Biochemical diversity of carboxyl esterases and lipases from Lake Arreo – a metagenomic approach

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The esterases and lipases from the α/β hydrolase superfamily exhibit an enormous sequence diversity, fold plasticity and activities. Herein, we present the comprehensive sequence and biochemical analyses of seven distinct esterases and lipases from the metagenome of Lake Arreo, an evaporite karstic lake in Spain (42º46′N, 2º59′W; 655 m altitude). Together with oligonucleotide usage patterns and BLASTP analysis, our study of esterases/lipases mined from the Lake Arreo suggests that its sediment contain moderately halophilic and cold-adapted Proteobacteria containing DNA fragments of distantly related plasmids or chromosomal genomic islands of plasmid and phage origin. This metagenome encodes esterases/lipases with broad substrate profiles (tested over a set of 101 structurally diverse esters) and habitat-specific characteristics as they exhibit maximal activity at alkaline pH (8.0-8.5), 16-40ºC and are stimulated (1.5-2.2 times) by chloride ions (0.1-1.2 M) reflecting an adaption to environmental conditions. Our work provides further insights into the potential significance of the Lake Arreo esterases/lipases for biotechnology processes (i.e. production of enantiomers and sugar esters) because these enzymes are salt-tolerant and are active at low temperatures and against a broad range of substrates. As example, the ability of a single protein to hydrolyze at similar extent triacylglycerols, (non) halogenated alkyl and aryl esters, cinnamoyl and carbohydrate esters, lactones and chiral epoxides was demonstrated.

Esterases and lipases from the α/β hydrolase family have received considerable attention, because they are widely distributed within the microbial communities operating in most of environments where they have important physiological functions (45) and because they are one of the most important groups of biocatalysts for biotechnological applications (12, 21, 35). Upon searching the list of 'genes with Pfam' (the protein family database; ref. 4) from the approximately 140 metagenomic projects in various stages of sequencing on the GOLD website (Genomes OnLine Database; http://www.genomesonline.org/) and the available sequences of esterases and lipases, more than 72,000 predicted esterases/lipases of the α/β hydrolase superfamily were retrieved which revealed the richness of uncultured biodiversity (16) to provide wide collections of such biocatalysts. This is one of the largest protein families with available sequences. In relation to the cultivation-independent methods used to identify them, it should be highlighted that sequence-based metagenomics only provide the "presumptive" compositional and functional blueprint represented in the community genome (26, 54) but at the same time causes serious problems regarding both sequencing errors (29) and the erroneous assignment of substrate specificity (22). By contrast, the activity-directed techniques have been shown to provide a direct view of known or new protein families and functionalities (for examples see 6-8, 18, 20, 24, 25, 38, 47, 49, 50, 57, 59). Whatever the platform used (i.e. either gene cloning from sequence resources or 'naïve' activity screens), in many cases, enzyme properties have been shown to be modulated by ecosystem characteristics (5, 14).

From the whole set of sequences available, only a limited number of the metagenomic derived esterases and lipases have been experimentally characterized (Table S1 in the supplemental material), and only 27% of them (or circa sixty) have been shown to possess characteristics likely needed for industrial operations. In this respect, useful features such as broad substrate specificity complemented with high activity levels under a wide range of

thermal and pH conditions and salt concentrations, stability in organic solvents and enantio- and stereoselectivity, are widely used for defining the potential application of esterases and lipases in biotechnology settings (35); however, not all characterized enzymes matches these criteria (for example see ref. 19). Table S1 in the supplemental material provided an exhaustive list (with biochemical characteristics) of about more than two hundred different esterases/lipases from the α/β hydrolase superfamily which have been identified by metagenomic methods from various environments including soils, compost piles, landfill leachate, bioreactors and activated sludges, marine water and sediment samples (including tidal flat sediments, deep sea and water column) and freshwater samples (including drinking water, pond water, rivers and hot springs).

In the present work, we identified and successfully cloned, expressed, purified and characterized seven esterases/lipases from the α/β hydrolase superfamily via an activity-centered metagenome analysis from a microbial community of an evaporite karstic lake (Lake Arreo) at NW Spain (42°46′N, 2°59′W; 655 m altitude). Our findings are pointing at the importance of evaporite karstic lakes as rich resources for novel low temperature- and salt-adapted α/β hydrolases potentially useful for biotechnological applications according to their remarkable and broad substrate profiles. The substrate spectrum of newly isolated enzymes was compared with that of commercially available preparations and structural models were also used to suggest molecular features that allow for the acceptance of different set of substrates. Additionally, our work further provided insights into the placing of esterases/lipases at Lake Arreo by using genome linguistics, sequence similarity and activity phenotyping.

MATERIALS AND METHODS

Materials, strains and esterase/lipase preparations. Chemicals, biochemicals and solvents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Aldrich (Oakville, ON) or Fluka (Oakville, ON.) and were of p.a. (pro-analysis) quality. The oligonucleotides used for DNA amplification and sequencing were synthesised by Sigma Genosys Ltd. (Pampisford, Cambs, UK) and are provided in Table S2 in the supplemental material. The Ni-NTA His·Bind chromatographic media were from Sigma Chemical Co. (St Louis, MO, USA). The *Escherichia coli* EPI300-T1^{R} strain (Epicentre Biotechnologies; Madison, WI, USA) used for the fosmid library construction and screening and *E. coli* GigaSingles for the gene cloning and BL21 (DE3) for the expression using the pET-46 Ek/LIC vector (Novagen, Darmstadt, Germany) were cultured and maintained according to the recommendations of the suppliers and standard protocols (53). Commercial esterase/lipase preparations Novozym 735 (lipase CalA from *Candida antarctica*), Novozym CALB L (lipase CalB from *C. antarctica*), Lipolase 100L (lipase from *Thermomyces lanuginosa*) and Lipozyme RM – Novozym 388L (lipase from *Rhizomocur miehei*), were provided by Novozymes A/S (Bagsvaerd, Denmark). Lipase from *Alcaligenes* sp. was kindly donated by Meito Sangyo Co. (Japan). Lipase from *Rhizopus oryzae* was obtained and prepared as described elsewhere (32).

Selection of hydrolases derived from the evaporite karstic lake (Lake Arreo) fosmid library. A large-insert pCCFOS1 fosmid library was created from the DNA of a microbial community inhabiting sediment samples from an evaporite karstic lake (Lake Arreo). Sediment sampling (10 cm deep) was carried out in February 2007 with a sterilized 50 ml polypropilene Falcon tube in the East shallow part of the lake (Fig. S1 in the supplemental material). Sample was maintained cold and dark during transportation to the laboratory, where it was frozen at -20°C until processing. Total DNA (5.2 μg DNA/g sediment) was extracted using the G'NOME® DNA Isolation Kit (Qbiogene, Heidelberg, Germany). Purified and size-fractioned DNA was ligated into the pCCFOS fosmid vector and further cloned in *E. coli*

EPI300-T1^R according to the instructions of Epicentre Biotechnologies (WI, USA) and a procedure described earlier (6). Fosmid clones (40,000; average insert size of 29.7 kbp) harbouring approximately 1 Gbp of community genomes were arrayed and grown in 384-microtitre plates containing Luria Bertani (LB) medium with chloramphenicol (12.5 μg/ml) and 15% (*v/v*) glycerol and stored at -80°C.

Approximately 11,520 clones, plated onto small (12.5 x 12.5 cm) Petri plates (each containing 96 clones) with LB agar containing chloramphenicol (12.5 μg/ml) and the induction solution (Epicentre Biotechnologies; WI, USA) as recommended by the supplier to induce a high fosmid copy number, were screened with α-naphthyl acetate under previously described conditions (51). The positive clones were selected and their DNA inserts sequenced with a Roche 454 GS FLX Ti sequencer (454 Life Sciences, Branford, CT, USA) at Life Sequencing S.L (Valencia, Spain). Upon completion of sequencing, the reads were assembled to generate non-redundant metasequences using Newbler GS De Novo Assembler v.2.3 (Roche). GeneMark software (34, 43) was employed to predict potential protein-coding regions (open reading frames, ORFs with ≥ 20 amino acids) from the sequences of each assembled contig and deduced proteins were screened via blastp and psi-blast (2). Sequences are available at NCBI under accession number SRA059294.

Cloning, expression, and purification of selected proteins. The cloning, expression, and purification of selected His_6-tagged proteins in the Ek/LIC 46 vector and *E. coli* BL21 were performed as described elsewhere (23), except that protein expression was performed using 1.0 mM isopropyl-β-D-galactopyranoside for 16 h at 16°C. After purification using a Ni-NTA His·Bind resin (Sigma Chemical Co.; St Louis, MO, USA), protein solutions were extensively dialyzed with 20 mM 4-(2-hydroxyethylpiperazine-1-ethanesulfonic acid (HEPES) buffer (pH 7.0) by ultra-filtration through low-adsorption hydrophilic 10,000 nominal molecular weight limit cutoff membranes (regenerated cellulose; Amicon, Madrid,

Spain) and stored at -86ºC, until use. Purity was assessed as >95% using SDS-PAGE performed on 12% (v/v) acrylamide gels as described by Laemmli (37) in a Bio-Rad Mini Protein system. Protein concentrations were determined according to Bradford (10).

