgabT::FRT           | This study    |
| KJ109             | SH639ΔgogA::kan+ΔgabT::FRT| This study    |
| SH639             | F′Δggt-2                 | 2             |
| SH703             | F′Δggt-2 ΔgabT::Tn10      | 30            |
| SK111             | SH703ΔycjLC::kan+        | This study    |
| SK112             | pBR-ycj/SK111            | This study    |
| SK121             | pBR322-ycjLC/SK111       | This study    |
| SK187             | SH639ΔgogA::kan+         | This study    |
| SK189             | ΔordHL-ΔgoaG::kan+       | This study    |
| SK247             | SH639ΔycjLC::FRTΔaldH-ordL::goaG::FRT | This study |
| SK263             | pACYC184/SK247           | This study    |
| SK279             | pACYC-aldH-ordL/SK247    | This study    |
| SK283             | pACYC-ordL/SK247         | This study    |
| SK303             | pBelo-ycjLC-aldH-ordL::goaG/SK247 | This study |
| SK306             | pBeloBAC11/SK247         | This study    |
| SO23              | SH639ΔycjJ::tet+         | This study    |
| SO24              | pBR-ycj/SO23             | This study    |
| SO358             | SH639ΔycjK::FRTΔaldH-ordL::goaG::kan+ | This study |
| SO60              | pmal-c2X/SO58            | This study    |
| SO61              | pmal-c2X-ycjK            | This study    |
| SO66              | pQE-80L-ordL/SK247       | This study    |
| SO67              | pQE-80L/SK247            | This study    |

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**Plasmids**

| Plasmids | Characteristic or sequence | Source or Ref. |
|----------|---------------------------|---------------|
| pACYC184 | p15A replicon cat+ tet+   | New England Biosibs |
| pACYC-aldH | p15A replicon cat+ aldH+ | This study    |
| pACYC-aldH-ordL | p15A replicon cat+ aldH-ordL+ | This study |
| pACYC-aldH-ordL::goaG | p15A replicon cat+ aldH-ordL::goaG+ | This study |
| pACYC-ordL | p15A replicon cat+ ordL+ | This study    |
| pACYC-ordL | encodes aldH were looped out | This study    |
| pBeloBAC11 | Mini-F replicon cat+ | New England Biosibs |
| pBelo-ycjLC::aldH-ordL::goaG | Mini-F replicon cat+ ycjL::ycjC+ aldH+ ordL+::goaG+ | This study |
| pBR322   | ColE1 replicon rop+ bl+ tet+ | New England Biosibs |
| pBR-ycjJ | ColE1 replicon rop+ bl+ bl+ tet+ ycjJ+ | This study |
| pBR-ycjLC | ColE1 replicon rop+ bl+ tet+ ycjL+ | New England Biosibs |
| pBelo-ycjLC | ColE1 replicon rop+ bl+::ycjL-C+ | This study |
| pmal-c2X | ColE1 replicon f1 ig rop+ bl+ lacP::malE-lacZa::(Hyb) (tac+)::(malE without signal peptide) | New England Biosibs |
| p mal-c2X-ycjK | ColE1 replicon f1 ig rop+ bl+ lacP::malE-ycjK::(Hyb) (tac+)::(malE without signal peptide) | This study |
| pQE-80L  | ColE1 replicon bl+ lacF::T5_{5}(His)_{6} | Qiagen |
| pQE-80L-ordL | ColE1 replicon bl+ lacF::T5_{5}(His)_{6}-ordL+ | This study |

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strains and plasmids used in this study are summarized in Table I. Genes were disrupted according to the method of Datskeno and Wanner (17) or as described previously (18). Analysis of γ-Glutamyl Compounds, Amino Acids, and Amines—The γ-glutamyl compounds, amino acids, and amines in the samples were measured by using an HPLC system (model LC-9A; 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. The gradient of the mobile phase was formed with buffer A (66.6 mM tri-sodium citrate dehydrate, 1% perchloric acid, 7% ethanol (pH 3.2)) and buffer B (200 mM tri-sodium citrate dehydrate, 200 mM boric acid, 0.12 N NaOH (pH 10)). The concentration of buffer B was kept at 0% until 9 min. It was linearly increased to 7% from 9 to 13 min, to 8% from 13 to 17.2 min, and then to 11%. α-Phthalaldehyde was used as the detection reagent (19) as described previously (20), and the fluorescence was detected with a fluorescence detector (model RF-535, Shimadzu) as the absorbance at 450 nm, with excitation at 366 nm. Standard compounds were purchased from Nacalai Tesque, Kyoto, Japan, and Sigma, except for γ-Glu-Put and γ-Glu-GABA, which were synthesized as described below. In our HPLC system, γ-Glu-GABA, glutamate, GABA, and γ-Glu-Put was eluted at 8, 11, 26, and 29 min, respectively. Before measuring amino acids from the cell-free extract, the extract was previously treated with trichloroacetic acid (final concentration, 10%) to precipitate its proteins.

