**A novel piperidine degradation mechanism in a newly isolated piperidine degrader Pseudomonas sp. strain KU43P**

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The degradation pathways in microorganisms for piperidine, a secondary amine with various applications, are not yet fully understood, especially in non-Mycobacterium species. In this study, we have identified a piperidine-degrading isolate (KU43P) from a soil sample collected in a cultivation field in Osaka, Japan, and characterized its mechanisms of piperidine degradation, thereby furthering current understanding of the process. The genome of isolate KU43P consists of a 5,869,691-bp circular chromosome with 62.67% GC content and with 5,294 predicted protein-coding genes, 77 tRNA genes, and 22 rRNA genes. 16S rRNA gene sequence analysis and average nucleotide identity analysis suggest that the isolate is a novel species of the Pseudomonas putida group in the genus Pseudomonas. The genomic region encoding the piperidine degradation pathway, designated as the pip gene cluster, was identified using transposon mutagenesis and reverse transcription polymerase chain reaction. Deletion analyses of pipA, which encodes a glutamine synthetase (GS)-like protein, and pipBa, which encodes a cytochrome P450 monoxygenase, indicate that pipA and pipBa are involved in piperidine metabolism and suggest that pipA is involved in the first step of the piperidine metabolic pathway. *Escherichia coli* whole cells overexpressing PipA converted piperidine and glutamate to γ-glutamylpiperidine, and crude cell extract enzyme assays of PipA showed that this reaction requires ATP and Mg²⁺. These results clearly show that pipA encodes γ-glutamylpiperidine synthetase and that piperidine is first glutamylated and then hydroxylated in the piperidine degradation pathway of Pseudomonas sp. strain KU43P. This study has filled a void in the general knowledge of the microbial degradation of amine compounds.

Key Words: γ-glutamylation; genome analysis; glutamine synthetase-like protein; heterocyclic secondary amine; piperidine; Pseudomonas

### Introduction

Piperidine, a heterocyclic secondary amine, is found in the structures of many natural products, such as alkaloid compounds, and piperidine derivatives exhibit interesting pharmacological properties (Eller et al., 2000; Rubiralta et al., 1991). Therefore, the compound is an important core structure in organic chemistry, is frequently employed as an intermediate for pharmaceuticals and plant protection agents, and is found in the environment (Eller et al., 2000). Piperidine can be converted to potent mutagenic and carcinogenic N-nitroso compounds in the environment and the gastrointestinal tract of mammals (Alam et al., 1971a, b; National Toxicology Program, 2016). Therefore, clarifying the mechanisms of the microbial degradation of piperidine is essential both for developing effective bioremediation programs and for the bioproduction of piperidine-related compounds.

Microbial degradation by Gram-positive bacteria of heterocyclic secondary amines, including piperidine, has been reported mostly for members of the genus Mycobacterium, with very few reports for non-Mycobacterium species (Combourey et al., 2000; Kim et al., 2006; Knapp et al., 1996; Poupin et al., 1999a, b; Trigui et al., 2003). The degradation pathways for compounds of all reported strains are proposed to be initiated by hydroxylation on a C atom adjacent to the amine group by cytochrome P450 monoxygenases, thereby forming unstable compounds.

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et al. (1994)

Enrichment and purification of the strain were carried out as medium (MSM) containing 10 mM piperidine as the carbon source, using classical enrichment techniques. 

Mineral salt components have been extensively studied; however, details of the entire degradation pathway and the complete genome sequences of the degrading strains are unavailable (Poupin et al., 1999a, b; Sielaff and Andreesen, 2005; Trigui et al., 2004). The compounds are transformed into amino aldehyde compounds by spontaneous linearization. In these pathways, the initial cytochrome P450 monooxygenase system and the genes encoding its components have been extensively studied; however, details of the entire degradation pathway and the complete genome sequences of the degrading strains are unavailable (Poupin et al., 1999a; Trigui et al., 2004).