Biochemical assays. If otherwise not stated, hydrolase reactions using *p*-nitrophenyl (*p*NP) esters were performed in a microplate reader (Synergy HT Multi-Mode Microplate Reader - BioTek) as described previously (23) with minor modifications. Briefly, reactions contained 2 μg pure enzyme and 0.8 mM *p*NP ester (from a 20 mM stock solution in acetone) in 20 mM HEPES buffer pH 7.0, at 30ºC, in a total volume of 190 μl. Hydrolase specific activity using other structurally diverse esters was also determined using *p*-nitrophenol as a pH indicator as described elsewhere (36) with small modifications. Briefly, specific activity was calculated by adding 2 μg pure enzyme (lower amount of enzyme for LAE6 (0.4 μg) were used) and 2 mM of 100 mM ester stock solution (in acetonitrile) in 2 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer (pH 7.2) containing 0.45 mM *p*-nitrophenol and 2.5% acetonitrile (v/v), at 30°C, in a total volume of 150 μl. In all assays, the reactions were followed every 2 min by spectrophotometrically measuring the absorbance *p*-nitrophenol at 410 (for *p*NP esters) or 404 nm (for other substrates rather than *p*NP esters) during 15 min (except for LAE6 with which 10 min incubation time was used). Under our experimental conditions, the absorption coefficient for *p*-nitrophenol was measured as 15,200 $M^{-1} \cdot cm^{-1}$. One unit (U) of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of substrate in 1 min under the assay conditions. In all cases, three independent experiments were performed and graphs were plotted using mean values; the standard deviation was lower than 5%. Kinetic parameters were calculated by using a conventional Lineweaver and Burk model, at 30ºC, as outlined above in 96-well microtiter plates where each well contained 0.388-0.882 μM enzyme solution and 0-100 mM substrate.

The optimal pH, temperature and salt concentration were determined using *p*NP butyrate (0.8 mM final concentration) as the substrate; pH values between 4.0 and 9.0, temperatures between 4 and 70°C and sodium chloride concentration ranging from 0.1 to 1.2 M, were tested. All of the following buffers were tested at 20 mM: sodium citrate (pH 4.0-4.5), sodium acetate (pH 5.0-6.0), 2-(N-morpholino)ethanesulfonic acid (pH 5.5-6.0), HEPES (pH 7.0-8.0), piperazine-N,N'-bis(ethanesulfonic acid) (pH 6.0-7.0), K/Na-phosphate (pH 7.5), Tris-HCl (pH 8.5) and glycine (pH 9.0-9.5). The pH was always adjusted at 25°C. The pH and temperature profiles were obtained at 30ºC and pH 7.0, respectively. The optimal anion concentration was obtained at 30ºC and pH 7.0. Under our experimental conditions, the absorption coefficients for *p*-nitrophenol were measured for each indicated temperature and pH and ranged from 132 (for pH 4.0) to 28,381 (for pH 9.5) $M^{-1} \cdot cm^{-1}$. These values were considered for calculating activity for each pH and temperature, as described above.

Oligonucleotide usage pattern analysis. DNA sequences of contigs were searched for oligonucleotide compositional similarity against all sequenced bacterial chromosomes, plasmids and phages by using GOHTAM web tool (46). Frequencies of tetranucleotides for compositional genome comparison were calculated as it was described earlier (27).

3D model analysis. Suitable protein structures to be used as templates for modeling were searched using the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/). The Swiss Model server (http://swissmodel.expasy.org/) was used to generate the enzyme models. DeepView - Swiss-PdbViewer (http://spdbv.vital-it.ch/) was used to analyze the structures and generate the figures.

RESULTS AND DISCUSSION

Study site description. Lake Arreo (42°46′N, 2°59′W; 655 m altitude) is one of the deepest karstic lakes on the Iberian Peninsula (maximum depth = 24.8 m) (15, 51) (Fig. S1 in the

supplemental material). Chemically, the lake is subsaline, with an electrical conductivity range of 703–1,727 μS/cm during the last 20 years. Most of the sediment showed anoxic conditions, except for a thin surface layer which has a Ca–(Mg)–(Na)–SO_4–HCO_3–(Cl) ionic composition (13, 30). The main water physicochemical data in sampling point were: Conductivity 1.079 μS/cm, temperature 6.9ºC, dissolved oxygen 11.5 mg/l (101%), pH 8.0. The mean values of major ionic concentrations (mg/l) are: sulfates (354.9), bicarbonates (269.1), chloride (99.6), calcium (188.4), magnesium (37.5), sodium (51.52) and potassium (14.08).

Anoxic sediment samples (10 cm deep) were taken and total DNA was purified and size-fractionated DNA was ligated into the pCCFOS fosmid vector and further cloned in *E. coli* EPI300-$T1^R$ to create a fosmid clone library harbouring approximately 1 Gbp of community genomes.

Metagenome library screening and sequence analysis. Approximately, 11,520 fosmid clones from the metagenomic library of Lake Arreo sediment (nearly 342 Mbp of metagenome DNA) were screened using plate-based screens for hydrolytic activity against α-naphthyl acetate, a model esterase substrate (51). Ten positive clones (incidence of positive clones: 1/1,152) were identified. Fosmid DNA isolated from each clone was mixed in equal amount and sequenced as a pool using a Roche GS FLX DNA sequencer (1/16 plate), which produced 77,841 reads with an average length per read of 454.69 bp. Accordingly, a total of 38.5 Mbp of raw DNA sequences were obtained, which were assembled into 735,686 bp (19 contigs with length ranging from 1,854 to 43,416 bp), with an average GC content of 58.75%. A total number of 378 open reading frames were identified which were analyzed and compared to the sequences available in the databases. Seven out of ten identified genes encoding the predicted esterases from the α/β hydrolase superfamily (named LAE1 to LAE7), were expressed and produced in soluble form in *E. coli* BL21 (DE3), and the corresponding

gene products were purified (Fig. S2 in the supplemental material), and their activities were characterized using 15 model substrates and an array of additional 86 structurally diverse esters (Fig. S3 in the supplemental material).

Sequence analysis of α/β hydrolases from Lake Arreo. Analysis of sequences of the Lake Arreo proteins showed that they all belong to the α/β hydrolase superfamily with the amino acid sequence identity ranging from 31 to 57%. As determined by Matcher (EMBOSS package), among Lake Arreo α/β hydrolases LAE4 and LAE7 were the most similar enzymes (71.4% sequence identity) whereas LAE5 and LAE6 were the most different at sequence level (18.2% identity) (Table S3 in the supplemental material). The LAE1 protein was structurally most similar to thermophilic esterase from *Thermotoga maritima* (31%; PDB code 3DOH_A (40)) and α/β hydrolase from *Agrobacterium tumefaciens* (24%; PDB code 2r8b); LAE2 to GDSL-like brain platelet activating factor acetylhydrolases (SGNH_hydrolase subfamily) (31%, PDB code 1FXW (55); 28%, PDB code 1ES9 (44)); LAE3 to thermostable esterase/lipase from uncultured bacterium (36%; PDB code 3V9A_A) and hormone sensitive esterase (35%; PDB code 3fak (48)); LAE4 to esterase from *Pyrobaculum calidifontis* (25%; PDB code 2YH2_A (34)) and bacterial α/β hydrolase (19%; PDB code 3k2i); LAE5 to GDSL family lipolytic protein (SGNH_hydrolase subfamily) from *Alicyclobacillus Acidocaldarius* subsp. *Acidocaldarius* Dsm 446 (54%; PDB code 3RJT_B); LAE6 to thermophilic and thermostable carboxylesterase from metagenomic library (41%; PDB code 2c7b (11)); and LAE7 to GDSL-like brain platelet activating factor acetylhydrolase (SGNH_hydrolase subfamily) (32%, PDB code 1ES9 (44)).

The deduced molecular mass and estimated *p*I values of Lake Arreo α/β hydrolases range from 24,328.83 to 34,002.96 Da and from 4.78 to 10.04, respectively. Suitable templates have been found for each enzyme and 3D models of the corresponding protein structures are represented in Fig. S4 in the supplemental material. The analysis of the seven

enzymes, which can be classified in the microbial lipase/esterase families described by Arpigny and Jaeger (3), allows the distinction of three structural groups. The first group (LAE2, LAE5 and LAE7) are GDSL esterases/lipases (1). A characteristic feature of GDSL enzymes is that the serine-containing motif is close to the protein N-terminus. However, whereas the presumptive nucleophile of LAE5 corresponds to Ser15, that of LAE2 and LAE7 correspond to Ser143 and Ser130, respectively. The structural analysis of the LAE2 and LAE7 enzymes shows that they are bi-modular enzymes in which the α/β hydrolase fold of the esterase catalytic unit is preceded at N-terminal position by a 100-120 amino acid module whose structure could not be modeled due to the absence of suitable templates. A remarkable feature of this module is the presence of a glycine/proline rich stretch, collagen-like sequence (GPGGPGGPRGGGFGAPPTPPGP in LAE2). These sequences, which are found in diverse bacterial proteins, are considered to be signatures of phage origin (28); this agrees with the analysis of DNA fragments using genome linguistics approach (see below). Whatever the case, the fact that the catalytic Ser located closer to the N-terminal in LAE5 as compared to LAE2 and LAE7 would possibly make the catalytic site more accessible to substrates; this was further confirmed (see below) by showing that LAE5 accepted a higher number of substrates and by showing that albeit all three enzymes hydrolyzed *p*NP acetate and *p*NP propionate, LAE5 did prefer *p*NP propionate while LAE2 and LAE7 preferred shorter *p*NP acetate (Table 1). Additionally, the fact that LAE5 retained circa 70% of the activity at 4ºC while LAE2 and LAE7 only 42 and 28%, respectively (see below; Fig. 1), may suggest a link between the differences in active center location and optimization of activity at low temperatures. Further structural analysis is in progress to confirm this hypothesis. The second group corresponds to common esterases/lipases with typical α/β hydrolase fold and -GxSxG- motif (LAE1, LAE4 and LAE6). LAE1 belongs to Family I and LAE4 and LAE6 to Family VI; with a molecular mass in the range 23-26 kDa, the enzymes in Family VI are among the

smallest esterases known, in agreement with the theoretical MW of LAE4 which is the smaller (24 kDa) among the Lake Arreo esterases. The third group, represented by LAE3, belongs to the hormone-sensitive lipase/esterase (HSL) type (42) and contained also a -GxSxG- motif. It is though that that HSL hydrolases retained high activity at low temperature, although sequence analysis indicates that temperature adaptation is not responsible for such sequence conservation (3); the fact that LAE3 did show maximal activity at 16ºC (Fig. 1) agrees with this hypothesis.