Assays for Enzymes—The γ-GpNA hydrolysis activity of YgJ and GGT was assayed as described previously (2). γ-Glu-Put synthetase activity was determined by measuring the γ-Glu-Put synthesized from glutamate and putrescine. The reaction mixture containing 50 mM sodium glutamate, 50 mM putrescine dihydrochloride, 15 mM ATP, 60 mM MgCl$_2$, and 200 mM imidazole chloride buffer (pH 7.0) was incubated at 37 °C.
buffer E (40% ethylene glycol in buffer C). In the purification step using Cellulofine GC-700, the enzyme solution was concentrated by the ammonium sulfate precipitation and applied to the column previously equilibrated with buffer C. The active fraction was applied to the MiniQ column previously equilibrated with buffer C. YcjL was eluted with a linear gradient formed between buffer C and 0.5 M NaCl in buffer C. YcjL was eluted when the concentration of NaCl was 0.15 M. MalE-YcjK was overexpressed after induction by isopropyl-β-D-thiogalactopyranoside and purified from the cell-free extract by anion exchange chromatography using Dowex 1-X8 (Dow Chemical) and purified by the cell-free extract by anion exchange chromatography using Dowex 50W-X8 (Dow Chemical) from the cell-free extract of SK247.

The purity was checked by HPLC. Purified YcjL hydrolyzed glutamine to glutamate and glutamine and glutamate using MalE-YcjK and purified by Dowex 50W-X8. Purified YcjL because the function of was not known, but the function of one of the, the ipuABCDEFGH operon in Pseudomonas sp., has been reported (25) (Fig. 2A). According to the report, the operon functions in isopropylamine (IPA) degradation. YcjK and IpuC showed 29.5% identity at the protein level, and both had homology with glutamate synthetase. YcjL, which is located at downstream of YcjK (Fig. 1), showed 19.5% identity with IpuG, which is thought to be an IPA transporter. Furthermore, IpuF had hydrolysis activity for the γ-glutamyl linkage similar to YcjL. In the degradation pathway encoded by the ipu operon, IPA is taken up by IpuG and γ-glutamylated by IpuC in Pseudomonas sp. cells. Subsequently, γ-glutamyl-IPA is oxidized by IpuD, and the product, γ-glutamyl-alaninol, is hydrolized by IpuF to glutamate and alaninol (Fig. 2A). Here we hypothesized that some amine is first imported into the E. coli cell by YcjJ, and its amino group is γ-glutamylated by YcjK. Subsequently, the amine part of γ-glutamyl-amine is oxidized by some oxidoreductase. The γ-glutamyl linkage of γ-glutamyl-oxidized amine is hydrolyzed to glutamate and oxidized amine by YcjL. The oxidized amine can probably be converted to the next catalobite more easily than the original amine. In this hypothesis, the oxidoreductase is important to make sense of the γ-glutamylamidation, and we searched for it near ycjJKLC. Downstream of the ycjLC operon, we found ordL predicted to encode oxidoreductase, ordL is a member of the aldH-ordL-goaG operon. This operon had already been reported, but its mRMA could not be detected by Northern analysis (26). Therefore, we hypothesized that the gene products of ycjJ, ycjK, the ycjLC operon, and the aldH-ordL-goaG operon (ycj gene cluster) work together in the uptake and degradation of some amine. Therefore, it was essential to identify this amine to elucidate the physiological role of the ycj gene cluster.

