Supporting Information

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Conversion of a carboxylesterase into a triacylglycerol lipase by random mutation**

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SUPPORTING EXPERIMENTAL SECTION

Reagents, strains and buffers: p-NP esters, triacylglycerols, Fast Blue RR, α-naphthyl acetate (αNA) and laurate (αNL), phenyl methyl sulphonyl fluoride (PMSF) and 1,3-dipalmitoyl-2-oleyl-glycerol, 1,3-dipalmitoyl-glycerol, 1-palmitoyl-glycerol and ethyl palmitate and ethyl oleate (for reactions and calibrations), were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Capryl-, lauryl- and palmitoyl-sulphonyl fluorides were synthesized as described by Deutsch et al.[1]

Sepabeds EC-EP3 (Batch No. SY1P8/39) was kindly provided by Resindion S.R.L (Mitsubishi Chemical Corporation, Milan, Italy). Molecular mass markers for SDS-PAGE were obtained from Novagen (Madison, Wisconsin, USA). Restriction and modifying enzymes were from New England Biolabs (Beverly, Massachusetts, USA). DNase I grade II was from Boehringer Mannheim (Mannheim, Germany). Chromatographic media and molecular markers for native electrophoresis, were from Amersham Pharmacia Biotech (Little Chalfont, UK). E. coli strains XL1-Blue MRF’ (for library construction and screening), XLOLR (for expression of the esterase from phagemid) (both-Stratagene; La Jolla, California, USA), and TOP 10 (for mutagenesis and expression of mutant esterases) (Invitrogen; Carlsbad, CA, USA), were maintained and cultivated according to the recommendations of suppliers and standard protocols described elsewhere.[2] Unlike otherwise indicated, the standard buffer used in the present study was 100 mM HEPES buffer, pH 7.5. DNA manipulations were according to Sambrook et al.[2] and according to manufacturer’s instructions for the enzymes and materials employed.

PCR mutagenesis: Error-prone PCR mutagenesis was carried out using Taq polymerase (Sigma Chemical Co.) and the phagemid pBKR.34 as template.[3] The reaction was performed in 50-µl mixture containing 3% dimethyl sulfoxide (DMSO), 5 µM MnCl₂, 1.5 mM MgCl₂, 0.3 mM dNTP’s, 2.5 U Taq polymerase, 5 ng template and 4.5 pmol of oligonucleotides. The concentration of template and MnCl₂ were adjusted to achieve a mutation rate from 1 to 3 mutations per kb. The amplification program was as follows: 2 min at 95 ºC, 27 seg at 94 ºC, 27 seg at 53 ºC, followed by 28 cycles of 3 min at 74 ºC, and 10 min at 74 ºC. Primers sequences were as follows: OligF sense (5’-CCT ATC CCT ATA CCA TTG C-3’) and OligR antisense (5’- CCG TCC ATA TAA TAC TTC AGG -3’). The amplified PCR products were purified from a 0.75% agarose gel using QIAEX II gel extraction kit from Qiagen, cloned into plasmid pCR2.1 (Invitrogen) and transformed into E. coli TOP10 (Invitrogen) as recommended by the supplier, and the resulting transformants plated onto fresh LB-
plate containing 50 µg/ml kanamycin. Plates were incubated for 12 h at 37 °C and then the plates were covered with a second layer containing the substrate (20 ml HEPES 50 mM, pH 7.5, 0.4% agarose, 320 µl of Fast Blue RR solution in DMSO [80 mg/ml] and 320 µl of αNL solution in acetone [20 mg/ml]). Positive clones appeared due to the formation of a brown precipitate. Using these conditions E. coli colonies expressing wild type R.34 produced no brown zones on αNL-plates (Figure S1).

Positive transformant of E. coli was pooled and the plasmid DNA was isolated using a QIAprep spin miniprep kit (Qiagen). To determine the biochemical properties of the R.34 and EL1 variant, the gene corresponding to the full-length protein was amplified and produced as fusion with a hexahistidine (6His) tag at the C-terminus as described below.

