# Evolving a lipase for hydrolysis of natural triglycerides along with enhanced tolerance towards a protease and surfactants.

Ana L. Alfaro-Chavez[1], Jian-Wei Liu[2], Bradley J. Stevenson[1], Adrian Goldman[3,4] and David L. Ollis[1].

[1]Research School of Chemistry, Australian National University, Canberra, ACT 2601, Australia

[2]CSIRO Land and Water, Black Mountain, Canberra, ACT, 2601, Australia

[3]School of Biomedical Sciences, Astbury Centre for Structural Molecular Biology, University of Leeds, LS2 9JT

[4]Molecular and Integrative Biosciences Program, University of Helsinki, FIN-0018.

Corresponding Author:

David L Ollis

Research School of Chemistry

Australian National University

Canberra, ACT, 2601, Australia

Tel: +61 254377

E-mail: david.ollis@anu.edu.au

**Abstract**

In the accompanying paper, we described evolving a lipase to the point where variants were soluble, stable and capable of degrading C8 triacylglycerides and C8 esters. These variants were tested for their ability to survive in an environment that might be encountered in a washing machine. Unfortunately, they were inactivated both by treatment with a protease used in laundry detergents and by very low concentrations of sodium dodecyl sulfate. In addition, all the variants had very low levels of activity with triglycerides with long aliphatic chains and with naturally occurring oils, like olive oil.

Directed evolution was used to select variants with enhanced properties. In the first ten rounds of evolution, the primary screen selected for variants capable of hydrolysing olive oil whereas the secondary screen selected for enhanced tolerance towards a protease and sodium dodecyl sulphate (SDS). In the final six rounds of evolution, the primary and secondary screens identified variants that retained activity after treatment with SDS. Sixteen cycles of evolution gave variants with greatly enhanced lipolytic activity on substrates that had both long (C16 and C18) as well as short (C3 and C8) chains. We found variants that were stable for more than 3 hours in protease concentrations that rapidly degrade the wild-type enzyme. Enhanced tolerance towards sodium dodecyl sulphate was found in variants that could break down naturally occurring lipid and resist protease attack. The amino acid changes that gave enhanced properties were concentrated in the cap domain responsible for substrate binding.

## Introduction

The object of this study was to identify lipase variants that could be used in industrial applications. To identify suitable variants, we used directed evolution (for reviews, see (Arnold 1998; Arnold and Volkov 1999; Arnold 2018; Porter et al. 2016; Powell et al. 2001; Turner 2009)). The starting point for this work was a collection of variants produced in a study described in the accompanying paper (Alfaro-Chavez et al. 2019). These variants were evolved from a lipase, Lip3 (wild-type - *wt*), from *Drosophila melanogaster* encoded by the gene CG8823. The previous study produced 30 variants with higher activities than *wt* as measured with crude lysate. The *wt* and five of these variants were purified and characterized. The *wt* expressed well, but had very little activity due, in part, to its poor solubility. The variants exhibited greatly enhanced solubility and thermal stability as well as modest increases in activity compared with *wt*. We decided that these variants were a suitable starting point for the current study, which aimed to identify variants that could withstand the harsh conditions found in laundry detergents.

Laundry detergents vary considerably in composition and application. A set of three quite different detergents was chosen for testing variants. The first was a traditional laundry powder, LD1, that relied mainly upon surface active agents to clean cloths. The second, LD2, was intended for washing in cold water and contained an enzyme that removed stains that were rich in protein; the enzyme was thought to be a variant of the protease subtilisin. The third detergent, LD3, is marketed as a liquid; it operates in warm water and is intended primarily to remove stains caused by lipids. The enzyme present is thought to be a lipase. There were predominantly two types of surface active agents in these detergents; anionic and non-ionic. There were many types of each of these surface-active agents in the detergents, too many to be used for selection in our experiments. We therefore used a single representative surface active agent, sodium dodecyl sulphate (SDS) in the experiments. As will be shown below, the starting variants exhibited good tolerance towards some non-ionic agents, while they showed poor tolerance towards SDS, an anionic surface-active agent. One of our aims was to identify variants of Lip3 that were stable in SDS and that retained tolerance to non-ionic surface-active agents.

In addition to surface-active agents, laundry detergents frequently contain enzymes. Proteases were the first and are still the most common enzyme additive (Kumar et al. 2008; Maurer 2004; Saeki et al. 2007). Lipases are also found in laundry detergents (Adrio and Demain 2014; Hasan et al. 2010; Niyonzima and More 2015), but are not used in conjunction with proteases that could degrade them. A commercial preparation of subtilisin known as Alcalase®, was one of a set that were available from commercial suppliers for use in laundry detergents. Our objective was to identify variants that could withstand treatment with this protease along with surface active agents. In addition, we wanted Lip3 variants that could degrade lipids that might be encountered in a washing cycle. The starting variants for this work had enhanced activity towards tri-octyl glyceride. We wanted to identify variants that could act on tri-acyl glycerides with long chains (C16 and C18) and natural oils that had variable length chains, specifically olive oil (16-22C) (Quintero-Flórez et al. 2015) and coconut oil (6-14C) (Bezard et al. 1971).

Our goal was to identify variants with enhanced tolerance to SDS and that were protease tolerant while exhibiting broad substrate specificity. A schematic work flow diagram of the experimental procedure is shown in Figure 1. The initial objective was to produce variants suitable for industrial applications. However, it is the methods used to identify variants that may be of practical use rather than the variants themselves. Our approach can be modified to deal with the features of a specific laundry detergent. If the detergent uses a protease other than Alcalase®, our approach can be used to evolve tolerance to that enzyme; and, of course, surfactants other than SDS could be used in the secondary selection process.

