Full Paper

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

(Received October 25, 2019; Accepted November 22, 2019; J-STAGE Advance publication date: July 8, 2020)

Taisei Yamamoto, Yaxuan Liu, Takaaki Sumiyoshi, Yoshie Hasegawa, and Hiroaki Iwaki*

Department of Life Science & Biotechnology, Kansai University, 3-3-35 Yamate-cho, Suita, Osaka 564-8680, Japan

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 monooxygenase, 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 -glutamylpiperidide, and crude cell extract enzyme assays of PipA showed that this reaction requires ATP and Mg^{2+} . These results clearly show that *pipA* encodes γ -glutamylpiperidide 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 (Combourieu 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 monooxygenases, thereby forming unstable compounds

*Corresponding author: Hiroaki Iwaki, Department of Life Science & Biotechnology, Kansai University, 3-3-35 Yamate-cho, Suita, Osaka 564-8680, Japan.

Tel: +81-6-6368-0779 E-mail: iwaki@kansai-u.ac.jp

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

Table 1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>Pseudomonas</i> sp.		
KU43P	Wild type, grows on piperidine	This study
KU43P-Tn	Transposon mutant of KU43P, <i>pipBc::Tn5-3/Tp</i> , no growth on piperidine	This study
KU43PΔ <i>pipA</i> -1 and -2	<i>pipA</i> deletion mutants of KU43P, no growth on piperidine	This study
KU43PΔ <i>pipBa</i> -1 and -2	<i>pipBa</i> deletion mutants of KU43P, no growth on piperidine	This study
<i>E. coli</i>		
KRX	Host strain for gene cloning and protein expression, [F', <i>traD36</i> , Δ <i>ompP</i> , <i>proA</i> ⁺ B ⁺ , <i>lacIq</i> , Δ(<i>lacZ</i>)M15] Δ <i>ompT</i> , <i>endA1</i> , <i>recA1</i> , <i>gyrA96</i> (Nal ^r), <i>thi</i> -1, <i>hsdR17</i> (rk ⁻ , mk ⁺), <i>e14</i> ⁻ (McrA ⁻), <i>relA1</i> , <i>supE44</i> , Δ(<i>lac-proAB</i>), Δ(<i>rhaBAD</i>)::T7 RNA polymerase	Promega
S-17-1	Donor strain for transconjugation, <i>recA pro thi hsdR</i> RP4-2-Tc::Mu-Km::Tn7 Tra ⁺ Tp ⁺ Sm ^r	Simon et al. (1983)
Plasmids		
pK19mobsacB	Mobilizable cloning vector, <i>oriT</i> , <i>sacB</i> , Km ^r	Schäfer et al. (1994)
pET-16b	Expression vector with T7 promoter, ColE1 ori, Ap ^r	Merck Millipore
pK19-Δ <i>pipA</i>	pK19mobsacB derivative carrying the <i>pipA</i> deletion cassette	This study
pK19-Δ <i>pipB1</i>	pK19mobsacB derivative carrying the <i>pipB1</i> deletion cassette	This study
pET- <i>pipA</i>	pET-16b derivative carrying the <i>pipA</i>	This study

(Poupin et al., 1999a, b; Sielaff and Andreessen, 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 Saulnier (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 N_{50} 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., 2007).

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

Table 2. Homology search of open reading frames that were in flanking regions of the transposon insertion site.

