Function of a Glutamine Synthetase-Like Protein in Bacterial Aniline Oxidation via γ -Glutamylanilide

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Acinetobacter sp. strain YAA has five genes (*atdA1* to *atdA5*) involved in aniline oxidation as a part of the aniline degradation gene cluster. From sequence analysis, the five genes were expected to encode a glutamine synthetase (GS)-like protein (AtdA1), a glutamine amidotransferase-like protein (AtdA2), and an aromatic compound dioxygenase (AtdA3, AtdA4, and AtdA5) (M. Takeo, T. Fujii, and Y. Maeda, J. Ferment. Bioeng. 85:17–24, 1998). A recombinant *Pseudomonas* strain harboring these five genes quantitatively converted aniline into catechol, demonstrating that catechol is the major oxidation product from aniline. To elucidate the function of the GS-like protein AtdA1 in aniline oxidation, we purified it from recombinant *Escherichia coli* harboring *atdA1*. The purified AtdA1 protein produced γ -glutamylanilide (γ -GA) quantitatively from aniline and L-glutamate in the presence of ATP and $MgCl_2$. This reaction was identical to glutamine synthesis by GS, except for the use of aniline instead of ammonia as the substrate. Recombinant *Pseudomonas* strains harboring the dioxygenase genes (*atdA3* to *atdA5*) were unable to degrade aniline but converted γ -GA into catechol, indicating that γ -GA is an intermediate to catechol and a direct substrate for the dioxygenase. Unexpectedly, a recombinant *Pseudomonas* strain harboring only *atdA2* hydrolyzed γ -GA into aniline, reversing the γ -GA formation by AtdA1. Deletion of *atdA2* from *atdA1* to *atdA5* caused γ -GA accumulation from aniline in recombinant *Pseudomonas* cells and inhibited the growth of a recombinant *Acinetobacter* strain on aniline, suggesting that AtdA2 prevents γ -GA accumulation that is harmful to the host cell.

Aniline and its derivatives are very important for the synthesis of chemical products such as dyes, resins, and medicines, but these compounds have toxic, mutagenic, and carcinogenic properties (1–4). Because anilines are widely used in industry and agriculture, they have frequently been detected in aquatic environments, and even in a drinking water treatment plant (5–7). Simple anilines such as aniline and monosubstituted anilines are known to disappear from the environment mainly via biodegradation (8, 9). To understand the mechanisms of aniline biodegradation, many aniline-degrading bacteria have been isolated and characterized (10–25).

Acinetobacter sp. strain YAA has an aniline degradation gene cluster on the plasmid pYA1; the cluster consists of 14 genes (*atdA1* to *atdA5* and *atdRBCDEFGH*) required for the conversion of aniline into tricarboxylic acid (TCA) cycle intermediates (14, 26, 27). The first five genes (*atdA1* to *atdA5*) encode proteins involved in the initial oxidation of aniline to catechol by the release of its amino group, and we tentatively designated them multicomponent aniline dioxygenase (AD) genes (26). The *atdA1* gene product shows approximately 30% amino acid (aa) sequence identity with bacterial glutamine synthetases (GSs), while the *atdA2* gene product has approximately 30% identity with bacterial glutamine amidotransferases (GATs) (26). The *atdA3* to *atdA5* gene products share considerable homology with the oxygenase components (large and small subunits) and the reductase component, respectively, of two-component Rieske-type aromatic compound dioxygenases (26, 28, 29). This dioxygenase homolog belongs to group II in the classification of Nam et al. (28), based on large-subunit aa sequence analysis, and to type I α in the later classification of Kweon et al. (29), adding information on the electron transfer chain components to large-subunit sequence analysis. Similar AD gene clusters have been cloned from *Pseudomonas putida* UCC22 (30), *Delftia acidovorans* 7N (23), *Delftia tsuruhatensis* AD9 (18), *Frateruia* sp. strain ANA-18 (31), *Delftia* sp. strain

AN3 (32), and *Comamonas testosteroni* I2 (33). However, the mechanism of aniline oxidation has not been defined, as there is little information on the functions of the gene products involved in the process. In particular, the GS-like and GAT-like proteins are quite unique; these proteins have so far not been identified in other aromatic compound dioxygenases, with the only exception being a GS-like protein gene (*sadB*) in the 4-aminobenzenesulfonate 3,4-dioxygenase gene cluster (*sadABD*) of *Hydrogenophaga* sp. strain PBC (34). However, the function of the gene product is still unknown.

de Azevedo Wäsch et al. (35) reported the conversion of isopropylamine into L-alaninol in *Pseudomonas* sp. strain KIE171. In this process, as shown in Fig. 1A, a GS-like protein (IpuC) catalyzes the formation of γ -glutamylisopropylamide from isopropylamine and L-glutamate, and a GAT-like protein (IpuF) hydrolyzes the amide after the oxidation of the isopropyl group by other enzymes (IpuABDE). These reactions resemble the protection and deprotection of a reactive amino group in chemical synthesis. Similar reactions via γ -glutamylated intermediates occur in the putrescine utilization pathway of *Escherichia coli* K-12 (36). Considering the functions of IpuC and IpuF and their similarity with AtdA1 (31%) and AtdA2 (26%), we predicted the functions of the Atd proteins in aniline oxidation (Fig. 1B). In this oxidation reaction, a GS-like protein, AtdA1, forms γ -glutamylanilide (γ -GA) from aniline and L-glu-

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FIG 1 Isopropylamine oxidation mechanism of *Pseudomonas* sp. KIE171 (35) (A) and putative aniline oxidation mechanism of *Acinetobacter* sp. YAA (B).

tamate, and the dioxygenase proteins AtdA3, AtdA4, and AtdA5 oxidize the aromatic-ring of γ -GA. A GAT-like protein, AtdA2, hydrolyzes the oxidized product into L-glutamate and an unstable cyclohexadiene with an amino group, which spontaneously forms catechol and ammonia.

