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Isolation and Biochemical Characterization of Two Novel Metagenome-Derived Esterases

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The metagenomes of uncultured microbial communities are rich sources for novel biocatalysts. In this study, esterase EstA3 was derived from a drinking water metagenome, and esterase EstCE1 was derived from a soil metagenome. Both esterases are approximately 380 amino acids in size and show similarity to β-lactamases, indicating that they belong to family VIII of the lipases/esterases. EstA3 had a temperature optimum at 50°C and a pH optimum at pH 9.0. It was remarkably active and very stable in the presence of solvents and over a wide temperature and pH range. It is active in a multimeric form and displayed a high level of activity against a wide range of substrates including one secondary ester, 7-[3-octylcarboxy-(3-hydroxy-3-methyl-butyloxy)]-coumarin, which is normally unreactive. EstCE1 was active in the monomeric form and had a temperature optimum at 47°C and a pH optimum at pH 10. It exhibited the same level of stability as EstA3 over wide temperature and pH ranges and in the presence of dimethyl sulfoxide, isopropanol, and methanol. EstCE1 was highly enantioselective for (+)-menthylacetate. These enzymes display remarkable characteristics that cannot be related to the original environment from which they were derived. The high level of stability of these enzymes together with their unique substrate specificities make them highly useful for biotechnological applications.

Modern biotechnology has a steadily increasing demand for novel biocatalysts, thereby prompting the development of novel experimental approaches to find and identify novel biocatalyst-encoding genes. Recently, there has been an increase in the number of studies using a metagenomic approach to investigate the catalytic potential of uncultured microorganisms (8, 28). The term metagenome was introduced to describe the genomes of complex microbial communities found in natural habitats, only a small fraction of which can be cultured (1, 17). Investigation of metagenomes became possible after the development of strategies for the isolation and cloning of environmental DNA (41, 47). Modern metagenomic developments and cloning strategies have recently been reviewed in detail (8, 9, 16, 46). Once constructed, metagenomic libraries can be screened for a wide range of ecologically and biotechnologically interesting phenotypes (44).

In the search for novel biocatalysts, there are various metagenomic strategies that are used for targeting specific catalyst characteristics such as substrate range or temperature and pH optima. One approach is to generate the metagenomic library from soils or sediments that are known to harbor a high level of microbial diversity and thus, potentially, a wide diversity of biocatalysts (7). This approach has been used successfully to find a wide variety of novel catalysts and secondary metabolites (9, 19, 20, 27, 30, 39). A further development of this approach is to create the metagenomic library from an environment that has been subjected to extreme conditions in the likelihood that enzymes from such an environment will be able to function under those extreme conditions. One example of this is the isolation of thermophilic enzymes from a hot spring metagenomic library (37). Another approach is to use selective preenrichment of those members of the microbial consortia that carry the specific trait of interest. This preenrichment strategy has been successfully applied in the search for polysaccharide-degrading enzymes and enzymes involved in the synthesis of vitamins and antibiotics (13, 18, 25, 48). While preenrichment increases the likelihood of finding genes that encode the target trait, this must be balanced against the concurrent decrease in genomic diversity. It is also important that the preenrichment step may limit the chances of finding an enzyme that displays optimal activity outside the range of conditions chosen for the preenrichment.

Esterases and lipases have a wide range of biotechnological applications, from the food industry to the production of perfumes and the development of pharmaceuticals (21, 23). These enzymes are often found using metagenomics (4, 20, 33, 35, 39, 48). Despite this, metagenomic esterases have only recently been sufficiently biochemically characterized to allow evaluation of their suitability for biotechnological applications (14, 24, 27, 37).

In the present paper, we report the isolation of two novel metagenome-derived esterase genes, *estCE1* from oil-contaminated soil and *estA3* from a drinking water biofilm, and the detailed biochemical characterization of their corresponding enzymes.

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MATERIALS AND METHODS

Environmental samples and enrichment cultures. The soil samples used for the generation of the metagenomic library were collected near the town of Wietze in northern Germany. There are crude oil springs close to Wietze, and these springs were exploited between 1850 and 1900. As a result of this industrial activity, the soil in the area is heavily contaminated. To obtain microbial consortia with lipolytic activities, medium composed of M9 medium with mineral oil (1%, vol/vol) was inoculated with a few grains of oil-contaminated soil and incubated at room temperature for 2 to 3 days. The second microbial consortium sample was collected from a biofilm growing within a drinking water network as described previously (40).

DNA isolation, library construction, and screening for lipolytic clones. DNA isolation methods were based on a method described previously (13).

Libraries were prepared in the cosmid vector pWE15, which has kanamycin and ampicillin resistance (Stratagene, La Jolla, CA), using protocols provided by the manufacturer. DNA fragments (20 to 40 kb) obtained after partial Sau3AI digestion were ligated into the BamHI restriction site of the cosmid vector. Phage packaging mixes were obtained from Stratagene (La Jolla, CA), and infection of *Escherichia coli* VCS257 was performed according to the manufacturer's protocol. When required, gaps in the DNA sequences were filled by PCR. Automated DNA sequencing was performed using ABI377 and dye terminator chemistry according to the manufacturer's instructions.

Clones carrying lipolytic activity were identified by the formation of clear halos surrounding the colonies after growth for 4 to 5 days on agar plates containing tributyrin as the indicator substrate (26). To avoid the isolation of false-positive clones, cosmid DNA was isolated from the positive clones obtained in the initial screening and retransformed, and the new clones were examined on the same type of indicator plates for esterase activity. The tributyrin-positive clones were then tested on triolein-rhodamine B agar, which is used to detect lipolytic activity against long-chain fatty acids (C_{18}) (50).

In vitro transposon mutagenesis. Detection of the open reading frames (ORFs) responsible for lipolytic activity was done by in vitro transposon mutagenesis using the EZ::TN <KAN-2> transposon kit (Epicenter, Madison, WI) according to the manufacturer's instructions.

