Improvement of the Glutaryl-7-Aminocephalosporanic Acid Acylase Activity of a Bacterial γ -Glutamyltranspeptidase[†]

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7-Aminocephalosporanic acid (7-ACA) is an important material in the production of semisynthetic cephalosporins, which are the best-selling antibiotics worldwide. 7-ACA is produced from cephalosporin C via glutaryl-7-ACA (GL-7-ACA) by a bioconversion process using D-amino acid oxidase and cephalosporin acylase (or GL-7-ACA acylase). Previous studies demonstrated that a single amino acid substitution, D433N, provided GL-7-ACA acylase activity for γ -glutamyltranspeptidase (GGT) of *Escherichia coli* K-12. In this study, based on its three-dimensional structure, residues involved in substrate recognition of *E. coli* GGT were rationally mutagenized, and effective mutations were then combined. A novel screening method, activity staining followed by a GL-7-ACA acylase assay with whole cells, was developed, and it enabled us to obtain mutant enzymes with enhanced GL-7-ACA acylase activity. The best mutant enzyme for catalytic efficiency, with a k_{cat}/K_m value for GL-7-ACA almost 50-fold higher than that of the D433N enzyme, has three amino acid substitutions: D433N, Y444A, and G484A. We also suggest that GGT from *Bacillus subtilis* 168 can be another source of GL-7-ACA acylase for industrial applications.

Semisynthetic cephalosporins are the best-selling antibiotics worldwide, with global sales of \$8.3 billion and almost 30% of the market for antibiotics in 2003 (34). These compounds are made by modification of the side chains at positions 3 and 7 of 7-aminocephalosporanic acid (7-ACA). Four thousand tons of 7-ACA is produced per year, and the original production process was chemical deacylation of fermented cephalosporin C (CPC) from the fungus *Acremonium chrysogenum* (19). This chemical process, however, has several problems: it requires very pure 7-ACA, several complicated reaction steps, and treatment of a lot of toxic waste. Therefore, a simpler and more environmentally sound process for enzymatic deacylation of CPC to 7-ACA has been of great interest. Since no enzyme catalyzes the direct bioconversion of CPC to 7-ACA at an acceptable rate (1), a two-step enzymatic deacylation process was proposed (26), which has now replaced the chemical process. In this process, D-amino acid oxidase catalyzes the oxidative deamination of CPC to 7- β -(5-carboxy-5-oxypentanamide)-cephalosporanic acid, which is followed by auto-conversion of the latter compound to glutaryl-7-ACA (GL-7-ACA). Cephalosporin acylase (CA) then deacylates GL-7-ACA to 7-ACA (Fig. 1A). CAs are found in various *Pseudomonas* species and are classified into five classes on the basis of their substrate specificity and primary structure (13). Class I to IV CAs

are reported to be members of the N-terminal nucleophile (Ntn) hydrolase superfamily (12).

Although the wild-type γ -glutamyltranspeptidase (GGT) (EC 2.3.2.2) of *Escherichia coli*, which is also a member of the Ntn hydrolase superfamily (10), has no detectable GL-7-ACA acylase activity, our previous study showed that *E. coli* GGT can acquire GL-7-ACA acylase activity by a single amino acid substitution, D433N (34). GGT catalyzes the hydrolysis of γ -glutamyl compounds and the transfer of their γ -glutamyl moieties to other amino acids or peptides (36) (Fig. 1B). As shown in Fig. 1, the γ -glutamyl moiety and the glutaryl moiety have similar chemical structures. Furthermore, the primary structure of GGT has a very high level of similarity to that of class IV CA. Of the 76 amino acid residues completely conserved among GGTs whose amino acid sequences are known, 58 are also conserved among class IV CAs (34). The D433 residue is conserved among GGTs, while the corresponding residue that is also conserved among class IV CAs is asparagine (Fig. 2). It has been suggested that the D423 residue of human GGT, which corresponds to the D433 residue of *E. coli* GGT, interacts with the α -amino group of the γ -glutamyl moiety of the substrate (9), which is why we introduced the D433N mutation into *E. coli* GGT (34). The GL-7-ACA acylase activity of the D433N mutant of *E. coli* GGT, however, was very low, and it was necessary to improve its activity for industrial application.

Recently, we determined the crystal structures of *E. coli* GGT in complex with γ -glutamyl compounds (20). The structures revealed that the γ -glutamyl moiety is held by extensive hydrogen bonds and charge interactions with several residues: D433, R114, and S462 interact with the α -carboxyl group,

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FIG. 1. (A) Enzymatic production of 7-ACA. (B) Reactions catalyzed by GGT. Reaction a, oxidative deamination reaction catalyzed by D-amino acid oxidase and autoconversion to GL-7-ACA; reaction b, deacylation reaction catalyzed by class IV CA; reaction c, hydrolysis reaction catalyzed by GGT; reaction d, transpeptidation reaction catalyzed by GGT.

N411, Q430, and D433 interact with the α -amino group, and G484 interacts with the γ -carboxyl group of the γ -glutamyl moiety (Fig. 3). Moreover, the substrate-binding pocket of GGT is covered by a loop (lid loop), which is formed by autocatalytic processing (21, 30) and contributes to recruiting γ -glutamyl substrates; the lid loop is flexible when the pocket is vacant, whereas it shields the pocket when the substrate is bound to the pocket (21). This structural information should be very helpful for introducing mutations in order to improve the activity and affinity for the substrate.

In this study, the residues involved in recognition and binding were rationally replaced or randomized on the basis of the crystal structures, and the desired mutants, which are very active with GL-7-ACA, were isolated by a novel method developed in this study.

MATERIALS AND METHODS

Strains, plasmids, and oligonucleotides. The *E. coli* strains, plasmids, and oligonucleotides used in this study are listed in Table 1. A 3.2-kb SmaI-SphI fragment of pSH253 was ligated with a 4.0-kb EcoRV-SphI fragment of pBR322 and with the 3.7-kb ClaI (blunt-ended)-SphI fragment of pACYC184 to obtain pSY43 and pCY131, respectively. pCY132 was constructed in the same way as pCY131, but pCM3 was used instead of pSH253.

