Novel Metagenome-Derived Carboxylesterase That Hydrolyzes β -Lactam Antibiotics^{∇†}

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It has been proposed that family VIII carboxylesterases and class C β -lactamases are phylogenetically related; however, none of carboxylesterases has been reported to hydrolyze β -lactam antibiotics except nitrocefin, a nonclinical chromogenic substrate. Here, we describe the first example of a novel carboxylesterase derived from a metagenome that is able to cleave the amide bond of various β -lactam substrates and the ester bond of *p*-nitrophenyl esters. A clone with lipolytic activity was selected by functional screening of a metagenomic library using tributyrin agar plates. The sequence analysis of the clone revealed the presence of an open reading frame (*estU1*) encoding a polypeptide of 426 amino acids, retaining an S-X-X-K motif that is conserved in class C β -lactamases and family VIII carboxylesterases. The gene was overexpressed in *Escherichia coli*, and the purified recombinant protein (EstU1) was further characterized. EstU1 showed esterase activity toward various chromogenic *p*-nitrophenyl esters. In addition, it exhibited hydrolytic activity toward nitrocefin, leading us to investigate whether EstU1 could hydrolyze β -lactam antibiotics. EstU1 was able to hydrolyze first-generation β -lactam antibiotics, such as cephalosporins, cephaloridine, cephalothin, and cefazolin. In a kinetic study, EstU1 showed a similar range of substrate affinities for both *p*-nitrophenyl butyrate and first-generation cephalosporins while the turnover efficiency for the latter was much lower. Furthermore, site-directed mutagenesis studies revealed that the catalytic triad of EstU1 plays a crucial role in hydrolyzing both ester bonds of *p*-nitrophenyl esters and amide bonds of the β -lactam ring of antibiotics, implicating the predicted catalytic triad of EstU1 in both activities.

Carboxylesterases (EC 3.1.1.1), which are widely distributed in bacteria, fungi, plants, and animals, catalyze both the hydrolysis and synthesis of carboxylic ester bonds. They share a characteristic α/β -hydrolase structure, including a catalytic triad composed of Ser-Asp (or Glu)-His and a consensus sequence (G-X-S-X-G) around the active-site serine residue (5, 23). These enzymes are currently used in a broad array of industrial applications, including organic chemical processing, detergent formulations, the synthesis of biosurfactants, the oleochemical industry, the dairy industry, the agrochemical industry, paper manufacturing, nutrition, cosmetics, and pharmaceutical processing because of their exquisite enantioselectivity and regioselectivity (15, 16, 24, 28). Their great variety of uses has prompted the search for novel carboxylesterases with

functional properties that are better suited to those industrial applications.

Microbial carboxylesterases have been classified into eight families (families I to VIII) based on their conserved sequence motifs and biological properties (2). Among them, the primary sequences of family VIII carboxylesterases were similar to those of class C β -lactamases and distinct from those of other carboxylesterases. The nucleophilic serine residue in family VIII carboxylesterases occurs in the S-X-X-K motif, like those in class C β -lactamases, instead of in the G-X-S-X-G motif (2). Furthermore, the two-domain structure composed of a small helical domain and a mixed α/β domain is similar to the structure of class C β -lactamases (31). In recent studies, family VIII carboxylesterases have been identified from metagenomic libraries (12, 19, 26, 33). Among these proteins, EstC, derived from a leachate metagenome library, and two carboxylesterases, EstM-N1 and EstM-N2, derived from an arctic soil metagenome library, displayed a notable catalytic feature: in addition to their carboxylesterase activity, they also exhibited hydrolyzing activity toward nitrocefin, a chromogenic substrate used to determine β -lactamase activity (26, 33). However, none of the carboxylesterases, including EstC, EstM-N1, and EstM-N2, has shown hydrolyzing activity toward β -lactam antibiotics. It is noteworthy that class C β -lactamases have been shown to catalyze the hydrolysis of linear acyclic substrates,

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such as depsipeptides and thioesters (10, 22, 32), as well. These observations raise an interesting question about the relationship between carboxylesterases and class C β -lactamases.