Tests for β-lactamase activity. To assay β-lactamase activity, the *estA3*, *estCE1*, and *ORF006* genes were cloned into the pET24c expression vector, which has kanamycin resistance (Novagen/Merck KGaA, Darmstadt, Germany), transformed into *E. coli* BL21(DE3), grown overnight, and plated onto LB plates containing 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to ensure expression of the genes. Small filter disks (Oxoid, Wesel, Germany) containing various concentrations of the different β-lactam antibiotics (amoxicillin, ampicillin, cefaclor, cefixime, ceftibuten, ceftriaxone, ceftazidime, cefoxitin, cefadroxil, cefotaxime, cefamandole, loracarbef, meropenem, mezlocillin, and penicillin) were put onto the bacterial lawn, and the diameter of the clearance zone around the discs was recorded after incubation overnight at 37°C.

Cloning, expression, and activity staining of EstCE1 and EstA3. The *estCE1* and the *estA3* genes were amplified from cosmid DNAs using PCR in 35 cycles with primer pairs ESTCE1-for (5′-AAG CTT TTA CGA GTA GGT TCG TTT GTC C-3′) and ESTCE1-rev (5′-GGA TCC ATG TCG ATA GCG GAT CAG TCA3′) and ESTA3-for (5′-GCG GAT CCA TGA GCG CCG AAG AAC TAG GG-3′) and ESTA3-rev (5′-CGA AGC TTG GCG GCG AGC GCG CTG TA-3′), respectively. Primers were designed to introduce a 3′ HindIII restriction site and a 5′ BamHI site into the cloned fragments. To increase cloning efficiency, the PCR fragments were first ligated into pBSK+ and then excised with HindIII and BamHI and ligated into pQE30, which has ampicillin resistance (QIAGEN, Hilden, Germany). The plasmids carrying the *estCE1* and the *estA3* sequence were designated pQE30-EstCE1 and pQE30-EstA3, respectively. To confirm that the correct genes had been amplified from the original cosmid DNA, the PCR fragments that were cloned into pQE30 were sequenced. Freshly transformed *E. coli* M15 cells carrying pQE30-EstCE1 or pQE30-EstA3 were used to overproduce the corresponding proteins. Cultures were grown at 37°C to an optical density at 600 nm of 0.5, and production of the recombinant protein was induced by the addition of 0.5 mM IPTG. After 4 to 5 h, cells were harvested and disrupted in a French pressure cell, and EstCE1 and EstA3 were purified from the soluble fraction. Extracts were loaded on Protino-Ni-2000 prepacked columns, and chromatography was done as specified by the manufacturer (Machery & Nagel, Düren, Germany). Protein extracts were then dialyzed against 10 mM Tris buffer (pH 7.2) overnight. The resulting protein fractions were analyzed by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting using His_6-specific antibodies.

EstA3 was further purified by size exclusion chromatography (Superdex 200 prep grade; column, HiLoad16/60; 50 mM NaH_2PO_4–300 mM NaCl, 1 ml/min).

The detection of lipolytic activity in SDS-polyacrylamide gels was done as described previously (43). Nondenatured protein was electrophoresed, and the gel was washed with isopropanol and water, placed on an activity agar plate (20 mM Tris, 5 mM $CaCl_2$, 1% [vol/vol] tributyrin, 1.5% [wt/vol] agar, pH 8.0), and incubated overnight at room temperature. Activity of the proteins was made visible by the formation of a clear halo surrounding the protein band on the test agar plate.

Site-directed mutagenesis of EstCE1 and EstA3. To confirm the location of the catalytic site of the enzymes, amino acid exchanges were introduced at position 65 (serine to alanine) for EstCE1 and position 61 (serine to alanine) for EstA3 using the Stratagene (La Jolla, CA) Quik Change kit according to the manufacturer's instructions. Primers used to introduce mutations in the variants EstCE1S65A and EstA3S61A were as follows: S65A_forward (5′-TTCGGCTG TCGGCGGTGTCCAAGCC-3′), S65A_reverse (5′-GGCTTGGACACCGCCG ACAGCCGAA-3′), S61A_forward (5′-ATTTTCCGCATTGCAGCGATGACC AAACCGGTC-3′), and S61A_reverse (5′-GACCGGTTTGGTCATCGCTGC AATGCGGAAAAT-3′). (The positions of the mutated codons are underlined.) Correctness of the isolated variants was verified by sequencing the corresponding clones, pQE30-EstA3-S61A and pQE30-EstCE1-S65A, using standard primers. The catalytic activity of the two variants was tested and compared to that of the original enzyme.

Catalytic activity measured using pNP substrates. Enzyme activity tests were performed by incubating the enzyme with 4 mM substrate at 45°C in 100 mM Tris-HCl or 100 mM potassium phosphate buffer at pH 7.2, unless otherwise indicated. The reaction was measured at 410 nm. One unit is defined as the amount of enzyme catalyzing the appearance of 1 μmol of free *p*-nitrophenol/min. Enzyme activity against *p*-nitrophenyl (pNP)–acylesters (butyrate, valerate, caproate, octanoate, decanoate, and palmitate) was tested under the above-described conditions.

The temperature ranges of EstCE1 and EstA3 were tested using pNP-caproate as a substrate. To determine the temperature optimum, the enzymes were tested at temperatures ranging from 25°C to 70°C. To assay the thermostability of the enzymes, they were incubated at different temperatures for 1 h, and the residual activity was assayed at 10-min intervals.

EstCE1 and EstA3 were tested for their stability in the presence of metal ions, detergents, and EDTA and under various pH conditions by measuring their residual activity. For pH stability, EstCE1 and EstA3 were diluted to 2 μg/ml in buffers (50 mM final concentration) at different pH adjustments (pH 2 to 3, glycine-HCl buffer; pH 4 to 6, sodium phosphate buffer; pH 7 to 9, Tris-HCl; pH 10 to 12, glycine-NaOH) and incubated for 48 h at room temperature. For EstCE1, the following metal ions were all tested at 10 mM: Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Rb^{2+}, and Zn^{2+}. For EstA3, the same metals were tested at 10 mM, except Fe^{3+} (1 mM), Cu^{2+} (1 mM), and Co^{2+} (5 mM). EDTA was tested at 10 mM, and SDS was tested at 1% (wt/vol). The assays were run using pNP-caproate as a substrate.