According to sequence and 3D model analyses (Fig. S4 in the supplemental material), the catalytic triads were tentatively identified: Ser166, Glu217 and His249 (in LAE1), Ser140, Glu292 and His295 (in LAE2), Ser143, Glu237 and His267 (in LAE3), Ser144, Asp248 and His281 (in LAE4), Ser15, Asp192 and His195 (in LAE5), Ser161, Asp256 and His286 (in LAE6) and Ser130, Glu282 and His285 (in LAE7).

Optimization of activity and anion stimulation of esterases/lipases from Lake Arreo. As shown in **Fig. 1**, purified proteins showed high hydrolytic activity against the model esterase substrate *p*NP butyrate at 16-40ºC and pH 8.0-8.5, with the hydrolases LAE3, LAE5 and LAE6 being the most active at temperatures as low as 4ºC as they retained from 64 to 78% of the activity as compared to the activity level at the optimal temperatures. Hydrolytic activities were stimulated (1.5-2.2 times) by the addition of NaCl to the reaction mixture (0.1-1.2 M NaCl) for all enzymes but one (LAE3), which was inhibited from 1.6 (at 0.1 M) to 3.8 (at 1.2 M)-times. This is consistent with the fact that Lake Arreo basin was formed by dissolved evaporites and thus represents a sub-saline environment (43). The fact that all but one of the esterases was activated by NaCl indicates that enzyme properties resembled habitat-specific characteristics and that activation by chloride may be common for enzymes from the Lake Arreo. Similar observations have been reported for the cold-active

and anion-activated carboxyl esterase OLEI01171 from the oil-degrading marine bacterium *Oleispira antarctica* (39).

Using model substrates that included 3 α-naphtyl, 6 *p*NP and 6 triacylglycerol esters and according to specific activity (units/mg) determinations (Table 1) we observed that LAE6 did show the highest capacity to accept longer esters (up to tricaprin, *p*NP laurate and α-naphthyl butyrate) which is in line with the highest lipase character of this enzyme compared to other Lake Arreo α/β hydrolases. *p*NP esters were preferred substrates for LAE3, whereas LAE1, LAE2, LAE4, LAE5 and LAE7 preferentially hydrolyzed α-naphthyl esters and LAE6 did show similar activity for both substrates; in all cases, the activity towards triacylglycerols was significantly lower (from 3.5-fold for LAE4 to 100-fold for LAE2, as compared to α-naphtyl esters). Overall, LAE6 (approx. 125 units/mg for α-naphtyl acetate) was the most active enzyme whereas LAE3 showed the lowest activity (0.74 units/mg with α-naphtyl butyrate) (Table 1).

Substrate fingerprinting of esterases/lipases from Lake Arreo. The substrate range of the purified esterases was characterized using a battery of 86 different esters (see Fig. S3 in the supplemental material) that included 25 halogenated alkyl and aryl esters, 34 alkyl esters, 12 aryl esters, 10 hydroxycinammic esters, 2 epoxides, 1 lactone and 2 carbohydrate esters, using a colorimetric method in which *p*-nitrophenol was used as pH indicator (36). The fingerprints of LAE1 to LAE7 are shown in Figs. 2 and 3, which showed that 52 out of the 86 (or 61%) esters used in the present study were accepted as substrates (for substrates not being hydrolyzed by any of the enzymes see Fig. S3 in the supplemental material).

Using the ester library substrates (Figs. 2 and 3) we observed that LAE6 exhibits the broader substrate spectrum, being able to hydrolyze 43 substrates, followed by LAE3 (28 substrates), LAE4 (27 substrates) and LAE1 (26 substrates) and to lower extent LAE5 (14 esters), LAE7 (10 esters) and LAE2 (7 esters). The majority of compounds were accepted by

at least two or more enzymes; however, 14 substrates were LAE6-specific (Fig. 3), that included ethyl (S)-(−)-4-chloro-3-hydroxybutyrate, (+)-methyl (R)-2-chloropropionate, methyl α-bromophenylacetate, methyl α-bromoisobutyrate, ethyl octanoate, vinyl decanoate, ethyl benzoate, vinyl benzoate, methyl cinnamate, methyl trans-cinnamate, isobutyl cinnamate, phenethyl cinnamate, (R)-(−)-glycidyl butyrate, (S)-(+)-glycidyl butyrate and γ-butyrolactone, and the flavor terpene compound geranyl acetate was only accepted as substrate by LAE5 (Fig. 2). Specific activity (units/mg) determinations were performed for the best representative substrates (Figs. 2 and 3). At 30ºC and pH 7.2, methyl-2-chloro-3-hydroxypropionate (for LAE1, 0.28 units/mg), phenyl acetate (for LAE2, 0.39 units/mg), tri-O-acetyl-D-glucal (for LAE3, 0.33 units/mg; for LAE7, 1.41 units/mg), methyl 4-bromobenzoate (for LAE4, 1.01 units/mg), α-D-glucose pentaacetate (for LAE5, 0.60 units/mg) and (S)-(+)-glycidyl butyrate (for LAE6, 17.45 units/mg) were the preferred substrates.

The ability to hydrolyze non-halogenated and halogenated (including those containing bromo, chloro, fluor and iodo) alkyl and aryl esters were demonstrated for all enzymes (Figs. 2 and 3). Of special significance is that long alkyl and aryl esters with vinyl and isoprenyl substituents such as vinyl decanoate, vinyl benzoate and isoprenyl acetate were mainly hydrolyzed by LAE6 (up to 4.23 units/mg; Fig. 3), and thus this enzyme may be potentially applied in transesterification reactions using vinyl esters as compared to the other enzymes from Lake Arreo. In addition, LAE1 was the only enzyme that did not accept aromatic halogenated esters such as methyl 4-bromobenzoate (Fig. 3), albeit it was able to hydrolyze the non-halogenated substrate (methyl benzoate). As shown in Figs. 2 and 3, four enzymes exhibited capacity to accept the carbohydrate ester α-D-glucose pentaacetate (LAE2, LAE3, LAE5 and LAE7) and all but one (LAE4) did show activity for tri-O-acetyl-glucal. Finally, four enzymes were able to hydrolyze one or several cinnamate esters (LAE1, LAE3, LAE4

and LAE5), four accept *p*-coumarate esters (LAE1, LAE3, LAE4 and LAE6) and LAE3 and LAE5 were the only ones accepting methyl sinnapinate esters; no activity towards methyl ferulate was observed for any of the enzymes. Of special significance is that LAE4 (Fig. 3) was the only enzyme that showed activity on cinnamate esters of tertiary alcohols such as tert-butyl cinnamate, although it was unable to accept primary and secondary alcohol substituents such as methyl or isobutyl substituents. Further, LAE6 exhibited activity towards cinnamate esters with methyl, ethyl, isobutyl, vinyl and the large aromatic phenethyl substituents for which low or now activity was detected for the majority of other esterases/lipases (Fig. 3).

In relation to the enantioselective character of Lake Arreo hydrolases we further observed that LAE1, LAE3 and LAE4 did show capacity to hydrolyze both enantiomers of methyl mandelate, whereas LAE6 was the only one accepting both enantiomers of glycidyl butyrate (Figs. 2 and 3); others chiral esters such as menthyl, neomenthyl or lactate esters, were not substrates for any of the enzymes. According to the Quick E assay (37) and k_{cat}/K_m determinations for separate enantiomers (Table S4 in the supplemental material), apparent enantiomeric ratios (E_{app} values) were calculated. At 30ºC and pH 7.2, E_{app} varied from 816 (for LAE3) to 15 (for LAE6), 8.3 (for LAE1) and 2.0 (for LAE4). LAE1 and LAE3 did show enantiopreference for methyl-(*R*)-(−)mandelate whereas LAE4 for methyl-(*S*)-(+) mandelate; LAE6 did prefer (*R*)-(−)-glycidyl butyrate. In addition, LAE6 further exhibited activity toward γ-butyrolactone, although the activity for this substrate was 108-fold lower as compared to (*S*)-(+)-glycidyl butyrate which was the best substrate (Fig. 3).