In this study, we report the identification of a novel family VIII carboxylesterase called EstU1 from a metagenomic DNA library of soil. Remarkably, EstU1 exhibits β -lactam hydrolytic activity toward nitrocefin and cephalosporins (cephaloridine, cephalothin, and cefazolin) as well as esterase activity toward *p*-nitrophenyl esters. We describe the expression of the *estU1* gene in *Escherichia coli* and the biochemical characterization of the recombinant protein and mutant proteins to elucidate the mechanisms of action of EstU1.

MATERIALS AND METHODS

Strains, library construction, and screening. *E. coli* strains DH5 α and BL21(DE3) were used for all cloning and expression studies. A soil sample was collected from the Upo wetland in South Korea. A metagenomic library was constructed in the vector pSuperCosI, as previously described (18).

Subcloning and sequence analysis. A cosmid clone (pCosU1) showing lipolytic activity on the tributyrin (TBN) agar plate was inoculated into 50 ml of LB broth containing 50 μ g/ml of ampicillin. After an overnight incubation at 30°C, the cells were harvested by centrifugation at 5,000 $\times$ g for 15 min and washed twice with distilled water. The cosmid DNA was purified using the alkaline lysis method (4) with minor modifications and was randomly sheared by nebulization according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). After nebulization, DNA fragments of 2 to 4 kb were isolated from a 0.6% low-melting-point agarose (FMC Bioproducts, Rockland, ME) gel and end repaired to generate blunt ends. The blunt-ended DNA was ligated into the plasmid pUC118/HincII/BAP purchased from Takara (Kyoto, Japan), and the ligation products were introduced into *E. coli* DH5 α cells (Takara, Kyoto, Japan). The *E. coli* transformants were plated onto LB agar plates containing 100 μ g/ml of ampicillin and 1% tributyrin. After incubation at 37°C for 24 h, a colony surrounded by a clear halo was selected. Nucleotide sequencing was performed with an ABI 3100 automated sequencer using a BigDye Terminator kit (PE Applied Biosystems, Foster City, CA). The DNA sequence was determined by primer walking in both directions and assembled using the ContigExpress program of the Vector NTI Suite, version 7, software package (Informa, North Bethesda, MD). The open reading frame (ORF) was detected using the ORF search tool provided by the National Center for Biotechnology Information (NCBI). Sequence homology searches were performed with the BLAST program (1). A signal sequence search was performed with the SignalP, version 3.0, program (13). Multiple alignments between protein sequences were performed with the ClustalW program (30). A phylogenetic tree was constructed by the neighbor-joining method (27) using Molecular Evolutionary Genetics Analysis (MEGA; version 4.1, software (29)).

Expression and purification of recombinant EstU1. The *estU1* gene was amplified without its signal sequence using pUCU1 as a template and the following primers: (5'-GACCTCCCATATGGAAGGGCCGGTTACG-3' and 5'-CTCTCTCGAGTCGATCAAACGCTCCATAGACAATATTTC-3' (NdeI and XhoI restriction enzyme sites are underlined). The *estU1* gene was cloned into the expression vector pET-24a(+), and the recombinant plasmid was transformed into *E. coli* BL21(DE3) cells. As cell density reached a turbidity of about 0.6 at 600 nm, 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to the culture to induce protein expression. After 10 h of cultivation at 25°C, the cells were harvested by centrifugation (5,000 $\times$ g for 20 min at 4°C) and resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl and 10% glycerol. The cells were disrupted by sonication and centrifuged (15,000 $\times$ g for 1 h at 4°C). To purify EstU1 with a His₆ tag, the resulting supernatant was applied to a column of Talon metal affinity resin (BD Biosciences Clontech, Palo Alto, CA) and washed with resuspension buffer containing 10 mM imidazole in 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl and 10% glycerol. EstU1 was eluted with buffer containing 300 mM imidazole, followed by size exclusion chromatography with a Superdex-75 (16/60) column, equilibrated with 20 mM Tris-HCl buffer, pH 7.8, and 150 mM NaCl at a 1 ml/min flow rate. Protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard (6). The purity of the protein was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions, as described by Laemmli (21).

Esterase assay. Enzyme activity was measured by a spectrophotometric method using *p*-nitrophenyl esters (Sigma, St. Louis, MO) as the substrate. After

incubation at each optimum temperature for 5 min, the absorbance at 405 nm was measured to detect the released *p*-nitrophenol. One unit of esterase activity was defined as the amount of enzyme required to release 1 μ mol of *p*-nitrophenol from *p*-nitrophenyl esters per min.