In this study, we first identified a new piperidine-degrading bacterium isolated from a soil sample in Japan. We then performed a series of characterization studies, including genome sequence analyses, mutagenesis analyses, and heterologous expression in Escherichia coli, in order to elucidate the pathway for piperidine degradation in this isolate. Our results revealed a novel piperidine degradation pathway in non-Mycobacterium species, thereby furthering our understanding of piperidine degradation mechanisms.

Materials and Methods

Isolation of piperidine-degrading bacterium. A piperidine-degrading bacterium, strain KU43P, was isolated from a soil sample collected from a cultivated field in Osaka, Japan, using classical enrichment techniques. Mineral salt medium (MSM) containing 10 mM piperidine as the carbon source was used as the enrichment medium, and enrichment and purification of the strain were carried out as previously described (Iwaki and Hasegawa, 2007).

Genome sequencing of isolate KU43P. KU43P cells were grown for 18 h in Miller’s LB medium (Merck Millipore, Darmstadt, Germany) at 30°C, and genomic DNA was extracted following the method described by Chachaty and Saultnier (2000). A 20-kb SMRTbell template library was prepared from approximately 8 μg of input genomic DNA, using the SMARTbell Template Prep Kit 1.0 (Pacific Biosciences, CA, USA). The SMRTbell library was sequenced using SMRT cell 8Pac version 3, with P6-C4 chemistry, and 240 min movies were captured for each SMRT cell, using the PacBio RS II platform (Pacific Biosciences). The subreads were filtered by PreAssembler Filter version 1 (minimum subread length, 500 bp; minimum polymerase read quality, 0.80; minimum polymerase read length, 100 bp) with SMRT analysis version 2.3.0 (Pacific Biosciences), and 108,679 reads that passed the filter were obtained, with a total of 1,112,426,305 bp and an N50 value of 15,256 bp. The reads were de novo assembled with the hierarchical genome assembly process protocol version 3 in SMRT analysis version 2.3.0 (Pacific Biosciences).

Genome analyses. The genome was annotated using DFAST: the DNA Data Bank of Japan (DDBJ) Fast Annotation and Submission Tool (Tanizawa et al., 2018; http://dfast.nig.ac.jp). The average nucleotide identity (ANI) was calculated with JSpeciesWS (http://jspecies.ribohost.com/jspeciesws/) using BLAST with default parameters (RichTer et al., 2016).

Transposon mutagenesis. Transposon mutagenesis and isolation of the mutant which is unable to grow on piperidine were performed as previously described (Muraki et al., 2003). The insertion site of the transposon was determined by inverse PCR with KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan) using two primer sets, BL/IR1 and BR/IR1 (Table S1), described previously (Iwaki et al., 2016). The insertion site of the transposon was determined by inverse PCR with KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan) using two primer sets, BL/IR1 and BR/IR1 (Table S1), described previously (Iwaki et al., 2003). The insertion site of the transposon was determined by inverse PCR with KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan) using two primer sets, BL/IR1 and BR/IR1 (Table S1), described previously (Iwaki et al., 2003).

Reverse transcription polymerase chain reaction (RT-PCR). Isolate KU43P was grown in MSM containing 10 mM piperidine or 5 mM glucose until the optical density at 600 nm (OD600) of the culture reached 0.5. RNAprotect Bacteria Reagent (QIAGEN, Hilden, Germany) was immediately added to the cells. Total RNA was isolated using ISOGEN II (Nippon Gene, Tokyo, Japan). In order to remove any contaminating genomic DNA, the RNA samples were incubated with 1 U of Deoxyribonuclease (RT Grade) for Heat Stop (Nippon Gene). The cDNA was obtained by a reverse transcription (RT) reaction using PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan) and random primers. The cDNA was used for...