**RESULTS**

A Novel γ-GpNA Hydrolysis Activity of GGT-deficient Strain of E. coli—γ-GpNA is an artificial substrate widely used to measure GGT activity (24). We found a novel γ-GpNA hydrolysis activity in the cytosol of E. coli strain SH639 (γ-gtt-2) grown at high aeration (60 ml of culture in a 300-ml Erlenmeyer flask shaken at 140 rpm). To identify the protein responsible for this activity, the relevant protein was purified, and its N-terminal amino acid sequence was determined by the Edman degadation method. The amino acid sequence obtained, MEN-IMNNPVIG, was compared with sequences in a databases. Colibri (genolist.pasteur.fr/Colibri/), and the relevant gene was found to be ycjL whose function was not known. Strain SK111 (ΔycjLC) completely lost the γ-GpNA hydrolysis activity, and SK112 (pBR-ycjL/ΔSK111) overproduced the protein responsible for the activity. Purified YcjL hydrolyzed glutamine to glutamate and thamine (γ-glutamylethylamine) to glutamate and ethylamine. These results suggested that YcjL is a novel enzyme that catalyzes the hydrolysis of the γ-glutamyl linkage.

**Hypothesis for Physiological Roles of ycjL and Genes Around ycjL**—It was not easy to determine the physiological role of YcjL because the ΔycjL mutant could grow on M9-glucose minimal medium as well as the wild-type strain. Several genes seem to function with ycjL in E. coli cells (Fig. 1). ycjC is located downstream of ycjL, and these two genes are suggested to compose an operon. YcjC has a helix-turn-helix motif, and the γ-GpNA hydrolysis activity of strain SK112 (pBR-ycjL/ΔycjLC) was 400 times that of SK121 (pBR-ycjL/ΔycjLC). The results indicate that YcjC is a repressor of ycjL, ycjK is located upstream of ycjL in a reverse orientation, and its transcription is suggested to overlap with that of ycjL (Fig. 1). The disposition of these three genes, ycjK, ycjL, and ycjC, suggested that they functioned together. To presume the function of the products of this gene cluster, a homologous gene cluster for ycjKLC was searched for in the database GenomeNet (www.genome.ad.jp/), and several were found in bacteria. The function of most of them was not known, but the function of one of them, the ipuABCDEFGH operon in Pseudomonas sp., has been reported (25) (Fig. 2A). According to the report, the operon functions in isopropylamine (IPA) degradation. YcjK and IpuC showed 29.5% identity at the protein level, and both had homology with glutamate synthetase. YcjL, which is located at downstream of YcjK (Fig. 1), showed 19.5% identity with IpuG, which is thought to be an IPA transporter. Furthermore, IpuF had hydrolysis activity for the γ-glutamyl linkage similar to YcjL. In the degradation pathway encoded by the ipu operon, IPA is taken up by IpuG and γ-glutamylated by IpuC in Pseudomonas sp. cells. Subsequently, γ-glutamyl-IPA is oxidized by IpuD, and the product, γ-glutamyl-alaninol, is hydrolized by IpuF to glutamate and alaninol (Fig. 2A). Here we hypothesized that some amine is first imported into the E. coli cell by YcjJ, and its amino group is γ-glutamylated by YcjK. Subsequently, the amine part of γ-glutamyl-amine is oxidized by some oxidoreductase. The γ-glutamyl linkage of γ-glutamyl-oxidized amine is hydrolyzed to glutamate and oxidized amine by YcjL. The oxidized amine can probably be converted to the next catalochrome more easily than the original amine. In this hypothesis, the oxidoreductase is important to make sense of the γ-glutamylamidation, and we searched for it near ycjJKLC. Downstream of the ycjLC operon, we found ordL predicted to encode oxidoreductase. ordL is a member of the aldH-ordL-goaG operon. This operon had already been reported, but its mRNA could not be detected by Northern analysis (26). Therefore, we hypothesized that the gene products of ycjJ, ycjK, the ycjLC operon, and the aldH-ordL-goaG operon (ycj gene cluster) work together in the uptake and degradation of some amine. Therefore, it was essential to identify this amine to elucidate the physiological role of the ycj gene cluster.
catalyzes the reaction of γ-Glu-Put synthesis in vivo, SO61 (pMal-c2X-ycjK/ΔycjKLC Δ(aldH-ordL-goaG)); ycjJ⁺ ycjK⁺ ycjL⁺ ycjC⁺ aldH⁺ ordL⁺ goaG⁺) and SO60 (pMal-c2X/ΔycjKLC Δ(aldH-ordL-goaG)); ycjJ⁺ ycjK⁺ ycjL⁺ ycjC⁺ aldH⁺ ordL⁺ goaG⁺) were grown in M9-tryptone, and the cell-free extract was subjected to HPLC. The strain with the plasmid encoding MalE-YcjK exhibited γ-Glu-Put accumulation, whereas the strain with the empty vector did not. To show γ-Glu-Put synthesis by YcjK in vitro, MalE-YcjK was purified and mixed with putrescine, glutamate, ATP, and Mg²⁺, and the mixture was incubated at 37 °C. The synthesis of γ-Glu-Put was confirmed by HPLC and was not observed without ATP and Mg²⁺. These results strongly indicated that YcjK is γ-Glu-Put synthetase, and the products of the ycj gene cluster catabolize putrescine.