To confirm this putative pathway, γ -GA was synthesized chemically as an authentic compound, and a *Pseudomonas* strain that does not degrade catechol was constructed by gene disruption to evaluate the quantitative formation of catechol from aniline or γ -GA. To investigate the first step of the oxidation reaction, AtdA1 was purified from recombinant *E. coli* harboring *atdA1*, and aniline conversion to γ -GA was investigated at the enzymatic level. Conversion of γ -GA to catechol was also examined in *Pseudomonas* strains expressing the remaining dioxygenase genes (*atdA3* to *atdA5* or *atdA2* to *atdA5*). These experiments revealed that aniline is oxidized via γ -GA into catechol in *Acinetobacter* sp. YAA and that the γ -GA formation is catalyzed by AtdA1. The putative function of AtdA2 in aniline oxidation is also discussed, based on the results of the gene deletion study.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, media, and cultivation conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. The primers used in this study are listed in Table S1 in the supplemental material. *Escherichia coli* strains were cultivated at 37°C, while *Pseudomonas* and *Acinetobacter* strains were cultivated at 30°C. Bacterial cultures were maintained on a rotary shaker at 140 rpm. Luria-Bertani (LB) medium was used as a rich medium for growth, and Terrific broth (TB) was used for protein production (37). MSB medium (pH 7.6), consisting of 1 g K_2HPO_4 , 1 g $(\text{NH}_4)_2\text{SO}_4$, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g FeCl_3 , 0.1 g NaCl, and 0.1 g CaCl_2 (liter⁻¹), was used for the growth test on aniline.

Chemical synthesis of γ -GA. γ -GA was chemically synthesized as shown in Fig. S1 in the supplemental material, and γ -GA and its synthetic intermediates were analyzed by ¹H nuclear magnetic resonance (¹H-NMR) and Fourier transform infrared spectroscopy (FTIR) as described previously (38, 39). The analytical data are shown in Table S2.

DNA purification, PCRs, electroporation, and other DNA techniques. Total DNA was extracted from *Pseudomonas putida* KT2440 (40) or its derivatives by the method of Saito and Miura (41). PCR was performed with a TaKaRa PCR Dice thermal cycler (TaKaRa Bio, Kyoto, Japan). The standard PCR mixture contained 10 ng of template DNA, 10 μmol of each primer, a 2.5 mM concentration of each deoxynucleoside triphosphate (dNTP), 5 μl of 10 $\times$ Ex Taq buffer, and 0.5 U of Ex Taq polymerase (TaKaRa Bio) in a total volume of 50 μl . The standard PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min kb^{-1} ; and 72°C for 5 min. For colony PCR, a small amount of bacteria was used instead of the template DNA. DNA fragments were purified from agarose gel by use of TaKaRa Suprec-01 cartridges (TaKaRa Bio), and ligation reactions were performed with a TaKaRa DNA ligation kit (ver.2.1; TaKaRa Bio). *P. putida* KT2440 was transformed with plasmids (electroporation) as described previously (38), but with a modified voltage (2.5 kV). Plasmid DNA preparation, restriction digestion, and transformation were performed according to standard methods (37).

Construction of recombinant plasmids pGS18 and pBA2. A 1.5-kb DNA fragment containing *atdA1* was amplified from pAS185 (Table 1) by a PCR using primers A1F and A1R and subsequently was digested with BamHI. The fragment was ligated into BamHI-digested pUC18 (Table 1) to form pGS18. Similarly, a 0.7-kb DNA fragment containing *atdA2* was amplified from pAS185 by using primers A2F-salI and A2R and then was doubly digested with BamHI and SalI. The fragment was ligated into doubly digested pBBR1MCS-2 (Table 1) to form pBA2. The sequences of the inserts in these plasmids were confirmed by nucleotide sequencing.