Biochemical properties of esterase. Substrate specificity was determined by using *p*-nitrophenyl esters with different aliphatic side chains: C₂ (acetate), C₄ (butyrate), C₆ (hexanoate), C₈ (octanoate), C₁₀ (decanoate), C₁₂ (laurate), C₁₄ (myristate), C₁₆ (palmitate), and C₁₈ (stearate). The kinetic parameters (k_{cat} and K_m) of enzymatic conversion were determined by analysis of the dependence of the initial reaction rates on the *p*-nitrophenyl butyrate concentration (10 to 200 μ M). Each protein was used at a concentration of around 34 nM. The molar extinction coefficient measured under the assay conditions was 13,500 M⁻¹ cm⁻¹. The kinetic parameters k_{cat} and K_m were determined by fitting the data to the Michaelis-Menten equation. The optimum temperature of the enzyme reaction was determined in the same substrate solution described above at various temperatures ranging from 5 to 70°C. The optimum pH was determined over a pH range of 4.0 to 10.0, using the following buffer systems: 50 mM sodium acetate (pH 4.0 to 6.0), 50 mM sodium phosphate (pH 6.0 to 7.5), 50 mM Tris-HCl (pH 7.5 to 8.5), and 50 mM CHES (*N*-cyclohexyl-2-aminoethanesulfonic acid; pH 8.5 to 10.0). Various metal ions (MnCl₂, MgCl₂, CaCl₂, CuCl₂, ZnSO₄, FeSO₄, CoSO₄, and NiSO₄) and enzyme inhibitors (phenylmethylsulfonyl fluoride [PMSF] and EDTA) at final concentrations of 1 mM were incubated with the enzyme in 50 mM Tris-HCl buffer (pH 7.5) at 35°C for 1 h, and then the enzyme activity was assayed.

β -Lactamase assay. The β -lactam hydrolytic activity of EstU1 was determined spectrophotometrically using a chromogenic β -lactam substrate such as nitrocefin [3-(2, 4 dinitro-5-phenyl)-(6R,7R-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E-isomer] as a substrate (Unipath, Basingstoke, United Kingdom). The enzyme was incubated with a 1 mM nitrocefin solution (in 0.1 M phosphate, 1 mM EDTA, pH 7.0) at 35°C, and the rate change at 486 nm was recorded. The molar extinction coefficient of nitrocefin at 486 nm is 20,500 M⁻¹ cm⁻¹. The hydrolyzing activity toward the β -lactam substrates was determined by a paper disc method as described previously with slight modification (11). Antibiotics (ampicillin, penicillin G, cephaloridine, cephalothin, cefazolin, cefuroxime, and cefotaxime) and class C β -lactamase from *Enterobacter cloacae* were obtained from Sigma-Aldrich (St. Louis, MO). A negative control with antibiotics only and a positive control incubated with the class C β -lactamase and antibiotics were included for comparison. The hydrolytic activity of the enzyme toward nonchromogenic β -lactam antibiotics was measured by incubating 200 μ M purified EstU1 with antibiotic substrates, such as 1 mM ampicillin, 4 mM penicillin G, 3 mM cephaloridine, 3 mM cephalothin, 3 mM cefazolin, 1 mM cefuroxime, and 1 mM cefotaxime, in 50 mM Tris-HCl (pH 8.0) for 2 h at 35°C. Then, the resulting reaction mixtures were put onto small paper discs placed on the *E. coli* BL21(DE3) lawn. To prepare the bacterial lawn of *E. coli* BL21(DE3) in advance, a suspension of *E. coli* BL21(DE3) grown in LB medium at 37°C to an optical density at 600 nm (OD₆₀₀) of 0.6 was added to 80 ml of LB agar. The inoculated medium was then poured into a flat-bottomed square dish (inner dimensions, 125 by 125 mm; SPL), and a thin filter paper disc 0.8 cm in diameter (Advantec, Japan) was carefully put onto the gelled plate. The diameters of the inhibition zones around the discs were recorded after an overnight incubation at 37°C.