Our results indicate that esterases/lipases from Lake Arreo are characterized by high activities and different substrates profiles and enantioselectivities, and therefore they are potentially useful for various biotechnological applications. The differences in hydrolytic capacities (by meaning of the half-saturation (Michaelis) coefficient (K_m), the catalytic rate constant (k_{cat}), the catalytic efficiency (k_{cat}/K_m) values and/or specific activities; Table S4 in

the supplemental material) with distinct acid and alcohol substituent of different length and nature may further indicate differences in active site. Having said that, it should be highlighted that, as compared to previously reported esterases/lipases (Table S1 in the supplemental material), some of the substrate profiles described herein are biologically relevant, such as the ability of a single esterase/lipase such as LAE6 to hydrolyze at similar extent triacylglycerols, halogenated and non halogenated alkyl and aryl esters, cinnamoyl and carbohydrate esters, lactones and chiral epoxides, which has not been previously described. It is to note also that enzymes LAE1 and LAE2, which belong to the same contig (i.e. produced by same bacterium; see below), have similar optimal parameters (pH 8.0-8.5, 30ºC and up to 1.2 M NaCl; Fig. 1) but have quite distinct substrate profile (Figs. 3 and 4). Thus, only 6 out 27 substrates were hydrolyzed by both enzymes, with LAE1 possessing the broader substrate spectrum; these distinct functionalities may suggest complementary metabolic and ecological capacities *in vivo*, together with different biotechnological capacities.

Comparison with commercial esterases and lipases. Lipases and esterases are one of the most important classes of hydrolytic enzymes in industrial settings (9, 12). Nowadays there are a wide range of available preparations that can be tested for particular applications and for comparative studies (21, 31, 35); in this context it is interesting to evaluate and compare the substrate spectrum of newly isolated enzymes with that of commercially available and most common esterases and lipases. Here we used five esterases/lipases commercial preparations, namely, Novozym 735 (lipase CalA from *Candida antarctica*), Novozym CALB L (lipase CalB from *C. antarctica*), Lipolase 100L (lipase from *Thermomyces lanuginosa*), Lipozyme RM – Novozym 388L (lipase from *Rhizomocur miehei*) and lipase from *Alcaligenes* sp. Lipase from *Rhizopus oryzae*, obtained and prepared as described elsewhere (32), was also added to the study.

The clustering analysis, generated from a binomial distribution based on the presence or absence of activity (at 30ºC and pH 7.2) against the set of 101 different esters herein used (Fig. 4), suggested that six of the Arreo Lake α/β hydrolases were functionally closer to each other as compared to the other preparations which clustered together. Lipases from *Rhizopus oryzae* and *Rhizomocur miehei* did show also similar profile and clustered together with lipases from *T. lanuginosa* and CalA from *C. antarctica*. Interestingly, the lipase LAE6 clusters more closely with the lipase CALB L from *C. antarctica*, both forming a separate group, which further indicate that both enzymes possess similar substrate spectrum; this is of special significance as Novozym CALB L is one of the most common commerically available lipase (Novozymes A/S, Bagsvaerd, Denmark). However, some minor differences were observed: (1) LAE6 hydrolyzed tri-O-acetyl-D-glucal (9.9 units/mg) and methyl 4-bromobenzoate (9.6 units/mg) for which no activity was detected with CalB; and (2) CalB hydrolyzed methyl 2-bromopropionate (0.74 units/mg) for which negiglible activity was found for LAE6. This suggests structural factors as determinants for substrate specificity when comparing both lipases.

To analyze the sequence similarity of known commercial and new discovered esterases/lipases belonging to different families a dendrogram was created in MEGA5 (56) by the Maximum Likelihood algorithms (Fig. 5). The dendogram was further used to evaluate whether the placing of the different enzymes in the phylogenetic tree related to their placing in the activity profile-based tree (Fig. 4). The sequences of reference and commercial esterases were obtained from the NCBI and PDB databases. The protein sequences were aligned by MUSCLE (17) and edited manually in JalView 2.5.1 (58) colleting with the alignment quality histogram. Due to an extreme diversity of selected sequences of enzymes, some of which were bacterial (PDB and Lake Arreo sequences) and others fungal (5 out of 6 sequences of commercial enzymes), the final alignment was reduced to 161 amino acid

residues by removal of ambiguously aligned regions constituting to 70% of the initial alignment. Analysis of the alignment by MEGA5 suggested WAG+G as the best evolutionary model. Robustness of the dendrogram was analyzed by the Bootstrap analysis based on 100 replicates of the initial dataset. As shown in Figs. 4 and 5, we made the following observations: (1) commercial lipases CalA, Lipolase 100L, Lipozyme RM, *Alcaligenes* sp., and *R. oryzae* formed a separate cluster at the sequence level, as was also found for the activity clustering; (2) similarly, lipases/esterases CalB, LAE4 and LAE6 clustered together both at the sequence and activity levels, which is of special significance taken into consideration the extreme diversity of selected sequences; (3) LAE1, LAE2, LAE5 and LAE7 formed a separate group from LAE4 and LAE6 at both levels; (4) no clear correlation between sequence and activity placing was observed for LAE3. Accordingly, under the experimental setting herein applied, the results suggest that, to some extent, an association between the phylogenetic/sequence positioning and the activity relationships exists.

Analysis of metagenomic DNA fragments using genome linguistics approach. Compositional similarity between the metagenomic fragments and the sequences of sequenced bacterial genomes and plasmids was analyzed by the comparison of frequencies of tetranucleotides in DNA sequences. A comprehensive analysis of the GOHTAM and BLAST analyses results is shown in Supplemental Text in the supplemental material. Five studied metagenomic DNA fragments containing genes encoding α/β hydrolases (excluding the LAE6-containing contig 19, which was too short for the compositional analysis) shared a significant level of tetranucleotide usage pattern similarity that indicated their origination from related organisms, which may be of *Burkholderia*/*Ralstonia* (Betaproteobacteria) and/or *Rhizobium*/*Methylobacterium* (Alphaproteobacteria) lineages. A search against GOHTAM database showed compositional similarities to multiple bacterial genomes, plasmids and phages. The protein blast of contig encoded proteins against plasmid proteins also showed

many hits but without gene syntenies. DNA fragment (contig 2) containing LAE1 and LAE2 enzymes, is most similar to several plasmids from *Cupriavidus* and *Ralstonia*, and it shares one Major Facilitator Superfamily (MFS_1) gene and an integrase fragment with a genomic island from *Ralstonia pickettii*. Accordingly, we may conclude that this fragment most likely is a mobilom associated either with a plasmid, or an integrated genomic island of a betaproteobacterium related to *Cupriavidus* and *Ralstonia*. Several genes of this fragment share similarity with the corresponding genes of *Arthrobacter aurescens* TC1 plasmid TC2 [NC_008713] and *Shewanella baltica* OS155 plasmid pSbal01 [NC_009035]. DNA fragment (contig 3) containing LAE3 enzyme shows some compositional similarity with alphaproteobacterial *Methylobacterium* plasmids and it shares several genes with *Nitrobacter hamburgensis* X14 plasmid 1 [NC_007959]. It also contains a gene for transposase IS4. DNA fragment (contig 4) containing LAE7 enzyme resembles betaproteobacterial *Burkholderia* phages and contains phage major capsid proteins. DNA fragment (contig 6) containing LAE4 enzyme has weak, but consistent, compositional similarity to alphaproteobacterial *Rhizobium* plasmids and multiple blast hits against *Rhizobium leguminosarum* bv. trifolii WSM1325 plasmid pR132504 [NC_012852], *Rhizobium leguminosarum* bv. trifolii WSM1325 plasmid pR132505 [NC_012854], *Rhizobium etli* CFN 42 plasmid p42c [NC_007764] and *Azospirillum* sp. B510 plasmid pAB510f [NC_013860]. DNA fragment (contig 7) containing LAE5 enzyme shows weak compositional similarity to gammaproteobacterial *Azotobacter* genome and alphaproteobacterial *Azospirillum* plasmids, and weak but persistent blast hits against several bacterial plasmids. DNA fragment (contig 19) containing LAE6 enzyme is too short for the compositional analysis but its genes are similar to those in the alphaproteobacterial *Sphingomonas* sp. KA1 plasmid pCAR3 [NC_008308]. According to the above analysis, DNA fragments containing Lake Arreo esterases may be plasmids or plasmid-

born genomic islands from Proteobacteria, although contig 4 containing LAE7 is most likely a pro-phage.

Taken together, in the present study an esterase-driven assay, based on a well-established screen with α-naphthyl acetate, was used to identify hydrolytic activity in ten *E. coli* clones. These clones harbor DNA plasmid fragments from a microbial community from evaporite karstic lake (Lake Arreo). Lake Arreo is one of the few relatively deep (z_{max} = 24 m) karstic lakes in Spain and developed in gypsum formations. Our study of α/β hydrolases mined from the Lake Arreo suggests they display habitat-specific characteristics and that cold-adapted or psychrophilic Proteobacteria occupy this ecological niche with average temperatures ranging from 4.7 to 19.8ºC as they produced low-temperature active and anion-activated enzymes with unusual substrate specificities. The production of highly promiscuous hydrolases with broad substrate profiles may have important metabolic and ecological implications, such as that the function of these enzymes lies in substrate scavenging in low temperature and substrate-poor karstic environments. A more comprehensive structural survey of enzymes from the Lake Arreo, currently in progress, will determine whether or not this hypothesis is correct. Having said that recent developments for the production of esterase and lipase improved commercial preparations have been achieved and many factors account for the cost-effective utilization of esterases and lipases (12), this study provides experimental evidences that enzymes from evaporite bacterial metagenomes may be of a great potential interest for biotechnological processes because they are salt-tolerant and are active at low temperatures and against a broad constellation of structurally diverse esters. This should be further evaluated and compared with commercial preparations under application conditions in a plethora of reactions according to the substrate specificity herein reported. As example, preliminary tests confirmed that LAE6 preparation had a high performance for esterification and transesterification reactions of sucrose with methyl and

vinyl esters in several organic solvents; also LAE3 have been preliminary found suitable for the production of enantiomers, such as (*R*)-mandelic acid, for synthetic purposes. Finally, this study may open research avenues into comparative catalysis models and structural-functional studies, and confirms the necessity of isolating and characterizing new enzymes from environmental metagenomes.