Preparation of *E. coli* cell extract and purification of AtdA1. *E. coli* SO58 harboring pGS18 was grown overnight in 300 ml of TB with 0.25 mM isopropyl- β -D-thiogalactoside (IPTG) and 100 mg liter⁻¹ ampicillin. Cells were harvested by centrifugation (4°C, 10,000 $\times$ g, 10 min), washed twice with 10 mM sodium phosphate buffer (pH 7.0), and suspended in 15 ml of the same buffer. The bacterial cells were disrupted using a model UD-200 ultrasonic disruptor (Tomy, Tokyo, Japan) (vol. 6, 10 times for 1 min each on ice). After pelleting of the cell debris and unbroken cells by centrifugation (4°C, 15,000 $\times$ g, 30 min), the supernatant was used as a cell extract for protein purification.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
Strains		
<i>Escherichia coli</i> JM109	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) e14 ⁻ (Δ <i>mcrA</i>) <i>supE44 relA1</i> Δ (<i>lac-proAB</i>) [F' <i>traD36 proAB⁺ lacI^qZ</i> Δ M15]	TaKaRa Bio, Kyoto, Japan
<i>E. coli</i> S17-1	<i>RP4-2</i> (Km::Tn7 Tc::Mu-1) <i>pro-82 recA1 endA1 thiE1 hsdR17 creC510</i>	46
<i>E. coli</i> SO58	K-12 derivative; F ⁻ Δ <i>ggd-2</i> Δ <i>ycjKLC::cat⁺FRT</i> Δ (<i>aldH-ordL-goagG</i>)::kan ⁺	36
<i>Pseudomonas putida</i> KT2440	mt-2 derivative; <i>hsdR1</i> (r ⁻ m ⁺) Cm ^r	40
<i>P. putida</i> KT2440- Δ <i>catA</i>	KT2440 mutant; Δ <i>catA1</i> Δ <i>catA2</i> ; catechol degradation negative	This study
<i>P. putida</i> KT2440- Δ <i>catA</i> Δ <i>ggd</i>	KT2440 mutant; Δ <i>catA1</i> Δ <i>catA2</i> Δ <i>ggd-2</i> ; catechol and γ -GA degradation negative	This study
<i>Acinetobacter baylyi</i> BD413	Unencapsulated mutant of <i>A. baylyi</i> BD4; ATCC 33305	53
Plasmids		
pUC18	Ap ^r ; cloning vector; 2.7 kb; <i>lacOP</i>	TaKaRa Bio, Kyoto, Japan
pUC19	Ap ^r ; cloning vector; 2.7 kb; <i>lacOP</i>	TaKaRa Bio, Kyoto, Japan
pAS185	Ap ^r ; 18.5-kb SalI fragment of pYA1 in pUC19; <i>atdA1</i> to <i>atdA5</i>	14
pGS18	Ap ^r ; 1.5-kb BamHI fragment containing <i>atdA1</i> cloned into pUC18	This study
pBBR1MCS-2	Km ^r ; broad-host-range vector; 5.1 kb; <i>mob lacOP</i>	58
pTB01	5.3-kb SalI-SmaI fragment containing <i>atdA1</i> to <i>atdA5</i> in pBBR1MCS-2	48
pK18 <i>mobsacB</i>	Km ^r ; vector for gene disruption; <i>mob sacB lacOP</i>	45
pKA1	1.1-kb fragment containing Δ <i>catA1</i> mutation (lacking the 394-bp central part of <i>catA1</i>) in pK18 <i>mobsacB</i>	This study
pKA2	1.2-kb fragment containing Δ <i>catA2</i> mutation (lacking the 389-bp central part of <i>catA2</i>) in pK18 <i>mobsacB</i>	This study
pKGGT-2	1.4-kb fragment containing Δ <i>ggd-2</i> mutation (lacking the 936-bp central part of <i>ggd-2</i>) in pK18 <i>mobsacB</i>	This study
pBA2	0.7-kb fragment containing <i>atdA2</i> in pBBR1MCS-2	This study
pTB01- Δ A1	pTB01 derivative lacking <i>atdA1</i>	This study
pTB01- Δ A2	pTB01 derivative lacking <i>atdA2</i>	This study
pTB01- Δ A1A2	pTB01 derivative lacking <i>atdA1A2</i>	This study

A Hi-Trap Q Sepharose column (5 ml) (GE Healthcare Japan, Tokyo, Japan) was preequilibrated with phosphate buffer, and the cell extract was loaded onto the column. After washing with phosphate buffer, the target protein was eluted at 2 ml min⁻¹ with an NaCl gradient (total volume, 180 ml) from 0 M to 0.6 M, using a Biologic LP chromatography system (Bio-Rad Japan, Tokyo, Japan), and the last 90 ml of eluate was fractionated into 30 3-ml fractions, which were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (26). Fractions containing the target protein were collected, concentrated with a Vivaspinn 6-10K concentrator (GE Healthcare), and desalted using a PD-10 desalting column (GE Healthcare). The desalted solution was concentrated again with the Vivaspinn 6-10K concentrator to a volume suitable for the next step. The described anion-exchange chromatography and concentration processes were repeated.

A Hi-Trap butyl FF column (1 ml) (GE Healthcare) was preequilibrated with 10 mM sodium phosphate buffer containing 1 M (NH₄)₂SO₄, and the concentrated protein solution was loaded onto the column. The target protein was eluted at 1 ml min⁻¹ with a gradient of (NH₄)₂SO₄ solution (total volume, 30 ml) from 1 M to 0 M, using the same chromatography system, and was fractionated into 30 1-ml fractions, which were analyzed and purified as described above. The protein concentration was determined by the method of Lowry et al. (42), using bovine serum albumin (Nacalai Tesque, Kyoto, Japan) as a protein standard.

Assay conditions for aniline conversion by AtdA1 and determination of its GS activity. The standard reaction mixture for the conversion of aniline to γ -GA by AtdA1 contained 0.5 mM aniline, 1 mM L-glutamate, 0.5 mM ATP, 5 mM MgCl₂, and 1 μ g of AtdA1 in 1 ml of 10 mM sodium phosphate buffer, pH 8.0. The reactions were performed at 40°C. The pH and temperature were optimized prior to fixing these assay conditions (see Fig. S5 in the supplemental material). The GS activity of AtdA1 was measured based on the method of Listrom et al. (43), but employing the above-mentioned reaction mixture and temperature, ex-

cept for the addition of hydroxylamine instead of aniline to the mixture and the use of an excess amount (10 μ g) of AtdA1.

Gel filtration chromatography. The native molecular mass of AtdA1 was determined at room temperature by use of a Shimadzu Prominent high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) (44) equipped with a TSKgel G3000SWXL column (300-mm length, 7.8-mm internal diameter [ID], 5- μ m particles) (Tosoh, Tokyo, Japan) and a refractive index detector (RI-8020; Tosoh). The HPLC conditions were as follows: mobile phase, 0.1 M KH₂PO₄, 0.1 M Na₂SO₄, 0.05% NaN₃; detection wavelength, 280 nm; flow rate, 1.0 ml min⁻¹; and injection volume, 20 μ l.

