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Special *Rhodococcus* sp. CR-53 esterase Est4 contains a GGG (A)X-oxyanion hole conferring activity for the kinetic resolution of tertiary alcohols

Arnau Bassegoda · Amanda Fillat · F. I. Javier Pastor · Pilar Diaz

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Abstract Rhodococci are highly adaptable bacteria, capable to degrade or transform a large number of organic compounds, including recalcitrant or toxic products. However, little information is available on the lipases of the genus *Rhodococcus*, except for LipR, the first lipase isolated and described from strain *Rhodococcus* CR-53. Taking into consideration the interest raised by the enzymes produced by actinomycetes, a search for new putative lipases was performed in strain *Rhodococcus* CR-53. We describe here the isolation, cloning, and characterization of intracellular esterase Est4, a mesophilic enzyme showing preference for short-chain-length acyl groups, without interfacial activation. Est4 displays moderate thermal and pH stability and low tolerance to most tested ions, being inhibited by detergents like sodium dodecyl sulfate and Triton X-100®. Nevertheless, the enzyme shows good long-term stability when stored at 4–20 °C and neutral pH. Amino acid sequence analysis of Est4 revealed a protein of 313 amino acids without a signal peptide, bearing most of the conserved blocks that define bacterial lipase family IV, thus being assigned to this family. Detection of a GGG(A)X oxyanion hole in the enzyme motivated the evaluation of Est4 ability to convert tertiary alcohol esters. The newly discovered esterase Est4 from *Rhodococcus* CR-53 successfully hydrolyzed the tertiary alcohol esters linalyl acetate, terpinyl acetate, and 1,1,1-trifluoro-2-phenyl-but-3-yn-2-yl acetate.

Keywords *Rhodococcus* · Esterase · Tertiary alcohols · Enantioselectivity

A. Bassegoda · A. Fillat · F. I. J. Pastor · P. Diaz (✉)
Department of Microbiology, Faculty of Biology,
University of Barcelona, Av. Diagonal 643,
08028 Barcelona, Spain
e-mail: pdiaz@ub.edu

Introduction

Lipases and esterases (EC 3.1.1.) are glycerol ester hydrolases acting on acyl glycerols to liberate free fatty acids and glycerol. Microbial lipases constitute the most important group of biocatalysts for synthetic organic chemistry due to their high specificity, regio-, and enantioselectivity (Bornscheuer et al. 2002; Bornscheuer and Kazlauskas 2005; Gupta et al. 2004; Reetz 2002; Rosenau and Jaeger 2000; Vadell and Cavender 2007). Accordingly, there is a substantial interest in developing new lipases isolated from a large variety of microorganisms (Bofill et al. 2010; Falcocchio et al. 2005, 2006; Prim et al. 2000, 2001, 2006; Ruiz et al. 2002, 2003, 2007) or from special ecologic niches. Recently, esterase-mediated resolution of enantiomerically pure tertiary alcohols has gained interest due to their application as valuable building blocks for pharmaceuticals production, and to the harsh conditions required for their chemical synthesis (Bartsch et al. 2008; Elenkov et al. 2007; Henke et al. 2002; Kourist et al. 2007a, b, 2008). Structure and function studies revealed that the majority of esterases found to exhibit activity towards the sterically demanding tertiary alcohols bear GGG(A)X motifs in the oxyanion hole next to their active sites, whereas the inactive ones bear G(X) motifs (Bassegoda et al. 2010; Henke et al. 2002; Herter et al. 2011; Kourist et

al. 2008). Although reaction rates and enantioselectivities are quite low, which still makes industrial application rather inefficient, these enzymes are promising biocatalysts since the chemical stereoselective production of tertiary alcohols remains still a challenge (Rehdorf et al. 2012).

Although metagenomics can provide a new variety of enzymes with desirable properties, well-known cultivable microorganisms can also be promisingly unexplored pools of new enzymes. At this respect, the wide range of chemicals transformed or degraded by many rhodococci (Bell et al. 1998; Larkin et al. 2005) makes them actually or potentially useful in environmental and industrial biotechnologies, as does their ability to synthesize several products such as surfactants, flocculants, amides, or polymers (Bell et al. 1998). In the last years, different enzymes from rhodococci showing interesting biotechnological applications have been cloned and characterized (Larkin et al. 2005, 2010). However, concerning lipases, rhodococci are poorly explored microorganisms, being the recently described LipR from *Rhodococcus* sp. CR-53, the first characterized lipase from this genus up to date (Bassegoda et al. 2012). The special properties shown by LipR, which constitutes the first member of the new bacterial lipase family X, plus the finding of an uncommon Y-type oxyanion hole, never found before in bacteria have boosted this genus as a promising pool of new lipases (Bassegoda et al. 2012; Falcocchio et al. 2005).