Gene locus	Protein length (aa) ^a	Database	Closest related protein [Organism]	Accession No.	Identity (%)	Proposed function
<i>KU43P_22470</i> (<i>pipE</i>)	470	UniProtKB/SwissProt	Gamma-aminobutyrate transaminase [<i>Solanum lycopersicum</i>]	Q84P54	50.3	γ-aminovaleate transaminase
		NR ^b	aspartate aminotransferase family protein [<i>Pseudomonas oryzae</i>]	WP_090348989	76.2	
<i>KU43P_22460</i> (<i>pipC</i>)	497	UniProtKB/SwissProt	Gamma-glutamyl-gamma-aminobutyraldehyde dehydrogenase [<i>Escherichia coli</i>]	P23883	55.5	γ-glutamyl-gamma-aminovalealdehyde
		NR	aldehyde dehydrogenase [<i>Pseudomonas lutea</i>]	WP_073450043	89.5	dehydrogenase
<i>KU43P_22450</i> (<i>pipD₂</i>)	226	UniProtKB/SwissProt	GMP synthase [glutamine-hydrolyzing]	Q8DGA5	35.1	γ-glutamyl peptidase
		NR	type 1 glutamine amidotransferase [<i>Pseudomonas</i> sp.]	WP_047526609	71.2	
<i>KU43P_22440</i> (<i>pipBa</i>)	403	UniProtKB/SwissProt	Cytochrome P450 CYP107DY1; Mevastatin hydroxylase [<i>Bacillus megaterium</i>]	D5E3H2	31.7	cytochrome P450
		NR	cytochrome P450 [<i>Pseudomonas mohnii</i>]	WP_090467611	88.1	
<i>KU43P_22430</i> (<i>pipBb</i>)	425	UniProtKB/SwissProt	Cindoxin reductase; NADPH-dependent flavodoxin/ferredoxin reductase [<i>Citrobacter braakii</i>]	Q8VQF5	48.1	NADPH-dependent flavodoxin/ferredoxin reductase
		NR	Oxidoreductase [<i>Pseudomonas aeruginosa</i>]	WP_088177255	57.0	
<i>KU43P_22420</i> (<i>pipBc</i>)	146	UniProtKB/SwissProt	Cindoxin; FMN-containing P450 redox partner [<i>Citrobacter braakii</i>]	Q8VQF4	43.0	FMN-containing P450 redox partner
		NR	nitric oxide synthase [<i>Pseudomonas aeruginosa</i>]	WP_088177256	69.2	
<i>KU43P_22410</i> (<i>pipD₁</i>)	251	UniProtKB/SwissProt	Gamma-glutamyl peptidase 3 [<i>Arabidopsis thaliana</i>]	Q9M 0A5	27.2	γ-glutamyl peptidase
		NR	type 1 glutamine amidotransferase [<i>Pseudomonas</i> sp. DP16D-R1]	WP_103406598	68.8	
<i>KU43P_22400</i> (<i>pipA</i>)	457	UniProtKB/SwissProt	Glutamine synthetase [<i>Sinorhizobium meliloti</i>]	O87393	31.1	γ-glutamyl piperidine
		NR	Glutamine synthetase [<i>Pseudomonas mohnii</i>]	WP_047526599	89.5	synthetase
<i>KU43P_22390</i>	237	UniProtKB/SwissProt	L-cystine-binding protein FliY [<i>Escherichia coli</i>]	P0AEN0	29.2	
		NR	amino acid ABC transporter substrate-binding protein [<i>Pseudomonas</i> sp.]	WP_119955251	73.3	
<i>KU43P_22380</i> (<i>pipR</i>)	252	UniProtKB/SwissProt	HTH-type transcriptional regulator RmpR [<i>Mycobacterium gastr</i>]	Q9LBW6	45.7	transcriptional regulator
		NR	FadR family transcriptional regulator [<i>Pseudomonas furukawaii</i>]	WP_036993670	75.6	
<i>KU43P_22370</i>	248	NR	histidine kinase [<i>Pseudomonas</i> sp.]	WP_119955253	64.7	
<i>KU43P_22360</i> (<i>pipT</i>)	502	UniProtKB/SwissProt	GABA permease [<i>Arabidopsis thaliana</i>]	Q9ZU50	27.1	permease
		NR	amino acid permease [<i>Pseudomonas furukawaii</i>]	WP_003455121	77.8	
<i>KU43P_22350</i>	421	NR	major facilitator family transporter [<i>Pseudomonas oleovorans</i>]	WP_074856440	66.9	

^aaa, amino acids, ^bNR, NCBI nonredundant protein sequence database.

GL Sciences, Tokyo, Japan) using methanol-H₂O (7:3) at a flow rate of 0.2 mL/min.

Phylogenetic analysis. 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 × 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 rRNA gene sequence similarity (99.3–99.7% identity) with type strains of *P. plecoglossicida*, *P. taiwanensis*, *P. monteilii*, and *P. entomophila* (Table S2), which belong to the *P. putida* group (Anzai et al., 2000; Yonezuka et al., 2017), which is well known for its ability to degrade various compounds. However, we were unable to identify the isolate KU43P at the species level. Therefore, we applied the average nucleotide identity (ANI) method to identify the isolate. ANI was calculated with JSpecies (<http://jspecies.ribohost.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 *pipBc*; 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 redox partner of P450cin,

Fig. 2. Metabolite analysis in the piperidine degradation by deletion mutant strains KU43PΔpipA-1 and KU43PΔpipBa-1.