Gene disruption by homologous recombination. Gene disruption by homologous recombination in *P. putida* KT2440 was performed as previously described by Schäfer et al. (45).

(i) Disruption of catechol 1,2-dioxygenase genes (*catA1* and *catA2*). Two DNA fragments (587 bp and 509 bp), which included the 5'-end region and the 3'-end region of the catechol 1,2-dioxygenase gene of KT2440 (*catA2*) (912 bp; product accession no. NP_745310), respectively, were amplified from the total KT2440 DNA by PCR with two primer sets: cA2F-F and cA2F-R for the 5'-end region and cA2R-F and cA2R-R for the 3'-end region (see Table S1 in the supplemental material). The PCR conditions were identical to those described above except for the annealing temperature (60°C). The 5'-end-region fragment was digested with EcoRI and BamHI, while the 3'-end-region fragment was digested with BamHI and XbaI. These fragments were cloned together into EcoRI- and XbaI-digested pK18*mobsacB* (Table 1) to form pKA2, which had an incomplete *catA2* gene (Δ *catA2*) lacking a 389-bp central region. pKA2 was used to transform *E. coli* S17-1. pKA2 was then transferred from *E. coli* S17-1 to *P. putida* KT2440 by biparental mating (46), and the transconjugants were selected on LB plates containing kanamycin (100 mg liter⁻¹) and chloramphenicol (50 mg liter⁻¹). Kanamycin resistance of the transconjugants originated from pK18*mobsacB*, whereas chloramphenicol resistance was

the original phenotype of *P. putida* KT2440 (47). This method selected the first-crossover mutants of KT2440, which had the pKA2 sequence inserted into the genome by homologous recombination; pKA2 cannot replicate in KT2440. After colony PCR using primers cA2F-F and cA2R-R to confirm the presence of *catA2* and the Δ *catA2* mutation in the transconjugants (see Fig. S2A), one clone was incubated in LB medium at 30°C for 12 h. The culture was spread on LB plates including 10% (wt/vol) sucrose, and the plates were incubated at 30°C for a few days. By this selection, second-crossover mutants or revertants of KT2440 were obtained (see Fig. S2), as the *sacB* gene in pKA2 is lethal to the host strain grown on sucrose-containing media (45). Colonies containing the Δ *catA2* mutation were checked by colony PCR (see Fig. S2A).

Similarly, two DNA fragments (541 bp and 611 bp) of another catechol 1,2-dioxygenase gene (*catA1*) (933 bp; product accession no. NP_745846) were amplified from the total KT2440 DNA as described above, using two primer sets: cA1F-F and cA1F-R for the 5'-end region and cA1R-F and cA1R-R for the 3'-end region (see Table S1 in the supplemental material). The resultant fragments were doubly digested with EcoRI and HindIII for the former or with HindIII and XbaI for the latter and were inserted together into EcoRI- and XbaI-digested pK18mobsacB to form plasmid pKA1, which had an incomplete *catA1* gene (Δ *catA1*) lacking a 394-bp central region. pKA1 was transferred into the Δ *catA2* mutant of KT2440 to make the Δ *catA1* Δ *catA2* double mutant. This *catA1* disruption was also confirmed by PCR (see Fig. S2B).

(ii) **Disruption of γ -glutamyltranspeptidase gene (*ggt-2*).** Two DNA fragments (458 bp and 902 bp) of the γ -glutamyltranspeptidase gene (*ggt-2*) of KT2440 (1,666 bp; product accession no. NP_746768) were amplified from the total DNA by PCR as described above, using two primer sets: ggt-2FF-H and ggt-2FR for the former fragment and ggt-2RF and ggt-2RR for the latter (see Table S1 in the supplemental material). The resultant fragments were doubly digested with XbaI and SalI for the former or with SalI and BamHI for the latter and were inserted together into XbaI- and BamHI-digested pK18mobsacB to form pKGGT-2, which had an incomplete *ggt-2* gene (Δ *ggt-2*) lacking a 936-bp central region. pKGGT-2 was introduced into the Δ *catA1* Δ *catA2* double mutant as described above to make the Δ *catA1* Δ *catA2* Δ *ggt-2* triple mutant of KT2440.

Construction of pTB01 derivatives lacking *atdA1*, *atdA2*, or *atdA1* and *atdA2* (*atdA1A2*). An 8.8-kb DNA fragment was amplified from pTB01 (Table 1) by a PCR using PrimeSTAR Max DNA polymerase (TaKaRa Bio) and primers F-pAS51 and pTB01-A2-5R (see Table S1 in the supplemental material). This primer set was designed to amplify all of the pTB01 sequence except for the *atdA1* gene. The fragment was self-ligated to construct pTB01- Δ A1, which was used to transform *E. coli* JM109, *P. putida* KT2440, and *P. putida* derivatives.

Similarly, 9.7-kb and 8.1-kb DNA fragments were independently amplified by PCRs using the same polymerase and two different primer sets (primers A3F and A1R2 and primers A3F and pTB01-A2-5R) (see Table S1 in the supplemental material). The former set was designed to amplify all of the pTB01 sequence except for the *atdA2* gene, while the latter was designed to amplify all of the pTB01 sequence except for the *atdA1* and *atdA2* genes. The amplified fragments were self-ligated to construct pTB01- Δ A2 and pTB01- Δ A12, respectively, and used to transform the host strains. The loss of *atdA1*, *atdA2*, or *atdA1A2* in these deletion plasmids was confirmed by nucleotide sequencing.