The stability of the two enzymes was determined in the presence of the following solvents: dimethyl sulfoxide (DMSO), isopropanol, methanol, dimethylformamide, acetone, and acetonitrile. The enzymes were incubated for 1 h at 30°C in the presence of either 15% (vol/vol) or 30% (vol/vol) of the solvents, and the residual activity was measured at 45°C. The substrate used was pNP-butyrate.

The substrate range of the two enzymes was determined using pNP esters of the following substrates: benzoate, 2-(4-isobutylphenyl)propanoate (ibuprofen), 2-phenylpropanoate, 3-phenylbutanoate, cyclohexanoate, 2-(3-benzoylphenyl)propanoate, 2-naphthoate, 1-naphthoate, adamantanoate, and 2-(6-methoxynaphthalene-2-yl)propanoate (naproxen). Activity was determined at 410 nm after 30 min of incubation at 40°C. The reaction mixture consisted of 950 μl buffer (100 mM Tris, pH 7.5), 50 μl substrate (5 mg/ml in DMSO), and either 1 μg EstCE1 or 1 μg EstA3.

Catalytic activity measured using titration assays. EstCE1 and EstA3 activities on the substrates triacetin, tripropionin, tributyrin, tricaprylene, and trilaurin and on vinyl substrates (vinyl acetate, vinyl propionate, vinyl butyrate, vinyl caproate, vinyl caprylate, and vinyl laurate) were determined using a standard titration assay with minor modifications (12). Tests were performed with a Methrom 718 STAT potentiometric titrator (Herisau, Switzerland) and by using 10 mM NaOH for titration. The substrates at a 10 mM concentration were emulsified in 20 ml reaction buffer containing 2 mM Tris-HCl, pH 7.0. After the addition of 7.5 μg of the purified enzyme, the NaOH consumption was recorded at a reaction temperature of 37°C. One unit was defined as the amount of enzyme that released 1.0 mmol of fatty acid per min.

The pH optimum for the two enzymes was determined using tributyrin as a substrate. The incubation conditions were 20 ml buffer (2.5 mM phosphate buffer [pH 4 to 7], 2.5 mM Tris-HCl buffer [pH 7 to 11], or 2.5 mM glycine buffer [pH 9 to 11], supplemented with 100 mM NaCl) stirred at a constant temperature of 37°C. The esterase substrate tributyrin (final concentration of 2 mM) was added

TABLE 1. Blast results of ORFs identified on the metagenome cosmid clones

ORF	Size (aa[a])	Possible function and GenBank accession no.	Microorganism	% Identity (no. of similar aa/total no.)
pCosCE1				
ORF001	271	Hypothetical protein, NP_669416	*Yersinia pestis*	48 (123/256)
estCE1	389	β-Lactamase class C, ZP_00486659	*Burkholderia pseudomallei*	51 (196/378)
ORF003	310	Probable regulator, NP_103137.1	*Mesorhizobium loti*	42 (124/293)
ORF004	224	Putative transcriptional regulator, *tetR* family, ZP_00235387	*Bacillus cereus*	40 (78/191)
ORF005	385	Arabinose efflux permease, ZP_00136708	*Pseudomonas aeruginosa*	45 (171/380)
ORF006	379	β-Lactamase class C, CAB69829.1	*Serratia marcescens*	77 (302/378)
pCosCS-A3				
ORF001	359	Aminoglycoside phosphotransferase, ZP_00579208.1	*Sphingopyxis alaskensis*	79 (280/353)
ORF002	253	Oxidoreductase, NP_421870.1	*Caulobacter crescentus*	73 (186/254)
ORF003	265	Short-chain dehydrogenase/reductase ZP_00579206.1	*Sphingopyxis alaskensis*	81 (216/265)
estA3	396	β-Lactamase, ZP_00579205.1	*Sphingopyxis alaskensis*	59 (236/396)
ORF005	301	Protein of unknown function, ZP_00577139.1	*Sphingopyxis alaskensis*	57 (158/277)
ORF006	171	Similar to aspartyl protease, ZP_00577809.1	*Sphingopyxis alaskensis*	40 (49/120)
ORF007	153	Putative protein disulfide isomerase, ZP_00375704.1	*Erythrobacter litoralis*	43 (58/133)
ORF008	108	Hypothetical protein, ZP_00567441.1	*Frankia* sp.	28 (29/101)

[a] aa, amino acids.

to 5 ml of the corresponding buffer at 37°C, emulsified, and added to the whole preparation. This substrate emulsion was held constant at the correct pH automatically by the titrator (718 STAT Titrino; Metrohm, Herisau, Switzerland) for 2 min, and this served as the control. The enzymes were added separately to the emulsion, and the consumption of 0.01 M NaOH was measured over 4 min, which allowed the calculation of the specific activity of EstCE1 and EstA3 at various pH adjustments.

Catalytic activity measured using GC. To assay the activity of EstCE1 and EstA3 on a range of acetic acid esters, gas chromatography was used. The samples were analyzed for the production of chiral alcohols by the gas chromatograph GC-17A (Shimadzu, Duisburg, Germany) equipped with a flame ionization detector and CP-Chirasil-DEX CB (Chrompack [internal diameter, 25 m by 0.25 mm]; Middelburg, The Netherlands) at a helium flow rate of 1.3 ml/min. The temperature program included 5 min at 60°C followed by an increase of 5°C per min until 190°C was reached. For the gas chromatography (GC) analysis, activity tests were run in 1 M Tris-buffered solutions and using 10 mM substrates dissolved in DMSO as 0.1 M stock solutions. Two micrograms of the enzyme was added, and test mixtures were incubated overnight before the corresponding alcohols could be extracted by treatment with acetic acid ethyl ester and analyzed by GC.

Enzyme activity fingerprinting with substrate cocktails. The enzyme activity fingerprinting used 10 substrates and was carried out as described previously (15). The assay conditions consisted of 75% phosphate-buffered saline buffer at pH 7.4, 25% DMSO, and 0.0025% SDS. Each substrate was present in the cocktail at 15 μM, and the total concentration of the substrate cocktail was 150 μM. EstA3 was added at a concentration of 0.75 mg/liter, and EstCE1 was added at a concentration of 0.2075 mg/liter. The reaction time was 30 min. The chemical background was measured by recording the products observed under the same assay conditions after a 5-day incubation in the absence of the enzyme. The blank value was taken from the reaction after 30 min of incubation in the absence of the enzyme.