The discovery of LipR allowed confirming that the genome of strain *Rhodococcus* sp. CR-53 is very similar to the known genome of *Rhodococcus erythropolis* PR4 (Bioproject accession, PRJNA59019). Analysis of *R. erythropolis* PR4 genome sequence revealed the presence of several open reading frames (ORFs) showing the general features of esterases (Fojan et al. 2000) and annotated as putative lipases. Based on *R. erythropolis* PR4 genome, we describe here the isolation, sequence analysis, and biochemical characterization of a new esterase—Est4—from *Rhodococcus* sp. CR-53, assigned to the bacterial lipase family IV (Arpigny and Jaeger 1999). The newly discovered esterase was also evaluated for the kinetic resolution of tertiary alcohols. Est4 is the first rhodococci family IV esterase described and, together with LipR, the only *Rhodococcus* lipases whose complete sequence and mode of action are known.

Materials and methods

Strains, plasmids, and growth conditions

Wild-type strain *Rhodococcus* sp. CR-53 (CECT 7058) was grown in Luria–Bertani (LB) medium for 3 days at 20 °C and pH6.8, under aerobic conditions (Falcocchio et al. 2005). *Escherichia coli* BL21 (Novagen®) was routinely cultured overnight at 37 °C in LB broth or on LB agar plates and was used as the host strain for cloning and expression of lipase-encoding genes. Plasmid pET101/D-TOPO® (Invitrogen®) was used as expression vector.

DNA manipulation and cloning

DNA manipulations were carried out according to Sambrook (Sambrook and Russell 2001). Plasmid DNA was purified using commercial kits (Illustra PlasmidPrep, GE Healthcare, UK). Thermostable polymerases *taq* and *pfu* (Biotools, Spain) were used according to the manufacturers' instructions. PCR amplifications were performed in a GeneAMP PCR system 2400 (Perkin Elmer) using different cycling periods, seldom including a hot start procedure (Sambrook and Russell 2001). Specific primers FW*est*4TOPO (5′ *CACCA*TGAGCACGTTCACCCTTCCGC 3′) and BK*est*4TOPO (5′ GTGACTGTTCGATCCCACTGC 3′) were used for *est*4 ORF amplification and cloning, using *Rhodococcus* sp. CR-53 genome as a template. Amplified DNA was purified through chromatography (Illustra™ PlasmidPrep, GE Healthcare). For ligation and cloning of the amplified DNA fragment, the pET101/D-TOPO® manual was used (Invitrogen®). To obtain the nucleotide sequences of DNA, PCR-amplified fragments were analyzed using the ABI PRISM® BigDye® Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems) and the analytical system CEQ™ 8000 (Beckman-Coulter) available at the Serveis Científico Tècnics of the Universitat de Barcelona. DNA samples were routinely analyzed by agarose gel electrophoresis (Sambrook and Russell 2001) and stained with GelRed™ 0.27 % (*v*/*v*). Nucleic acid concentration and purity were measured using a Spectrophotometer ND-100 NanoDrop®.

Bioinformatics tools

Blast searches were routinely performed for DNA or protein sequence analysis (Altschul et al. 1997). Alignments were performed using the Multiple Alignment Fast Fourier Transform server (http://mafft.cbrc.jp/alignment/server). BioEdit Sequence Alignment Editor v.7.0.1 (Hall 1999) was used for restriction pattern determination. The webtool ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to identify the open reading frames, and the software Contig Express was used to assemble DNA sequences (Vector NTI version 8; Invitrogen, Carlsbad, CA). Identification of putative signal peptide and transmembrane regions was performed through SignalP 3.0 (Emanuelsson et al. 2007) (http://www.cbs.dtu.dk/services/SignalP/). Secondary structure prediction was achieved using the PSIPRED protein structure prediction server (Bryson et al. 2005) (http://bioinf.cs.ucl.ac.uk/psipred/). A structural three-dimensional (3D) homology model was generated with software Yasara (version

9.6.28, www.yasara.org), using default settings. Pymol software (PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC) was used to visualize the 3D protein model. Enzyme classification and family assignment were performed with cluster analysis of sequences (http://toolkit.tuebingen.mpg.de/clans) (Frickey and Lupas 2004). High-scoring segment pairs with *P* values better than 0.05 were considered to be significant. The ExPASy proteomics server (http://us.expasy.org/tools/protparam.html) was used to analyze the protein physicochemical parameters (ProtParam tool). The DNA sequence of Est4 was submitted to EMBL-EBI databank at EBI and given the accession no. GenBank, HE860514.1.

Activity assays and protein analysis

Lipase activity assays were performed using a previously described colorimetric microassay (Bofill et al. 2010; Ruiz et al. 2004) based on measuring the release of *para*-nitrophenol (*p*-NP) from *p*-NP-fatty-acid-derivative substrates. One unit of activity was defined as the amount of enzyme that released 1 μmol of *p*-NP per minute under the assay conditions used. Substrate specificity assays were performed on several *p*-NP-derivative substrates at pH7. Optimum temperature was determined in the range from 4 to 70 °C at pH7, using 1 mM *p*-NP butyrate as a substrate (Ruiz et al. 2004). Optimum pH was established analyzing the activity in a pH range from 3 to 10 with Britton Robinson buffer (Bofill et al. 2010; Britton 1952) at a final concentration of 50 mM. To subtract the effect of pH on color release after incubation, 100 μl 2 M Tris–HCl pH7 was added to 100 μl reaction mixture to equal the different pHs of each reaction sample (1 M Tris–HCl final concentration). For inhibition studies, assays were performed on *p*-NP butyrate in the presence of several metal ions, used at 1 and 10 mM concentrations. In order to determine thermal and pH stability, enzyme samples were preincubated in a temperature range of 4 to 80 °C and in a pH range of 3.0 to 10.0, and the residual activity was analyzed under the standard reaction conditions (Bofill et al. 2010; Ruiz et al. 2004). Enzyme kinetics parameters were calculated using the optimum substrate, pH, and temperature, from the activity vs. substrate concentration curves by regression analysis performed using the BioFitWeb tool (http://biofitweb.cox-thurmond.net/FittingRoom/FittingTools.html). Protein analysis was performed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by zymogram, as previously described (Diaz et al. 1999; Prim et al. 2003). Isoelectric focusing was performed in a Pharmacia Phast System unit, using gels with a pH range from 3 to 9, followed by zymogram analysis. The Bradford method was used for protein concentration determination (Bassegoda et al. 2010).