Expression and purification of enzyme variants: To determine the biochemical properties of the R.34 and EL1 variant, the gene corresponding to the full-length protein was amplified and produced as fusion with a hexahistidine His6 tag at the C-terminus (R.34His or EL1His) as follows. The esterase-encoding gene was amplified from pBKR.34 or pCREL1 plasmids, by PCR with oligonucleotide primers designated Mut34FpCR sense: 5´-CCT ATC CCT ATA CCA TTG CTT-3' and Mut34RpCR antisense: 5´- TTT AGT GGT GGT GGT GGT GCT TGA TCC TGA TCT TTT TCC CGG T-3’. Reactions were carried out in a total volume of 50 µL and were catalyzed by 25 U of Taq polymerase (Qiagen). The amplification program was as follows: 1 min 94 °C followed by 25 cycles of 20 seg 94 °C, 60 seg 40 °C, 1 min 72°C; the final elongation step was 5 min 72 °C and 15 min 10 °C. The amplified fragments, purified from a 0.75% agarose gel, were cloned into plasmid pCR2.1 (Invitrogen) and electroporated into E. coli TOP10 (Invitrogen) as recommended by the supplier. E. coli TOP10 transformed with the expression plasmids (pCRR.34His and pCREL1His) were grown overnight at 37°C in Luria–Bertani medium supplemented with 50 µg/ml kanamycin. Isopropyl thiogalactoside (IPTG) was added to a concentration of 1 mM and cultivation was continued for an additional 4 h. Cells were collected by centrifugation (30 min, 8000 g, 4 °C) and resuspended in 20 mM NaH2PO4 pH 7.4, 150 mM NaCl and 20 mM imidazole. After the addition of lysozyme (1 mg/ml), the suspension was incubated on ice for 30 min and then sonicated four times for 30 s. The cell lysate was centrifuged for 20 min at 4°C (25000 g). The His6-tagged enzyme was purified at 4°C on HisTrap HP column (Amersham Pharmacia Biotech; Little Chalfont, UK). After washing with 4 mL of 20 mM NaH2PO4 pH 7.4, 150 mM NaCl and 20 mM imidazole, the recombinant enzyme was eluted at pH 7.4 with 10 mM of 20 mM NaH2PO4, 150 mM NaCl and 500 mM imidazole. Purification of the recombinant proteins was monitored spectrophotometrically following the increase in absorbance at 405 nm due to hydrolysis of p-NP propionate. About 2.2 and 3.1 mg of pure recombinant proteins (R.34 and EL1, respectively) per g wet weight cells were recovered by a one-step purification method involving metal-chelating chromatography (Figure S2).
**Protein characterization:** SDS PAGE was performed using 12\% (v/v) acrylamide gels according to Laemmli.[4] The protein concentration was determined according to Bradford[5] with BSA as the standard.

Esterase activity was determined spectrophotometrically following the increase in absorbance at 410 nm at 40 °C, using p-NP esters as substrates, as described by Ferrer et al.,[6] although with small modifications. Briefly, the reaction mixture (3 ml) contained 0.2 mM p-NP esters with 0.2\% (w/v) arabic gum and 5 µg/ml enzyme. p-NP propionate was the esterase substrate for activity determination, if not otherwise stated. Standard assays were performed in 100 mM HEPES buffer, pH 7.5. The absorption coefficients were measured at each indicated temperature and pH and ranged over 14000 – 21000 M⁻¹·cm⁻¹. Lipase activity was determined at 40 °C in a pH-stat assay[7] by titrating fatty acids released from triacylglycerols ranging from triacetin to triolein, with 0.1 M sodium hydroxide in a pH-stat Mettler Toledo (model DL50-Graphix) (Metrohm). The reaction mixture (20 ml of 1 mM Tris-HCl, pH 8.0) contained emulsions of 80 mM triacylglycerols (C₂–C₄) or 40 mM (C₆–C₁₈:1) with 0.2\% (w/v) arabic gum and 5 µg/ml enzyme. Tripropionin was the lipase substrate for activity determination, if not otherwise stated. In all cases, one unit of enzyme is defined as the amount of enzyme liberating 1 µmol product per min, under experimental conditions. All values were determined in triplicate and were corrected considering the autohydrolysis of the substrate.

**Lipase immobilization:** EL1 enzyme was immobilized on Sepabeds EC-EP3, a polymethacrylate-based carrier. EC-EP3 is a epoxy-activated resin with a high reactive group density.[8] Protein immobilization was carried out as described by Ghazi et al.,[8] although with small modifications. Briefly, 7.5 ml of 0.5 M sodium phosphate, pH 8.0 containing 10 mg pure EL1 protein and Sepabeads EC-EP3 (1.5 g) were mixed and incubated for 72 h at 4 °C with roller shaking. The biocatalyst was then filtered using a glass filter (Whatman), washed (3 x 10 ml) with 0.5 M sodium phosphate, pH 8.0, dried under vacuum and stored at 4 °C until use. The hydrolytic activity of the immobilized lipase was 89 U/mg, as determined in the pH-stat assay using triolein as substrate.