Nucleotide sequence accession numbers. The nucleotide sequences obtained have been deposited in the GenBank database under accession numbers DQ022078 (pCosCS-A3) and DQ022079 (pCosCE1).

RESULTS

Construction of metagenomic libraries and screening for clones encoding lipolytic activity. To obtain genes associated with esterase activity, metagenomic libraries were generated using DNA isolated from oil-contaminated soil and from a drinking water biofilm. The soil library contained 2,500 clones, and the drinking water library contained 1,600 clones. The quality and size of inserts were verified by analyzing 30 randomly picked clones from each library. The majority of analyzed clones contained inserts of approximately 25 to 40 kb, and restriction analysis revealed a high level of diversity of the cloned DNA fragments.

Cosmid clones encoding esterase activity were identified by transfer to agar plates containing tributyrin. The soil library yielded one positive clone, pCosCE1, and the drinking water biofilm library yielded six positive clones from which clone pCosCS-A3 is described here in more detail. When the tributyrin-positive clones were then screened on triolein/rhodamine B agar, they displayed no activity, indicating that these enzymes are not lipases (50).

Genetic characterization. Cosmids pCosCE1 and pCosCS-A3 were characterized by partial DNA sequencing together with additional restriction analysis. Insert sizes of approximately 27 kb for the pCosCE1 clone and 16.7 kb for the pCosCS-A3 clone were observed, with pCosCS-A3 containing a total of 17 ORFs. In vitro transposon mutagenesis verified the ORFs responsible for the hydrolytic activities on each cosmid. The putative esterase genes were designated *estCE1* and *estA3*.

BlastP analysis of the amino acid sequence of EstCE1 indicated that it was similar (51% identity) to a class C β-lactamase from *Burkholderia pseudomallei* (Table 1). Further analysis of EstCE1 identified a conserved motif with similarity to class C β-lactamases. EstCE1 was also similar (61% similarity and 45% identity) to a functional esterase (GenBank accession number AAF59826) from *Burkholderia gladioli*. The amino acid sequence of EstA3 was similar (59% identity) to a β-lactamase from *Sphingopyxis alaskensis* (Table 1). The deduced amino acid sequence also contained a β-lactamase class C motif. Within the class C β-lactamase motifs of both enzymes, there was the conserved peptide sequence Ser-X-X-Lys, which is known to be of importance for catalytic activity of esterases with similarity to penicillin binding proteins (32).

Alignment of EstCE1 with EstB from *B. gladioli* also revealed a weak similarity at positions 137 to 141 (G-I-K-D-G) with the conserved G-I-S-D-G motif at positions 147 to 151 from EstB, but EstCE1 lacks the nucleophilic serine residue, which is in the classical lipase or esterase motif (G-X-S-X-G).

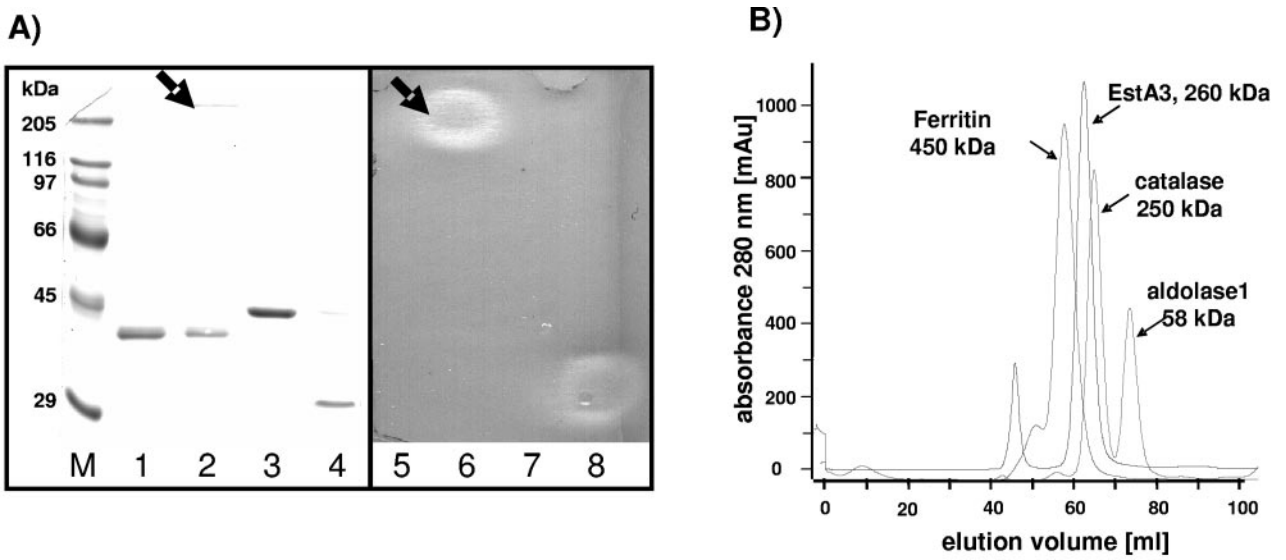

FIG. 1. (A) Overexpressed and purified His_6-tagged EstCE1 and EstA3 after denaturing SDS-PAGE analysis and activity staining. Lane M, marker; lane 1, purified and denatured EstA3; lane 2, nondenatured EstA3; lane 3, purified and denatured EstCE1; lane 4, nondenatured EstCE1. Lanes 5 to 8 are in the same order as lanes 1 to 4. The unstained SDS gel on the right (lanes 5 to 8) was placed on a tributyrin-containing agar plate and incubated at room temperature overnight. Note the small amount of protein larger than 205 kDa in lane 2 and the corresponding halo indicating activity in lane 5 (arrows). Approximately 1.5 μg of the recombinant protein was loaded in each lane. (B) Elution profile of Superdex 200 size exclusion chromatography with the corresponding standard proteins. The elution volume of EstA3 corresponded to a molecular mass of approximately 260 kDa.

This serine residue is not important in the *B. gladioli* protein (32). Because of these observations, EstCE1 and EstA3 most likely belong to family VIII of esterases and lipases, which contains mostly enzymes with approximately 380 residues that often show sequence homology to class C β-lactamases and do not possess the mandatory lipase motif (2).