Est4 purification

Purification of Est4 was performed with an ÄKTA™ FPLC™ system from Amersham Biosciences, in a single step of His-tag affinity chromatography. Crude cell extract samples (2 ml) of recombinant *E. coli* BL21/pET101Est4 prepared in 50 mM phosphate buffer (pH7.5), 50 mM NaCl, and 30 mM imidazole were injected into a 1 ml HP His-Trap column containing immobilized nickel. Two different elution buffers were used: 50 mM phosphate buffer (pH7.5) with 500 mM NaCl and 50 mM phosphate buffer (pH7.5) with 0.5 mM NaCl and 300 mM imidazole. The buffers were automatically mixed to generate an imidazole gradient from 1 to 100 %. Eluted fractions were collected continuously at a rate of 0.5 ml per fraction and analyzed by using SDS-PAGE, followed by zymogram analysis (Diaz et al. 1999). Selected samples were pooled and desalted in order to remove the accompanying imidazole and NaCl. The desalting buffer for Est4 purification was 20 mM Tris–HCl (pH7), supplemented with 0.02 % sodium azide.

Kinetic resolution of tertiary alcohols

Linalyl and terpinyl acetates were purchased from Aldrich Biochemicals. 1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate (2Ac) was produced by the Unitat de Química Combinatòria from the Scientific Park of Barcelona. Stock solutions of tertiary alcohol acetates were prepared in DMSO at 460 mM. Substrate stock solutions (6.5 μl) were added to the esterase solution (1.1 U cell free enzyme solution in 20 mM Tris–HCl pH7) or to the same volume of buffer (20 mM Tris–HCl pH7) for autohydrolysis control. The reaction mixtures (500 μl, 6 mM substrate concentration) were stirred in a thermoshaker (Eppendorf, Germany) at 37 °C, and 400 μl samples were taken after 2 h and extracted twice with 400 μl dichloromethane (Bassegoda et al. 2010). The organic layers extracted were dried over anhydrous sodium sulfate (Na_2SO_4), filtered to remove solid impurities, and the organic solvent was removed under nitrogen, up to 50 μl, and transferred to GC vials. Samples were analyzed by using gas chromatography/mass spectrometry (GC-MS) on a chiral phase GC-MS Finnigan Trace DSQ chromatographer (Thermo Electro Corporation) equipped with a BetaDEX™ 120 column made of β-cyclodextrin (30 m×0.25 mm×0.25 μm film thickness) from Supelco. Retention times for the analyzed products of linalyl and terpinyl compounds were as follows: (±)-linalyl acetate=39.1 min, (*S*)-(+)-linalool=27.6 min, (*R*)-(−)-linalool=26.9 min, (±)-(α)-terpinyl acetate=26.6 min, (*S*)-(−)-terpineol=23.9 min, and (*R*)-(+)-terpineol=24.2 min. Esters and alcohols derived from 2Ac displayed retention times of 16.6/16.8 and 20.2/20.6 min, respectively. Enantioselectivity was calculated from the enantiomeric excess of the product (ee_p)

and from the enantiomeric excess of product vs. enantiomeric excess of substrate (ee_s) values, according to Straathof and Jongejan (1997).

Results

Est4 identification and cloning

Analysis of *R. erythropolis* PR4 proteome revealed 26 ORFs annotated as putative esterase/lipase proteins (Bioproject accession, PRJNA59019). Among them, ORF YP_002767573 encodes for a protein displaying high-sequence identity with putative esterases from *R. erythropolis* SK121 (99 %), *R. erythropolis* (99 %), *Rhodococcus opacus* B4 (72 %), *Rhodococcus jostii* RHA1 (72 %), *Rhodococcus equi* (67 %), and *Nocardia farcinica* IFM10152 (51 %). In addition, a BLASTpdb search revealed 38 % sequence identity of ORF YP_002767573 with the crystal structure of a hormone-sensitive lipase from a metagenome (Nam et al. 2009; Straathof and Jongejan 1997). ORF YP_002767573 has 942 bp and encodes for a protein of 313 amino acids, with an estimated molecular mass of 33 kDa, bearing the typical features of lipolytic enzymes (Fojan et al. 2000; Jaeger et al. 1999). Accordingly, ORF YP_002767573 was named *est*4, and the encoded protein, selected for further isolation and characterization, was designated Est4. For isolation, gene *est*4 was amplified from *Rhodococcus* sp. CR-53 using specific primers (FW*est*4TOPO and BK*est*4TOPO), ligated into pET101 vector, and the resulting construction was transformed into strain *E. coli* BL21 (DE3), producing recombinant clone *E. coli* BL21/pET101Est4.