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FOOTNOTES

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FIGURE LEGENDS

FIG 1 Effect of NaCl concentration (upper panel), pH (middle panel) and temperature (lower panel) on the hydrolytic activity of Lake Arreo enzymes. The heat map colours represent the relative percentages of specific activity (units/mg) as compared to the maximum (100%) within each enzyme using *p*NP butyrate as substrate. The effect of NaCl concentration was measured in 20 mM HEPES buffer pH 7.0 and 30°C and varied from 0 to 1.2 M; the pH dependence was tested in the range of pH 4.0–9.5 at 30°C in 20 mM buffers; and the temperature dependence in the range of 4–70°C at pH 7.0. Reactions conditions as described in Table 1 and Material and Method section.

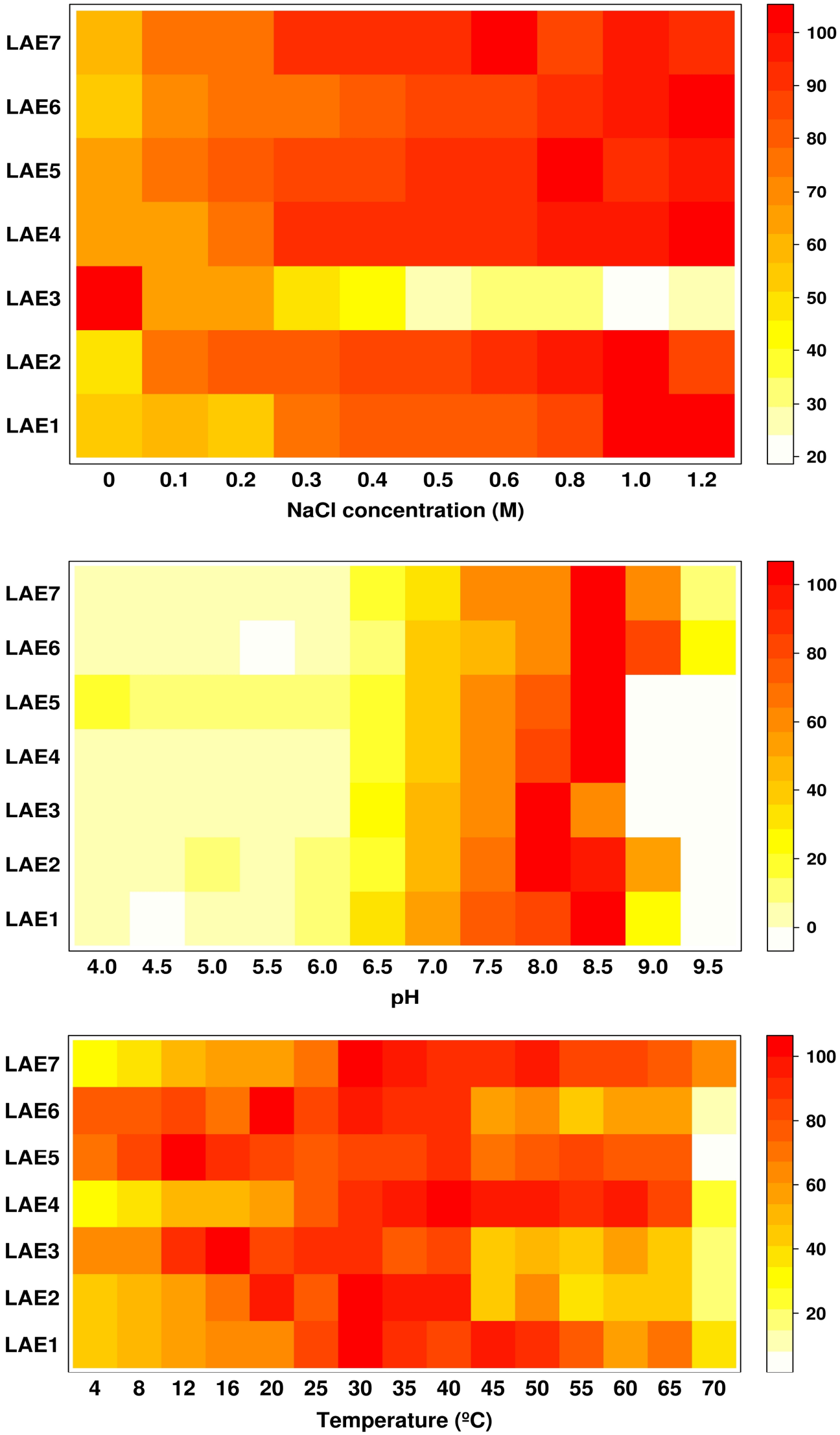
FIG 2 Substrate profiles of the wild-type LAE2, LAE5 and LAE7 α/β hydrolases from Lake Arreo against a set of structurally diverse ester substrates. LAE2, LAE5 and LAE7 were the α/β hydrolases characterized by a restricted substrate spectrum. Reactions (30ºC and pH 7.2) were performed as for the α-naphtyl and triacylglycerol esters as described in Table 1 and followed during 15 min and the absorbance of *p*-nitrophenol at 404 nm was recorded and ploted. For best and/or representative substrates, specific activities (units/mg) were calculated as described in Table 1 and Material and Methods section and are specifically shown on the top of bars. In all cases, three independent experiments were performed for each parameter and graphs were plotted using mean values; the standard deviation was lower than 5%.

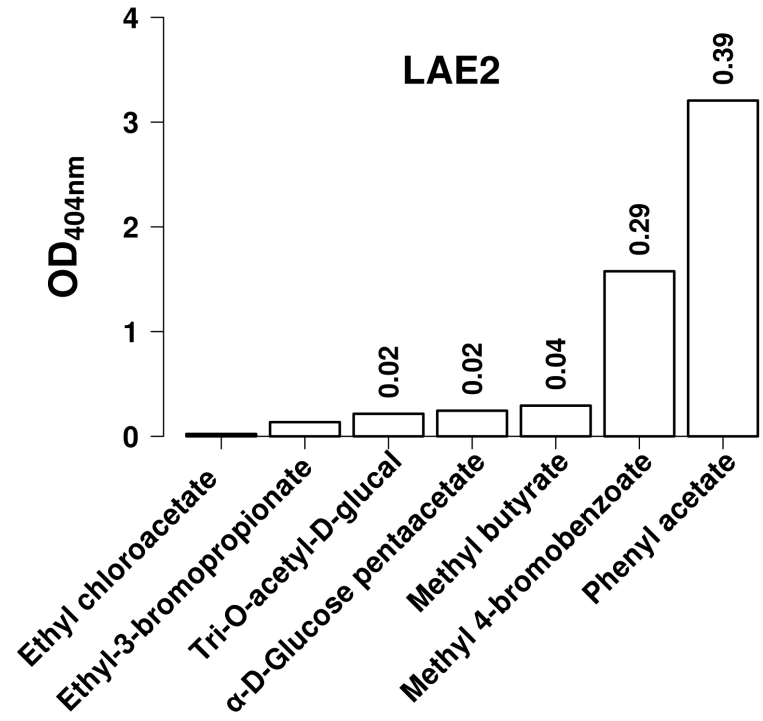
FIG 3 Substrate specificity of the wide substrate spectra LAE1, LAE3, LAE4 and LAE6 α/β hydrolases from Lake Arreo against a set of structurally diverse ester substrates. LAE1, LAE3, LAE4 and LAE6 were the hydrolases characterized by the widest substrate spectrum. Reaction conditions (30ºC and pH 7.2) and activity parameter determinations as

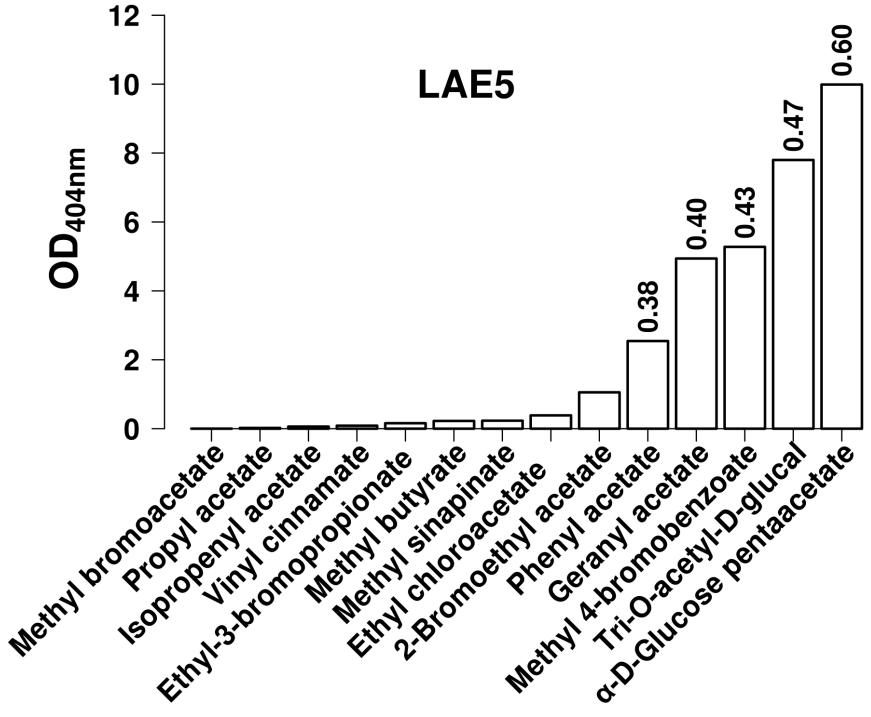
described in Table 1 and Figure 2. Abbreviations as follows: M2Cl3HP, methyl-2-chloro-3-hydroxypropionate.