Apart from *estCE1*, there were other ORFs on cosmid pCosCE1 that showed homology to proteins involved in antibiotic resistance. ORF006 showed a high similarity (77% identity) to a β-lactamase class C protein from *Serratia marcescens*, and one of the homologies produced for ORF005 was 33% identity to a protein involved in chloramphenicol resistance from *Streptomyces venezuelae*.

Also, while ORFs on pCosCE1 showed homologies to proteins from a wide range of microorganisms, five of the eight ORFs analyzed on cosmid pCosCS-A3 showed homology to proteins from the same microorganism, *Sphingopyxis alaskensis* (Table 1).

β-Lactamase activity of EstCE1, EstA3, and ORF006. Since the EstCE1 and EstA3 amino acid sequences contained a possible β-lactamase consensus motif, one of the first tests performed was to overexpress these genes and investigate the activity of the enzymes towards β-lactam antibiotics. Neither EstCE1 nor EstA3 was able to cleave the β-lactam ring of any of the antibiotics tested. In contrast, ORF006, which also had a very high homology to a β-lactamase (Table 1) and which was tested in the same way as EstCE1 and EstA3, was able to cleave the β-lactam ring of cefamandole, cefadroxil, and loracarbef. This provided an indication of the possible function of this gene in antibiotic resistance.

Overexpression, purification, and size of EstCE1 and EstA3. In order to confirm the functions of EstCE1 and EstA3, the corresponding genes were cloned and overexpressed. The recombinant proteins were purified from culture supernatants of *E. coli* cells harboring the pQE30-estCE1 and pQE30-estA3 constructs.

The EstCE1 protein was purified in a single step using a Protino-Ni-2000 column. SDS-PAGE analysis indicated that the protein was homogenous, and further analysis on SDS gels indicated a molecular mass of approximately 44 kDa for the EstCE1 His_6-tagged protein (Fig. 1A). The estimated molecular mass of 44 kDa is in accordance with the theoretical molecular mass of 42.1 kDa. Also, activity staining of purified native protein loaded and electrophoresed by SDS-PAGE using tributyrin as a substrate revealed a single active band at the position of EstCE1 (Fig. 1A).

The EstA3 protein was purified in a first step using Protino-Ni-2000 columns. For further purification, it was separated by size exclusion chromatography using a Superdex 200 gel filtration column. Interestingly, the protein eluted from the column at an elution volume according to a size of approximately 260 kDa (Fig. 1B), whereas SDS-PAGE analysis of the denatured protein revealed a molecular mass of 42 kDa, which is in accordance with the theoretical mass of 43.3 kDa (Fig. 1A). Additional tests using activity staining and Western blot analysis using His_6-specific antibodies indicated that only the multimeric protein with a molecular mass of at least 260 kDa was active on tributyrin (Fig. 1A). Based on these findings, EstA3 is a multimer in its active form.

Activity of EstCE1 and EstA3 on short-chain fatty acid substrates. Substrate specificity of both enzymes was initially assayed using pNP-fatty acid substrates. Both enzymes showed the highest activity towards short-chain fatty acids (C_4), while activity towards long-chain fatty acids ($>C_8$) was much lower

TABLE 2. Specific enzyme activities of EstCE1 and EstA3

Substrate	Sp act (U/mg)[a]	
	EstCE1	EstA3
pNP-acylesters		
Butyrate (C_4)	31.1	513.6
Valerate (C_5)	22.9	425.7
Caproate (C_6)	7.4	425.7
Octanoate (C_8)	1.0	3.6
Decanoate (C_{10})	0.9	0
Palmitate (C_{16})	0.0	0
Triglycerides		
Triacetin (C_2)	26.7	147.5
Tripropionin (C_3)	33.3	ND
Tributyrin (C_4)	54.7	167.5
Tricaprylin (C_8)	0	ND
Trilaurin (C_{12})	0	ND
Vinyl acids		
Vinyl acetate (C_2)	13.3	107.5
Vinyl propionate (C_3)	13.3	380.0
Vinyl butyrate (C_4)	16.0	387.5
Vinyl caproate (C_6)	0	237.5
Vinyl caprylate (C_8)	0	155.0
Vinyl laurinate (C_{12})	0	7.5

[a] ND, not determined.

(Table 2). Further titration tests using triglycerides and vinyl acids as substrates confirmed the observation that only short-chain substrates are converted by EstCE1 (Table 2). The specific activity of EstA3 was 5 to 60 times higher than that of EstCE1 against most of the tested substrates, and EstA3 was able to hydrolyze longer-chain fatty acids than EstCE1 (Table 2). These biochemical tests indicate that the enzymes belong to the esterase class of enzymes rather than to the lipases.

Site-directed mutagenesis of the catalytic sites of EstCE1 and EstA3. In both EstA3 and EstCE1, a conserved peptide sequence, Ser-X-X-Lys, was found, which is known to be of importance for the catalytic activity of esterases with similarity to penicillin binding proteins (32). To test the importance of this sequence for the catalytic activity of EstCE1 and EstA3, site-directed mutagenesis, where serine 65 of EstCE1 and serine 61 of EstA3 were replaced with an alanine, was carried out. Mutations at the indicated sites were verified by DNA sequencing. The catalytic activities of the two variants were compared to those of the wild-type enzyme, and it was found that both variants were inactive when tested at 20°C and 45°C using the substrates pNP-butyrate, pNP-valerate, pNP-caproate, and tributyrin. This indicates that the serine residues are absolutely required for the catalytic activity of both of these enzymes.