Est4 purification and characterization

For characterization purposes, Est4 was purified by fast protein liquid chromatography from crude cell extracts of recombinant *E. coli* BL21/pET101Est4 clone. Presence of the histidine tag facilitated one-step purification. The purification process (Table 1) rendered a yield of 3 %, allowing to get 2.8-fold purified Est4 with a specific activity on *p*-NP butyrate of 0.64 Umg^{-1} protein. Purified Est4 migrated in SDS-PAGE/zymograms as a single band with an estimated molecular mass of 36 kDa (not shown), corresponding to the theoretical weight (33 kDa) plus the histidine tag. Isoelectric focusing gels of crude cell extracts from *E. coli* BL21/pET101Est4 coupled with zymogram analysis allowed identification of a protein band with activity on *p*-NP butyrate and an approximate *p*I of 6, confirming the predicted parameters for Est4 (5.7) fused with the his tag (not shown).

Est4 substrate specificity was tested on several *p*-NP derivatives, exhibiting the highest activity (100 %) on *p*-NP butyrate (Table 2), a short-chain-length substrate. However, the enzyme also kept very high activity on *p*-NP valerate ($C_{5:0}$), and 50 % activity was detected on *p*-NP caprylate ($C_{8:0}$). A dramatic activity reduction was observed when medium and long-chain-length substrates were used. The effect of temperature and pH on the activity of Est4 was also determined (Fig. 1a, b), using *p*-NP butyrate as a substrate. The enzyme displayed optimum temperature at 30 °C, and activity decreased rapidly at higher temperatures, showing 50 % activity at 40 °C. It is interesting to note that the enzyme displays 80 and 40 % of the maximum activity at 20 and 4 °C, respectively, bearing good activity under mesophilic environmental conditions. Regarding the optimum pH, the enzyme exhibited maximum activity at pH7 and kept 80 % activity at pH6, 6.5, and 7.5. However, very low activity was observed at alkaline pHs. Analysis of the kinetic parameters of Est4 on *p*-NP butyrate under optimum conditions showed a typical Michaelis–Menten behavior without interfacial activation. The calculated apparent V_{max} and K_m values found for the enzyme on *p*-NP butyrate were 1.1 ± 0.031 Umg^{-1} and 0.17 ± 0.038 mM, respectively.

Concerning stability, Est4 kept maximum activity after 1 h incubation in a range of temperatures from 4 to 40 °C. However, incubation at higher temperatures produced a dramatic decrease of Est4 activity, which displayed high inactivation after 1 h incubation at 50 °C. When thermal stability was tested for longer incubation periods, the enzyme maintained almost full activity after 24-h incubation at 20 °C, while more than 80 % residual activity was detected after 24 h at 30 °C. Nevertheless, activity was completely lost after 24-h incubation at 40 °C. On the contrary, Est4 retained 93 and 85 % residual activity after incubation at 4 °C for 15 and 30 days, respectively. An 88 and 67 % residual activity was detected after incubation at 20 °C for 15 and 30 days. Est4 exhibited moderate stability after 1-h incubation at alkaline pH conditions, showing maximum stability at pH6 and 7 when stored at 4 °C (not shown).

Table 1 Summary of Est4 purification procedure and yield

	U/ml	Fraction volume (ml)	Total activity (U)	Protein (mg/ml)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude cell extract	0.37	50	18.4	1.6	0.23	1	100
Purified Est4	0.021	25	0.54	0.033	0.64	2.8	2.94

Table 2 Substrate profile of Est4

*p*NP derivatives	Specific activity (mU/mg prot; %)
*p*NP-butyrate ($C_{4:0}$)	159 (100)
*p*NP-valerate ($C_{5:0}$)	151 (95)
*p*NP-caprylate ($C_{8:0}$)	77 (50)
*p*NP-caprate ($C_{10:0}$)	34 (23)
*p*NP-laurate ($C_{12:0}$)	40 (27)
*p*NP-palmitate ($C_{16:0}$)	4 (5)

Activity values are the mean of at least three independent assays using purified Est4

The effect of different ions and chemicals on the activity of purified Est4 was analyzed using *p*-NP butyrate as a substrate (Fig. 1c). In general, Est4 was not significantly affected by the presence of ions in the reaction mixture, as none of the tested ions increased the lipolytic activity of the enzyme. However, Cu^{2+}, Zn^{2+}, and Ag^{2+} completely inhibited Est4 at low concentration (1 mM). On the other hand, Fe^{2+} and Co^{2+} completely abolished activity at high concentration (10 mM). The enzyme was strongly inhibited by 0.1 % SDS (2.5 % residual activity), whereas a 50 % residual activity was observed in the presence of 1 % Triton X-100®; however, no activity was detected at 5 % concentrations of this detergent (not shown).