| Strain or Plasmid | Relevant characteristic(s) | Source or reference |
|-------------------|---------------------------|---------------------|
| **Strains**       |                           |                     |
| Pseudomonas sp.   |                           |                     |
| KU43P             | Wild type, grows on piperidine | This study          |
| KU43P-Tn          | Transposon mutant of KU43P, pипB::Tn5-37Pp, no growth on piperidine | This study          |
| KU43P-pипA-1 and -2 | pипA deletion mutants of KU43P, no growth on piperidine | This study          |
| KU43P-pипB-1 and -2 | pипB deletion mutants of KU43P, no growth on piperidine | This study          |
| E. coli KRX       | Host strain for gene cloning and protein expression, [F’, traD36, ΔompP, proA’B*, lacI, Δ(lacZΔM15) ΔompT, endA1, recA1, gyrA96 (Nal’), thi-1, hsdR17 (rk-, mK+), cI4’ (McrA’), relA, supE44, Δ(lac-proAB), Δ(rhaBAD):T7 RNA polymerase | Promega              |
| S-17-1            | Donor strain for transconjugation, recA pro thi hsdR KP4-2-Tc::Mu-Knc::Tn7 Tra7’Tp’Sn’ | Simon et al. (1983) |
| **Plasmids**      |                           |                     |
| pK19mob sacB      | Mobilizable cloning vector, oriT, sacB, Km’ | Schäfer et al. (1994) |
| pET-16b           | Expression vector with T7 promoter, ColE1 ori, Ap’ | Merck Millipore     |
| pK19-ApipA        | pK19mob sacB derivative carrying the pипA deletion cassette | This study          |
| pK19-ApipB1       | pK19mob sacB derivative carrying the pипB deletion cassette | This study          |
| pET-pипA          | pET-16b derivative carrying the pипA | This study          |
as the template for subsequent PCRs with specific primers (Table S1). PCR samples were run on 0.8% agarose gel by electrophoresis and visualized by staining with ethidium bromide.

**Construction of deletion mutants.** Construction of the pipA and the pipBa deletion mutants was performed by two-step homologous recombination methods using pK19mobsacB as a vector, as previously described (Iwaki et al., 2007). The deletion cassettes of pipA and pipBa were assembled, and cloned into pK19mobsacB, using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs Japan, Tokyo, Japan), for the construction of deletion plasmids pK19–ΔpipA and pK19–ΔpipBa, respectively (Table 1). The deletion plasmids were transferred to isolate KU43P from *Escherichia coli* S17-1 by transconjugation. The primer sets used are listed in Table S1. The MSM plates containing 10 mM *p*-hydroxybenzoate and 100 µg/mL kanamycin were used for selection of single crossover mutants, and the MSM plates containing 10 mM *p*-hydroxybenzoate and 10% sucrose were used for selection of double crossover mutants. The deletion mutants were analyzed by PCR.

**Chemical synthesis of γ-glutamylpiperidine.** γ-Glutamylpiperidine was chemically synthesized as shown in Fig. S1, and the synthesized γ-glutamylpiperidine was analyzed by 1H nuclear magnetic resonance (1H-NMR).

**Expression of pipA gene in *E. coli*.** The DNA fragment carrying pipA was amplified by KOD-Plus-Neo DNA polymerase (Toyobo) using the primers shown in Table S1, and the gel-purified DNA was ligated to the expression vector, pET-16b (Merck Millipore). The forward primer was designed to contain an Ndel restriction site with an ATG start codon and the reverse primer contains a HI restriction site, to facilitate directional cloning in the pET-16b expression vector. The resulting plasmid was designated pET-pipA (Table 1). For protein production, all *E. coli* KRX (Promega KK, Tokyo, Japan) strains carrying the expression plasmid and the empty pET-16b vector (negative control), were cultivated in Miller’s LB medium (Merck Millipore) supplemented with 100 µg/mL ampicillin at 37°C. When the culture reached an OD600 of 0.4–0.5, the incubation temperature was shifted to 25°C from 37°C, and the cells were further cultured until an OD of 0.5 to 0.6 was reached, in order to adjust the medium temperature to 25°C. At this point (OD600 of 0.5 to 0.6), rhamnose was added to the medium to a final concentration of 0.1%, in order to induce protein expression, and the cells were further cultured for 16 h. The resulting cells were harvested by centrifugation, washed twice with 21 mM sodium-potassium phosphate buffer (pH 7.1), and used as whole cells. Crude cell extracts were obtained by ultrasonication with a Sonifier 250 apparatus (Branson, CT, USA) for three 40-s bursts on ice, followed by centrifugation 30 min at 18,000 × g at 4°C to remove unbroken cells and debris.