Temperature range of activity and thermostability of EstCE1 and EstA3. Both enzymes were active over a wide temperature range, retaining a minimum of 50% relative activity between 37.6°C and 55.8°C for EstA3 and between 36.3°C and 51.2°C for EstCE1 when pNP-caproate was used as a substrate. Maximum activity was measured at 47°C for EstCE1 and 50°C for EstA3 (Fig. 2A and B). Both enzymes displayed similar overall activity patterns, with a rapid decrease in activity when the enzymes were incubated at temperatures higher than their optimum temperatures (Fig. 2A and B). In order to test the thermostability of both enzymes, they were incubated at various temperatures, and the residual activities were assayed. The enzymes remained relatively stable at temperatures below 40°C and could be incubated for at least 60 min without a major loss of activity at 40°C. However, incubation at temperatures above 45°C for more than 60 min resulted in a rapid inactivation of both enzymes (data not shown).

pH range and stability of EstCE1 and EstA3. The activities of EstCE1 and EstA3 were tested under buffered conditions over the pH range of 4 to 11. Both enzymes displayed high levels of activity under neutral to alkalophilic conditions, with no activity below pH 5 for EstCE1 and below pH 4 for EstA3. The highest level of activity was measured at pH 10 for EstCE1 and pH 9 for EstA3 (Fig. 2C and D). Interestingly, both enzymes were extremely stable, with little loss of activity after incubation for up to 48 h under alkaline conditions up to pH 12 (data not shown).

Stability of EstCE1 and EstA3 against solvents, metal ions, detergents, and EDTA. EstCE1 was quite stable in the presence of 15% (vol/vol) DMSO, methanol, and isopropanol, retaining 103%, 90%, and 75% residual activity, respectively. At the same concentration, dimethylformamide, acetone, and acetonitrile strongly inhibited EstCE1 activity, with the enzyme displaying only 34%, 14%, and 0% residual activity, respectively. When EstCE1 was tested against a higher concentration, 30% (vol/vol), of the solvents, it was completely inhibited and able to retain only 17% and 23% activity in the presence of DMSO and isopropanol, respectively.

In contrast, EstA3 proved to be very stable, displaying increased activity in the presence of all the solvents tested. With 15% and 30% (vol/vol) DMSO, it retained full activity at 102% and 104%, respectively. EstA3 activity was stimulated to the same level by both concentrations of dimethylformamide and acetone, with activity measured at levels up to 128% and 155%, respectively. When the concentration of methanol was increased from 15% (vol/vol) to 30% (vol/vol), there was an increase in the activity of EstA3 from 111% to 130%. When concentrations were increased from 15% (vol/vol) to 30% (vol/vol), there was a decrease in the activity of EstA3 from 155% to 117% in the presence of isopropanol and from 110% to 87% in the presence of acetonitrile.

The influence of cations and EDTA on the activity of EstCE1 and EstA3 was tested by measuring the residual enzyme activity in their presence. EstCE1 activity was unchanged by the presence of Mn^{2+} (100%) and Fe^{3+} (100%), strongly inhibited by the presence of Cu^{2+} (0%) and Zn^{2+} (66%), and only slightly increased by the presence of Mg^{2+} (108%), Ca^{2+} (118%), and EDTA (120%). EstA3 activity was increased up to 133% in the presence of Co^{2+}. In the presence of Mn^{2+} and Fe^{3+}, a low level of inhibition was recorded, with 70% and 76% residual activity, respectively. In the presence of the following additives, a stronger inhibition, residual activity of between 0% and 46%, was detected: Ca^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Rb^{2+}, and EDTA. These results indicate that EstA3 is more likely to be dependent on cofactors than EstCE1.

Short-term incubation with SDS (0.1%, wt/vol) did not affect the enzymes' activity. However, a prolonged incubation for a period of 60 min or longer resulted in a complete inactivation of both enzymes.

Substrate range and enantioselective activity of EstCE1 and EstA3. Using GC analysis, it was shown that EstCE1 was highly