A. HPLC chromatogram of the culture medium (MSM with 10 mM piperidine and 5 mM glucose) before inoculation. B. HPLC chromatogram of the KU43PΔpipA-1 culture supernatant 18 h after piperidine degradation, showing no accumulation of any product. C. HPLC chromatogram of the KU43PΔpipBa-1 culture supernatant 18 h after piperidine degradation, showing an accumulation of γ -glutamylpiperidine in the culture supernatant. D. IT-TOF-MS spectrum of the product in panel C. A molecular ion $[M+H]^+$ peak at 215 m/z corresponds to γ -glutamylpiperidine.

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 redox 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 type 1 glutamine amidotransferase, γ -aminobutyrate transaminase, and γ -glutamyl- γ -aminobutyraldehyde dehydrogenase, respectively (Fig. 1, Table 2). Some GS-like proteins have been reported to catalyze the formation of γ -glutamyl compounds from amine compounds and L-glutamate (de Azevedo Wäsch et al., 2002; Kurihara et al., 2005; Takeo

Fig. 3. Functional analysis of the *pipA* gene with whole *E. coli* cells transformed with pET-*pipA*.

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.

et al., 2013; Yamamoto et al., 2008). The amino acid sequence of PipA showed similarities with those of known enzymes that catalyze γ -glutamylation of amine compounds, e.g., γ -glutamylmethylamide synthetase (GMSA) from *Methylovorus mays* (30.6% identity) (Yamamoto et al., 2008), γ -glutamylanilide synthase (AtdA1) from *Acinetobacter* sp. strain YAA (27.1%) (Takeo et al., 2013), γ -glutamylisopropylamide synthetase (IpuC) from *Pseudomonas* sp. strain KIE171 (26.8%) (de Azevedo Wäsch et al., 2002), and γ -glutamylputrescine synthetase (PuuA) from *E. coli* K-12 (24.8%) (Kurihara et al., 2005), suggesting that γ -glutamylation is involved in the piperidine degradation pathway. The presence of genes encoding a γ -glutamyl peptidase homolog and a γ -glutamyl- γ -aminobutyraldehyde dehydrogenase homolog supports the hypothesis that γ -glutamyl compounds are involved in the piperidine degradation pathway. In general, γ -glutamylation 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). There-

Fig. 4. Phylogenetic tree of PipA with GS-like proteins.

The scale bar corresponds to a genetic distance of 0.1 substitution per position. PipA_KU43P, PipA from *Pseudomonas* sp. KU43P; MSMEG_6693, 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 *Methylovorus mays* strain No. 9 (BAF99006); GMAS_METUF, γ -glutamylmethylamide synthetase from *Methyloversatilis 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 (AAG08504); IPUC_PSESP, γ -glutamylisopropylamide synthetase from *Pseudomonas* sp. strain KIE171 (CAC81335); PUUA_ECOLI, γ -glutamylputrescine synthetase from *Escherichia coli* K-12 (BAA14857).

fore, γ -glutamylation 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*, *pipBb-pipBc*, and *pipBc-pipD₁* intergenic regions were successfully amplified (Fig. 1), the *pipD₁-pipA* intergenic region was not amplified, indicating that *pipBaBbBcD* but not *pipA* are co-transcribed. The *pipD₂-pipBa* 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 permease *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 Δ *pipA*-1 and KU43P Δ *pipA*-2 for *pipA*, and KU43P Δ *pipBa*-1 and KU43P Δ *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 Δ *pipBa*-1, however, no product accumulation was similarly observed for the *pipA* deletion mutant, KU43P Δ *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 -glutamylated piperidine 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 -glutamylolation 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 -glutamylamide synthetases are known to require ATP and Mg^{2+} (Kurihara et al., 2005; Takeo et al., 2013). To test the ATP and Mg^{2+} requirements, crude cell extract enzyme assays of PipA overexpressed *E. coli* were performed. When Mg^{2+} 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 Mg^{2+} for the reaction, and is a γ -glutamylpiperidide synthetase. To our knowledge, PipA is the first example of an enzyme that catalyzes γ -glutamylolation 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 -glutamylolation 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 (Sielaff and Andreesen, 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 -glutamylolation.

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 -glutamylolation 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 -glutamylolation 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 -glutamylolation 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.

Acknowledgments

This work was supported by a Grant-in-Aid from Kansai University for progress of research in a graduate course, 2018.