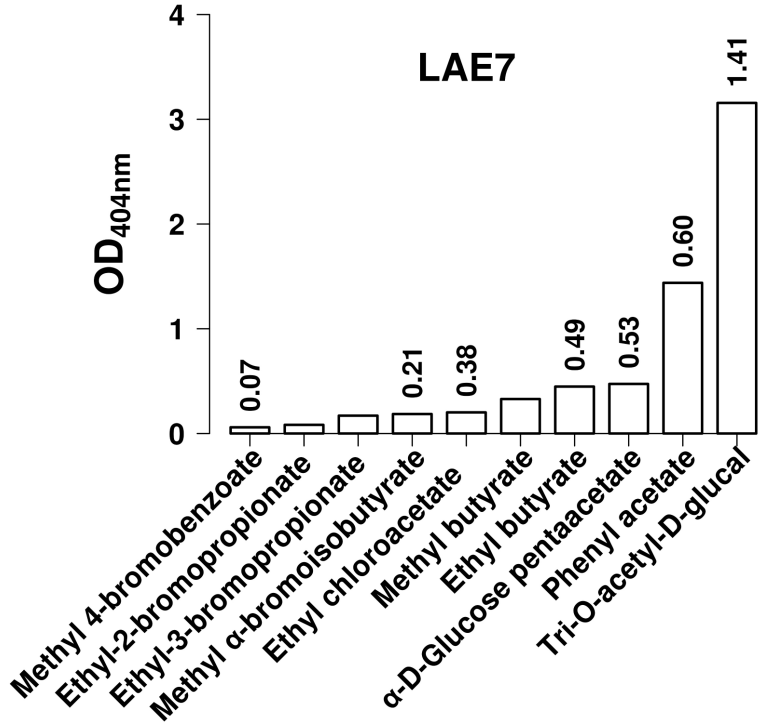
FIG 4 Clustering of substrate spectrum profile of Lake Arreo enzymes and commercial preparations applying the Pearson's correlation to calculate the distances. Hierarchical clustering was based on binomial distribution based on the presence or absence of activity against particular substrates (activity data not shown) measured as described in Table 1 using 2 μg protein extracts.

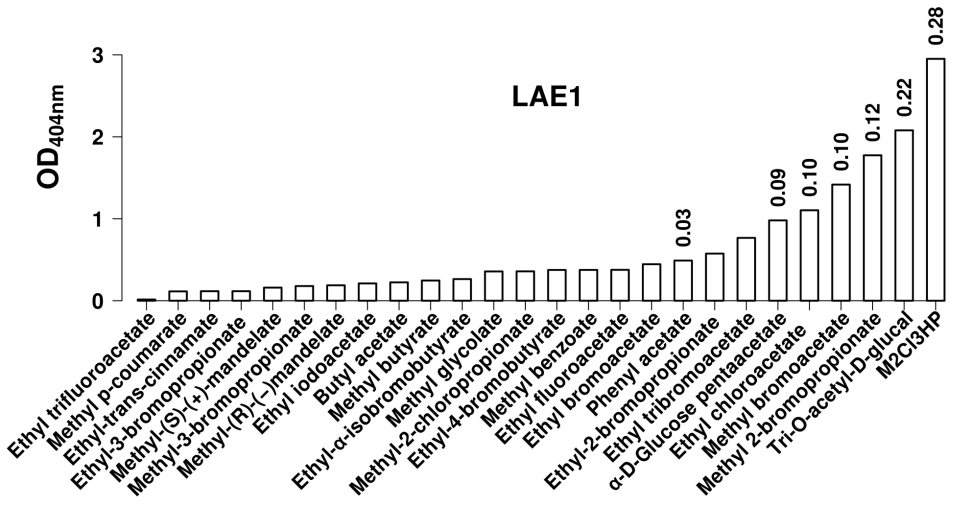
FIG 5 Dendrogam of protein sequence similarity relationships between newly identified and reference esterases/lipases. Enzyme families are depicted, according to the Arpigny and Jaeger classification (3).

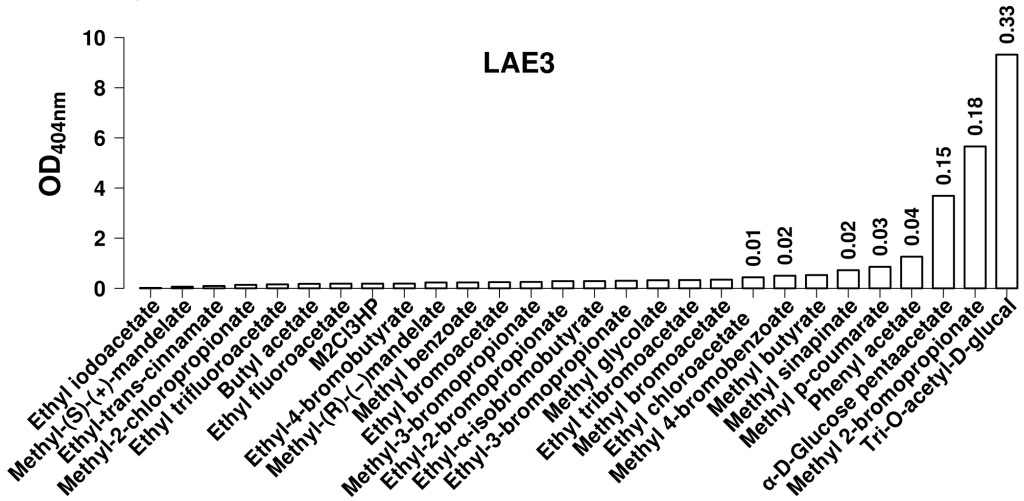

LAE7
LAE6
LAE5
LAE4
LAE3
LAE2
LAE1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.8
1.0
1.2
NaCl concentration (M)
100
90
80
70
60
50
40
30
20
LAE7
LAE6
LAE5
LAE4
LAE3
LAE2
LAE1
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH
100
80
60
40
20
0
LAE7
LAE6
LAE5
LAE4
LAE3
LAE2
LAE1
4
8
12
16
20
25
30
35
40
45
50
55
60
65
70
Temperature (ºC)
100
80
60
40
20

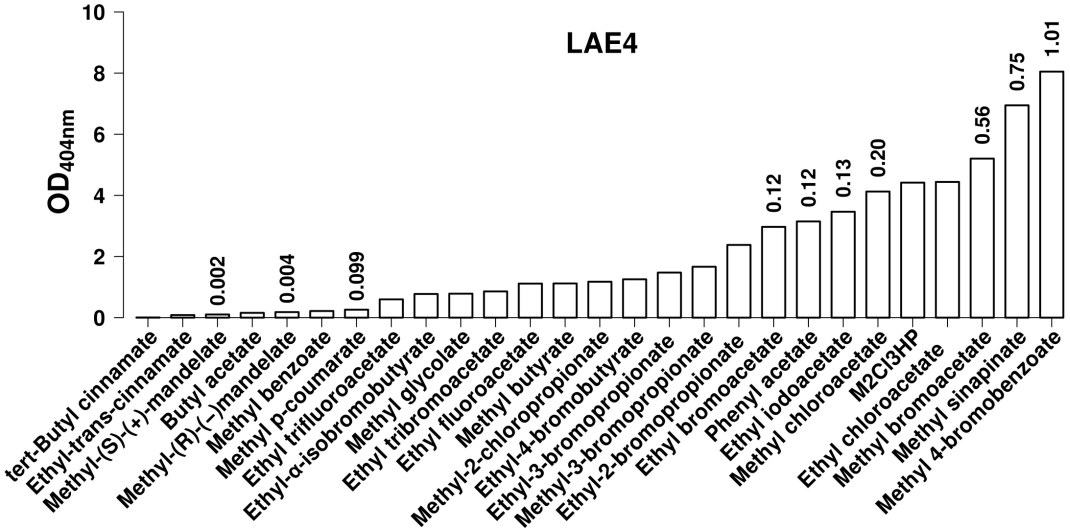

LAE2
OD404nm
0
1
2
3
4
0.02
0.02
0.04
0.29
0.39
Ethyl chloroacetate
Ethyl-3-bromopropionate
Tri-O-acetyl-D-glucal
α-D-Glucose pentaacetate
Methyl butyrate
Methyl 4-bromobenzoate
Phenyl acetate