**Transesterification of 1,3-dipalmitoyl-2-oleyl-glycerol (POP):** Transesterification reaction were performed as described by Schmid et al.,[9] although with small modifications. Briefly, POP (47 mg, 0.056 mmol) was dissolved in 1 ml 2-methyl-2-butanol adjusted to a water activity (a_w) of 0.22 in a closed vessel. Dry ethanol (27 mg, 0.58 mmol) was added and the mixture incubated at 30 °C with orbital shaking at 1000 rpm. The mixture was allow to equilibrate for 5 min, after which 5 mg of immobilized EL1 lipase (a_w= 0.22) were added. Water activity was determined using a humidity and temperature digital indicator Novasina Thermoconstanter TH200 (Novasina, Switzerland). The humidity sensor was calibrated with control saturated salts solutions (LiCl, a_w=0.11; potassium acetate, a_w=0.22; NaBr, a_w=0.57; NaCl, a_w=0.75) at 25 °C. All solvents were stored over molecular sieves (3 Å) previously heated 16 h at 250°C, at least 24 h prior to use. The a_w for both solvent and
enzyme preparation were optimized prior to the assay, being the optimal 0.22 (not shown). Other solvents such as acetone, methyl-\textit{t}-butylether, hexane and iso-octane were tested; however, the conversion was unsatisfactory under the same reaction conditions used for 2-methyl-2-butanol (conversion: ≤ 48%).

Aliquots were taken at intervals over a 240 min period, centrifuged and filtered using Ultrafree-MC filter (0.45 µm) devices (Millipore). Substrate-product (from mono- to triglycerides) analyses were carried out by HPLC (model Varian 9012) using a Nucleosil 100-C18 column (250 x 4.6 mm) (Análisis Vínicos, Tomelloso, Spain) and methanol:acetone as the mobile phase. The conditions of the analysis were: methanol:water 95:5, 1.2 ml/min, 6 min; increase to methanol 100%, 1.2 ml/min, 1 min; held methanol 100%, 1.7 ml/min, 2 min; goes to methanol:acetone 50:50, 1.7 ml/min, 2 min; held methanol:acetone 50:50, 1.7 ml/min, 6 min; increase to methanol 100%, 1.7 ml/min, 2 min; held methanol 100%, 1.2 ml/min, 1 min; methanol:water 95:5, 1.2 ml/min, 1 min; methanol:water 95:5, 1.2 ml/min, 1 min. The column was kept constant at 45 °C. Integration was carried out using the Millennium software. Evaporative light-scattering detector (Alltech) was used for the analyses. Calibration analyses were performed using pure standards provided by Sigma Chemical Co. Fatty acid ethyl esters (FAEEs) were analyzed with a gas chromatograph (GC, model Varian 3400CX, Varian Chromatography Systems, Sugar Land, TX), equipped with a 30 m x 0.25 mm HP-5 (5% diphenyl and 95% dimethylpolysiloxane) fused silica capillary column and flame-ionization detector (FID); flow rate 1 ml/min; sample input temperature from 230 °C (2 min) to 360 °C, at a rate of 8 °C/min. Hydrogen was used as carrier gas.

Site-directed mutagenesis: R.34 and EL1 esterase mutants were prepared using a QuikChange XL site-directed mutagenesis kit (Stratagene), according to the vendor’s instructions. The oligonucleotides used for mutagenesis were as follows. R49D: 5’- GCC TCA AGA TAT TCG ACG CAC CTG ATG ACA AGG -3’ and 5’- CCT TGT CAT CAG GTG CGT CGA ATA TCT TGA GGC -3’; R49N: 5’- GCC TCA AGA TAT TCA ACG CAC CTG ATG ACA AGG -3’ and 5’- CCT TGT CAT CAG GTG CGT TGA ATA TCT TGA GGC -3’; N33R: 5’- CGA AGT CGG CGT GCC CAG GGT CAG CAA CAT CAA GC-3’ and 5’- GCT TGA TGT TGC TGA CCC TGG GCA CGC CGA CTT CG-3’. R.34 and EL1-derived plasmids containing mutations were introduced into \textit{E. coli} TOP10 by electroporation.