All amino acid substitution reactions were performed in basically the same way, as reported previously (29). A template, double-stranded plasmid DNA, was subjected to PCR with the oligonucleotides listed in Table 1 and their complements, including the intended mutation sequence. After DpnI treatment, the reaction mixture was subjected to transformation, and the DNA sequence of the plasmid was confirmed. The oligonucleotides used for site-directed random amino acid substitutions included the “NNN” sequence at the intended codons to cover all genetic codes.

To obtain pCY137, pCY139, pCY140, and pCY147, a 1.1-kb ClaI fragment of each plasmid obtained from selected mutants by simultaneous randomization of the N411, Q430, and D433 residues was ligated with a 7.2-kb ClaI fragment of pCY131.

Genes were disrupted by the method of Datsenko and Wanner (5). Using oligonucleotides ampC-1 and ampC-2 as the primers and pKD3 as the template, ampC of DH5 α was disrupted. Elimination of the chloramphenicol resistance

gene marker was carried out at 30°C by introducing plasmid pCP20 as described previously (5), and then the growth temperature was shifted to 37°C to cure this plasmid and strain KY76 was obtained. Strain CY128 was made in a slightly different way. A 5.2-kb blunt-ended ClaI fragment of pCY3 was ligated with a 1.3-kb FRT-kan-FRT fragment obtained by PCR using oligonucleotides pKD13-1 and pKD13-4 and the template plasmid pKD13. As a result, *ggt* on pCY3 was disrupted. The resultant plasmid, pCY126, was used as the template for PCR with oligonucleotides ecoggt-1 and ecoggt-2, and then the PCR product was used to disrupt *ggt* of strain KY76. The kanamycin resistance gene marker was eliminated using pCP20 as described above, and strain CY128 was obtained.

Purification of GGT. Native GGT was purified basically as described previously (31). Strain SH641 transformed with the series of plasmids carrying the *ggt* genes listed in Table 1 was used. Cells were grown at 20°C for 2 days in 100 ml LB medium supplemented with 100 μ g/ml ampicillin for strains carrying pSY43 derivatives or with 30 μ g/ml chloramphenicol for strains carrying pCY131 derivatives. The periplasmic fraction was prepared essentially by the method of Suzuki et al. (33). For strains carrying pCY131 derivatives, however, lysozyme treatment on ice for 10 min was more successful. The periplasmic fraction was subjected to chromatofocusing with a PBE94 column (0.6 by 6.5 cm; GE Healthcare Bio-Sciences). Fractionation with ammonium sulfate was omitted because mutants could be precipitated at unexpected percentages of saturation.

Purification of His₆-GGT. DH5 α carrying the plasmid derived from pCY2 was grown at 37°C in 2 ml of LB medium with 100 μ g/ml ampicillin until the optical density at 600 nm (OD_{600}) was 0.5 to 0.7, and then 10 μ l of 0.1 M isopropyl- β -D-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After 4 h of reciprocal shaking at 20°C, cell extracts were prepared with BugBuster reagent (Novagen), which was followed by affinity column chromatography with Profinity immobilized metal ion affinity chromatography resin (200 μ l; Bio-Rad) in Micro Bio-Spin chromatography columns (Bio-Rad). His₆-GGT was eluted with elution buffer containing 150 mM imidazole.

Synthesis of GL-7-ACA, glutaryl-p-nitroanilide (GL-pNA), and glutaryl- α -naphthylamide (GL- α -naphthylamide). GL-7-ACA was synthesized as described previously from glutaric anhydride and 7-ACA, which were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Sigma-Aldrich, respectively (27).

GL-pNA was synthesized as described previously (6). Solution B (1 g of p-nitroaniline in 10 ml of dioxane) was added to solution A (1.25 g of glutaric anhydride in 5 ml of dioxane) and then incubated at room temperature for 10 to 20 min. The mixture was refluxed for 8 h in an oil bath, and the dioxane was evaporated. The residue was dissolved in saturated sodium hydrogen carbonate, and ethyl acetate was added. GL-pNA was extracted with water, and the pH was adjusted to 3 with 2 N hydrochloric acid. Ethyl acetate was added to extract the

FIG. 2. Multiple-sequence alignment of class IV CAs and GGTs. The sequences shown are the sequences of class IV CAs of *Pseudomonas* sp. strain SE82 (SE82) (Swiss-Prot accession number P15557) and *Pseudomonas* sp. strain V22 (V22) (Q05053) and GGTs of *E. coli* (ECO) (P18956), *B. subtilis* (BSU) (P54422), *Helicobacter pylori* (HPY) (Q9ZK95), *Pseudomonas* sp. strain A14 (PSU) (P36267), rat (RAT) (P07314), mouse (MOU) (Q60928), pig (PIG) (P20735), and human (HUM) (P19440). Residues completely conserved among class IV CAs and GGTs are indicated by gray shading. Residues with a black background are conserved among GGTs and also conserved among class IV CAs, but the properties of their side chains are quite different. The lid loop consists of the residues P438 to G449 of *E. coli* GGT and is enclosed by a dotted box. Residues of *E. coli* GGT involved in recognition of the γ -glutamyl moiety (20), the catalytic center (T391) (10), and the D423 residue of human GGT (9) are shown above the sequences. The alignment was performed with CLUSTALW (37).

product. Finally, magnesium sulfate anhydride was added to absorb the water, and the ethyl acetate was evaporated in vacuo to dryness.

GL- α -naphthylamide was synthesized basically in the same way as GL-*p*NA, except that solution B (0.72 g of α -naphthylamine in 10 ml of dioxane) was added to solution A (0.57 g of glutaric anhydride in 5 ml of dioxane) and incubated at 60°C.

Enzyme assay and kinetics. The hydrolysis activity of GGT was measured using γ -glutamyl-*p*-nitroanilide (γ -GpNA) as a substrate (32). To measure glutaryl acylase activity, 0.25 mM GL-*p*NA was used instead of 0.5 mM γ -GpNA.