**Whole cell transformation.** The *E. coli* whole cells were resuspended in 21 mM sodium-potassium phosphate buffer (pH 7.1) supplemented with 10 mM glucose, adjusted to an OD600 of 1.0, and incubated with 5 mM piperidine and 5 mM glutamate. Cell suspensions were incubated with shaking at 30°C for 10 h, and supernatants were analyzed by high-pressure liquid chromatography (HPLC) and liquid chromatography ion trap/time-of-flight mass spectrometry (LC-IT-TOF-MS). The mass spectrometer was operated in the positive ion mode.

**Crude cell extract enzyme assay.** The standard reaction mixture for the crude cell extract enzyme assay, for the conversion of piperidine to γ-glutamylpiperidine, contained 2 mM piperidine, 4 mM L-glutamate, 4 mM ATP, 20 mM MgCl2, and 0.4 mg of crude extracts in 1 mL of 21 mM sodium/potassium phosphate buffer, pH 7.1. After the reaction, the reaction mixtures were analyzed by HPLC.

**Analytical methods.** HPLC analysis was performed on a CAPCELL PAK C18UG120 column (column size of 4.6 by 250 mm and particle size of 5 µm; Shiseido, Tokyo, Japan) connected to an LC-6AD pump and an SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan). The mobile phase consisted of methanol/H2O (7:3) with 0.07% perchloric acid at a flow rate 1.0 mL/min. LC-IT-TOF-MS analysis was performed using a Shimadzu LC-IT-TOF instrument connected to a Shimadzu LC-20 series HPLC system. Separation was performed on an Inertsil ODS-3 column (column size, 2.1 × 50 mm; particle size, 3 µm;
Table 2. Homology search of open reading frames that were in flanking regions of the transposon insertion site.

| Gene locus      | Protein length (aa) | Database                          | Closest related protein [Organism]                                      | Accession No. | Identity (%) | Proposed function                  |
|-----------------|---------------------|-----------------------------------|------------------------------------------------------------------------|---------------|--------------|------------------------------------|
| KU43P_22470     | 470                 | UniProtKB/SwissProt               | Gamma-aminobutyrate transaminase [Solanum hirciscum]                    | Q84IP54       | 50.3         | γ-aminovaleate transaminase        |
|                 |                     | **NR**                            | aspartate aminotransferase family protein [Pseudomonas aeruginosa]      | WP_090348989  | 76.2         |                                    |
| KU43P_22460     | 497                 | UniProtKB/SwissProt               | Gamma-glutamyl-gamma-aminobutyraldehyde dehydrogenase [Escherichia coli] | P23883        | 55.5         | γ-glutamyl-gamma-aminobutyaldehyde dehydrogenase |
| KU43P_22450     | 226                 | UniProtKB/SwissProt               | GMP synthase [glutamin-hydroryzing]                                   | Q8DG59        | 35.1         | γ-glutamyl peptidase               |
| KU43P_22440     | 403                 | UniProtKB/SwissProt               | Cytochrome P450 CYP107DY1; Mecvastatin hydroxylase [Bacillus megaterium] | DSEH2         | 31.7         | cytochrome P450                    |
| KU43P_22430     | 425                 | UniProtKB/SwissProt               | Cindoxin reductase; NADPH-dependent flavodoxin/ferredoxin reductase [Citrobacter braakii] | Q8VQF5        | 48.1         | NADPH-dependent flavodoxin/ferredoxin reductase |
| KU43P_22420     | 146                 | UniProtKB/SwissProt               | Cindoxin; FMN-containing P450 redox partner [Citrobacter braakii]       | Q8VQF4        | 43.0         | FMN-containing P450 redox partner  |
| KU43P_22410     | 251                 | UniProtKB/SwissProt               | Gamma-glutamyl peptidase 3 [Arabidopsis thaliana]                      | Q8M0A5        | 27.2         | γ-glutamyl peptidase               |
| KU43P_22440     | 457                 | UniProtKB/SwissProt               | Glutamine synthetase [Sporothrix mellisseri]                           | O87393        | 31.1         | γ-glutamyl peptidase               |
| KU43P_22430     | 245                 | UniProtKB/SwissProt               | Glutamine synthetase [Pseudomonas aeruginosa]                         |               |              |                                    |
| KU43P_22390     | 237                 | UniProtKB/SwissProt               | L-cystine-binding protein FLX [Escherichia coli]                       | P0AEN0        | 29.2         |                                    |
| KU43P_22380     | 253                 | UniProtKB/SwissProt               | HTH-type transcriptional regulator RmpR [Myxobacterium gssi]           | Q8LBW6        | 45.7         | transcriptional regulator          |
| KU43P_22350     | 421                 | UniProtKB/SwissProt               | major facilitator family transporter [Pseudomonas oleovorans]          | WP_07456440   | 66.9         |                                    |