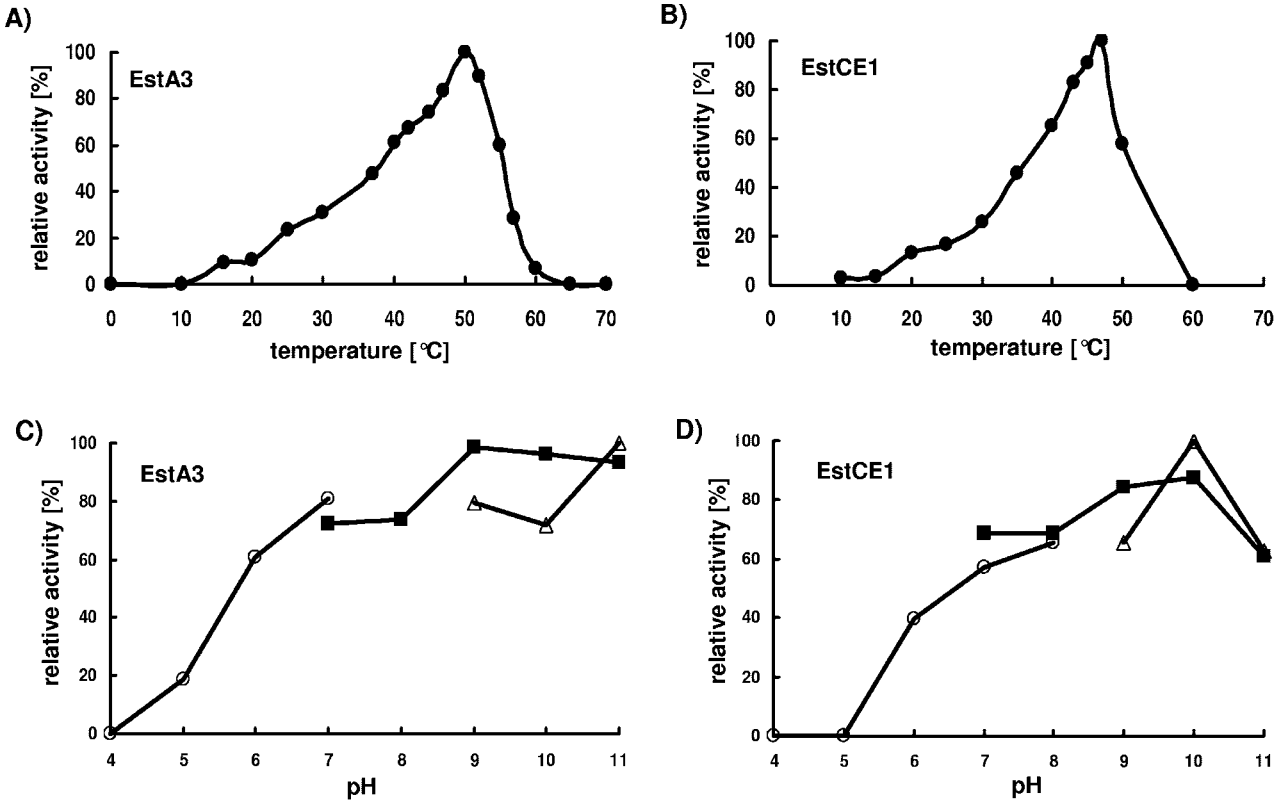

FIG. 2. Effects of temperature and pH on the activity of EstCE1 and EstA3. In A and B, the enzymes were incubated with a mixture containing 100 mM Tris buffer (pH 7.2) and 4 mM *p*-nitrophenyl caproate for 10 min at the temperature indicated, and the *p*-nitrophenol formed was measured. The highest value of each enzyme was set as 100%. In C and D, enzyme activity was measured using tributyrin as a substrate at different pHs. Tests were done using three different buffer systems. ○, Na-phosphate buffer, pH 4 to 7; ■, Tris buffer, pH 7 to 11; △, glycine buffer at pH 9 to 11.

enantioselective for (+)-menthylacetate, with only the (+) enantiomer being converted (enantiomeric excess [%], 100). EstCE1 and EstA3 showed different enantioselectivity when tested against the substrate *cis*-3,5-diacetoxy-1-cyclopentene, but due to a lack of pure reference compounds, the enantiomers could not be identified. Both EstCE1 and EstA3 were able to hydrolyze (+/−)-1-octin-3-ol, R-(+)-3-chlor-1-phenyl-1-propanol, and trimethylsilylbutinol. Only EstCE1 was able to hydrolyze *cis*/*trans*-1,2-cyclohexanediol and isopropylidenglycerol acetate, and neither enzyme was able to hydrolyze tetrahydronaphthylamine and phenylethylamine. In these tests, no stereospecificity could be observed.

Using spectrophotometric analyses, EstA3 was able to hydrolyze cyclohexanoate (50 U/mg), benzoate (9.4 U/mg), 2-phenylpropanoate (21 U/mg), 3-phenylbutanoate (31.6 U/mg), and 2-(3-benzoylphenyl)propanoate (26.7 U/mg) as well as the nonsteroidal anti-inflammatory drugs ibuprofen [2-(4-isobutylphenyl)propanoate] (7.2 U/mg) and naproxen [2-(6-methoxynaphthalen-2-yl)propanoate] (26.7 U/mg). EstCE1 was able to hydrolyze benzoate (12.1 U/mg), 3-phenylbutanoate (1.0 U/mg), cyclohexanoate (16.8 U/mg), and 2-(3-benzoylphenyl)propanoate (0.8 U/mg). Neither enzyme was able to hydrolyze 1-naphthoate, 2-naphthoate, and adamantanoate. EstCE1 and EstA3 displayed overlapping but quite different substrate spectra.

Fingerprint analysis of EstCE1 and EstA3. EstCE1 and EstA3 were analyzed by cocktail fingerprinting using a series of 10 monoacyl glycerol analogs optimized for reactivity with lipases and esterases (15). This substrate series contains octanoyl esters of four primary alcohols (Fig. 3, panels 1 to 4), four neopentylic alcohols (Fig. 3, panels 5 to 8), and two sterically hindered secondary alcohols (Fig. 3, panels 9 and 10) and can be analyzed by a single high-performance liquid chromatography analysis, which separates all products. Interestingly, EstA3 was able to convert the secondary ester 7-[3-octylcarboxy-(3-hydroxy-3-methyl-butyloxy)]-coumarin, which is very unreactive and is almost never converted to any measurable extent by other enzymes.

DISCUSSION

Metagenomics has broadened the field of enzymology by providing enzymologists with access to a wider range of natural biocatalysts. As more enzymes are characterized, the factors that determine their level of activity under different conditions, their substrate range, and their potential product range can be elucidated. In the search for the ideal biocatalyst for industrial applications, enzymologists employ such diverse techniques as directed evolution and gene reassembly (22, 38). As enzymes are known to display habitat-related characteristics, metagenomics is an excellent strategy to obtain novel natural biocatalysts with unusual properties (14, 45). In the present study, EstCE1 and EstA3 are the two metagenome-derived esterases

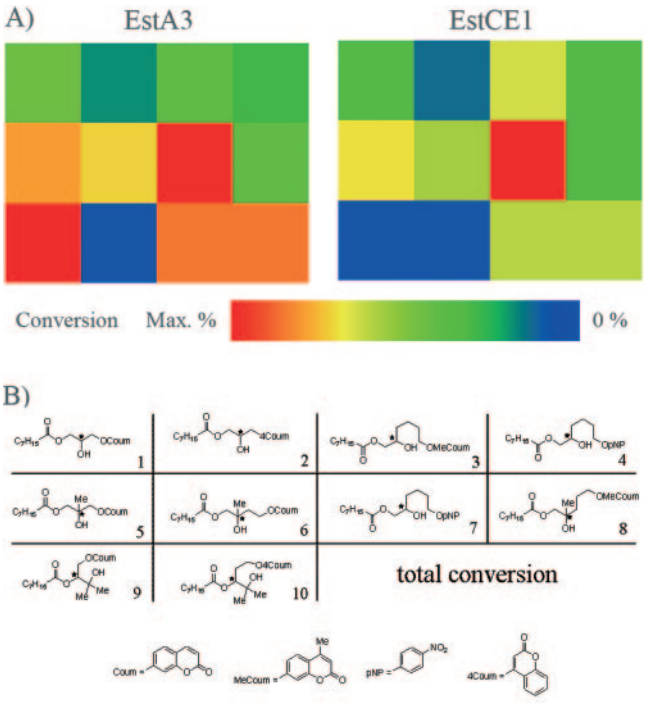

FIG. 3. Fingerprint analysis of the conversion of 10 different model substrates by EstCE1 and EstA3. (A) The substrate with highest conversion was set as the maximum (red), and the strength of conversion of the other substrates is indicated according to the color scheme. The colored boxes represent substrates 1 to 10 according to the order shown (B); the stereocenter of each molecule is indicated with an asterisk. The percent total conversion is color coded in the two squares at the lower right-hand side.

that were characterized to determine their applicability for industrial processes.