To determine k_{cat} and K_m values for GL-7-ACA, the reaction was carried out with purified mutant enzymes and the optimum buffers for each mutant enzyme. In order to determine the optimum buffers that were most effective for 7-ACA production, mutant enzymes were subjected to the reaction with 5 mM GL-7-ACA in the following buffers: 50 mM potassium phosphate buffer (pH 5.5 to 7.6), 50 mM Tris-HCl (pH 7.5 to 8.73), and 50 mM $\text{NH}_3\text{-NH}_4\text{OH}$ buffer (pH 8.5 to 10). To determine the initial rate of 7-ACA production, 50 μ l was removed from the reaction mixture after at least four incubation periods and mixed with the same volume of 40% acetic acid to terminate the enzyme reaction. The amount of 7-ACA liberated was measured by high-performance liquid chromatography (HPLC) as described previously (34). Kinetic parameters were obtained from Lineweaver-Burk plots using at least four different substrate concentrations.

The *p*-dimethylaminobenzaldehyde (*p*DMAB) method was used as a simple method when the 7-ACA concentration liberated was expected to be more than

0.04 mM (23). Fifteen microliters of 1% *p*DMAB in methanol was added to 80 μ l of each sample containing 30 μ l of 40% acetic acid and incubated at room temperature for 20 min. The OD₄₁₅ was then measured by using a Power Scan HT (DS Pharma Biomedical, Osaka, Japan). The 7-ACA concentration was determined by using a standard curve.

Activity staining of colonies. Activity staining was used as a rough guide of glutaryl acylase activity. Colonies on an LB agar plate were transferred to a Hybond N⁺ membrane filter (GE Healthcare Bio-Science), and then the membrane was placed on a paper filter presoaked with a reaction mixture containing 2.0 mg/ml Fast Garnet GBC sulfate salt (Sigma-Aldrich), 0.5 mM GL- α -naphthylamide, and 50 mM Tris-HCl (pH 8.73). Fast Garnet GBC sulfate salt was dissolved on ice to prevent degradation of the diazonium salt.

GL-7-ACA acylase assay with whole cells. All assays were performed as follows unless indicated otherwise. Cells were grown at 20°C for 2 days, and 500 μ l of culture was harvested and washed with 0.9% NaCl. The reaction was performed at 37°C and was started by resuspending cells with 100 μ l of the reaction mixture containing 5 mM GL-7-ACA and 50 mM Tris-HCl (pH 8.73), and then it was terminated by adding 60 μ l of 40% acetic acid.

The 7-ACA concentration in the reaction mixture was divided by the OD₆₀₀ of the strain used, and then the value for the negative control strain was subtracted. Relative activity was defined as the percentage of 7-ACA production by a certain number of cells. The value for the positive control strain, carrying *ggt*(D433N) on its plasmid, was defined as 100%.

FIG. 3. Structure around the substrate-binding pocket of *E. coli* GGT. The γ -glutamyl moiety is indicated by dark gray cylinders. The catalytic center (T391), residues surrounding the γ -glutamyl moiety (R114, N411, Q430, D433, Y444, S462, G483, and G484), and the lid loop are indicated by light gray cylinders. Hydrogen bonds between these residues and the γ -glutamyl moiety are indicated by dotted lines.

RESULTS

Effect of mutation of the Y444 residue located in the middle of the lid loop. The Y444 residue is located in the middle of the lid loop that consists of residues P438 to G449, which opens to allow substrate binding (Fig. 3) (17, 20, 21). The Y444 residue was replaced by histidine and amino acids with nonpolar side chains of various sizes. Also, one mutant enzyme in which most of the lid loop was not present was produced, in which the segment from V440 to V447 was selected to be deleted on the basis of the *E. coli* GGT structure (20), so perturbation of the enzyme structure would be minimal. The mutant enzymes were purified and used for the GL-7-ACA acylase assay. Among the mutations, the Y444A substitution gave the best result (Table 2). The catalytic efficiency of the D433N Y444A enzyme was 43.5-fold higher than that of the D433N enzyme. Other mutant enzymes also had increased k_{cat} values; an exception was the D433N Y444W enzyme, which had almost no activity.

Effect of mutation of the R114, Q430, D433, S462, and G484 residues. The R114, Q430, D433, S462, and G484 residues, located in the substrate-binding pocket of *E. coli* GGT for substrate recognition (20), are conserved among GGTs (Fig. 2). These residues were replaced by the equivalent residues of class IV CA; that is, R114L, Q430Y, D433N, PLS460-462VFT, and G484A mutations were generated, and these mutations were combined in various ways. Relative GL-7-ACA acylase activities were measured using whole cells of the strains that had the mutant GGT genes in pBR322 in the presence of benzo(b)thiophene-2-boronic acid (BZBTH2B) (24). The pro-

duction of 7-ACA by strain CM7, which synthesizes D433N mutant GGT, could be detected by this method. Strain KY8, which synthesizes D433N G484A mutant GGT, had much higher GL-7-ACA acylase activity than strain CM7, but all other mutants had less activity (Fig. 4A).

Effects of mutation of the NQD residues. The glutaryl moiety differs from the γ -glutamyl moiety only by the absence of an α -amino group. Based on the fact that three residues, N411, Q430, and D433 (NQD residues), interact with this α -amino group (20), residues in the His-tagged *ggt* gene in pCY2 (35) were randomly replaced to obtain a NQD random mutant library. Nearly 26,500 mutants were spread on LB agar plates containing 100 $\mu\text{g/ml}$ ampicillin and 0.05 mM IPTG to mildly induce the expression of His₆-GGT. Screening was performed using GL- α -naphthylamide, as described in Materials and Methods. Most of the colonies formed were white because the template, pCY2, contains the wild-type *ggt* gene, although it has no signal sequence but is His tagged at the N terminus. Colonies showing even the slightest red color when activity staining was used were then picked because some mutants that are less active with GL- α -naphthylamide may have high activity with GL-7-ACA (see Discussion). From this mutant library, 178 mutants were isolated. The enzyme activities of purified His₆-GGT mutant enzymes with GL-7-ACA were measured by the pDMAB method, as described in Materials and Methods, and 12 mutant enzymes with activity higher than that of the D433N enzyme were obtained (data not shown). The N411 residue of mutants with high GL-7-ACA acylase activity was replaced by glycine, histidine, or tyrosine, the Q430 residue

TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study

Strain(s), plasmid, or oligonucleotide	Relevant genotype, characteristics, construction, sequence, and/or function	Reference
<i>E. coli</i> K-12 strains		
CM7	DH5 α /pCM7	This study
CM9	DH5 α /pSH641	34
CY128	DH5 α but Δ ggt Δ ampC	This study
CY133	CY128/pCY131	This study
CY134	CY128/pCY132	This study
CY137, CY139, CY140, CY144, CY147, CY149, CY154 to CY161, CY163, CY171 to CY176, CY188 to CY195, CY198 to CY205, CY207, CY212 to CY214	CY128 derivatives; CY strain numbers and pCY plasmid numbers correspond to each other (for example, strain CY137 is strain CY128 containing pCY137)	This study
DH5 α	F ⁻ λ^- <i>deoR endA1 gyrA96 hsdR17</i> (r _K ⁻ m _K ⁻) <i>recA1 relA1 supE44 thi-1</i> Δ (<i>lacZYA-argF</i>) <i>U169</i> ϕ 80 <i>lacZ</i> Δ M15	8
KY4, KY6, KY8 to KY11, KY26, KY28, KY32, KY43 to KY45	DH5 α derivatives; KY strain numbers and pKY plasmid numbers correspond to each other (for example, strain KY4 is strain DH5 α containing pKY4)	This study
KY19, KY22, KY37, KY48, KY50, KY63 to KY66	SH641 transformed with pKY18, pKY20, pKY31, pKY47, pKY49, and pKY55 to pKY58, respectively	This study
KY76	DH5 α but Δ ampC	This study
SH641	F ⁻ Δ ggt-2 <i>rpsL recA56 srl300::Tn10</i>	31
SY43	DH5 α /pSY43	This study
Plasmids		
pACYC184	p15A replicon, <i>cat</i> ⁺ <i>tet</i> ⁺	2
pBR322	ColE1 replicon, <i>bla</i> ⁺ <i>tet</i> ⁺ <i>rop</i> ⁺	
pCP20	pSC101 replicon(Ts), <i>bla</i> ⁺ <i>cat</i> ⁺ Flp(λ Rp) <i>cI857</i>	3
pCM3	pUC replicon, <i>f1 ori bla</i> ⁺ <i>ggt</i> ⁺	34
pCM7	ColE1 replicon, <i>bla</i> ⁺ but <i>ggt</i> (D433N)	34
pCY2	ColE1 replicon, <i>bla</i> ⁺ <i>lacI</i> ^q His ₆ - <i>ggt</i> (T5p) <i>rmBT1</i>	35
pCY3	ColE1 replicon, <i>bla</i> ⁺ <i>lacI</i> ^q <i>ggt</i> (T5p) <i>rmBT1</i>	This study
pCY126	pCY3 but <i>ggt::FRT-kan</i> ⁺ -FRT	This study
pCY131	p15A replicon, <i>cat</i> ⁺ <i>ggt</i> ⁺	This study
pCY132	pCY131 but <i>ggt</i> (D433N)	This study
pCY137	pCY131 but <i>ggt</i> (N411G Q430I D433A)	This study
pCY139	pCY131 but <i>ggt</i> (N411G Q430I D433L)	This study
pCY140	pCY131 but <i>ggt</i> (N411H Q430I D433L)	This study
pCY144	pCY131 but <i>ggt</i> (D433G)	This study
pCY147	pCY131 but <i>ggt</i> (N411Y Q430V D433L)	This study
pCY149	pCY131 but <i>ggt</i> (D433N Y444A G484A)	This study
pCY154	pCY131 but <i>ggt</i> (N411G Q430I D433G)	This study
pCY155	pCY131 but <i>ggt</i> (N411G Q430V D433A)	This study
pCY156	pCY131 but <i>ggt</i> (N411H Q430V D433L)	This study
pCY157	pCY131 but <i>ggt</i> (N411Y Q430V D433A)	This study
pCY158	pCY131 but <i>ggt</i> (N411Y Q430I D433A)	This study
pCY159	pCY131 but <i>ggt</i> (N411H Q430V D433A)	This study
pCY160	pCY131 but <i>ggt</i> (N411Y Q430I D433L)	This study
pCY161	pCY131 but <i>ggt</i> (N411H Q430I D433A)	This study
pCY163	pCY131 but <i>ggt</i> (N411G Q430V D433L)	This study
pCY171	pCY131 but <i>ggt</i> (N411G Q430I D433A Y444A)	This study
pCY172	pCY131 but <i>ggt</i> (N411G Q430I D433A G484A)	This study
pCY173	pCY131 but <i>ggt</i> (N411G Q430I D433G Y444A)	This study
pCY174	pCY131 but <i>ggt</i> (N411G Q430I D433G G484A)	This study
pCY175	pCY131 but <i>ggt</i> (N411G Q430I D433A Y444A G484A)	This study
pCY176	pCY131 but <i>ggt</i> (N411G Q430I D433G Y444A G484A)	This study
pCY188	pCY131 but <i>ggt</i> (N411H Q430I D433G)	This study
pCY189	pCY131 but <i>ggt</i> (N411Y Q430I D433G)	This study
pCY190	pCY131 but <i>ggt</i> (N411H Q430I D433L Y444A)	This study
pCY191	pCY131 but <i>ggt</i> (N411H Q430V D433L Y444A)	This study
pCY192	pCY131 but <i>ggt</i> (N411H Q430V D433L G484A)	This study
pCY193	pCY131 but <i>ggt</i> (N411Y Q430I D433L Y444A)	This study
pCY194	pCY131 but <i>ggt</i> (N411Y Q430I D433L G484A)	This study
pCY195	pCY131 but <i>ggt</i> (N411Y Q430V D433L Y444A)	This study
pCY198	pCY131 but <i>ggt</i> (N411H Q430I D433L Y444A G484A)	This study
pCY199	pCY131 but <i>ggt</i> (N411Y Q430I D433L Y444A G484A)	This study
pCY200	pCY131 but <i>ggt</i> (N411Y Q430V D433L Y444A G484A)	This study
pCY201	pCY131 but <i>ggt</i> (N411H Q430I D433L G484A)	This study
pCY202	pCY131 but <i>ggt</i> (N411Y Q430V D433L G484A)	This study
pCY203	pCY131 but <i>ggt</i> (N411H Q430V D433G)	This study
pCY204	pCY131 but <i>ggt</i> (N411Y Q430V D433G)	This study
pCY205	pCY131 but <i>ggt</i> (N411G Q430V D433G)	This study
pCY207	pCY131 but <i>ggt</i> (N411H Q430V D433L Y444A G484A)	This study
pCY212	pCY131 but <i>ggt</i> (D433G Y444A)	This study
pCY213	pCY131 but <i>ggt</i> (D433G G484A)	This study
pCY214	pCY131 but <i>ggt</i> (D433G Y444A G484A)	This study
pKD3	Ry replicon, <i>bla</i> ⁺ FRT- <i>cat</i> ⁺ -FRT	5
pKD13	Ry replicon, <i>bla</i> ⁺ FRT- <i>kan</i> ⁺ -FRT	5
pKD46	R101 replicon, <i>repA101</i> (Ts) <i>bla</i> ⁺ <i>araC</i> ⁺ γ^+ - β^+ - <i>exo</i> ⁺ (<i>araBp</i>)	5
pKY4	pSY43 but <i>ggt</i> (G484A)	This study
pKY6	pSY43 but <i>ggt</i> (P460V L461F S462T)	This study
pKY8	pSY43 but <i>ggt</i> (D433N G484A)	This study