*a.a., amino acids. ** NR, NCBI nonredundant protein sequence database.*
Alignments were generated using the CLUSTALW tool in MEGA version 7 (Kumar et al., 2016). Phylogenetic trees were generated as previously described by Iwaki et al. (2018), and with the neighbor-joining method (Saitou and Nei, 1987), in MEGA version 7. A distance matrix was produced on the basis of Kimura's 2-parameter model (Kimura, 1980).

Data availability. The genome sequence annotated using DFAST was deposited at DDBJ with the accession number AP019365, with the associated BioProject and BioSample accession numbers PRJDB7650 and SAMD00151124, respectively.

Results and Discussion

Genome features and identification of isolate KU43P

The assembled complete genome sequence of isolate KU43P consisted of a 5,869,691-bp circular chromosome (158 x depth of coverage), with a GC content of 62.67%. No plasmid was found. In total, 5,294 protein-coding sequences (CDSs), 77 transfer RNA (tRNA) genes, and 22 ribosomal RNA (rRNA) genes were detected.

We then attempted to identify the isolate KU43P using the 16S rRNA loci. KU43P was found to share a high 16S ribosomal RNA genes were detected. KU43P consisted of a 5,869,691-bp circular chromosome

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We then attempted to identify the isolate KU43P using the 16S rRNA loci. KU43P was found to share a high 16S rRNA gene sequence similarity (99.3–99.7% identity) with the 16S rRNA gene of the isolate. ANI was calculated with JSpecies (http://jspecies.ribhost.com/jspeciesws/) using BLAST with default parameters (Richter et al., 2016). ANI values between isolate KU43P and the aforementioned species were far below the threshold of 94–95% recommended for species delineation (Richter and Rosselló Móra, 2009) (Table S3), indicating that KU43P should be classified as a novel species of the genus Pseudomonas. Therefore, we tentatively identified isolate KU43P as Pseudomonas sp.