Putrescine is reported to be degraded after acetylation in mammalian cells (27). Referring to that report (27), we hypothesized that putrescine is degraded in E. coli after γ-glutamylation instead of acetylation in mammalian cells. The entire novel putrescine utilization pathway of E. coli proposed here is summarized in Fig. 2B.

OrdL Is γ-Glu-Put Oxidase and AldH Is γ-Glu-γ-aminobutyraldehyde Dehydrogenase—After the YcjK reaction, γ-Glu-Put was expected to be oxidized to γ-Glu-γ-aminobutyraldehyde by OrdL and then to γ-Glu-γ-aminobutyrate (γ-Glu-GABA) by AldH (Fig. 2B). To show these reactions in vivo, four strains, SK263 (pACYC184ΔycjLC Δ(aldH-ordL-goaG)); ycjJ⁺ ycjK⁺ ycjL⁺ ycjC⁺ aldH⁺ ordL⁺ goaG⁺), SK285 (pACYC-aldH/ΔycjLC Δ(aldH-ordL-goaG)); ycjJ⁺ ycjK⁺ aldH⁺ ycjL⁺ ycjC⁺ ordL⁺ goaG⁺), SK283 (pACYC-ordL/ΔycjLC Δ(aldH-ordL-goaG)); ycjJ⁺ ycjK⁺ ordL⁺ ycjL⁺ ycjC⁺ aldH⁺ goaG⁺), and SK279 (pACYC-aldH-ordL/goaG); ycjJ⁺ ycjK⁺ aldH⁺ ordL⁺ ycjL⁺ ycjC⁺ goaG⁺) were constructed. Cell-free extracts of these strains were obtained and analyzed by HPLC. SK263 (ycjJ⁺ ycjK⁺) exceedingly accumulated γ-Glu-Put (Fig. 3A). The addition of aldH⁺ to this strain did not influence the chromatogram (SK285) (Fig. 3B), whereas the addition of ordL⁺ to SK263 diminished the peak of γ-Glu-Put (SK283) (Fig. 3C). When both ordL⁺ and aldH⁺ were added to SK263, a new compound that eluted at 7.5 min in our HPLC system was accumulated (SK279) (Fig. 3D). This compound was purified and subjected to NMR analysis. The results indicated that the new compound is γ-Glu-GABA. The compound was also hydrolyzed to GABA and glutamate by GGT which is well known to hydrolyze the γ-glutamyl linkage. γ-Glu-Put decreased with the addition of the cell-free extract of SO66 (pQE-80L-ordL/SK247) but not with that of SO67 (pQE-80L/SK247). These results suggested that γ-Glu-Put is catabolized to γ-Glu-GABA by the sequential reactions involving OrdL and AldH.

YcjL Is γ-Glu-GABA Hydrolase—To elucidate the physiological role of YcjL in vivo, two strains were constructed, SK189 (Δ(aldL-goaG)) and SK187 (ΔgoaG). OrdL is located upstream, and GoaG downstream, of YcjL in the hypothetical catabolic pathway (see Fig. 2B). The cell-free extract of SK189 (Δ(aldL-goaG)) exhibited only 23% γ-GpNA hydrolysis activity compared with SK187 (ΔgoaG) which exhibited the same level of activity as the wild-type strain. This result can be interpreted to mean that the metabolite upstream of YcjL, γ-glutamyl-γ-aminobutyraldehyde, and/or γ-Glu-GABA could induce ycjL ex-
pression and that the deletion of goaG, the product of which is located downstream of YcjL in the metabolic pathway, did not influence ycjL expression. YcJK catalyzes the γ-glutamylamination of putrescine to form the γ-glutamyl linkage of γ-Glu-Put, whereas YcjL catalyzes the hydrolysis of the γ-glutamyl linkage of γ-Glu-GABA. If γ-Glu-Put is a better substrate for YcjL than γ-Glu-GABA, there is no good reason for the γ-glutamylate putrescine consuming ATP. To determine which is the better substrate, $K_m$ and $k_{cat}$ values of YcjL for γ-Glu-Put and γ-Glu-GABA were determined. The $K_m$ for γ-Glu-Put was 18.5 mM and that for γ-Glu-GABA was 2.93 mM. The $k_{cat}$ for γ-Glu-Put was 434 min$^{-1}$ and that for γ-Glu-GABA was 2490 min$^{-1}$. The $k_{cat}/K_m$ for γ-Glu-Put was 23.5 and that for γ-Glu-GABA was 850, i.e. the $k_{cat}/K_m$ for γ-Glu-GABA was 36 times higher than that for γ-Glu-Put. The results showed that γ-Glu-Put rather than γ-Glu-GABA is the natural substrate of YcjL. The YcjL activity measured as γ-GpNa hydrolysis activity was 16 times higher when E. coli was grown in the W-Put medium rather than in the W salts minimal medium containing 0.2% NH$_4$Cl instead of putrescine. These results indicated that YcjL is γ-Glu-GABA hydrolase.