HPLC analysis. EstU1 (100 μ M or 200 μ M) was incubated with 2 mM cephaloridine, 1 mM cephalothin, or 2 mM cefazolin in 50 mM Tris-HCl (pH 8.0) for 1 h at 35°C, and the resulting mixtures were analyzed by high-performance liquid chromatography (HPLC). The standard samples containing cephalosporins (cephaloridine, cephalothin, and cefazolin) were used as a reference, and a positive control incubated with class C β -lactamase and cephalosporins was included. HPLC analysis of the reaction mixtures was conducted on an Atlantic dC₁₈ column (particle size, 5 μ m; inner dimensions, 4.6 by 150 mm; Waters, Ireland) using water containing 0.1% trifluoroacetic acid as mobile phase A and methanol containing 0.1% trifluoroacetic acid as mobile phase B. The gradient started at 0% B and increased to 100% B over 30 min at a flow rate of 1 ml/min. The retention times of cephaloridine, cephalothin, and cefazolin were 12.191, 12.331, and 13.488 min, respectively. The retention times of the reaction products of cephaloridine and cefazolin were 3.482 and 11.073 min, respectively.

Determination of kinetic parameters of β -lactamase activity. All the kinetic measurements of antibiotic substrates were performed at 35°C in 100 mM sodium phosphate (pH 7.0). The initial rates of hydrolysis were determined by following the absorbance variation, using a UV-2401PC spectrophotometer (Shimadzu, Japan). For cefazolin (extinction coefficient at 273 nm [ϵ_{273}] = 6,600 M⁻¹ cm⁻¹), the K_m and k_{cat} values were determined by fitting the data according to the methods of Hanes-Woolf (7). The K_m values of cephaloridine and cephalo-

EstU3	EPMTAETIFRIYSMSK [*] PIT [*] TVAAMMLVEEGKLT-121	ERIAKLPLVYQPGIT [*] TWVG-HSTDILG-226
EstC	IPVSEDTIFRIYSMTK [*] PVTGVALMMLVEEGKLT-124	DKLARIPLRQPGITLWHS-VSVDVQG-227
EstB	RPMREDTIFRLASVTK [*] PIVALAVRLVARGELAL-96	RRLASAPLSFAPGSGWQYS-LALDVLG-189
Lac-1	QPV [*] TQQTIFELG [*] SVSK [*] FTTGVLGDAIARGEIKL-101	RFYQNWQPAWAPG [*] QRIYANSISIGLEF-175
Lac-2	KPVT [*] PQTIFELG [*] SISK [*] FTTGVLGDAIARGEISL-105	RFYQNWQPWKPG [*] ITRIYANASIGLEF-179
PBP-1	RAITTTDFRFRVCSVTK [*] SFSAVLLQLVDEGKLDL-114	TLSLKHGVTNAPCAAYS [*] SNFVAVAG-199
PBP-2	KPMKTDFFERICSVTK [*] FTTATVVLQLVGENRLKL-125	KMGISFPPDFAPCKGWS [*] YSNTGYVLLG-210

FIG. 1. Conserved sequence blocks from a multiple sequence alignment of EstU1 and related Family VIII carboxylesterases, class C β -lactamases, and penicillin-binding proteins (PBPs). Family VIII carboxylesterases are represented by EstC (uncultured bacterium; accession number ACH88047) and EstB (*Burkholderia gladioli*; AAF59826). Class C β -lactamases are represented by Lac-1 (*E. coli*; AAA23441) and Lac-2 (*E. cloacae*; P05364), and penicillin-binding proteins are represented by PBP-1 (*Streptomyces* sp. strain R61; P15555) and PBP-2 (*Bacillus cereus*; CAA09676). The conserved S-X-X-K motif is boxed, and asterisks indicate conserved catalytic residues. Identical residues are shown as white letters on a dark background.

thin were determined as competitive inhibition constants, K_s , in the presence of *p*-nitrophenyl butyrate as a reporter substrate.

Site-directed mutagenesis of EstU1. To understand whether a single catalytic triad is important for both enzyme activities, site-directed changes to alanine were made at S100A, K103A, and Y218A using a Stratagene (La Jolla, CA) Quik Change kit according to the manufacturer's instructions. The primers used to introduce the S100A, K103A, and Y218A mutations were as follows: S100A_forward (5'-CGATCTTCCGCATCTACGCGATGTGGAAGCCAATC ACG-3'), S100A_reverse (5'-CGTGATTGGCTTCGACATCGCGTAGATGC GGAAGATCG-3'), K103A_forward (5'-CATCTACTCGATGTGCGGCCCA ATCAGACGGTGG-3'), K103A_reverse (5'-CCACCGTCGTGATTGGCGC CGACATCGAGTAGATG-3'), Y218A_forward (5'-CACGACCTGGGATGC CGGCCACAGCACTGAC-3'), and Y218A_reverse (5'-GTCAGTGCTGTGG CCGGATCCCAGTGTGCTG-3'). The positions of the mutated codons are underlined. The catalytic activities of the three variants were tested and compared to the activity of the wild-type enzyme.