Identification and sequence characterization of the piperidine utilization locus

To understand the molecular basis for piperidine degradation in this strain, identification of the genes involved in piperidine utilization, designated as the piperidine utilization (pip) locus, was performed by the generation of transposon mutant derivatives of strain KU43P, that were unable to grow with piperidine as the sole carbon source. A mutant strain was isolated from approximately 1,000 mutants by growth deficiency on piperidine and was designated as strain KU43M1. PCR analysis showed that the transposon was inserted into the intragenic region of KU43P_22420 (named pipBa; Fig. 1), which has approximately 43.0% amino acid sequence identity with cindoxins (Table 2). Cindoxin is a flavin mononucleotide (FMN)-containing a cytochrome P450 reduct partner of P450cin, which catalyzes 1,8-cineole hydroxylation (Hawkes et al., 2002, 2010). Sequence analysis of flanking regions of KU43P_22420 revealed that the KU43P_22440 (named pipBa) and KU43P_22430 (named pipBb) genes (Fig. 1), which appear to code for a cytochrome P450 monooxygenase and an NADH-dependent flavodoxin/ferredoxin reductase, respectively, are located in the upstream region of KU43P_22420. These results suggest that these three gene products form a cytochrome P450 system, and the growth deficiency of the transposon mutant strain KU43M1 on piperidine may be due to the disruption of the cytochrome P450 system by transposon insertion to the gene that encodes the reduct partner. In the flanking regions of the genes encoding the cytochrome P450 system, a gene cluster predicted to be involved in the degradation of piperidine was present. KU43P_22400 (named pipA) and KU43P_22410 (named pipD1), which were similar to GS and γ-glutamyl peptidase, respectively, were found downstream of pipBaBbBc (Fig. 1, Table 2). Genes encoding a putative GntR family transcriptional regulator, KU43P_22380 (named pipR), and an amino acid permease homolog, KU43P_22360 (named pipT), were also found in the downstream region (Fig. 1, Table 2). Upstream of pipBaBbBc, we found KU43P_22450 (named pipD2), KU43P_22460 (named pipC), and KU43P_22470 (named pipE), which were similar to typical glutamine compounds from amine compounds and t-glutamate (de Azevedo Wäsch et al., 2002; Kurihara et al., 2005; Takeo
The cell density of the reaction was adjusted to an OD₆₀₀ of 1.0 in 21 mM sodium-potassium phosphate buffer (pH 7.1) supplemented with 10 mM glucose and appropriate substrates. A. The HPLC chromatogram of the reaction supernatant (collected after 10 h), indicates that γ-glutamylpiperidide was formed from piperidine and l-glutamate. The reaction mixture contained 5 mM piperidine and 5 mM l-glutamate as substrates. B. The HPLC chromatogram of the reaction supernatant (collected after 10 h), indicates that trace amounts of γ-glutamylpiperidide were formed from l-glutamate without exogenous l-glutamate. The reaction mixture contained 5 mM piperidine as substrate. C. The HPLC chromatogram of the reaction supernatant (collected after 10 h), indicates that γ-glutamylpiperidide was not formed without piperidine. The reaction mixture contained 5 mM l-glutamate as substrate. D. IT-TOF-MS spectrum of the product of panel A. A molecular ion [M+H]⁺ peak at 215 m/z corresponds to γ-glutamylpiperidide.

The scale bar corresponds to a genetic distance of 0.1 substitution per position. PipA, KU43P; PipA from Pseudomonas sp. KU43P; MSEM6693, GS-like protein of strain mc²155 (accession no. ABK75399). The gene encoding the protein presents downstream of the cytochrome P450 gene involved in piperidine utilization; GMAS_METMY, γ-glutamylmethylamide synthetase from Methylorovus mays strain No. 9 (BAF99006); GMAS_METUF, γ-glutamylmethylamide synthetase from Methylotversatilis universalis strain FAM5 (ADH10360); ATDA1_ACISP, γ-glutamylanilide synthetase from Acinetobacter sp. strain YAA (BAA13010); GLN1B_MYCS2, glutamine synthetase from Mycobacterium smegmatis strain mc²155 (ABK70139); GLN1B_ECOLI, glutamine synthetase from Escherichia coli strain K-12 (BAE77439); GLN1B_PSEAE, glutamine synthetase from Pseudomonas aeruginosa strain PAO1 (AA980504); IPUC_PSESP, γ-glutamylisopropylamide synthetase from Pseudomonas sp. strain KIE171 (CAC8135); PUUA_ECOLI, γ-glutamylputrescine synthetase from Escherichia coli K-12 (BAA14857).