Est4 sequence and structure analysis

Analysis of the amino acid sequence of Est4 revealed a protein of 313 amino acids without a signal peptide, with an estimated molecular mass of 33 kDa, a deduced *p*I of 5.7, and a high content of nonpolar amino acids (58.2 %). The secondary structure prediction of the protein revealed the typical α/β fold of lipases and the presence of a conserved pentapeptide Gly-Asp-Ser-Ala-Gly containing the putative catalytic serine residue (Ser^{150}) which constitutes the characteristic "nucleophilic elbow" between strand β5 and the following α-helix (Fojan et al. 2000; Jaeger et al. 1999).

Like *R. erythropolis* ORF YP_002767573, Est4 revealed high amino acid sequence identity with putative esterases from *R. erythropolis* SK121 (99 %), *R. erythropolis* (99 %), *R. opacus* B4 (72 %), *R. jostii* RHA1 (72 %), *R. equi* (67 %), and *N. farcinica* IFM10152 (51 %). Classification of the newly isolated Est4 was performed using the webtool cluster analysis of sequences. Comparison of all sequences that define each bacterial lipase family (Arpigny and Jaeger 1999) with that of Est4 showed significant similarities between Est4 and lipase sequences of family IV, also known as the hormone-sensitive lipase family (HSL) (Arpigny and Jaeger 1999). The amino acid sequence of Est4 was aligned with six annotated or described lipolytic enzymes from distantly related prokaryotes but belonging to family IV

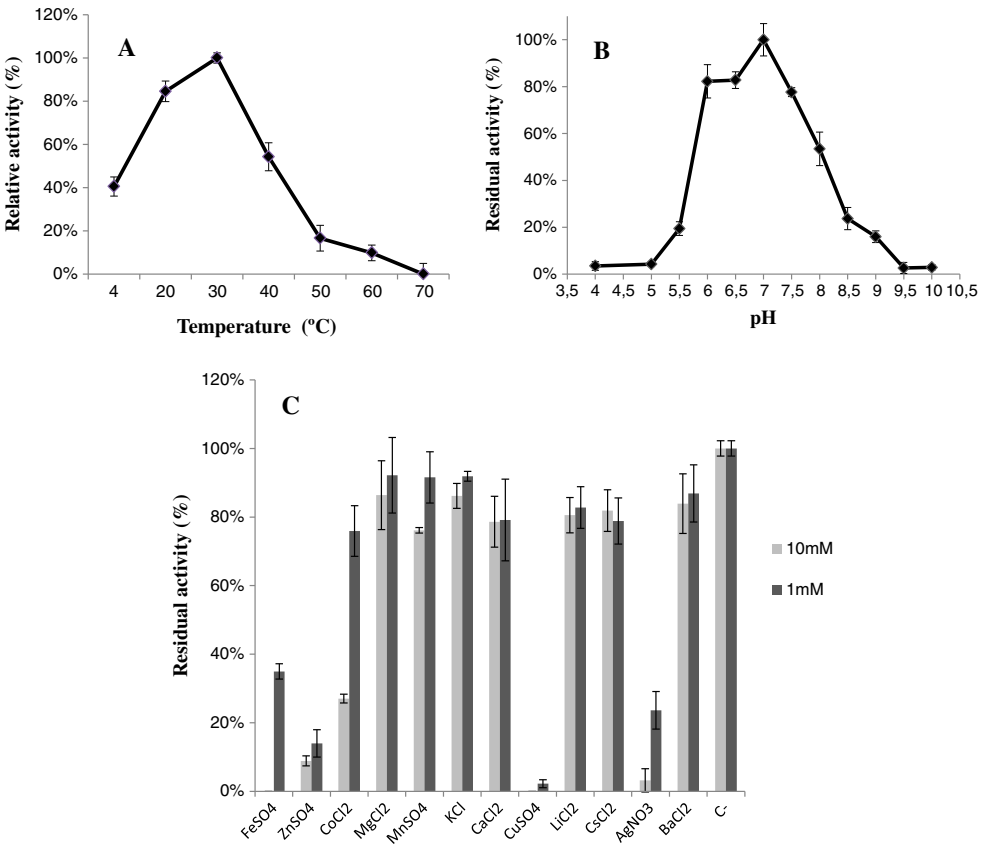

Fig. 1 Selected aspects of Est4 characterization, assayed on *p*NP butyrate. **a** Optimum temperature. **b** Optimum pH. **c** Effect of several ions on Est4 activity. *C* corresponds to the activity of Est4 without any ion added, but containing the corresponding volume of buffer. The activity values are the mean of at least three independents

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AF034088    2 -------PL-DKQIAAVLQQFSELPA-------PDFSQLDAA-QYRQFCDNLLPA-----
AE000985    4 ------MPI-DPVYYQLAEYFDSL---------PKFDQFSSAREYREAINRIYEERNRQL
L36817     38 TPGLPSSPL-DPQVAALLELIARAKR-------PPIHAMEPED-----AKIAYEKSAPIL
X53868     61 AEQ---YPLADAHLRLILAISNKLKRPLAIDKLPKLRQKFGTDAVSLQAPSVWQQNADAS
AE000153    7 ------LPVLDLISAEMKTVVNTLQPDLPPW--PATGTIAEQRQYYTLERRFWNA-----