Degradation of aniline and γ -GA in cell suspensions. Recombinant *P. putida* KT2440 or its gene-disrupted strains were cultivated in LB medium containing kanamycin (100 mg liter⁻¹) for 24 h, and cells were harvested by centrifugation (4°C, 8,000 $\times$ g, 10 min). The cells were washed three times with cold sterile water and suspended in a small amount of 10 mM sodium phosphate buffer (pH 7.0). The suspension was diluted with MSB medium to adjust the optical density at 600 nm (OD₆₀₀) to 5.0. Aniline or γ -GA was added to the suspension, and degradation experiments were performed at 30°C and 150 rpm on a rotary shaker. Samples were taken at specific intervals, and the substrates and their metabolites were analyzed by HPLC. A Shimadzu Prominent HPLC system

(Shimadzu) equipped with a Mightysil RP18 GP Aqua column (150- to 4.6-mm ID by 5 μ m) (Kanto Kagaku Kogyo, Tokyo, Japan) (44) was used for the analysis, and the HPLC conditions were as follows: mobile phase, ratio of solution A (5% CH₃CN, 95% H₂O, 0.1% CH₃COOH) to solution B (95% CH₃CN, 5% H₂O, 0.1% CH₃COOH) of 61.9:38.1 or 70:30; detection wavelengths, 254 nm and 277 nm; flow rate, 0.5 ml min⁻¹; and injection volume, 10 μ l.

RESULTS

Construction of non-catechol-degrading *Pseudomonas putida* KT2440. We previously constructed the recombinant plasmid pTB01 (48), which had a 5.3-kb DNA fragment including the complete AD gene cluster (*atdA1* to *atdA5*), in the broad-host-range plasmid pBBR1MCS-2. An *E. coli* strain harboring pTB01 showed a strong brown color around the colonies on aniline-containing plates (most likely from catechol formation and auto-oxidation). However, in liquid cultures, a significant decrease in aniline concentration was not detected by HPLC analysis, and only a trace amount of catechol was detected by gas chromatography-mass spectrometry analysis due to the poor enzymatic activity of the gene products expressed in *E. coli*. In contrast, *P. putida* KT2440 harboring pTB01 degraded aniline rapidly (48) (the cell suspension at an OD₆₀₀ of 2 degraded 100 mg liter⁻¹ of aniline within 70 min). Catechol was not detected as the metabolite by HPLC, because this strain could further degrade catechol by use of endogenous catechol 1,2-dioxygenases (encoded by *catA1* and *catA2* on the genome) (49). To evaluate the degradation of aniline or its intermediates into catechol, it was necessary to quantify the catechol. Therefore, we generated a mutant of KT2440 that could not degrade catechol by disrupting *catA1* and *catA2*.

We constructed two recombinant plasmids, pKA1 and pKA2, which had Δ *catA1* and Δ *catA2* mutations lacking the central segments of *catA1* and *catA2*, respectively, in pK18mobsacB. pKA2 was first introduced into *E. coli* S17-1 and then transferred into *P. putida* KT2440 by mating. After the selection of kanamycin- and chloramphenicol-resistant strains (first-crossover mutants) and subsequent sucrose-resistant strains (second-crossover mutants), several Δ *catA2* mutants were obtained, and the deletion in *catA2* was confirmed by PCR (see Fig. S2A in the supplemental material). Furthermore, pKA1 was introduced into one of the Δ *catA2* mutants in the same manner. Finally, several Δ *catA1* Δ *catA2* double mutants were obtained (see Fig. S2B). The double mutant was designated *P. putida* KT2440- Δ *catA*. The KT2440- Δ *catA* strain accumulated catechol from benzoate stoichiometrically (data not shown), as it had a benzoate degradation pathway via catechol (49). This phenotype strongly supported the disruption of *catA1* and *catA2*.

Conversion of aniline using KT2440- Δ *catA* harboring pTB01. To investigate aniline conversion in KT2440- Δ *catA*, pTB01 including the complete AD gene cluster (*atdA1* to *atdA5*) was introduced into this strain, and the resultant strain was used for aniline degradation studies. As shown in Fig. 2, the cell suspension of this strain converted aniline into catechol almost quantitatively, indicating that catechol is a major oxidation product from aniline.

Conversion of aniline by *AtdA1*. To examine the function of *AtdA1* in aniline oxidation, we planned a similar aniline conversion experiment using cell suspensions of KT2440- Δ *catA* harboring only *atdA1*. However, our preliminary study revealed that the putative product, γ -GA, was easily degraded into aniline and L-

FIG 2 Conversion of aniline into catechol by a cell suspension of *P. putida* KT2440- Δ catA harboring pTB01 (*atdA1* to *atdA5*). Symbols: open circles, aniline; closed circles, catechol. The experiment was performed in triplicate, and averages $\pm$ standard deviations are shown.

glutamate through an unknown endogenous hydrolytic activity of this strain (this problem was essentially solved, as described later). Thus, it was difficult to quantify γ -GA in the degradation tests using this strain. To overcome this limitation, purified AtdA1 was used in an *in vitro* aniline conversion experiment.

A recombinant *E. coli* SO58 strain containing *atdA1* (in plasmid pGS18) was grown in TB in the presence of IPTG, and its cell extract was prepared. SDS-PAGE of the cell extract showed a 57-kDa protein that was absent in the cell extract of the empty vector control strain and that was very close in size to the predicted molecular mass of AtdA1 (55 kDa). The protein was purified to a single band in SDS-PAGE gels through two cycles of anion-exchange chromatography and hydrophobic chromatography (see Fig. S3 in the supplemental material). The specific aniline conversion activity of the purified protein solution was 22-fold higher than that of the cell extract (see Table S3) (measured under the optimized assay conditions shown below). Gel filtration chromatography showed that its native size was approximately 350 kDa (see Fig. S4), indicating that AtdA1 forms a hexameric structure.