Continued on following page

TABLE 1—Continued

Strain(s), plasmid, or oligonucleotide	Relevant genotype, characteristics, construction, sequence, and/or function	Reference
pKY9	pSY43 but <i>ggt</i> (P460V L461F S462T G484A)	This study
pKY10	pSY43 but <i>ggt</i> (D433N P460V L461F S462T)	This study
pKY11	pSY43 but <i>ggt</i> (D433N P460V L461F S462T G484A)	This study
pKY18	pSY43 but <i>ggt</i> (D433N Y444F)	This study
pKY20	pSY43 but <i>ggt</i> (D433N Y444A)	This study
pKY26	pSY43 but <i>ggt</i> (Q430Y D433N)	This study
pKY28	pSY43 but <i>ggt</i> (R114L D433N)	This study
pKY31	pSY43 but <i>ggt</i> (D433N Y444H)	This study
pKY32	pSY43 but <i>ggt</i> (R114L Q430Y D433N S462T G484A)	This study
pKY43	pSY43 but <i>ggt</i> (Q430Y D433N G484A)	This study
pKY44	pSY43 but <i>ggt</i> (Q430Y D433N S462T G484A)	This study
pKY45	pSY43 but <i>ggt</i> (R114L Q430Y D433N G484A)	This study
pKY47	pSY43 but <i>ggt</i> (D433N Y444W)	This study
pKY49	pSY43 but <i>ggt</i> (D433N Δ (440–447))	This study
pKY55	pSY43 but <i>ggt</i> (D433N Y444G)	This study
pKY56	pSY43 but <i>ggt</i> (D433N Y444I)	This study
pKY57	pSY43 but <i>ggt</i> (D433N Y444L)	This study
pKY58	pSY43 but <i>ggt</i> (D433N Y444V)	This study
pSH253	pUC replicon, fl ori bla ⁺ <i>ggt</i> ⁺	4
pSY43	pCM7 but <i>ggt</i> ⁺	This study
Oligonucleotides		
ampC-1	5'-AAGCGGAGAAAAGGTCCGAAAATTCGGACCCGATGGAATTGTGTAGGCTGGAGCTGCTTC-3'	
ampC-2	5'-CCCGCTATGCGGGCCGTTTGTATGGAACACAGACCTCATATGAATATCCTCCTTAG-3'	
ecoggt-1	5'-CCCGCATGCATGATAAAACCGACGTTTTTACGCCGGGTG-3'	
ecoggt-2	5'-CCCGTGCAGTTAGTACCCCGCGTTAAATCATCCAC-3'	
G484A	5'-CGGTAGCCAGGCCAGCCAGCCGATCATC-3', for G484A substitution of <i>ggt</i>	
N411X Q430X D433X	5'-GTGACCTATACGCTGNNNACCACCTTCGGTACGGGCATTGTCGCGGGCGAGAGCGGTATTCTGCTTAATAACNNNATGGATNNNTTCTCCGCCAACCG-3', for random amino acid substitution at N411, Q430, and D433 residues of <i>ggt</i>	
pKD13-1	5'-GTGTAGGCTGGAGCTGCTTC-3'	5
pKD13-4	5'-ATTCCGGGGATCCGTCGACC-3'	5
PLS460-462VFT	5'-GCCGTCCGGCCGAACAAACGCGTGTTTACCTCGATGTCGCCGACC-3', for P460V, L451F, and S462T substitutions of <i>ggt</i>	
R114L	5'-GGCTATCGATTTCCTGGAATGGCACCCGC-3', for R114L substitution of <i>ggt</i>	
Q430I D433A	5'-GCTTAATAACATTATGGATGCATTCTCCGC-3', for Q430I and D433A substitutions of <i>ggt</i>	
Q430I D433G	5'-GCTTAATAACATTATGGATGGCTTCTCCGC-3', for Q430I and D433G substitutions of <i>ggt</i>	
Q430I D433L	5'-GCTTAATAACATTATGGATGTTCTCTCCGC-3', for Q430I and D433L substitutions of <i>ggt</i>	
Q430V D433A	5'-GCTTAATAACGTGATGGATGCATTCTCCGC-3', for Q430V and D433A substitutions of <i>ggt</i>	
Q430V D433G	5'-GCTTAATAACGTGATGGATGGCTTCTCCGC-3', for Q430V and D433G substitutions of <i>ggt</i>	
Q430V D433L	5'-GCTTAATAACGTGATGGATGTTCTCTCCGC-3', for Q430V and D433L substitutions of <i>ggt</i>	
S462T	5'-CAACGCCCCGCTGACCTCGATGTCGCCG-3', for S462T substitution of <i>ggt</i>	
Δ (V440–V447)	5'-CCGCCAACCCGGCGCGGTGATGCCAAC-3', for deletion from V440 to V447 of <i>ggt</i>	
Y444A	5'-GGCGTACCGAACGTTTGCCGGCTGGTGGGC-3', for Y444A substitution of <i>ggt</i>	
Y444F	5'-GGCGTACCGAACGTTTTCGGGCTGGTGGGC-3', for Y444F substitution of <i>ggt</i>	
Y444G	5'-GGCGTACCGAACGTTTGCCGGCTGGTGGGC-3', for Y444G substitution of <i>ggt</i>	
Y444I	5'-GGCGTACCGAACGTTATGGGCTGGTGGGC-3', for Y444I substitution of <i>ggt</i>	
Y444L	5'-GGCGTACCGAACGTTCTGGGCTGGTGGGC-3', for Y444L substitution of <i>ggt</i>	
Y444V	5'-GGCGTACCGAACGTTGTGGGCTGGTGGGC-3', for Y444V substitution of <i>ggt</i>	
Y444W	5'-GGCGTACCGAACGTTTGGGGCTGGTGGGC-3', for Y444W substitution of <i>ggt</i>	