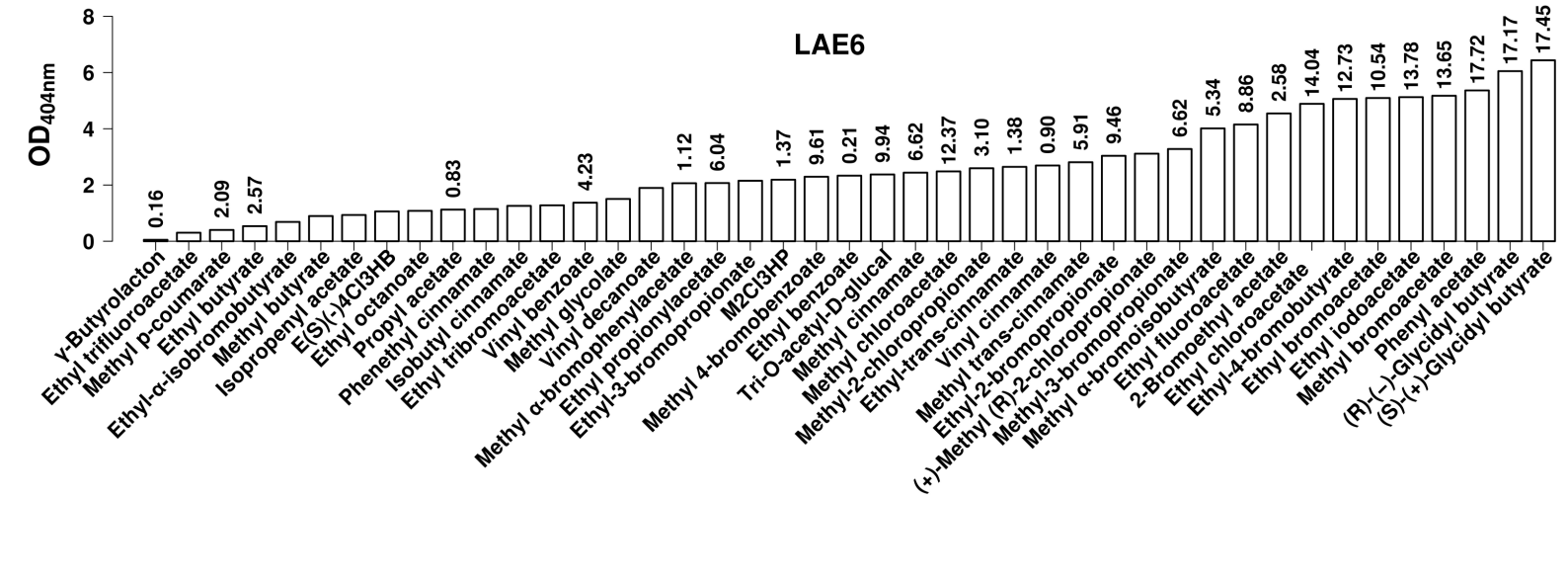

LAE5
OD404nm
0
2
4
6
8
10
12
0.38
0.40
0.43
0.47
0.60
Methyl bromoacetate
Propyl acetate
Isopropenyl acetate
Vinyl cinnamate
Ethyl-3-bromopropionate
Methyl butyrate
Methyl sinapinate
Ethyl chloroacetate
2-Bromoethyl acetate
Phenyl acetate
Geranyl acetate
Methyl 4-bromobenzoate
Tri-O-acetyl-D-glucal
α-D-Glucose pentaacetate


LAE7
OD404nm
0
1
2
3
4
0.07
0.21
0.38
0.49
0.53
0.60
1.41
Methyl 4-bromobenzoate
Ethyl-2-bromopropionate
Ethyl-3-bromopropionate
Methyl α-bromoisobutyrate
Ethyl chloroacetate
Methyl butyrate
Ethyl butyrate
α-D-Glucose pentaacetate
Phenyl acetate
Tri-O-acetyl-D-glucal


LAE1
OD404nm
0
1
2
3
0.03
0.09
0.10
0.10
0.12
0.22
0.28
Ethyl trifluoroacetate
Methyl p-coumarate
Ethyl-trans-cinnamate
Ethyl-3-bromopropionate
Methyl-(S)-(+)-mandelate
Methyl-3-bromopropionate
Methyl-(R)-(−)-mandelate
Ethyl iodoacetate
Butyl acetate
Methyl butyrate
Ethyl-α-isobromobutyrate
Methyl glycolate
Methyl-2-chloropropionate
Ethyl-4-bromobutyrate
Methyl benzoate
Ethyl fluoroacetate
Ethyl bromoacetate
Phenyl acetate
Ethyl-2-bromopropionate
α-D-Glucose pentaacetate
Ethyl tribromoacetate
Ethyl chloroacetate
Methyl bromoacetate
Methyl 2-bromopropionate
Tri-O-acetyl-D-glucal
M2Cl3HP


LAE3
OD404nm
0
2
4
6
8
10
0.01
0.02
0.02
0.03
0.04
0.15
0.18
0.33
Ethyl iodoacetate
Methyl-(S)-(+)-mandelate
Ethyl-trans-cinnamate
Methyl-2-chloropropionate
Ethyl trifluoroacetate
Butyl acetate
Ethyl fluoroacetate
M2Cl3HP
Ethyl-4-bromobutyrate
Methyl-(R)-(−)-mandelate
Methyl benzoate
Ethyl bromoacetate
Methyl-3-bromopropionate
Ethyl-2-bromopropionate
Ethyl-α-isobromobutyrate
Ethyl-3-bromopropionate
Methyl glycolate
Ethyl tribromoacetate
Methyl bromoacetate
Ethyl chloroacetate
Methyl 4-bromobenzoate
Methyl butyrate
Methyl sinapinate
Methyl p-coumarate
Phenyl acetate
α-D-Glucose pentaacetate
Methyl 2-bromopropionate
Tri-O-acetyl-D-glucal


LAE4
OD404nm
0
2
4
6
8
10
0.002
0.004
0.099
0.12
0.12
0.13
0.20
0.56
0.75
1.01
tert-Butyl cinnamate
Ethyl-trans-cinnamate
Methyl-(S)-(+)-mandelate
Butyl acetate
Methyl-(R)-(−)-mandelate
Methyl benzoate
Methyl p-coumarate
Ethyl trifluoroacetate
Ethyl-α-isobromobutyrate
Methyl glycolate
Ethyl tribromoacetate
Ethyl fluoroacetate
Methyl butyrate
Methyl-2-chloropropionate
Ethyl-4-bromobutyrate
Ethyl-3-bromopropionate
Methyl-3-bromopropionate
Ethyl-2-bromopropionate
Ethyl bromoacetate
Phenyl acetate
Ethyl iodoacetate
Methyl chloroacetate
M2Cl3HP
Ethyl chloroacetate
Methyl bromoacetate
Methyl sinapinate
Methyl 4-bromobenzoate


LAE6
OD404nm
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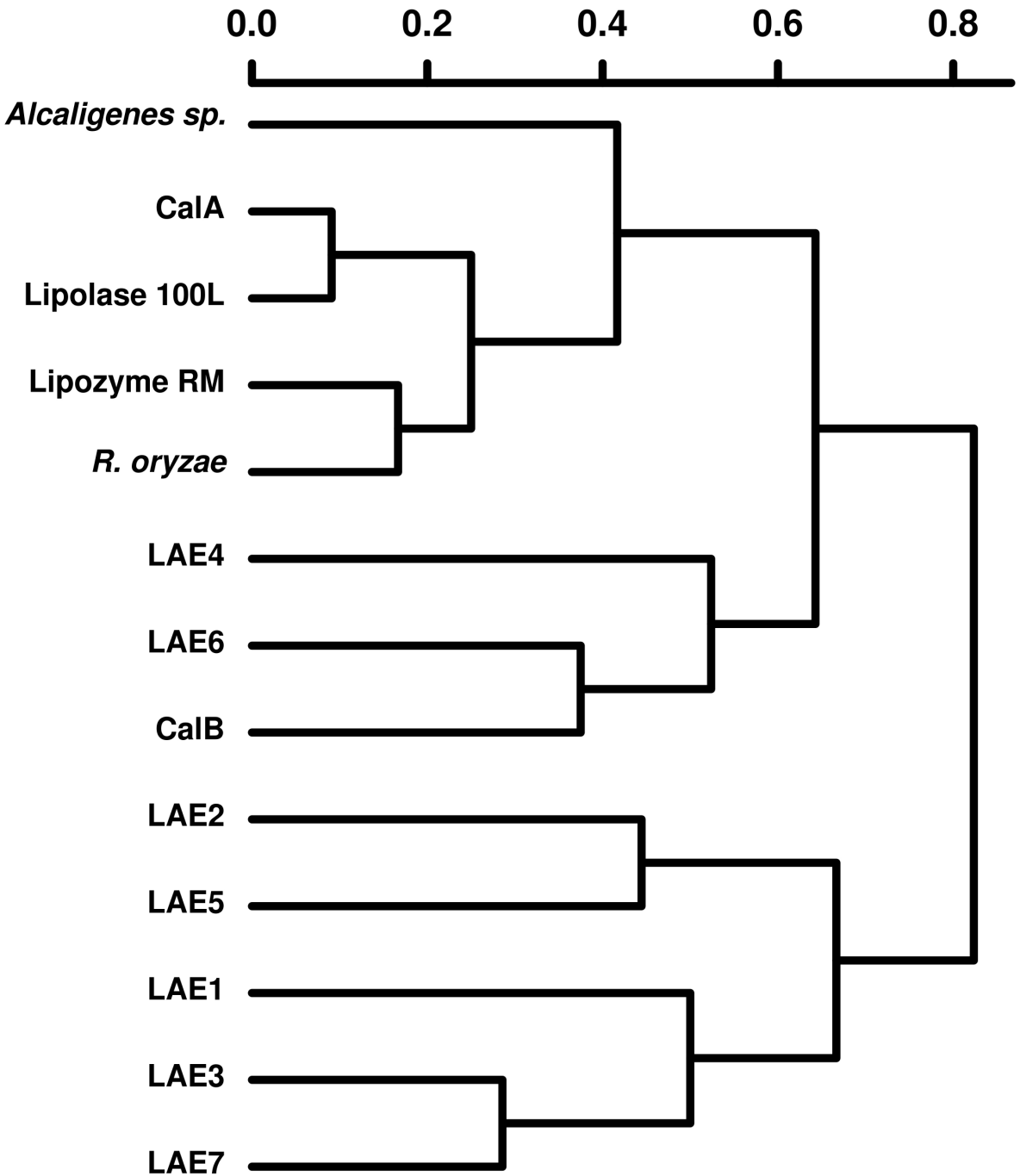
2
4
6
8
0.16
2.09
2.57
0.83
4.23
1.12
6.04
1.37
9.61
0.21
9.94
6.62
12.37
3.10
1.38
0.90
5.91
9.46
6.62
5.34
8.86
2.58
14.04
12.73
10.54
13.78
13.65
17.72
17.17
17.45
γ-Butyrolacton
Ethyl trifluoroacetate
Methyl p-coumarate
Ethyl butyrate
Ethyl-α-isobromobutyrate
Methyl butyrate
Isopropenyl acetate
E(S)(-)4Cl3HB
Ethyl octanoate
Propyl acetate
Phenethyl cinnamate
Isobutyl cinnamate
Ethyl tribromoacetate
Vinyl benzoate
Methyl glycolate
Vinyl decanoate
Methyl α-bromophenylacetate
Ethyl propionylacetate
Ethyl-3-bromopropionate
M2Cl3HP
Methyl 4-bromobenzoate
Ethyl benzoate
Tri-O-acetyl-D-glucal
Methyl cinnamate
Methyl chloroacetate
Methyl-2-chloropropionate
Ethyl-trans-cinnamate
Vinyl cinnamate
Methyl trans-cinnamate
Ethyl-2-bromopropionate
(+)-Methyl (R)-2-chloropropionate
Methyl-3-bromopropionate
Methyl α-bromoisobutyrate
Ethyl fluoroacetate
2-Bromoethyl acetate
Ethyl chloroacetate
Ethyl-4-bromobutyrate
Ethyl bromoacetate
Ethyl iodoacetate
Methyl bromoacetate
Phenyl acetate
(R)-(−)-Glycidyl butyrate
(S)-(+)-Glycidyl butyrate