As the aa sequence of AtdA1 showed approximately 30% identity with bacterial GSs (26), aniline conversion by AtdA1 was investigated in a reaction mixture similar to that used for the measurement of GS activity (43, 50), which included L-glutamate, ATP, and MgCl_2 . Aniline was added to the reaction mixture instead of ammonia. As shown in Fig. 3, aniline (0.5 mM) was reduced by 80% in 8 h, and an almost equal amount of γ -GA was formed. Removal of any of the components (L-glutamate, ATP, or MgCl_2) resulted in no conversion (data not shown). Therefore, like GS, AtdA1 requires these components to convert aniline. Maximum activity occurred between pH 8 and pH 10, and the optimum temperature was approximately 40°C (see Fig. S5 in the supplemental material). Under the same conditions, AtdA1 converted the following anilines (with percent conversion listed): aniline (100%), *o*-chloroaniline (92%), *m*-chloroaniline (69%), *p*-chloroaniline (92%), *o*-methylaniline (40%), *m*-methylaniline (27%), and *p*-methylaniline (45%). These results indicate that AtdA1 has broad substrate specificity and prefers *o*- and *p*-substituted anilines. AtdA1 showed no GS activity ($<0.001 \text{ U mg}^{-1}$), although a positive-control GS from *Bacillus stearothermophilus* (Unitika, Osaka, Japan) showed considerable GS activity (0.200 U mg^{-1}).

FIG 3 Conversion of aniline into γ -GA by AtdA1. Symbols: open circles, aniline; closed circles, γ -GA. The experiment was performed in triplicate, and averages $\pm$ standard deviations are shown.

Construction of *ggt-2*-disrupted mutant of KT2440- Δ catA.

As described above, in *P. putida* KT2440, γ -GA was degraded into aniline by an unknown endogenous hydrolytic activity (see Fig. S6 in the supplemental material). Thus, this strain was not an ideal host for performing γ -GA degradation experiments. Kurihara et al. (36) used *E. coli* SO58 with a mutation in the γ -glutamyltranspeptidase gene (*ggt-2* mutant) to study the putrescine utilization pathway via γ -glutamylated intermediates. γ -Glutamyltranspeptidase catalyzes the hydrolysis of the γ -glutamyl linkage of glutathione (γ -glutamylcysteinylglycine) and uses γ -GA and γ -glutamyl-*p*-nitroaniline as substrates in colorimetric assays (51, 52). In fact, γ -GA was not degraded in *E. coli* SO58 (*ggt-2*) (data not shown). We found two putative γ -glutamyltranspeptidase genes (*ggt-1* and *ggt-2*) and some homologous genes in the registered genome sequence of KT2440 (accession no. NC_002947). These genes were independently disrupted in the same way as that utilized for the *catA1* and *catA2* disruptions. As expected, when *ggt-2* was disrupted in KT2440- Δ catA, the mutant strain no longer degraded γ -GA (see Fig. S6). Thus, we named this triple mutant KT2440- Δ catA Δ ggt and used it for γ -GA degradation studies.

Conversion of γ -GA by KT2440- Δ catA Δ ggt harboring pTB01- Δ A1. We constructed pTB01- Δ A1, which was an *atdA1*-deleted version of plasmid pTB01. This plasmid was introduced into KT2440- Δ catA Δ ggt. From the proposed pathway shown in Fig. 1B, this recombinant strain was expected to convert γ -GA into catechol, because it contained *atdA2* to *atdA5*. Unexpectedly, γ -GA was almost quantitatively converted into aniline (Fig. 4A), although a small amount of catechol was detected ($\leq 0.02 \text{ mM}$). We suspected that AtdA2 catalyzed this unexpected conversion, because AtdA3, AtdA4, and AtdA5 are putative members of the two-component dioxygenase (26, 28, 29). In fact, the cell suspension of KT2440- Δ catA Δ ggt harboring only *atdA2* (in plasmid pBA2) efficiently degraded γ -GA into aniline (Fig. 4B). To prevent this activity, we constructed an *atdA1A2*-deleted plasmid, named pTB01- Δ A12, and γ -GA degradation was again carried out, using KT2440- Δ catA Δ ggt cells harboring pTB01- Δ A12. As a result, the cell suspension was able to convert γ -GA into catechol ($\leq 0.05 \text{ mM}$), but aniline was not formed as expected (Fig. 4C). This result shows that the two-component dioxygenase (AtdA3, AtdA4, and AtdA5) can convert γ -GA into catechol. The same cell suspension never degraded aniline (data not shown), indicating that aniline is not a direct substrate for the dioxygenase. When the cell suspension of KT2440- Δ catA Δ ggt harboring pTB01 (*atdA1* to

FIG 4 Degradation of γ -GA by cell suspensions of KT2440- Δ catA Δ ggt harboring pTB01- Δ A1 (A), pBA2 (B), or pTB01- Δ A12 (C). Symbols: open circles, γ -GA; closed circles, catechol; closed triangles, aniline. The experiments were performed in triplicate, and averages $\pm$ standard deviations are shown.

atdA5) was tested for γ -GA degradation, aniline accumulated from γ -GA at the beginning of the reaction, but γ -GA was almost completely converted into catechol (Fig. 5). The presence of both the *atdA1* and *atdA2* genes significantly improved γ -GA degradation.