The amino acid sequence of PipA showed similarities with those of known enzymes that catalyze γ-glutamylamination of amine compounds, e.g., γ-glutamylmethylamide synthetase (GMSA) from Methylorovus mays (30.6% identity) (Yamamoto et al., 2008), γ-glutamylanilide synthetase (Atda1) from Acinetobacter sp. strain YAA (27.1%) (Takeo et al., 2013), γ-glutamylisopropylamide synthetase (IpuC) from Pseudomonas sp. strain YAA (27.1%) (de Azevedo Wäsch et al., 2008), and γ-glutamylputrescine synthetase (PuuA) from E. coli K-12 (24.8%) (Kurihara et al., 2005), suggesting that γ-glutamylamination is involved in the piperidine degradation pathway. The presence of genes encoding a γ-glutamyl peptidase homolog and a γ-glutamyl-γ-aminobutylaldehyde dehydrogenase homolog supports the hypothesis that γ-glutamyl compounds are involved in the piperidine degradation pathway. In general, γ-glutamylamination of amine compounds occurs prior to hydroxylation. Therefore, we proposed that piperidine may be first glutamylated and then hydroxylated (Fig. 1). When piperidine is hydroxylated directly, an amino aldehyde compound is formed. However, the amino aldehyde compound is assumed to be unstable, and its amino group and aldehyde group may form cyclic compounds nonenzymatically (Kurihara et al., 2005; Shaibe et al., 1985). Therefore, γ-glutamylamination of piperidine may facilitate piperidine degradation by preventing cyclization.

**Transcriptional analysis of the pip locus**

To investigate the operon structure of pip locus in strain KU43P, and whether the genes were highly transcribed in response to piperidine, RT-PCR was performed using the total RNA derived from the KU43P cells grown on piperidine. Total RNA extracted from strain KU43P grown on glucose was used as a control. While the pipBa-pipBb-pipBc, pipBc-pipD1, and pipD2-pipD3 intergenic regions were successfully amplified (Fig. 1), the pipD2-pipA intergenic region was not amplified, indicating that pipBaBbBcD but not pipA are co-transcribed. The pipD2-pipBb intergenic region was not amplified as expected from the direction of transcription. The pipD2-pipC and pipC-pipE intergenic regions were also amplified (Fig. 1), indicating that pipD2 and pipCE are co-transcribed. The transcription levels of the pip genes significantly increased upon piperidine induction (Fig. 1). On the other hand, the transcription levels of the gene encoding the putative transcriptional repressor pipR and of the gene encoding the putative permutase pipT were the same in the presence and absence of piperidine (Fig. 1). Taken together, these results suggest that the pip genes are involved in piperidine degradation.
Analysis of pipA and pipBa deletion mutants

To confirm that the pip genes are involved in piperidine degradation, we constructed pipA and pipBa deletion mutants, two positive colonies for each gene (KU43P-pipA-1 and KU43P-pipA-2 for pipA, and KU43P-pipBa-1 and KU43P-pipBa-2 for pipBa) were selected, and these mutants were found to be incapable of utilizing piperidine as a sole carbon source. In addition, a product from piperidine was observed to accumulate when glucose was the sole carbon source, in the pipBa deletion mutant, KU43P-pipBa-1, however, no product accumulation was similarly observed for the pipA deletion mutant, KU43P-pipA-1, suggesting that PipA is the first enzyme in the piperidine degradation pathway (Fig. 2). The product was identified as γ-glutamylpiperidide since the product had an identical retention time as the chemically synthesized γ-glutamylpiperidide in HPLC analysis and had a molecular ion [M+H]+ peak at 215 m/z, corresponding to glutamylpiperidide in LC-IT-TOF-MS analysis (Fig. 2). These results indicate that piperidine is first glutamylated and then hydroxylated by strain KU43P. To the best of our knowledge, this is the first report for γ-glutamylated of a secondary amine by a microorganism and for the presence of a γ-glutamyl intermediate in a secondary amine degradation pathway.