The deduced amino acid sequences of EstA3 and EstCE1 showed significant similarities to β-lactamases (Table 1). Both enzymes lacked the classical lipase motif but instead had the Ser-X-X-Lys motif. Mutation of the serine in the Ser-X-X-Lys conserved motif of EstCE1 and EstA3 resulted in the loss of catalytic activity of both enzymes, proving that this serine residue is essential for enzyme function. This, together with the fact that they are both approximately 380 amino acids in size, indicates that they belong to family VIII of esterases and lipases (2). Similar to other family VIII esterases such as EstB from *Burkholderia gladioli*, EstCE1 and EstA3 showed no β-lactamase activity (49). As both metagenome-derived enzymes belong to family VIII, it is possible that this type of enzyme is more widespread than previously thought.

Interestingly, there are ORFs flanking both *estCE1* and *estA3* that have functions related to antibiotic resistance (Table 1). *ORF006* on pCosCE1 encoded a functional enzyme that displayed class C β-lactamase activity, while *ORF001* on pCosCS-A3 was similar to a possible aminoglycoside transferase. It is quite possible that both metagenome esterases are part of ancient antibiotic resistance clusters. This hypothesis is supported by previous reports of ester-hydrolyzing activities of β-lactamases and DD-peptidases (11, 36). While cosmid clone pCosCS-A3 was most likely derived from alphaproteobacteria, the origin of pCosCE1 is more difficult to place, as the genes presented low homologies to genes from a wide range of bacteria belonging to two different phyla, *Proteobacteria* and *Firmicutes* (Table 1). In the case of pCosCE1, this may indicate a greater level of horizontal gene transfer.

The majority of esterases characterized to date are active in their monomeric form (2). While EstCE1 is active as a monomer, EstA3 was active only as a multimer of at least six subunits (Fig. 1). The initial data from crystallization studies indicate that EstA3 is most likely an octamer (data not shown). There are reports of tetrameric esterases (3, 5, 6, 10, 31, 34) and hexameric esterases from prokaryotes (29, 42), but this is the first report of an esterase with a possible octameric form.

When EstCE1 and EstA3 were characterized biochemically, they displayed quite unique properties. While their broad pH range and temperature range of activity are comparable to those of a lipase derived from a hot spring, the preference of both EstCE1 and EstA3 for short-chain substrates indicates that they are esterases and not lipases (4). A comparison of the substrate range of EstA3 with that of another family VIII esterase, EstB from *Burkholderia gladioli*, showed that EstA3 was active on esters with an acyl chain length up to C_8 (Table 2), whereas EstB activity was limited to C_6 substrates and shorter (32). In comparison to other recently published metagenome-derived esterases, EstCE1 and EstA3 are much more stable and active under alkaline conditions (24, 37). While the high level of stability in the presence of solvents displayed by EstA3 is comparable to that of the esterases recently derived from a deep-sea, hypersaline, anoxic basin, the size and substrate range of the enzyme are completely different (14). These facts all indicate that the EstCE1 and EstA3 are novel esterases that display unique characteristics.

EstCE1 and EstA3 differed from each other particularly in terms of their ability to catalyze industrially relevant substrates. EstCE1 was highly enantioselective for (+)-menthylacetate but did not hydrolyze (−)-menthylacetate. Menthylacetate is a compound that has many uses in the food industry and in the production of perfumes. EstCE1 was also stable over a wide pH range and in the presence of solvents such as DMSO, methanol, and isopropanol (Fig. 2). The stability and specificity of EstCE1 indicate that it is a good candidate for application in industrial processes.

EstA3, which was derived from a drinking water biofilm metagenome, displayed remarkable activity and stability over a wide range of conditions, which indicates that it has the potential to be used in many biotechnological processes. It was highly active and in some cases even enhanced in the presence of diverse solvents. It was also active over a wide pH range, being quite stable under alkaline conditions (Fig. 2). It displayed a broad substrate range and was able to hydrolyze substrates such as 7-[3-octylcarboxy-(3-hydroxy-3-methyl-butyloxy)]-coumarin, which is normally quite an unreactive secondary ester (Fig. 3) (15). Structural analysis is required to gain information about the unique catalytic mechanism of this enzyme. The stability and high level of activity against a broad range of substrates of EstA3 make it an ideal candidate for a wide range of industrial applications.

Recently, there has been an increase in the number of metagenomic lipases and esterases being reported (4, 14, 20, 24, 27, 33, 35, 37, 48). Only four of those reports included biochemical characterizations of the novel enzymes, and they tended to

concentrate on characteristics related to the environmental origin of the enzyme (4, 14, 24, 37). Among the best-characterized esterases is a thermophilic esterase derived from a hot spring (37) and the esterases derived from a deep-sea hypersaline anoxic basin (14). These esterases display habitat-related characteristics that make them highly useful for biotechnological applications. In contrast to those recently reported metagenome esterases, the remarkable characteristics of EstA3 cannot be explained by the environment from which it was obtained. This indicates that while it is interesting to investigate the enzymes from extreme environments, there is enough genetic and catalytic diversity within readily accessible environmental biofilms to provide unusual novel biocatalysts. For example, both EstCE1 and EstA3 displayed alkaliphilic properties that have been previously detected only in enzymes isolated from extreme environments (35). As one would not expect enzymes from nonextreme environments to display extreme characteristics, researchers often do not investigate the full potential of novel enzymes. In many cases, this leads to an underestimate of the enzyme's potential.

It is also interesting that EstA3 was obtained without enrichment and that EstCE1 was obtained with enrichment. Of the two enzymes, EstA3 shows the greater range of novel and biotechnologically useful characteristics. The enrichment step may have enhanced the soil sample for esterase activity but at the same time reduced the genetic diversity of the original soil sample to such an extent that the chance of discovery of novel esterases was reduced.

In summary, EstA3 and EstCE1 are both very interesting enzymes with a high potential for downstream biotechnological applications. This was confirmed by their extensive biochemical characterization, which revealed their wide substrate spectra, and their stability towards solvents, pH, and other parameters. Future work will establish the structure of both of these enzymes to gain more information about their catalytic mechanism.

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