0.0
0.2
0.4
0.6
0.8
Alcaligenes sp.
CalA
Lipolase 100L
Lipozyme RM
R. oryzae
LAE4
LAE6
CalB
LAE2
LAE5
LAE1
LAE3
LAE7

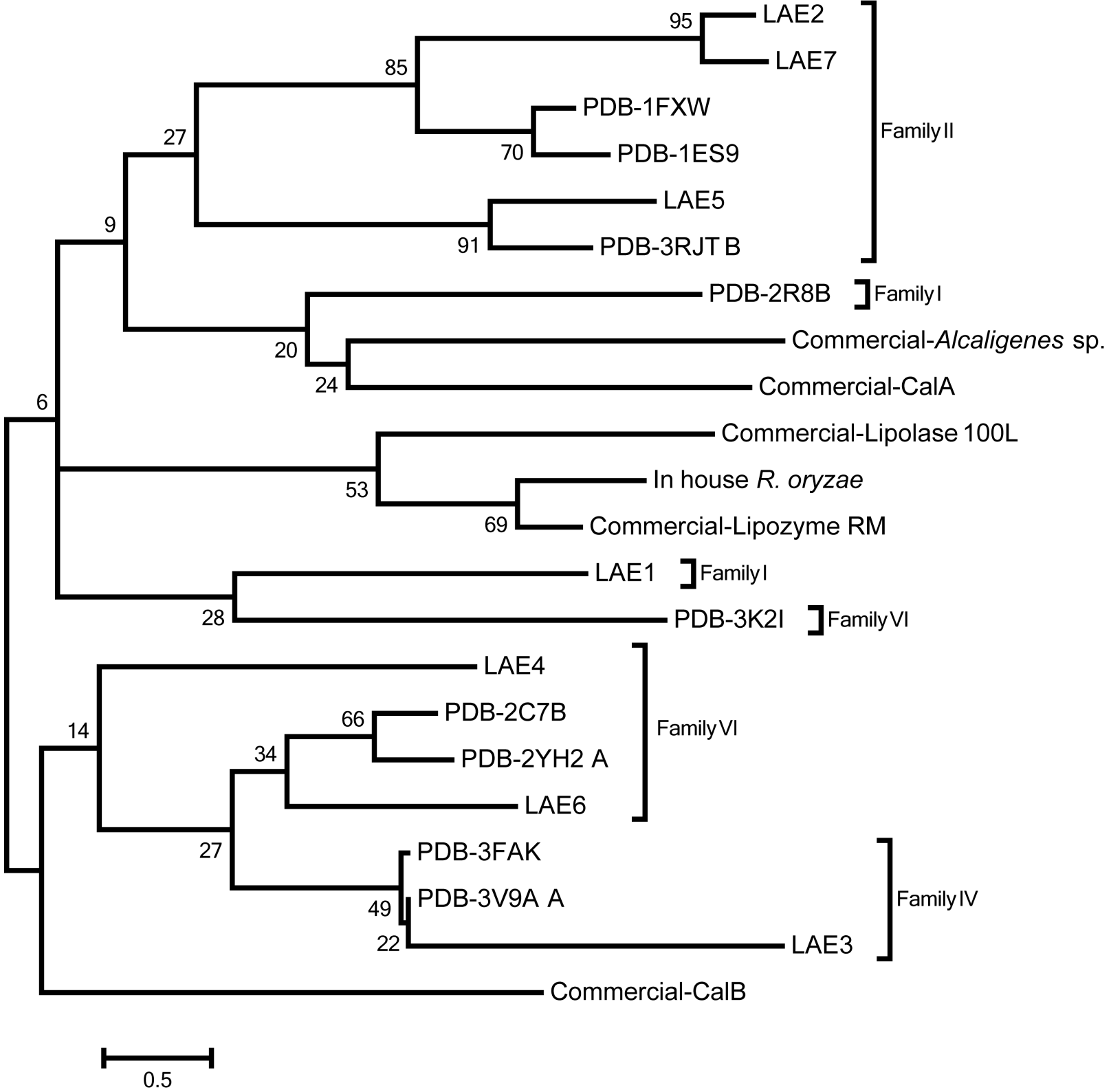

95
LAE2
LAE7
85
PDB-1FXW
27
70
PDB-1ES9
Family II
LAE5
9
91
PDB-3RJT B
PDB-2R8B
Family I
Commercial-Alcaligenes sp.
20
24
Commercial-CalA
6
Commercial-Lipolase 100L
In house R. oryzae
53
69
Commercial-Lipozyme RM
LAE1
Family I
28
PDB-3K2I
Family VI
LAE4
66
PDB-2C7B
14
Family VI
34
PDB-2YH2 A
LAE6
27
PDB-3FAK
PDB-3V9A A
49
Family IV
22
LAE3
Commercial-CalB
0.5

TABLE 1 Specific activities of the wild-type α/β hydrolases from Lake Arreo against a set of model α-naphtyl, *p*NP and triacylglycerol ester substrates.

	Activity (units/mg)[1]						
	LAE1	**LAE2**	**LAE3**	**LAE4**	**LAE5**	**LAE6**	**LAE7**
α-Naphthyl acetate	n.d	111.04	n.d	1.63	83.36	124.8	99.22
α-Naphthyl propionate	3.58	16.01	n.d	8.34	24.35	55.1	11.73
α-Naphthyl butyrate	3.36	0.92	0.74	3.93	1.23	28.3	1.21
***p*NP acetate**	0.133	5.57	23.80	0.05	1.23	34.3	0.64
***p*NP propionate**	0.365	0.29	0.84	0.15	2.05	135.5	0.13
***p*NP butyrate**	1.072	0.27	0.29	0.11	0.01	105.2	0.02
***p*NP octanoate**	0.129	n.d	0.04	n.d	n.d	2.6	n.d
***p*NP decanoate**	0.028	n.d	0.02	n.d	n.d	1.0	n.d
***p*NP laurate**	n.d	n.d	n.d	n.d	n.d	0.2	n.d
Triacetin	0.015	1.07	0.01	1.01	1.14	18.7	1.19
Tripropionin	0.080	n.d	0.01	2.33	0.02	14.9	0.39
Tributyrin	0.005	n.d	0.09	0.83	0.00	11.3	0.14
Tricaprin	0.001	n.d	n.d	0.06	0.00	5.5	n.d
Tricaprylin	n.d	n.d	n.d	n.d	n.d	3.4	n.d
Trilaurin	n.d	n.d	n.d	n.d	n.d	n.d	n.d

[1]For α-naphtyl and triacylglycerol esters, reactions contained 2 μg pure enzyme (lower amount of enzyme for LAE6 (0.4 μg) were used), 2 mM substrate, 0.45 mM *p*-nitrophenol and 2.5% acetonitrile (v/v) in 2 mM BES buffer (pH 7.2), at 30°C, in a total volume of 150 μl. For *p*NP esters, reactions contained same amount of enzymes and 0.8 mM *p*NP ester in 20 mM HEPES buffer (pH 7.0), at 30°C, in a total volume of 190 μl. In all cases, the reactions were followed every 2 min during 15 min and the absorbance of *p*-nitrophenol at 410 (for *p*NP esters) or 404 nm (for other substrates rather than pNP esters) was measured. In all cases, one unit (U) of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of substrate in 1 min under the assay conditions. Three independent experiments were performed for each parameter and mean values are given; the standard deviation was lower than 5%.

n.d. No activity detected under experimental conditions assayed.