was replaced by isoleucine or valine, and the D433 residue was replaced by glycine, alanine, or leucine. All combinations of these substitutions were obtained either by site-directed mutagenesis of pCY131 or by insertion of DNA fragments, including fragments with mutations obtained by the random NQD mutagenesis procedure described above, into pCY131. pCY131 is a pACYC184 derivative containing a wild-type *ggt* gene without an ampicillin resistance marker, and Δ ampC strain CY128 was transformed with the plasmids to eliminate the influence of the two β -lactamases from *E. coli* cells on the GL-7-ACA acylase assay with whole cells (see Discussion). The GL-7-ACA acylase activities of the transformants were determined from the amount of 7-ACA released from GL-7-ACA by spectrophotometry with pDMAB. The results (Fig. 4B) suggest that amino acid substitutions at NQD residues collaborate to improve GL-7-ACA acylase activity.

Effects of combinations of the Y444A and G484A mutations with mutations in the N411, Q430, and D433 residues. Finally, Y444A and G484A, which were effective for improving the

GL-7-ACA acylase activity of the D433N enzyme, were superimposed in NQD mutants, and then the GL-7-ACA acylase assay with whole cells was performed by using the pDMAB method. Although many NQD mutant strains showed moderate or reduced activity with one or two superimposed amino acid substitutions, triple mutant D433N/G Y444A G484A and double mutant D433G Y444A strains had significantly greater activity than D433N/G and D433G mutants, respectively (Fig. 4C).

Kinetic parameters of mutant enzymes. For mutants showing high GL-7-ACA acylase activity, the kinetic parameters with GL-7-ACA were determined using purified native-form enzymes (Table 2). Most mutant enzymes showed increased k_{cat} values. The D433N Y444A G484A enzyme gave the best k_{cat} value and catalytic efficiency (k_{cat}/K_m). Its K_m value was 2.5-fold lower than that of the D433N enzyme, and its k_{cat} value was 18-fold higher. Consequently, the k_{cat}/K_m value of this mutant enzyme was almost 50-fold higher than that of the D433N enzyme.

TABLE 2. Kinetic parameters of mutant GGT enzymes with GL-7-ACA

Enzyme ^a		K_m		k_{cat} ^b		k_{cat}/K_m	
Mutation	Source ^c	Value (mM)	Change (fold) ^d	Value (s ⁻¹)	Change (fold) ^d	Value (s ⁻¹ mM ⁻¹)	Change (fold) ^d
D433N ^e	CM9	0.198	1.00	0.0122	1.00	0.0616	1.00
D433N Y444A	KY22	0.026	0.131	0.0703	5.76	2.68	43.5
D433N Y444F	KY19	0.300	1.51	0.0203	1.66	0.067	1.09
D433N Y444G	KY63	0.036	0.182	0.0368	3.02	1.03	16.7
D433N Y444H	KY37	0.077	0.389	0.0220	1.80	0.286	4.64
D433N Y444I	KY64	0.062	0.313	0.0469	3.84	0.76	12.3
D433N Y444L	KY65	0.081	0.409	0.0375	3.07	0.46	7.47
D433N Y444V	KY66	0.018	0.0909	0.0446	3.66	2.48	40.3
D433N Δ (440–447)	KY50	0.85	4.29	0.0104	0.852	0.0118	0.192
D433N G484A	KY8	0.0360	0.182	0.0121	0.992	0.336	5.45
N411G Q430I D433A	CY137	6.61	33.4	0.0559	4.58	0.00846	0.137
N411G Q430I D433G	CY154	0.929	4.69	0.174	14.3	0.187	3.04
N411H Q430I D433L	CY140	0.124	0.626	0.0503	4.12	0.406	6.59
N411H Q430V D433L	CY156	0.675	3.41	0.0728	5.97	0.108	1.75
N411Y Q430I D433L	CY160	0.0570	0.288	0.0418	3.46	0.734	11.9
N411Y Q430V D433L	CY147	0.117	0.591	0.0546	4.48	0.466	7.56
D433G	CY144	0.445	2.25	0.0121	0.992	0.00278	0.0451
D433N Y444A G484A	CY149	0.0751	0.379	0.223	18.3	3.06	49.7
D433G Y444A	CY212	0.420	2.12	0.0676	5.54	0.161	2.61
D433G Y444A G484A	CY214	0.767	3.87	0.168	13.8	0.219	3.56
N411G Q430I D433A G484A	CY172	0.0196	0.0990	0.0404	3.31	2.06	33.4
N411G Q430I D433G G484A	CY174	0.0501	0.253	0.0481	3.94	0.959	15.6
N411H Q430V D433L Y444A G484A	CY207	0.183	0.924	0.116	9.51	0.634	10.3
N411Y Q430V D433L Y444A G484A	CY200	0.0564	0.285	0.112	9.18	1.99	32.3

^a All enzymes are non-His-tagged forms.^b The initial velocity of the release of 7-ACA from GL-7-ACA was measured by HPLC as described in Materials and Methods.^c Strain from which the mutant enzyme was purified to electrophoretic homogeneity.^d Change compared with the value for the D433N enzyme.^e Values from reference 34, with correction of the k_{cat} value. The k_{cat} value was previously reported to be 0.122 s⁻¹; however, we found a calculation error, and the actual value was 0.0122 s⁻¹.