DNA sequencing: Plasmids containing mutant genes were sequenced at the Sequencing Core Facility of the Instituto de Investigaciones Biomédicas (CSIC, Madrid) using an Applied Biosystems 377 automated fluorescent DNA sequencer. The primers used were as follows. F1: 5’-AAC AAC AAG GCC TTC CTG CGC-3’, F2: 5’-TGG GCG TGC TTA CCT ACA CCG-3’, and F3: 5’-ACA TCT GCT GGG CAG ACA ACG-3’.
**Sequence alignment:** Multiple sequence alignments of protein homologues to R.34 esterase were generated by GenTHREADER\[10\] using the following hydrolase sequences: beta-1,4-D-xylanase from *Butyrivibrio fibrisolvens* (accession number X61495.1), acetyl esterase family enzyme from *Clostridium acetobutylicum* ATCC 824 (accession number NC_003030.1), EST2 – esterase from *A. acidocaldarius*\[11\] (PDB accession code 1EVQA) and R.34 (R.34, this work).\[15\] The alignment featuring the highest score was obtained using the Blosum matrix\[12\] and standard CLUSTALX parameters.

**Molecular modelling:** The structure of esterase EST2 from *A. acidocaldarius* was chosen as the most suitable template to generate a model for R.34. The degree of sequence identity between these two proteins is 19%. Model coordinates were obtained from the Swiss-Model server\[13,14\] and analysed with Swiss-PDB Viewer program.\[14\] Ramachandran plots of both model and template proteins (included as supplementary information) were obtained to assess the overall stereochemical quality of the model. The model is not reliable at the N-terminal part of the structure (firsts 32 residues of esterase R.34) where sequence similarity between model and template is very low. However, the results of threading\[10\] indicate a consistent structural similarity in the region starting at residue 33.

**Nucleotide sequence accession numbers:** Mined gene coding for R.34 esterase has been submitted to the EMBL/DDBJ/GenBank databases under accession number AM050329.


**Supporting Figures**

**Figure S1.** Esterase-lipase phenotype of *E. coli* TOP10 cells expressing EL1 improved variant (left, A) and wild type R.34 (right, A) (both in PCR2.1 cloning vector). Cells were plated onto fresh LB-plate containing kanamycin (50 µg/ml). Plates were incubated for 12 h at 37 ºC and then the plates were covered with a second layer containing the substrate (20 ml 50 mM HEPES, pH 7.5, 0.4 % agarose, 320 µl of Fast Blue RR solution in DMSO [80 mg/ml] and 320 µl of αNL or αNA solution in acetone [20 mg/ml]). Positive clones appeared due to the formation of a brown precipitate. As can be seen, *E. coli* colonies expressing wild type R.34 produced no brown zones on αNL-plates (A), but it produced on αNA-plates (B). In contrast, EL1 produced brown zones on both αNA- and αNL-plates.
**Figure S2.** SDS-PAGE of the purified R.34 and EL1 proteins. Samples were loaded as follows: MW, molecular mass markers (15-100 kDa, Novagen); lane 1, crude extract of *E. coli* TOP10 harboring PCRR.34<sub>His</sub> after induction with IPTG; lane 2, crude extract of *E. coli* TOP10 harboring PCREL1<sub>His</sub> after induction with IPTG; lane 3, purified R.34 with His<sub>6</sub> tag at the C-terminus; lane 4, purified EL1 with His<sub>6</sub> tag at the C-terminus.
Figure S3. Effect of pH (A) and temperature (B) on R.34 (——) and EL1 (-----) enzymes. The optimal pH and temperature for enzyme activity was monitored spectrophotometrically following the increase in absorbance at 410 nm due to hydrolysis of p-NP propionate. The buffers (100 mM) used were: citrate (pH 5.0-5.5: ●), MES (pH 5.5-7.0: ▲), HEPES (pH 7.0-8.0: ■), Tris-HCl (pH 8.0-9.0: ○) and glycine-NaOH (pH 9.0-12: ▲). The activity:pH and activity:temperature relationships were determined by incubating the enzyme:substrate mixtures at different pH values and constant temperature (40 °C), and at different temperatures (15-80 °C) and constant pH (7.5), respectively.
Figure S4. Sequence alignment of R.34 esterase and other xylanases and esterases. Source organisms and accession numbers are as follows: R.34 (this work); B.fib- beta-1,4-D-xylanase from *Butyrivibrio fibrisolvens* (accession number X61495.1); C.ace- acetyl esterase family enzyme from *Clostridium acetobutylicum* ATCC 824 (accession number NC_003030.1); EST2 – esterase from *A. acidocaldarius* (PDB Acc. number 1EVQA; crystal structure resolved). The amino-acid sequence shares 22, 25 and 19% identity to the sequences of beta-1,4-D-xylanase from *B. fibrisolvens*, acetyl esterase family enzyme from *C. acetobutylicum* and esterase from *A. acidocaldarius*. Esterases/lipases typically have a Ser-Asp-His catalytic triad where the active site serine is located within the middle of the conserved consensus GXSXG or GDS(L) motifs [J.L. Arpigny, K.E. Jaeger. *Biochem. J.* 1999, 343, 177-183]. R.34 contained in its sequence the motif GDS(L), which is typical for family II of ester hydrolases. Sequence inspection allowed the identification of residues Ser137, Asp215 and His247 as the catalytic residues (shown in asterisk). Mutated residue is shown by an arrow (▲).