Supplementary Materials

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

References

- Alam, B. S., Saporoschetz, I. B., and Epstein, S. S. (1971a) Formation of *N*-nitrosopiperidine from piperidine and sodium nitrite in the stomach and the isolated intestinal loop of the rat. *Nature*, **232**, 116–118.

- Alam, B. S., Saporoschetz, I. B., and Epstein, S. S. (1971b) Synthesis of nitrosopiperidine from nitrate and piperidine in the gastro-intestinal tract of the rat. *Nature*, **232**, 199–200.
- Anzai, Y., Kim, H., Park, J. Y., Wakabayashi, H., and Oyaizu, H. (2000) Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *Int. J. Syst. Evol. Microbiol.*, **50**, 1563–1589.
- Berg, D. E. and Berg, C. M. (1983) The prokaryotic transposable element Tn5. *Biotechnology*, **1**, 417–435.
- Chachaty, E. and Saulnier, P. (2000) Isolation chromosomal DNA from bacteria. In *The Nucleic Acid Protocols Handbook Vol. 1*, ed. by Rapley, R., Humana Press, New Jersey, pp. 29–32.
- Combourieu, B., Besse, P., Sancelme, M., Godin, J. P., Monteil, A. et al. (2000) Common degradative pathways of morpholine, thiomorpholine, and piperidine by *Mycobacterium aurum* MO1: evidence from ¹H-nuclear magnetic resonance and ionspray mass spectrometry performed directly on the incubation medium. *Appl. Environ. Microbiol.*, **66**, 3187–3193.
- de Azevedo Wäsch, S. I., van der Ploeg, J. R., Maire, T., Lebreton A., Kiener, A. et al. (2002) Transformation of isopropylamine to L-alaninol by *Pseudomonas* sp. strain KIE171 involves N-glutamylated intermediates. *Appl. Environ. Microbiol.*, **68**, 2368–2375.
- Eller, K., Henkes, E., Rossbacher, R., and Höke, H. (2000) Amines, aliphatic. In *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA.
- Hawkes, D. B., Adams, G. W., Burlingame, A. L., Ortiz de Montellano, P. R., and De Voss, J. J. (2002) Cytochrome P450 (cin) (CYP176A), isolation, expression, and characterization. *J. Biol. Chem.*, **277**, 27725–27732.
- Hawkes, D. B., Slessor, K. E., Bernhardt, P. V., and De Voss, J. J. (2010) Cloning, expression and purification of cindoxin, an unusual FMN-containing cytochrome P450 redox partner. *ChemBioChem*, **11**, 1107–1114.
- Iwaki, H. and Hasegawa, Y. (2007) Degradation of 2-nitrobenzoate by *Burkholderia terrae* strain KU-15. *Biosci. Biotechnol. Biochem.*, **71**, 145–151.
- Iwaki, H., Muraki, T., Ishihara, S., Hasegawa, Y., Rankin, K. N. et al. (2007) Characterization of a pseudomonad 2-nitrobenzoate nitroreductase and its catabolic pathway-associated 2-hydroxylaminobenzoate mutase and a chemoreceptor involved in 2-nitrobenzoate chemotaxis. *J. Bacteriol.*, **189**, 3502–3514.
- Iwaki, H., Yamamoto, T., and Hasegawa, Y. (2018) Isolation of marine xylene-utilizing bacteria and characterization of *Halioxenophilus aromaticivorans* gen. nov., sp. nov. and its xylene degradation gene cluster. *FEMS Microbiol. Lett.*, **365**, fny042.
- Kim, Y. H., Kang, I., Bergeron, H., Lau, P. C., Engesser, K. H. et al. (2006) Physiological, biochemical, and genetic characterization of an alicyclic amine-degrading *Mycobacterium* sp. strain THO100 isolated from a morpholine-containing culture of activated sewage sludge. *Arch. Microbiol.*, **186**, 425–434.
- Kimura, M. (1980) A simple method of estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, **16**, 111–120.
- Knapp, J. S., Emtiazi, G., Yusoff, S., and Heron, S. T. (1996) The utilization of morpholine as a sole nitrogen source by Gram-negative bacteria. *Lett. Appl. Microbiol.*, **23**, 334–338.
- Kumar, S., Stecher, G., and Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.*, **33**, 1870–1874.
- Kurihara, S., Oda, S., Kato, K., Kim, H. G., Koyanagi, T. et al. (2005) A novel putrescine utilization pathway involves γ -glutamylated intermediates of *Escherichia coli* K-12. *J. Biol. Chem.*, **280**, 4602–4608.