Conversion of aniline by *P. putida* KT2440- Δ catA Δ ggt harboring pTB01- Δ A2. To understand the function of *atdA2* in aniline oxidation, *atdA2* was deleted from pTB01 to make pTB01- Δ A2. As shown in Fig. 6A, KT2440- Δ catA Δ ggt harboring pTB01 reduced 0.4 mM aniline (from 0.6 mM to 0.2 mM) in 4 h, and a major amount of catechol was detected. A small amount of γ -GA was also detected. In contrast, KT2440- Δ catA Δ ggt harboring pTB01- Δ A2 degraded aniline similarly, but a major amount of γ -GA was accumulated (Fig. 6B). This clearly shows that *AtdA2* prevents γ -GA accumulation in the cell.

Growth of *Acinetobacter baylyi* BD413 harboring pTB01- Δ A2 on aniline. To simulate the growth of *Acinetobacter* sp. YAA lacking *atdA2* on aniline, *A. baylyi* BD413 (formerly termed *Acinetobacter* sp. or *Acinetobacter calcoaceticus*) (53) was used as a host strain for pTB01- Δ A2, because in strain YAA, with several cryptic plasmids (14), it was difficult to delete only *atdA2* on the aniline degradation plasmid pYA1. The host BD413 strain was able to assimilate catechol but unable to degrade γ -GA (data not shown), indicating that there was no endogenous γ -GA hydrolytic activity. pTB01 and pTB01- Δ A2 were independently introduced into BD413 as described previously (48). The resultant BD413 strains were inoculated into MSB medium containing aniline as the sole

carbon source at an OD₆₀₀ of 0.1 and then cultivated with shaking. BD413 harboring pTB01 completely degraded 0.15 mM aniline within 1 day, as well as additional spikes of aniline administered day by day (Fig. 7A). The growth reached an OD₆₀₀ of approximately 0.4 after 5 days. During the degradation, γ -GA was not detected at all. In contrast, BD413 harboring pTB01- Δ A2 was unable to degrade 0.15 mM aniline completely even after 2 days of incubation, and a small amount of γ -GA was detected (Fig. 7B). The remaining aniline at day 2 was not reduced during a further 3-day incubation. In a repeated experiment, we added 0.2 mM aniline at day 2, but no further growth was observed (data not shown).

DISCUSSION

To date, several AD genes have been cloned and characterized (14, 18, 23, 30–33), but the aniline oxidation mechanisms have not been elucidated, that is, the concrete functions of the products of the AD genes in aniline oxidation are unknown. All the AD gene clusters found so far encode GS-like and GAT-like proteins, which have not been found in other dioxygenases, with one exception (34). In this study, we proposed an aniline oxidation mechanism for *Acinetobacter* sp. YAA that occurs via γ -GA, referring to the isopropylamide conversion mechanism of *Pseudomonas* sp. KIE171 (Fig. 1), in which GS-like and GAT-like proteins are involved in the formation and hydrolysis of the γ -glutamyl intermediates (35).

We first proved that catechol is the major oxidation product from aniline by using a Δ catA1 Δ catA2 mutant of *P. putida*

FIG 5 Conversion of γ -GA into catechol by a cell suspension of *P. putida* KT2440- Δ catA Δ ggt harboring pTB01 (*atdA1* to *atdA5*). Symbols: open circles, γ -GA; closed circles, catechol; closed triangles, aniline. The experiment was performed in triplicate, and averages $\pm$ standard deviations are shown.

FIG 6 Conversion of aniline into catechol by cell suspensions of *P. putida* KT2440- Δ catA Δ ggt harboring pTB01 (*atdA1* to *atdA5*) (A) or pTB01- Δ A2 (*atdA1* and *atdA3* to *atdA5*) (B). The experiments were performed in triplicate, and averages $\pm$ standard deviations are shown. Symbols: open circles, aniline; closed circles, catechol; closed triangles, γ -GA.

FIG 7 Growth of *Acinetobacter baylyi* BD413 harboring pTB01 (*atdA1* to *atdA5*) (A) or pTB01-ΔA2 (*atdA1* and *atdA3* to *atdA5*) (B) on aniline. Arrows indicate the addition of aniline. Symbols: open circles, aniline; closed circles, growth (OD₆₀₀); closed triangles, γ-GA.

KT2440 (Fig. 2). Catechols are known to be the oxidation products from aniline, methylaniline, and chloroaniline (14, 18, 32, 33), but this is the first report to have detected a major amount of catechol from aniline. Next, to prove the presence of γ-glutamyl intermediates in aniline oxidation, we purified the GS-like protein AtdA1 and showed that it can produce γ-GA from aniline and L-glutamate in the presence of ATP and MgCl₂ (Fig. 3). γ-GA was previously detected as a water-soluble metabolite in the aniline metabolism of a cattle tick, *Boophilus microplus* (54), but the formation mechanism was unknown. This enzymatic reaction was almost identical to glutamine synthesis by GS (L-glutamate: ammonia ligase; EC 6.3.1.2) (43, 50), except that aniline was the substrate instead of ammonia. Thus, AtdA1 could be designated an “L-glutamate:aniline ligase,” but here we call it a “γ-GA synthetase” to emphasize the product name γ-GA. Bacterial GS forms a dodecamer consisting of two face-to-face hexameric rings of identical subunits (55). The gel filtration chromatography result suggested that γ-GA synthetase forms a hexameric structure. Therefore, in addition to the sequence similarity with GS, γ-GA synthetase may be structurally similar to GS. Nevertheless, it showed no GS activity. Fukumori and Saint (30) showed that the *tdnQ* gene of *P. putida* UCC22 (encoding a GS-like protein) was unable to complement the glutamine requirement of an *E. coli* *glnA* (GS gene) mutant. This result is in good agreement with our result.