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<th>Source Organism</th>
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**Figure S5.** Analysis of reaction products (mono- to triglycerides). (A) HPLC chromatograms of reaction products (from mono to triglycerides). (B) Time course of the transesterification reaction of POP with ethanol. Conditions as described in Supporting Experimental Section. Initial conditions: 0.056 mmol of POP, 0.58 mmol ethanol, 5 mg of immobilized EL1 lipase ($a_w=0.22$), 30 ºC, 1 ml 2-methyl-2-butanol.
Figure S6. Esterase-lipase phenotype of *E. coli* TOP10 cells expressing R.34, EL1 and EL1 variants containing single R49D and R49N mutations (all in pCR.2.1 cloning vector). Cells were plated onto fresh LB-plate containing 50 µg/ml kanamycin. Culture and screening conditions as in Figure S1. As can be seen, *E. coli* colonies expressing wild type R.34, EL1_{R49D} and EL1_{R49N} produced no brown zones on αNL-plates (A), but they produce on αNA-plates (B).
**Figure S7.** Esterase-lipase phenotype of *E. coli* TOP10 cells expressing R.34 wild type, EL1 and R.34 variant with the reverse mutation (N33R-R49D). Culture and screening conditions as in Figure S1. As can be seen, *E. coli* colonies expressing only EL1 and the double mutant N33R-R49D produced brown zones on αNL-plates (A); however, all were found to be positive on αNA-plates (B).
**Figure S8.** Identification and quantification of lipase activity on rhodamine B agar plates using *E. coli* TOP10 cells expressing R.34 wild type, EL1 and mutant variants. All mutants were cloned into pCR.2.1 cloning vector. Cells were plated onto fresh LB-plate containing 50 µg/ml kanamycin, 0.001% rhodamine B and 3% triolein prepared as described [G. Kouker, K.-E. Jaeger, *Appl. Environ. Microbiol.* 1987, 53, 211-213]. Plates were incubated for 12 h at 37 °C and then subjected to UV irradiation (350 nm) and photographed. Positive lipase clones appeared due to the formation of orange fluorescent halo. As can be seen, only *E. coli* colonies expressing EL1 (containing N33D mutation) and the R.34 variant with the reverse mutation N33R-R49D produced halo zones on rhodamine-triolein plates.
RAMPAGE by Paul de Bakker and Simon Lovell available at http://www-cryst.bioc.cam.ac.uk/rampage/


Number of residues in favoured region (~98.0% expected) : 235 (86.7%)
Number of residues in allowed region (~2.0% expected) : 27 (10.0%)
Number of residues in outlier region : 9 (3.3%)
General Favored: 235 (86.7%)
General Allowed: 27 (10.0%)
Pre-Pro Favored: 9 (3.3%)
Pre-Pro Allowed: 6 (2.0%)
Glycine Favored: 235 (86.7%)
Glycine Allowed: 27 (10.0%)
Proline Favored: 9 (3.3%)
Proline Allowed: 6 (2.0%)

RAMPAGE by Paul de Bakker and Simon Lovell available at http://www-cryst.bioc.cam.ac.uk/rampage/

Number of residues in favoured region (~98.0% expected) : 289  (94.4%)
Number of residues in allowed region (~2.0% expected) : 15  (4.9%)
Number of residues in outlier region : 2  (0.7%)

RAMPAGE by Paul de Bakker and Simon Lovell available at http://www-cryst.bioc.cam.ac.uk/rampage/
Structure validation by Cα geometry: φ/ψ and Cβ deviation. Proteins: Structure, Function & Genetics. 50: 437-450
General

A15 ASN

Glycine

Pre-Pro

Proline

Number of residues in favoured region (~98.0% expected) : 289 (94.4%)
Number of residues in allowed region (~2.0% expected) : 15 (4.9%)
Number of residues in outlier region : 2 (0.7%)

RAMPAGE by Paul de Bakker and Simon Lovell available at http://www-cryst.bioc.cam.ac.uk/rampage/