Nucleotide sequence accession number. The nucleotide sequence of EstU1 was deposited in the GenBank database under accession number JF791800.

RESULTS

Screening and sequence analysis of a clone with lipolytic activity. A metagenomic library from a soil sample from Upo, South Korea, consisting of 6,912 cosmid clones had previously been constructed (18). To screen for an esterase-producing clone, the cosmid library clones were plated on LB agar containing 1% tributyrin (TBN). Sequence analysis of the pUCU1 insert DNA showed the presence of a 1,281-bp ORF (*estU1*) encoding a polypeptide of 426 amino acids. BlastP analysis of the amino acid sequence of EstU1 indicated that it was similar to a β -lactamase (YP_004154831) from *Variovorax paradoxus* EPS (58% identity), a hypothetical protein (NP_772348) from *Bradyrhizobium japonicum* USDA 110 (54% identity), a β -lactamase (YP_531482) of *Rhodopseudomonas palustris* BisB18 (51% identity), and a β -lactamase (YP_674851) from *Mesorhizobium* sp. strain BNC1 (48% identity). A multiple sequence alignment of EstU1 and its homologs showed that the S-X-X-K motif is well conserved in class C β -lactamases (20), penicillin-binding proteins (PBPs) (17), and family VIII carboxylesterases (12, 19, 26) (Fig. 1).

Purification and characterization of EstU1. To investigate its functionality in hydrolyzing esters and β -lactam antibiotics, the *estU1* gene was overexpressed in *E. coli*. A putative signal peptide of 25 amino acids in the EstU1 amino acid sequence was found by the SignalP, version 3.0, program, and the gene was amplified with primer pairs designed to remove the signal peptide. SDS-PAGE analysis of purified EstU1 showed a single band corresponding to approximately 44 kDa, which correlates well with the size of the mature protein (Fig. 2).

Purified EstU1 could hydrolyze a wide range of substrates (C_2 to C_{10}), with the highest activity toward *p*-nitrophenyl butyrate (C_4), while no enzyme activity was detected toward longer *p*-nitrophenyl esters (C_{12} to C_{18}), indicating that this protein is a bona fide esterase (Table 1). The enzyme appeared to have maximum hydrolytic activity at 45°C in the range of 5 to 75°C (Fig. 3A) and was active in the range of pH 7.5 to 9.5, with maximal activity at pH 8.5 (Fig. 3B). To determine the resistance to various chemical agents that might affect its activity, the enzyme was incubated under various conditions with compounds that might inhibit its activity. EstU1 activity was not affected by the presence of Ca^{2+} (91%), Co^{2+} (89%), Cu^{2+} (88%), Fe^{2+} (84%), Mg^{2+} (91%), Mn^{2+} (74%), Ni^{2+} (89%), Zn^{2+} (78%), or EDTA (94%), but it lost approximately 70% of its activity in the presence of 1 mM PMSF, which is known to bind specifically to the active-site serine residue in serine proteases and inhibit their activity.

Determination of β -lactamase activity. Because the amino acid sequence of EstU1 is also similar to the sequences of class C β -lactamases, we examined whether EstU1 has β -lactam hydrolytic activity toward nitrocefin and antibiotics such as ampicillin, penicillin G, and several first-generation cephalo-

FIG. 2. SDS-PAGE of the purified EstU1 protein. M, molecular size markers; T, whole-cell extracts; P1, EstU1 purified by Ni-nitrilotriacetic acid column; P2, EstU1 purified by a Superdex 75 gel filtration column. The purified EstU1 corresponded to a molecular mass of approximately 44 kDa.