Est4        6 ------LPL--PVVAFLLK--------------PYFRLAFNSRLPYRIQRLLLEAGAPL-

AF034088   41 -IPGDPMIEVRNLRVAAAAGE-LDARLYRPLE------EDNLPL-LVFFHGGGFVMGNLD
AE000985   48 -SQHERVERVEDRTIKGRNGD-IRVRVYQQ--------KPDSPV-LVYYHGGGFVICSIE
L36817     85 DINPPPVYMAEDLLAPARDGHAIPLRLYTPR---EASWTEPLPL-LVYFHGGGFTVGSVD
X53868    118 -GSTENAVSWQDKTIANADGGDMTVRCYQKSTQNSERKSTDEAA-MLFFHGGGFCIGDID
AE000153   54 ---GAPEMATRAYMVPTKYGQ-VETRLFCP--------QPDSPATLFYLHGGGFILGNLD

Est4       43 -QQVPAGAVVRPLELACRPAEKITVG------------ATERPTAVLYLHGGAYTLGSLA

AF034088   92 THDNLCRSLASQTEAVVVSVAYRLAPENHFPAAPLDCYAATCWLVEHAAELGVDGRRLAL
AE000985   97 SHDALCRRIARLSNSTVVSVDYRLAPEHKFPAAVYDCYDATKWVAENAEELRIDPSKIFV
L36817    141 SHDPLCRLLCGQADCMVLSVDYRLGPQWRFPTAANDAFDVLHWVFAEAGRLGADPARIAV
X53868    176 THHEFCHTVCAQTGWAVVSVDYRMAPEYPAPTALKDCLAAYAWLAEHSQSLGASPSRIVL
AE000153  102 THDRIMRLLASYSQCTVIGIDYTLSPEARFPQAIEEIVAACCYFHQQAEDYQINMSRIGF

Est4       90 THRSLAAHLARESSSAVFTLDYRLAPENPYPAALDDAVSAFMQLV---TEFDYSEDRVAI

AF034088  152 AGDSAGGNLALAVSRL----------------AAQRQ-----------GPKISYQCLFYP
AE000985  157 GGDSAGGNLAAAVSIM----------------ARDSG-----------EDFIKHQILIYP
L36817    201 GGDSAGGTLAAACAVE----------------ARNAG-----------LAPVLQLLIYP
X53868    236 SGDSAGGCLAALVAQQVIKPIDALWQDNNQAPAADKKVNDTFKNSLADLPRPLAQLPLYP
AE000153  162 AGDSAGAMLALASALW----------------LRDKQ---------IDCGKVAGVLLWYG

Est4      147 AGDSAGGGLAAATALR----------------LIERH-----------GVTPGALALISP
                 *

AF034088  185 VTDARCDSQSYEEFA---EG-YFLTGAMMYWFWQQYLQDTG-QGDDPLASPL----RAET
AE000985  190 VVNFVAPTPSLLEFG---EGLWILDQKIMSWFSEQYFSREE-DKFNPLASVI----FAD-
L36817    233 GTCARQDTPSHRALA---DG-YLLTADMIRWFFAQYLDQEA-SRDDWRFAPLDGGGAGAE
X53868    296 VTDYEAEYPSWELYG---EG-LLLDHNDAEVFNSAYTQHSGLPQSHPLISVM----HGD-
AE000153  197 LYGLR-DSVTRRLLGGVWDG---LTQQDLQMYEEAYLSNDA-DRESPYYCLF----NNDL

Est4      180 WVDPGDRGATK-------KADLVVNTAWSFDAADAYLA--GGDRTDPGYAPM----HGT-

AF034088  236 LADLPPTTLITAEFDPLRDEGEAFALRLQQAGVSVRVQRCEGMIHGFISMAPFVERAAHA
AE000985  241 LENLPPALIITAEYDPLRDEGEVFGQMLRRAGVEASIVRYRGVLHGFINYYPVLKAARDA
L36817    288 VRGTCPAWIAVAGYDPLHDEGVAYAEKLRAAGVAATLADYPGMIHDFFKLGRFVPAVAQA
X53868    347 NTQLCPSYIVVAELDILRDEGLAYAELLQKEGVQVQTYTVLGAPHGFINLMSVHQGLGNQ
AE000153  248 TREVPPCFIAGAEFDPLLDDSRLLYQTLAAHQQPCEFKLYPGTLHAFLHYSRMMKTADEA

Est4      226 LDGLPPTRIHVGSEEVLYPQIMTFTDKLSAAGVDVSLVEYARLWHVAHAQASLVKEAADA
                          *                                 *
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(Fig. 2). The multiple alignment revealed that Est4 bears most of the conserved blocks that define family IV (Fig. 2, shaded in black). Therefore, the new identified lipase Est4 was classified as a member of the bacterial lipase family IV.