Without *atdA1*, aniline was never oxidized, even if all other AD genes were present in KT2440 derivatives (e.g., KT2440-Δ*catA*Δ*ggt* containing pTB01-Δ*A1*). This suggests that γ-GA formation is necessary prior to the oxygenation of the aromatic ring of aniline. Fukumori and Saint (30) deleted the *tdnQ* region from the AD gene cluster of strain UCC22. A recombinant *P. putida* KT2442 strain harboring *tdnTA1A2B* no longer showed oxygen uptake for aniline or ammonia-releasing activity. This strain failed to grow on aniline. Murakami et al. (31) also reported that the loss of *tdnQ* resulted in no oxygen uptake for aniline in recombinant *E. coli* cells harboring the AD genes from *Frateriuria* sp. ANA-18. Moreover, *tdnQ*, TdnQ, and their homologs have always been detected in many aniline-degrading bacteria by PCR and proteomic analysis (13, 23, 56). These facts show that GS-like proteins (γ-GA synthetase) are indispensable for aniline oxidation.

The KT2440 derivatives harboring the dioxygenase genes (*atdA3* to *atdA5*) converted γ-GA into catechol (Fig. 4A and C). This is strong evidence demonstrating that γ-GA formation is the starting point of the proposed aniline oxidation mechanism (pathway). Recently, Król et al. (33) reported that recombinant *E.*

coli cells harboring only the dioxygenase genes (*dcaA1A2B*) from the AD gene cluster (*dcaQTA1A2B*) of *Comamonas testosteroni* produced a small amount of 4-chlorocatechol from 3-chloroaniline. Therefore, this dioxygenase may be able to attack anilines directly without GS-like and GAT-like proteins. However, we believe that the major pathway is via γ-glutamylated intermediates, because the gene cluster still keeps *dcaQT*, encoding the GS-like and GAT-like proteins.

Considering the function of a GAT-like protein, IpuF, in the isopropylamide conversion mechanism of *Pseudomonas* sp. KIE171 (35) (Fig. 1A), we expected that AtdA2 would catalyze the hydrolysis of the putative oxidized product of γ-GA (Fig. 1B). However, unexpectedly, in the presence of *atdA2*, γ-GA was easily degraded into aniline (Fig. 4B). This is a reverse reaction against the formation of γ-GA at the expense of ATP by AtdA1 (Fig. 3). Thus, the action of AtdA2 has a confounding effect. One possible explanation for this reverse reaction is control of the cellular γ-GA concentration. In fact, the deletion of *atdA2* from *atdA1* to *atdA5* caused the accumulation of a considerable amount of γ-GA from aniline in recombinant *Pseudomonas* cells (Fig. 6B). Thus, *atdA2* obviously contributes to the prevention of γ-GA accumulation in the host cell. Moreover, *A. baylyi* BD413 containing the AD gene cluster without *atdA2* lost its aniline degradation ability when grown on aniline (Fig. 7B), probably due to γ-GA formation. This host strain has no γ-GA-hydrolyzing activity and thus cannot scavenge γ-GA once it is formed. γ-GA and/or its putative oxidized product must inhibit the dioxygenase activity and be harmful to host cells, because γ-GA degradation always stops at the beginning of the degradation (Fig. 4A and C). The addition of *atdA1A2* to the *atdA3* to *atdA5* genes significantly improved γ-GA degradation (cf. Fig. 4C and 5), as AtdA1 should provide γ-GA for the dioxygenase, while AtdA2 might keep the γ-GA concentration suitable for (or not enough to inactivate) the dioxygenase in the cell.

When aniline conversion was compared between KT2440 strains with and without *ggt-2* (cf. Fig. 1 and 6A), the former was more efficient and rapid than the latter under similar conditions. γ-GA was detected only in the latter. This observation suggests that like *atdA2*, the host *ggt-2* gene also prevents γ-GA accumulation and improves aniline degradation. Fukumori and Saint (30) reported that recombinant *P. putida* KT2442 harboring the AD gene cluster of strain UCC22 without *tdnT* showed a comparable specific growth rate ($\mu = 0.16$) in mineral salt medium including aniline as the sole carbon source to that of cells with the intact AD gene cluster ($\mu = 0.17$). KT2442 is a spontaneous rifampin-resistant mutant of KT2440 (57) and still has *ggt-2* in the genome. Thus, the host *ggt-2* gene may have compensated for the lack of *tdnT*. *Hydrogenophaga* sp. PBC has the *sadABD* gene cluster for 4-aminobenzenesulfonate oxidation (34), which encodes a GS-like protein but not a GAT-like protein. However, intriguingly, this strain contains a DNA region encoding a GAT-like protein and a part of an AD large-subunit homolog (whole-genome shotgun sequence contig 46; accession no. AJWL01000044). Thus, this gene product may contribute to the 4-aminobenzenesulfonate oxidation to 4-sulfocatechol by release of the amino group.

At present, two cyclohexadiene diol intermediates (shown in brackets in Fig. 1B) are still hypothetical compounds. Like IpuF, AtdA2 may have activity hydrolyzing one of them to another, with release of L-glutamate, but there is no experimental information on the reaction. To understand the bacterial aniline oxidation

mechanism more completely, the involvement of these hypothetical compounds in aniline oxidation and the detailed functions of AtdA2 should be confirmed experimentally.

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