TABLE 1. Substrate preference of the purified EstU1 toward *p*-nitrophenyl esters

Substrate	Specific activity (U/mg)
<i>p</i> -Nitrophenyl acetate (C ₂).....	6.24 $\pm$ 0.61
<i>p</i> -Nitrophenyl butyrate (C ₄).....	22.24 $\pm$ 0.66
<i>p</i> -Nitrophenyl hexanoate (C ₆).....	11.78 $\pm$ 0.57
<i>p</i> -Nitrophenyl octanoate (C ₈).....	7.46 $\pm$ 0.45
<i>p</i> -Nitrophenyl decanoate (C ₁₀).....	3.75 $\pm$ 0.07
<i>p</i> -Nitrophenyl laurate (C ₁₂).....	ND ^a
<i>p</i> -Nitrophenyl myristate (C ₁₄).....	ND
<i>p</i> -Nitrophenyl palmitate (C ₁₆).....	ND
<i>p</i> -Nitrophenyl stearate (C ₁₈).....	ND

^a ND, not detected.

sporins (cephaloridine, cephalothin, and cefazolin). EstU1 showed β -lactam hydrolytic activity against nitrocefin, a chromogenic β -lactamase substrate (data not shown). Based on this observation, we tested the β -lactam hydrolytic activity of EstU1 toward various β -lactam antibiotic substrates. As shown in Fig. 4, the diameters of the inhibition zones around the discs containing penicillin G or cephaloridine together with EstU1 were slightly decreased compared to the disc diameter of the negative control, implying that the efficacy of the antibiotic was affected by EstU1 activity. The decrease in the size of the clear zone around the disc was even more obvious when cephalothin or cefazolin was incubated with EstU1 (Fig. 4). No inhibition zone was observed around the disc of cefazolin incubated with EstU1, indicating that cefazolin became completely ineffective against the bacteria. On the other hand, EstU1 addition did not seem to alter the antibiotic efficacy of ampicillin (Fig. 4), cefuroxime (second-generation cephalosporin), or cefotaxime (third-generation cephalosporin) (see Fig. S1 in the supplemental material) because the sizes of the clear zones did not change at all compared to the clear zone of the negative control. EstU1 seems to have β -lactam hydrolytic activity toward first-generation cephalosporins and penicillin G.

FIG. 4. Disc diffusion assay for confirming the hydrolysis of antibiotics by EstU1. This assay was performed by incubating the purified EstU1 with antibiotics such as 1 mM ampicillin (AMP), 4 mM penicillin G (PEN), 3 mM cephaloridine (LOR), 3 mM cephalothin (LOT), and 3 mM cefazolin (ZOL) in 50 mM Tris-HCl (pH 8.0) for 2 h at 35°C. The reaction mixtures were adsorbed onto a paper disk and put onto agar seeded with *E. coli* BL21(DE3). The negative controls are samples containing antibiotics, and the positive controls are reaction mixtures containing class C β -lactamase of *E. cloacae* and antibiotics.

To verify the β -lactam-hydrolyzing activity of EstU1 toward first-generation cephalosporins (cephaloridine, cephalothin, and cefazolin), the changes in cephalosporins or reaction products made by EstU1 were analyzed by reverse-phase HPLC. The HPLC spectra of cephalosporins or reaction products made by class C β -lactamase were used as references (Fig. 5). When cephaloridine (or cefazolin) was incubated with EstU1, the peak pattern was consistent with peaks of reaction mixtures resulting from the incubation of class C β -lactamase and cephaloridine (or cefazolin). The peaks corresponding to antibiotics were decreased with the appearance of a product peak (Fig. 5A and C). Hydrolysis of cephaloridine (or cefazolin) by a β -lactamase opens the β -lactam ring and thus generates an acidic functional group in the molecule. Consequently, the reaction product is eluted earlier than the substrate. In the case

FIG. 3. Effects of temperature and pH on the activity of EstU1. The enzyme activity was measured using *p*-nitrophenyl butyrate as a substrate at various temperatures. Buffers used were 50 mM sodium acetate buffer (closed circles; pH 4.0 to 6.0), 50 mM sodium phosphate buffer (open circles; pH 6.0 to 7.5), 50 mM Tris-HCl buffer (closed triangles; pH 7.5 to 8.5), and 50 mM CHES buffer (open triangles; pH 8.5 to 10.0). The highest value of each enzyme activity was set as 100%.