◀ **Fig. 2** Multiple sequence alignment of Est4 and other annotated or described family IV bacterial lipases (Arpigny and Jaeger 1999). The conserved blocks described for family IV lipases are *shaded in black*. Other conserved amino acids, both totally conserved and same amino acid type, not included formerly as representative of family IV have been *shaded in gray*. The catalytic residues are *highlighted with an asterisk*. AF034088, lipase (LipP) *Pseudomonas* sp. B11-1; AE000985, putative lipase *Archaeoglobus fulgidus*; L3681, putative lipase *Alcaligenes eutrophus*; AE000153, *E. coli* carboxylesterase; X53868, *Moraxella* sp. Lip2

The alignment also revealed other conserved residues which had not initially been included in the characteristic motifs of family IV, highlighted in Fig. 2 (Arpigny and Jaeger 1999).

A 3D homology model (Fig. 3) was constructed using templates of different crystal structures derived from the closest sequences: 3FAK, 3DNM (Nam et al. 2009), 1QZ3 (De Simone et al. 2004), and 1LZK (Zhu et al. 2003). The homology model [QMEAN score 0.57 (Arnold et al. 2006)] allowed identification of the catalytic triad Ser^{150}, Glu^{240}, and His^{270} and confirmed the presence of an oxyanion hole composed by two glycines and one alanine (Gly^{80}-Gly^{81}-Ala^{82}), a type of oxyanion hole that has been correlated to the activity of certain esterases towards tertiary alcohols (Bassegoda et al. 2010; Henke et al. 2002, 2003; Kourist et al. 2007b, 2008).

Kinetic resolution of tertiary alcohols

The ability of Est4 for the kinetic resolution of tertiary alcohol esters was evaluated using the esters linalyl acetate, terpinyl acetate, and 1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate as substrates. Est4 successfully hydrolyzed linalyl and terpinyl acetates to the corresponding alcohols, with a conversion rate of 35 and 26 %, respectively, after 2-h incubation at 37 °C. Longer incubation periods were not considered for these substrates due to their high autohydrolysis. The higher stability of 2Ac allowed incubation at 2 and 4 h, where a conversion rate of 6 and 10 %, respectively, was achieved. When assayed for enantioselectivity in the kinetic resolution of tertiary alcohol esters, Est4 showed low but significant enantioselectivity. Linalyl and terpinyl acetates were hydrolyzed with an enantiomeric excess of product of 39 and 20 % respectively, whereas the enantiomeric excess of product for compound 2Ac was 75 %. Calculated enantioselectivity (*E*) for linalyl acetate, terpenyl acetate, and 2Ac was 4.5, 2.4, and 7.3, respectively, indicating a low but significant activity of Est4 on such compounds. Further enzyme improvement through rational design will be performed for better performance of Est4 on these and other tertiary alcohol esters.

Discussion

Concerning lipases, rhodococci are poorly explored microorganisms. The discovery of the novel lipase LipR from strain *Rhodococcus* sp. CR-53 (Bassegoda et al. 2012; Falcocchio et al. 2005) increased our interest to use this strain to prospect for novel lipases from this genus. Moreover, the cloning of LipR revealed a high similarity between the genome of strain *Rhodococcus* sp. CR-53 and the published genome of strain *R. erythropolis* PR4 (Bioproject accession, PRJNA59019). Therefore, based on the *R. erythropolis* PR4 genome, we performed the isolation of new lipolytic enzymes from strain *Rhodococcus* sp. CR-53.

Analysis of the whole *R. erythropolis* PR4 proteome revealed 26 ORFs annotated as putative esterase/lipase enzymes. The criterium to select the best ORF to be amplified from *Rhodococcus* sp. CR-53 was on the basis of highest sequence identity. This was the case for ORF YP_002767573 that encodes for a highly conserved esterase among members of the genus *Rhodococcus*. Therefore, this ORF was selected as the best candidate to be identified and isolated from strain *Rhodococcus* sp. CR-53. The use of specific primers obtained from the sequence of ORF YP_002767573 allowed amplification of gene *est*4 from *Rhodococcus* CR-53, whose encoded protein was designated Est4. The amplified ORF was cloned in *E. coli*, and presence of lipolytic activity in the recombinant clone was

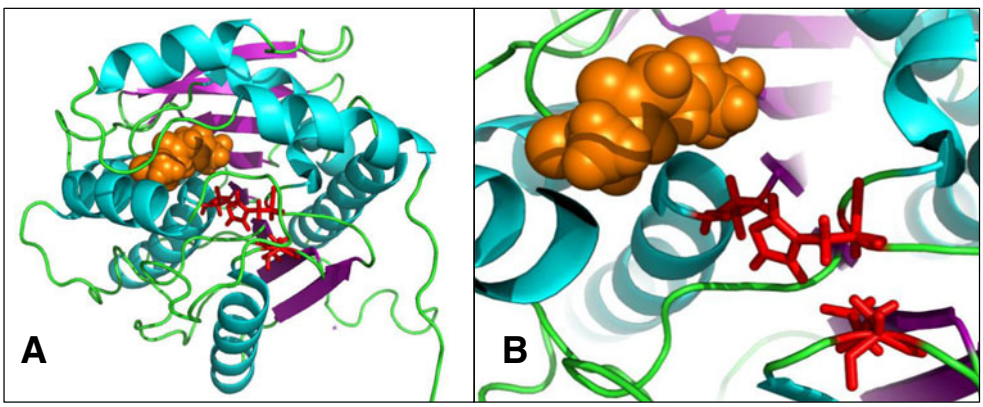

Fig. 3 **a** A 3D structure homology model obtained for Est4. **b** Detail of the catalytic triad, Ser^{150}, Glu^{240}, and His^{270}, in sticks. The amino acids constituting the oxyanion hole are depicted as *spheres*