DISCUSSION

Rational replacement of amino acid residues of *E. coli* GGT on the basis of the three-dimensional structure. The three-dimensional structure of the γ -glutamyl enzyme intermediate of *E. coli* GGT (20) revealed the detailed interaction between the γ -glutamyl moiety and GGT (Fig. 3). Moreover, it was demonstrated that the lid loop is flexible when its substrate-binding pocket is vacant, suggesting that the lid loop is involved in recruiting the substrates (21). The hydroxyl group of the side chain of the Y444 residue is hydrogen bonded with the hydroxyl group of the side chain of the N411 residue (Fig. 3) (20). We predicted that if the Y444 residue was replaced by an amino acid residue that cannot form a hydrogen bond, such as phenylalanine, the flexibility of the lid loop would increase. Moreover, we predicted that the side chain of the Y444 residue interacts with the hydrophobic cephalosporin nucleus and is involved in size exclusion of the substrates, although the three-dimensional structure of the D433N mutant enzyme bound with GL-7-ACA has not been determined. Y444 was replaced by A, F, G, H, I, L, V, and W, and Δ (440–447) was introduced. It was found that replacement of Y444 by an amino acid with a nonpolar smaller side chain, alanine, increased the k_{cat} value for GL-7-ACA and that replacement by valine decreased the K_m value (Table 2). The mutant enzymes had different substrate specificities than the D433N enzyme. For example, the purified D433N Y444H enzyme showed lower activity with γ -GpNA and GL-pNA and higher activity with GL-7-ACA

than the D433N enzyme, while the two enzymes showed almost the same activity with GL- α -naphthylamide (data not shown). These results indicated that the acylase activity for GL-7-ACA should be evaluated during screening of mutants.

R114, Q430, D433, S462, and G484, which are responsible for recognition/binding of the γ -glutamyl moiety in the substrate-binding pocket (Fig. 3), were replaced by the equivalent residues of class IV CA (Fig. 4A). Among the mutants, only the D433N G484A mutant strain showed much higher GL-7-ACA acylase activity than the D433N strain. In the GL-7-ACA acylase assay, an AmpC inhibitor, BZBTH2B (24), was added to the reaction mixture, and the AmpC-derived degradation of the 7-ACA nucleus was almost completely suppressed (data not shown).

Random mutagenesis of the N411, Q430, and D433 residues and development of effective screening methods. The N411, Q430, and D433 residues of *E. coli* GGT interact with the α -amino group of the γ -glutamyl moiety of its substrate. The absence of the α -amino group is the only difference between the glutaryl moiety and the γ -glutamyl moiety. These three residues were simultaneously randomized and then screened with the novel plate assay. The activity staining method was developed because it was difficult to use GL-7-ACA itself to screen numerous mutated strains in the primary screening. GL- α -naphthylamide, the substrate for this plate assay, was synthesized as described in Materials and Methods. Hydrolysis of GL- α -naphthylamide by a mutant strain results in a diazo

FIG. 4. Relative activities of various mutants with GL-7-ACA. (A) Relative activities for various combinations of the R114L, Q430Y, D433N, PLS460-462VFS, and G484A mutations. The bars indicate the relative activities of the following strains: bar 1, CM7; bar 2, KY4; bar 3, KY6; bar 4, KY8; bar 5, KY9; bar 6, KY10; bar 7, KY11; bar 8, KY26; bar 9, KY28; bar 10, KY32; bar 11, KY43; bar 12, KY44; and bar 13, KY45. Strain SY43 was used as the negative control strain, and 7-ACA production by CM7 was defined as 100%. One milliliter of culture was harvested and washed with 0.9% (wt/vol) NaCl. The reaction was started by resuspending cells with 200 μ l of the reaction mixture containing 5 mM GL-7-ACA, 50 mM Tris-HCl (pH 8.73), and 1 mM BZBTH2B. After 4 h of incubation at 37°C, the reaction was terminated by adding the same volume of 3.5 N acetic acid. The amount of 7-ACA was determined by HPLC. The concentration of 7-ACA (in mM) was divided by the OD₆₀₀ of each strain used, and the relative activities were calculated by defining the activity of strain CM7 (in mM/OD₆₀₀) as 100%. (B) Relative activities of NQD mutants. The bars indicate the relative activities of the following strains: bar 1, CY134; bar 2, CY154; bar 3, CY137; bar 4, CY139; bar 5, CY205; bar 6, CY155; bar 7, CY163; bar 8, CY188; bar 9, CY161; bar 10, CY140; bar 11, CY203; bar 12, CY159; bar 13, CY156; bar 14, CY189; bar 15, CY158; bar 16, CY160; bar 17, CY204; bar 18, CY157; and bar 19, CY147. (C) Relative activities of NQD mutants combined with Y444A and/or G484A. The bars indicate the relative activities of the following strains: bar 1, CY134; bar 2, CY149; bar 3, CY144; bar 4, CY212; bar 5, CY213; bar 6, CY214; bar 7, CY154; bar 8, CY173; bar 9, CY174; bar 10, CY176; bar 11, CY137; bar 12, CY171; bar 13, CY172; bar 14, CY175; bar 15, CY140; bar 16, CY190; bar 17, CY201; bar 18, CY198; bar 19, CY156; bar 20, CY191; bar 21, CY192; bar 22, CY207; bar 23, CY160; bar 24, CY193; bar 25, CY194; bar 26, CY199; bar 27, CY147; bar 28, CY195; bar 29, CY202; and bar 30, CY200. For panels B and C, strain CY133 was used as the negative control strain. The amount of 7-ACA was determined by spectrophotometry with pDMAB. The concentration of 7-ACA (in mM) was divided by the OD₆₀₀ of each strain used, and the relative activities were calculated by defining the activity of strain CY134 (in mM/OD₆₀₀) as 100%. ND, not detectable.