FIG. 5. HPLC analysis of the turnover of the cephalosporins cephaloridine (A), cephalothin (B), and cefazolin (C) by EstU1 (green) and class C β -lactamase of *E. cloacae* (red). The chromatogram of cephalosporins is shown as a black line. The retention times of cephaloridine, cephalothin, and cefazolin are 12.191, 12.331, and 13.488 min, respectively. The retention times of the reaction products of cephaloridine and cefazolin are 3.482 and 11.073 min, respectively.

of cephalothin hydrolysis, the cephalothin peak was significantly decreased by EstU1 as the incubation time elapsed. The peak profile was identical to that made by class C β -lactamase although the reaction product could not be detected in either EstU1 or class C β -lactamase (Fig. 5B). Taken together, we concluded that EstU1 hydrolyzes the cephalosporins in the same way class C β -lactamase does.

Determination of kinetic parameters. The kinetic parameters of EstU1 toward *p*-nitrophenyl butyrate and the first-generation cephalosporins (cephaloridine, cephalothin, and cefazolin) were investigated (Table 2). The K_m values for *p*-nitrophenyl butyrate and cefazolin were determined to be within a similar range; however, the k_{cat} values toward cefazolin were approximately 5 orders of magnitude lower than those toward *p*-nitrophenyl butyrate. Because the enzyme activities of EstU1 toward cephaloridine and cephalothin were too low to allow an accurate determination of kinetic parameters, we estimated the K_i values (~ 215 and ~ 140 μ M) for these two compounds by using them as competitive inhibitors of *p*-nitrophenyl butyrate.

Site-directed mutagenesis. The inhibition by PMSF and the presence of the conserved S-X-X-K motif implicate the involvement of serine in both esterase and β -lactamase activities. Additionally, EstU1 does not seem to harbor a separate active site for each activity, given the relatively small size of the enzyme. To confirm whether a single nucleophilic serine or catalytic triad can play a crucial role in both activities, serine 100, lysine 103, and tyrosine 218 of EstU1 were each separately replaced with an alanine residue, and the activities of mutant proteins were investigated. The three variants constructed were expressed as soluble and properly folded proteins, as analyzed by SDS-PAGE and column purification, with the

same retention times as the wild type (data not shown). Three mutants of EstU1 were inactive toward *p*-nitrophenyl butyrate and nitrocefin, confirming that the three residues are essential for both activities (data not shown). A multiple sequence alignment revealed that these three residues are highly conserved in family VIII carboxylesterases, class C β -lactamases, and penicillin-binding proteins (Fig. 1).

DISCUSSION

The discovery and characterization of carboxylesterases, including family VIII, have previously been reported in other metagenomic studies (12, 19, 26, 33). A relationship between family VIII carboxylesterases and β -lactamase has often been proposed due to the presence of the S-X-X-K motif; however, no single enzyme has been demonstrated to have both esterase and β -lactamase activities. Recently, it has been reported that proteins of metagenomic origin belonging to family VIII carboxylesterases (26, 33) could hydrolyze nitrocefin, which is a chromogenic substrate for assessing β -lactamase activity. However, none of these enzymes was able to hydrolyze β -lactam antibiotic substrates such as ampicillin, carbenicillin, cephalosporin C, or cephalothin. Based on phylogenetic analysis (see Fig. S2 in the supplemental material), EstU1 clearly grouped with nitrocefin-hydrolyzing enzymes, unlike other well-characterized family VIII carboxylesterases. Further, it is noteworthy that EstU1 formed a subbranch with hypothetical homologs, deviating from the nitrocefin-hydrolyzing enzymes as well.

In this study, EstU1 was isolated by functional screening of a metagenomic library constructed from a soil sample from the Upo swamp area. The deduced amino acid sequence of EstU1 showed the conserved S-X-X-K motif that is characteristic of

TABLE 2. Kinetic parameters for hydrolysis of *p*-nitrophenyl butyrate and cephalosporins by EstU1

Substrate	k_{cat} (s^{-1})	K_m (μ M)	k_{cat}/K_m ($s^{-1} M^{-1}$)	K_i (μ M)
<i>p</i> -Nitrophenyl butyrate	15.72 ± 0.174	6.03 ± 0.269	$(2.608 \pm 0.087) \times 10^6$	
Cefazolin	$(2.38 \pm 0.036) \times 10^{-4}$	78.49 ± 3.772	3.035 ± 0.098	
Cephaloridine				215 ± 14
Cephalothin				140 ± 10

family VIII carboxylesterases. The characterization of EstU1 revealed that the enzyme hydrolyzed the amide bond of the β -lactam ring of several antibiotics as well as nitrocefin. The β -lactam hydrolytic activity of EstU1 toward first-generation cephalosporins (cephaloridine, cephalothin, and cefazolin) was clearly demonstrated by the disc diffusion assay and HPLC analysis.