confirmed, thus indicating that the annotation of such ORF as a putative lipase was indeed correct. Recombinant clone *E. coli* BL21/pET101Est4 was used for Est4 production and purification for further biochemical characterization. Activity assays performed on several *p*-NP-derivative substrates revealed that Est4 displays the typical substrate profile of an esterase (Arpigny and Jaeger 1999; Jaeger et al. 1999), showing preference for short-chain acyl substrates, with optimum temperature and pH similar to those of most mesophilic, neutral enzymes. These results agree with the autochthonous environment of *Rhodococcus* sp. CR-53, isolated from a subtropical soil sample (Falcocchio et al. 2006; Ruiz et al. 2005) with an average pH of 6 and a range of temperatures between 15 and 24 °C. The kinetic parameters of Est4 were assayed at the optimum temperature (30 °C) and pH, using *p*-NP butyrate as a substrate, showing the typical profile of esterases without interfacial activation, indicating that no configurational changes occur during catalysis (Jaeger et al. 1999; Prim et al. 2000). Stability assays of Est4 were found to be also similar to previously described mesophilic esterases (Prim et al. 2000; Ruiz et al. 2003). Est4 showed moderate-low tolerance for the presence of ions in the reaction mixture, as most ions did not have any significant effect on the enzyme activity, although like for other esterases from Gram-positive bacteria, Cu^{2+}, Zn^{2+}, and Ag^{2+} drastically inhibited the enzyme (Prim et al. 2000, 2001). Divalent cations, which frequently contribute to increase lipase activity (Bofill et al. 2010; Falcocchio et al. 2006; Gupta et al. 2004; Prim et al. 2006; Ruiz et al. 2002), had no relevant effect on Est4.

As a newly identified bacterial esterase, Est4 was classified into one of the previously described bacterial lipase families (Arpigny and Jaeger 1999; Jaeger and Eggert 2002). Sequence and conserved motifs comparison revealed that protein Est4 bears most of the conserved amino acid sequence motifs that define family IV (Fig. 2), also known as the hormone-sensitive lipase family. This is the first esterase from the genus *Rhodococcus* belonging to HSL family. However, its physiological role is still far from our knowledge. The catalytic triad predicted by the 3D homology model matches with the catalytic residues found in family IV lipases (Arpigny and Jaeger 1999). Nevertheless, it should be noted that in the case of Est4, the acidic residue is a glutamic acid (Glu^{240}), while the acidic residue of other sequences used in the multiple alignment is an aspartic acid. It is interesting to observe that one of the conserved motifs of Est4 corresponds to the amino acid set of residues GGG(A)X that constitute the oxyanion hole (Pleiss et al. 2000). In the Est4 3D model structure, these conserved amino acids (Gly^{80}-Gly^{81}-Ala^{82}) are located in a loop near the catalytic serine, which corresponds with the typical architecture of a GGG (A)X-type oxyanion hole (Pleiss et al. 2000).

Presence of a GGG(A)X motif in the oxyanion hole has been associated with the ability to hydrolyze tertiary alcohols esters. Therefore, the presence of this motif in Est4 strongly suggested that Est4 could be used in the kinetic resolution of tertiary alcohols. Accordingly, we evaluated Est4 for the kinetic resolution of the tertiary alcohol esters linalyl acetate, terpinyl acetate, and 2Ac. Although with low efficiency, due probably to the small amount of enzyme used, Est4 successfully hydrolyzed all esters tested after 2-h incubation at 37 °C. Therefore, we can link the observed sequence and structure of the Est4 GGGAX motif to the ability of this esterase to hydrolyze tertiary alcohol esters, as has been described for other esterases (Henke et al. 2002, 2003; Kourist et al. 2008; Pleiss et al. 2000). Despite the observed low conversion and enantioselectivity values obtained, the ability of Est4 to hydrolyze tertiary alcohol esters suggests that this new enzyme is a good candidate for further analysis of tertiary alcohol conversion using a range of substrates since it has been observed that the conformation of the two enantiomers in the binding site depends on the details of the structures of both, substrate and enzyme, and the enantioselectivity has to be considered for each substrate–enzyme pair, independently (Henke et al. 2003). Moreover, different strategies for enantioselectivity optimization have been developed, ranging from tuning the reaction parameters (Heinze et al. 2007; Kourist et al. 2007a; Nguyen et al. 2010) to directed evolution (Bartsch et al. 2008) or rational protein design of the esterase (Bassegoda et al. 2010; Gall et al. 2010; Heinze et al. 2007) which could be applied to improve Est4 performance on the enantiomeric resolution of tertiary alcohols. Therefore, Est4 increases our knowledge about lipolytic enzymes of the genus *Rhodococcus* and expands the catalytic toolbox for the kinetic resolution of tertiary alcohols.

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