coupling reaction of α -naphthylamine (the product of the reaction) and Fast Garnet GBC sulfate salt, and the product is deep red (Fig. 5). Using this method, a mutant with glutaryl acylase activity is easily distinguishable because its colonies turn deep red. From 26,500 mutated strains, 178 mutants which turned red were picked successfully and were subjected to the second screening. Glutaryl-leucine was known to be a substrate for primary screening and was used for growth selection with a leucine-deficient *E. coli* host strain (22). This substrate, however, is not preferred because a mutant enzyme whose activity is too low for glutaryl-leucine to support formation of a colony on a selection plate might have high activity for

GL-7-ACA. Further, it takes several days for colonies of the desired mutant to form. Activity staining with GL- α -naphthylamide solved these problems; this compound requires only overnight incubation and allows selection of a mutant with the slightest activity. For a second screening, we developed the following method. Since NQD mutants were isolated with pCY2 plasmids and His tagged, 178 mutant enzymes were purified using a Ni column to measure GL-7-ACA acylase activity, and 12 mutants were obtained. Various NQD mutations were transferred to pCY131, and strain CY128 was transformed with the resulting plasmids to eliminate the influence of two β -lactamases from

FIG. 5. Mechanism of activity staining. α -Naphthylamine is produced by deacylation of GL- α -naphthylamide, followed by the diazo coupling reaction with Fast Garnet GBC sulfate salt. The reaction product gives a deep red color.

E. coli cells on the GL-7-ACA acylase assay with whole cells. This was done because degradation of the 7-ACA nucleus was not negligible, although the AmpC inhibitor BZBTH2B used as described above was very effective. Therefore, the GL-7-ACA acylase assay with whole cells was refined using a $\Delta ampC$ strain and a plasmid with a chloramphenicol resistance marker but not an ampicillin resistance marker. With these improvements, the amount of 7-ACA liberated by positive control strain CY134 could be measured by a spectrophotometric method using *p*DMAB. This method is most acceptable as a second screening method because it is both simple and relatively accurate. In a wild-type *E. coli* K-12 strain β -lactamase is encoded by the *ampC* gene in the genome. AmpC (formerly AmpA) has very high activity for CPC but very low activity for ampicillin (7, 14, 18); therefore, its contribution to the degradation of cephalosporins is great, while it does not interfere with selection of strains containing the pBR or pUC series of plasmids using ampicillin resistance. It was reported previously that an *E. coli* $\Delta ampC$ strain had no destructive activity with CPC (38). In contrast, the β -lactamase encoded on the pBR and pUC series of multicopy cloning vectors is TEM 1 β -lactamase, which has high activity and a low K_m value for ampicillin. Although TEM 1 β -lactamase has a rather high K_m value for cephem antibiotics (18, 25), it still interferes with β -lactam acylase assays (28). By using random mutagenesis of the NQD residues and then our new screening methods, we isolated mutant enzymes which do not have the D433N substitution. This indicates that the combination of these three residues is important for acquiring GL-7-ACA acylase activity.

Evaluation of all combinations of NQD mutations. Mutant enzymes with all combinations of replacement of N411 with G, H, or Y, replacement of Q430 with I or V, and replacement of D433 with G, A, or L were made, and their GL-7-ACA acylase activities were compared with that of the D433N mutant enzyme. Considering that in selected mutants the Q430 and D433

residues were replaced by hydrophobic amino acids, it is easier to capture the glutaryl moiety when the charge around these areas is reduced because the glutaryl moiety has no α -amino group, unlike the γ -glutamyl moiety. The three-dimensional structure of the D433N enzyme was not much different than that of the wild-type enzyme (data not shown), implying that reduction of polar or charged side chains in the substrate-binding pocket plays an important role in binding GL-7-ACA. The actual structures of mutant enzymes binding with GL-7-ACA, however, remain to be elucidated.

Effective combination of NQD mutations with Y444A and G484A mutations. Amino acid substitutions at two residues, Y444 and G484, increased the GL-7-ACA acylase activity of the D433N enzyme, although they did not result in GL-7-ACA acylase activity by themselves. In contrast, NQD mutants, obtained by simultaneous randomization of NQD residues, exhibited high GL-7-ACA acylase activity. We combined mutations at these five residues and obtained the D433N/G Y444A G484A triple mutant and the D433G Y444A double mutant, which had significantly increased catalytic efficiencies. It was found that the mutations obtained separately had synergistic effects in appropriate combinations.

Catalytic efficiency of the D433N Y444A G484A mutant enzyme. The K_m and k_{cat} values for GL-7-ACA of purified mutant enzymes were evaluated (Table 2), and the D433N Y444A G484A enzyme had the best catalytic efficiency for GL-7-ACA. Its k_{cat} and k_{cat}/K_m values were 18- and 50-fold higher than those of the D433N enzyme, respectively. Although the reaction conditions used in different studies are different and direct comparison is difficult, the k_{cat}/K_m value of this mutant enzyme is comparable to that of GL-7-ACA acylase from *Pseudomonas* sp. strain V22 (class IV CA) (11).

The γ -glutamyl moiety and the glutaryl moiety have similar chemical structures, and we could obtain GGT mutants with high GL-7-ACA acylase activities without gross structural

changes. Although the δ -aminoadipoyl moiety of CPC and the γ -glutamyl moiety also have similar chemical structures, the δ -aminoadipoyl moiety is one methylene longer than the γ -glutamyl moiety. From the three-dimensional structure of GGT, we predicted that the δ -aminoadipoyl group of CPC would not fit in the γ -glutamyl binding pocket without a gross structural change, and we did not screen for GGT mutants that exhibited CPC acylase activity in this study.

Recently, we found that wild-type GGT from *Bacillus subtilis* 168 has little GL-7-ACA acylase activity, although the wild-type GGT from *E. coli* has no activity (34). *B. subtilis* GGT is more similar to class IV CA in that it has no lid loop, unlike *E. coli* GGT (Fig. 2), and the potential GGT activity of *B. subtilis* GGT is greater than that of *E. coli* GGT. The hydrolysis activity of *B. subtilis* GGT with γ -GpNA is more than 30-fold higher than that of *E. coli* GGT (15, 16); therefore, *B. subtilis* GGT can be another target for modification to obtain an industrially applicable GL-7-ACA acylase. Improvement of the GL-7-ACA acylase activity of *B. subtilis* GGT will be reported elsewhere.

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