GoaG Is the Second GABA Aminotransferase—Previously, putrescine was reported to be oxidized directly to GABA (8) and that the strain ΔγabT did not grow in W salts minimal medium containing 0.2% GABA as a sole nitrogen source, whereas this strain grew in W-Put medium (10). Moreover, the cell-free extract of ΔγabT grown in W-Put medium exhibited significant GABA aminotransferase activity. These results suggested that besides GabT, there is another GABA aminotransferase that is induced by putrescine. We suspected that this second aminotransferase was encoded by goaG in the ycj gene cluster. The cell-free extract of KJ107 (ΔγabT), SK187 (ΔgoaG), and KJ109 (ΔγabT ΔgoaG) grown in M9-tryptone supplemented with 5 mM putrescine exhibited 68, 35, and 1.3% GABA aminotransferase activity, respectively, compared with SH639 (γabT$^+$ goaG$^+$). These results indicate that both GabT and GoaG are important in the catabolism of GABA. In the cell-free extracts of the ΔgoaG strain, accumulation of GABA was observed. These results agreed well with the putrescine-catabolizing pathway postulated in Fig. 2B.

YcjL Is a Novel Putrescine Importer—Downstream of ycjK, there is a gene, ycjL, whose product is suggested to be an amine transporter from its amino acid sequence. From the results described so far, the substrate of YcjL is expected to be putrescine.

To show that putrescine is imported by YcjL, a transporter assay using [14C] putrescine was performed. The putrescine transport activity of SO23 (ΔycjL) was reduced compared with that of SH639 (ycjL$^+$), whereas SO24 (pBR-ycjL/ΔycjL, squares) imported putrescine over 24 times more effectively than SH639 (ycjL$^+$) (Fig. 4). The result indicated that YcjL is a novel putrescine importer. In SO23 (ΔycjL), putrescine import activity was very much diminished, although the strain is potF$^+$G$^+$H$^+$I$^+$. The result indicates that under our growth conditions for E. coli, the putrescine importer mainly expressed is YcjL. A more detailed analysis of YcjL will be published elsewhere.

Disruption and Complementation of ycj Gene Cluster Influence the Utilization of Putrescine in E. coli—To show that the products of the ycj gene cluster play an important role in the catabolism of putrescine, various parts of the ycj gene cluster were disrupted or complemented, and the mutants and transformants were grown on W-Put medium. Whereas SO23 (ΔycjJ) and SO25 (pBR-ycjJ/ΔycjJ) were not able to grow on the W-Put plates, SH639 (ycjL$^+$) and SO24 (pBR-ycjJ/SO23) were (Fig. 5A). These results suggested that YcjJ is the main putrescine importer when E. coli grows on medium containing putrescine as a sole nitrogen source. Whereas SK247 (ΔycjLC-aldH-ordL-goaG$^+$) and SK306 (pBeloBAC11/SK247) could not grow on the W-Put plates, SH639 (ycjL$^+$C$^+$ aldH$^+$ ordL$^+$ goaG$^+$) and SK303 (pBelo-ycjLC-aldH-ordL-goaG/SK247) could (Fig. 5B). These results suggested that the catabolic pathway shown in Fig. 2B is the main one when E. coli grows in the medium containing putrescine as the sole source of nitrogen. KJ109 (ΔγabT ΔgoaG) did not grow on W-Put plates, whereas KJ107 (ΔγabT), SK187 (ΔgoaG), and SH639 (goaG$^+$ gabT$^+$) did (Fig. 5C). The result suggested that both GoaG and GabT are important in the catabolism of GABA. The two enzymes can substitute for each other in the catabolic pathway. This is consistent with the results from the assay of the GABA aminotransferase activity of SK187 (ΔgoaG), KJ107 (ΔγabT), and KJ109 (ΔgoaG ΔγabT).
FIG. 5. Physiological role of ycj gene products in E. coli cells grown in minimal medium containing putrescine as a sole nitrogen source. A, strains relevant to ycjJ were streaked on plates of W salts minimal medium containing 0.2% putrescine as a sole nitrogen source (W-Put plates). The vector used was pBR322. *p* in the figure indicates the plasmid vector. B, strains relevant to the (ycjL-ald-ordL-goaG) gene cluster were streaked on W-Put plates. The vector used was pBeloBAC11. C, strains relevant to goaG and gabT were streaked on W-Put plates.