- Muraki, T., Taki, M., Hasegawa, Y., Iwaki, H., and Lau, P. (2003) Prokaryotic homologs of the eukaryotic 3-hydroxyanthranilate 3,4-dioxygenase and 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase in the 2-nitrobenzoate degradation pathway of *Pseudomonas fluorescens* strain KU-7. *Appl. Environ. Microbiol.*, **69**, 1564–1572.
- National Toxicology Program (2016) N-Nitrosamines. In *Report on Carcinogens*, 14th ed., U.S. Department of Health and Human Services, Public Health Service, Research Triangle Park, NC2011.
- Poupin, P., Ducrocq, V., Hallier-Soulier, S., and Truffaut, N. (1999a) Cloning and characterization of the genes encoding a cytochrome P450 (PipA) involved in piperidine and pyrrolidine utilization and its regulatory protein (PipR) in *Mycobacterium smegmatis* mc²155. *J. Bacteriol.*, **181**, 3419–3426.
- Poupin, P., Godon, J. J., Zumstein, E., and Truffaut, N. (1999b) Degradation of morpholine, piperidine, and pyrrolidine by mycobacteria: evidences for the involvement of a cytochrome P450. *Can. J. Microbiol.*, **45**, 209–216.
- Richter, M. and Rosselló-Móra, R. (2009) Shifting the genomic gold standard for the prokaryotic species definition. *Proc. Natl. Acad. Sci. USA*, **106**, 19126–19131.
- Richter, M., Rosselló-Móra, R., Glöckner, F. O., and Peplies, J. (2016) JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics*, **32**, 929–931.
- Rubiralta, M., Giralt, E., and Diez, A. (1991) Piperidine: Structure, Preparation and Synthetic Applications of Piperidine and Its Derivatives, Elsevier, Amsterdam.
- Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406–425.
- Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G. et al. (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene*, **145**, 69–73.
- Shaibe, E., Metzger, E., and Halpern, Y. S. (1985) Metabolic pathway for the utilization of L-arginine, L-ornithine, agmatine, and putrescine as nitrogen sources in *Escherichia coli* K-12. *J. Bacteriol.*, **163**, 933–937.
- Sielaff, B. and Andreessen, J. R. (2005) Analysis of the nearly identical morpholine monooxygenase-encoding mor genes from different *Mycobacterium* strains and characterization of the specific NADH: ferredoxin oxidoreductase of this cytochrome P450 system. *Microbiology*, **151**, 2593–2603.
- Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technology*, **1**, 784–791.
- Takeo, M., Ohara, A., Sakae, S., Okamoto, Y., Kitamura, C. et al. (2013) Function of a glutamine synthetase-like protein in bacterial aniline oxidation via γ -glutamylanilide. *J. Bacteriol.*, **195**, 4406–4414.
- Tanizawa, Y., Fujisawa, T., and Nakamura, Y. (2018) DFAST: a flexible prokaryotic genome annotation pipeline for faster genome publication. *Bioinformatics*, **34**, 1037–1039.
- Trigui, M., Pulvin, S., Poupin, P., and Thomas, D. (2003) Biodegradation of cyclic amines by a *Pseudomonas* strain involves an amine mono-oxygenase. *Can. J. Microbiol.*, **49**, 181–188.
- Trigui, M., Pulvin, S., Truffaut, N., Thomas, D., and Poupin, P. (2004) Molecular cloning, nucleotide sequencing and expression of genes encoding a cytochrome P450 system involved in secondary amine utilization in *Mycobacterium* sp. strain RP1. *Res. Microbiol.*, **155**, 1–9.
- Yamamoto, S., Wakayama, M., and Tachiki, T. (2008) Cloning and expression of *Methylovorus mays* No. 9 gene encoding γ -glutamylmethanamide synthetase: an enzyme usable in theanine formation by coupling with the alcoholic fermentation system of baker's yeast. *Biosci. Biotechnol. Biochem.*, **72**, 101–109.
- Yonezuka, K., Shimodaira, J., Tabata, M., Ohji, S., Hosoyama, A. et al. (2017) Phylogenetic analysis reveals the taxonomically diverse distribution of the *Pseudomonas putida* group. *J. Gen. Appl. Microbiol.*, **63**, 1–10.