Expression of the pipA gene in E. coli

To understand the function of PipA, it was produced in E. coli containing the plasmid pET-pipA. Analysis of the overproduced protein by SDS-PAGE verified the expected molecular mass of the protein (Fig. S2). The experimental mass of the overproduced PipA was estimated to be 51.5 kDa, which is close to the calculated molecular weight of 53,404.94. HPLC analysis showed that E. coli whole cells harboring pET-pipA produced γ-glutamylpiperidide from piperidine and L-glutamate (Fig. 3), and the control E. coli whole cells harboring the empty pET-16b vector did not produce the product. Whereas γ-glutamylpiperidide was not produced without piperidine, the trace amounts of γ-glutamylpiperidide produced without L-glutamate are probably due to the utilization of endogenous L-glutamate in E. coli (Fig. 3). The reactions of γ-glutamylation and γ-glutamylamidase synthetases are known to require ATP and Mg2+ (Kurihara et al., 2005; Takeo et al., 2013). To test the ATP and Mg2+ requirements, crude cell extract enzyme assays of PipA overexpressed E. coli were performed. When Mg2+ was omitted from the reaction mixture, γ-glutamylpiperidide was not formed (Fig. S3). When ATP was omitted, γ-glutamylpiperidide was produced in trace amounts, but this was probably due to the utilization of endogenous ATP in the crude cell extracts (Fig. S3). These results indicate that PipA requires ATP and Mg2+ for the reaction, and is a γ-glutamylpiperidide synthetase. To our knowledge, PipA is the first example of an enzyme that catalyzes γ-glutamylation of a secondary amine.

PipA homologs in Gram-positive piperidine-degraders

A GS-like gene can also be found downstream of a cytochrome P450 gene involved in piperidine utilization in the Gram-positive piperidine-degrader Mycobacterium smegmatis strain mc2155 (Poupin et al., 1999a), and this gene has a 60.1% amino acid sequence identity with KU43P PipA. Furthermore, the GS-like gene from strain mc2155 and KU43P PipA are in the same branch of the phylogenetic tree we constructed (Fig. 4). These results suggest that the GS-like protein of strain mc2155 may also be involved in γ-glutamylation of piperidine. Interestingly, a partially sequenced GS-like protein with high similarity to that in mc2155 is present downstream of the cytochrome P450 gene involved in piperidine utilization in other piperidine-degraders, namely, Mycobacterium sp. strain RP1 (Trigui et al., 2004) and Mycobacterium sp. strain HE5 (Siehoff and Andreessen, 2005). As of now, although the piperidine degradation pathway in Gram-positive bacteria is thought to be initiated by hydroxylation on a C atom adjacent to the amine group by a cytochrome P450 monooxygenase, the universal presence of the gene encoding a GS-like protein suggests that Gram-positive bacteria also possess a degradation pathway that is initiated by γ-glutamylation.

Conclusions

In this study, we have identified the pip locus from the genome sequence of a newly isolated Pseudomonas sp. KU43P by transposon mutagenesis analysis and RT-PCR analysis. We propose the unexpected novel degradation pathway, which involves γ-glutamylation of piperidine, based on sequence analysis of the locus. The pathway has been verified using deletion mutants of genes encoding a GS-like protein (pipA) and cytochrome P450 monooxygenase (pipBa). Transformation analyses of piperidine using whole cells and crude cell extracts of E. coli harboring pipA indicate that PipA is γ-glutamylpiperidide synthetase. At present, the pathway after γ-glutamylation of piperidine is still a hypothetical pathway, and the enzymological characterization of PipA is not known. To understand the bacterial piperidine degradation mechanisms more completely, the function of pip genes should be confirmed experimentally and enzymological analysis of PipA should be performed in future studies. This study has filled a void in our knowledge of the microbial degradation mechanisms of xenobiotic amine compounds in general. Especially, with the preliminary evidence suggesting that γ-glutamylation may be involved in xenobiotic amine degradation more than previously thought, and that the addition of glutamate might be effective for the microbial treatment of amine containing pollutants.

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Supplementary Materials

Supplementary figures and tables are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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