The crystal structure of a carboxylesterase, EstB from *Burkholderia gladioli*, that is homologous to serine β -lactamases and DD-peptidases has been reported (31). The EstB crystal structure revealed that the catalytic residues, Ser75, Lys78, and Tyr181, of EstB play an important role in hydrolyzing esters. The corresponding catalytic residues, Ser100, Lys103, and Tyr218, were also conserved in EstU1. Because EstU1 clearly hydrolyzes both substrates, we wondered whether a single nucleophile was involved or whether the enzyme harbored two active sites. The latter possibility was regarded with suspicion because the enzyme was not big enough to contain two separate sets of catalytic machinery although we also considered the possibility that EstU1 somehow underwent a substantial change to harbor two different active sites for dual activity. The site-directed mutagenesis of the three catalytic residues of EstU1 clearly proved that the catalytic residues conserved in EstB were crucial for both the esterase and β -lactamase activities.

It is not certain what makes EstU1 hydrolyze β -lactam antibiotics, distinct from very homologous proteins, such as EstC carboxylesterase from an uncultured bacterium (39% identity; accession number ACH88047). The hydrolysis of β -lactam antibiotics by β -lactamases with the nucleophilic serine residue occurs through successive acylation and deacylation steps. In the acylation step, the serine residue attacks the carbonyl carbon of the lactam ring to form an acyl-enzyme intermediate. In the next deacylation step, the acyl-enzyme adduct is attacked by a water molecule, releasing hydrolyzed antibiotics (8, 14). Generally, it is thought that the deacylation is the rate-limiting step in the hydrolysis of poor substrates, like third-generation β -lactam antibiotics, by β -lactamases. Poor substrates adopt catalytically incompetent conformations, precluding the deacylation step in the acyl-enzyme intermediate due to their bulky size relative to the active site of general β -lactamases (3, 9, 25). It seems obvious that the active pocket of EstU1 is more or less changed to be suitable to accommodate β -lactam antibiotics like the class C β -lactamase, distinct from other carboxylesterases, including EstC, EstM-N1, and EstM-N2, and the change may allow EstU1 to bind the antibiotics and cleave the β -lactam rings of the antibiotics. It is noteworthy that nitrocefin is a little smaller than the β -lactam antibiotics we tested. Whether the β -lactamase activity of EstU1 is caused by an increase in substrate binding or changes in other catalytic machinery is not certain at this stage and needs further investigation. Despite the hydrolyzing activity of EstU1 toward β -lactam antibiotics, the kinetic study of EstU1 clearly indicates that esters are preferable substrates for the enzyme, and the first-generation cephalosporins (cephaloridine, cephalothin, and cefazolin) are poor substrates for EstU1. It will also be interesting to determine what further changes in EstU1 increase the enzyme's β -lactamase activity. A structural determination of the complex between EstU1 and β -lactam substrates can help to address these issues.

In conclusion, a metagenomic clone screened for lipolytic activity was identified to have the conserved S-X-X-K motif belonging to family VIII of bacterial lipolytic enzymes. The purified EstU1 protein showed hydrolyzing activity toward two different types of chemical bonds: the amide bond in β -lactams and the ester bond in *p*-nitrophenyl ester. Furthermore, EstU1 contains only one catalytic triad responsible for the hydrolyzing activity toward both *p*-nitrophenyl esters and β -lactam antibiotics. EstU1 is the first example of an enzyme that is able to cleave the β -lactam ring of antibiotics as well as ester substrates. To date, there have been few reports addressing the evolutionary relationship between family VIII carboxylesterases and class C β -lactamases. Based on the data in this study, we propose that EstU1 may be a model enzyme to explain the phylogenetic linkage between class C β -lactamases and family VIII carboxylesterases. The enzyme may provide a tool to delineate how two enzymes with shared motifs have different enzymatic activities. To elucidate the relationship between the two families, further studies will be required, including the crystallographic determination of EstU1.

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