### TABLE II

| Previous gene | Function                      | New gene names |
|---------------|-------------------------------|----------------|
| ycjJ          | Putrescine importer           | puuP           |
| ycjK          | γ-Glu-Put synthetase           | puuA           |
| ordL          | γ-Glu-Put oxidase              | puuB           |
| aldH          | γ-Glu-γ-aminobutyraldehyde dehydrogenase | puuC |
| ycjL          | γ-Glu-GABA hydrolase           | puuD           |
| goaG          | GABA aminotransferase          | puuE           |
| ycjC          | Transcriptional regulator      | puuR           |

### DISCUSSION

The Significance of γ-Glutamylation of Putrescine—In the novel pathway we proposed, putrescine is first γ-glutamylated and then catabolized (Fig. 2B), but previously, putrescine had been thought to be degraded directly to γ-aminobutyraldehyde without any modification and then to GABA (8). However, γ-aminobutyraldehyde is unstable, and its amino group and aldehyde group form cyclic Δ1-pyrroline nonenzymatically (8). γ-Glutamylation of putrescine may facilitate its catabolism by masking the amino group in advance and by preventing cyclization.

**Regulation of the ycj Gene Cluster**—Nac is a σ70-dependent transcriptional regulator under nitrogen-limited conditions (28). All of the members of the ycj gene cluster are expected to be transcribed from σ70-dependent promoters, and many results obtained so far were under nitrogen-limited conditions. Therefore, Nac was expected to control the expression of the ycj gene cluster; however, there is no Nac-binding site upstream of any of these genes. Furthermore, in a previous study using microarrays (29), no member of the ycj gene cluster was recognized as a Nac-controlled gene. However, the addition of putrescine to the medium as the sole nitrogen source (W-put medium) induced the expression of ycjL (data not shown). This result suggested that a new system or protein (for example, YcjC) involved in the response to the nitrogen starvation controls the expression of the ycj gene cluster. The addition of glucose to the M9-tryptone medium repressed the expression of ycjL (data not shown). However, there is no CRP-binding site upstream of any of these genes. Furthermore, the expression of ycjL was also repressed by the addition of glycerol and succinate to the M9-tryptone medium. These results suggested that the regulation of the expression of ycjL is independent of CRP. YcjL was highly expressed in the early stationary phase (data not shown) when the nutrient in the medium is drying up. These results suggested that the products of the ycj gene cluster are expressed when E. coli grows under conditions of starvation, and the products of the ycj gene cluster catabolize putrescine both as the carbon source and as the nitrogen source. We could not perform the experiment to detect the growth of E. coli on medium containing putrescine as a sole carbon source because wild-type E. coli could not grow on that medium.

In summary, the expression of ycjL (and maybe the other members of the ycj gene cluster) is repressed in the presence of ycjC and by the addition of another carbon or nitrogen source more easily utilized than putrescine, and is induced by nutrient starvation and by the addition of putrescine to the medium. A more detailed analysis of the regulation of the ycj gene cluster will be published elsewhere.

**Proposed New Gene Name for the ycj Gene Cluster**—We propose the name puu after putrescine utilization pathway for the ycj gene cluster. ycjJ, ycjK, aldH, ycjL, goaG, and ycjC would be renamed puuP, puuA, puuB, puuC, puuD, puuE, and puuR, respectively (Table II).

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