**Materials and Methods**

The materials and kits used for molecular biology are described in the accompanying paper (Alfaro-Chavez et al. 2019). Chemicals used were of analytical grade and obtained from Merck Pty. Ltd. (Australia) unless otherwise stated. Tris buffer was obtained from BioStrategy (Australia), sodium dodecyl sulfate (SDS) from Amresco (Australia) and Bromophenol blue from LabChem Inc. (PA). Materials to prepare culture media were from Bacto Laboratories Pty Ltd (Australia). Olive oil, coconut oil and commercial detergents (LD1, LD2 and LD3) were purchased from a Canberran supermarket (2016).

*Phase 1 - Initial characterization*

Lip3 *wt* and variants R7_59A (Round 7, Tray 5, Well 9A) and R7_82E were selected for initial characterization. These enzymes were purified as described in the accompanying paper (Alfaro-Chavez et al. 2019). The object of the initial characterization was to determine the ability of the enzymes to degrade esters with long chains; four substrates were used: pNP-C3, pNP-C8, pNP-C16 and pNP-C18. This assay was essentially the same as described in the accompanying paper (Alfaro-Chavez et al. 2019) and will only be briefly described here. All assays were done in triplicate. In addition, the ability of wt and variants to tolerate commercial detergents with agar plates was tested.

*Esterase activity*

The esterase activity was measured spectrophotometrically as described in the accompanying paper (Alfaro-Chavez et al. 2019). All the esterase assays were done with 0.4 mM of substrate and the hydrolysis reaction carried out in 96 well plates. The final volume of the reaction mix was 200 μL consisting of 100 μL of enzyme solution – either purified protein or crude lysate. As noted in the previous paper (Alfaro-Chavez et al. 2019), the purified enzyme was stored as a concentrate in buffer A (see protein purification) and diluted into assay buffer (50 mM Tris-HCl Buffer, pH 8). 100 μL of substrate solution was added to the enzyme solution and the reaction followed at 405 nm for 5 minutes at 22 $^{o}$C. Each substrate was prepared as follows: a quantity of substrate was dissolved in 10 mL of isopropanol before mixing with 90 ml of assay buffer to give twice the final substrate concentration. 20 μL/mL Triton X-100 was required in the final substrate preparation to prevent the precipitation of pNP-C16 and pNP-C18 when they were mixed with the assay buffer (Gupta et al. 2002). The activity was taken as the initial velocity of the reaction, as described in our accompanying paper (Alfaro-Chavez et al. 2019).

*Tolerance to Detergent, SDS and Tolerance to Alcalase*

Two esterase assays were carried out. In the first, the assay was as described in the previous paragraph. In the second, the 100 μL of enzyme also contained either: 5 g/L LD1, LD2, or LD3, or 2% (v/v) Triton X100 or 20 g/L SDS, or 0.4% (v/v) Alcalase®. The solutions of enzyme and

additive were incubated at 22 °C for one hour and the activity measured as described in the previous paragraph using pNP-C16. The tolerances were calculated by dividing the rate of the second reaction by the first.

*Tolerance to commercial detergents as determined with agar plates.*

The agar plates were prepared in essentially the same way as described in the accompanying paper (Alfaro-Chavez et al. 2019). However, in addition to C8-TAG, various concentrations of the commercial detergents (0.1%, 0.5%, 1% and 2%) were added.

*Phase 2 - Evolution*

*Stage 1 - Library creation*

The library creation employed error prone Polymerase Chain Reaction (epPCR) as described previously (Alfaro-Chavez et al. 2019; Stevenson et al. 2008). This protocol used short extension times so that the genes would be shuffled with the Staggered extension process (StEP) (Stemmer 1994; Zhao et al. 1998). R8 was generated from 50-60 ng of each of the 30 genes isolated in R7 variants. Randomized mutations were introduced using $MnCl_2$, high concentrations of $MgCl_2$ and *Taq* DNA polymerase (Alfaro-Chavez et al. 2019). The libraries for R8 to R23 were produced by mixing the product of three PCR runs, each with different amounts of $MnCl_2$. The amplified mixed product was cleaned using the Wizard® PCR purification kit (Promega) and digested with 20 U of each *Nde*I and *Eco*RI-HF. The digestion proceeded at 37 °C for three hours in the Cutsmart® buffer. The digested product was isolated by agarose gel electrophoresis and the Wizard purification kit. The ligation was set up with T4 DNA ligase and the purified PCR product ligated into the pETMCSIII plasmid cut with the same restriction enzymes. The reaction was carried out overnight at 4 °C. The resultant plasmids were purified with the Promega® PCR purification kit and eluted into 30 μL of water before transformation into *E. coli* BL21 (DE3) by electroporation. Transformants were plated on LB-agar plates with 100 mg/L ampicillin to determine the concentration of colony forming units before plating out all of the library on selection agar plates at 300-500 colonies per plate.

The initial R14 library gave a smaller than expected number of colonies when it was tested, so library creation was repeated with the addition of the wt gene to the mixture of genes to perform this epPCR cycle. In R19 a different approach was taken. The digested gene was

ligated into pET26b to reduce the expression level. However, the transformation efficiency was still lower than required. The problem with library size was eventually solved by introducing a new primer for sequencing and epPCR: 5'-GAA GGA GAT ATA CAT ATG GG-3'. This primer included the *Nde*I site and was used in R19 to R23. R23 was prepared as for previous rounds except that the parent genes used as templates in epPCR were R18_211H, R21_112B, R21_411C, R22_73H, R22_512G and R22_311H.

*Stage 2 - Library screening*

This was the done with a large primary screen that gave a few hundred variants for testing in secondary screens. The primary screen was done in three different ways: A) agar plates with lipid (R8 through 17), B) 96-well plate culture (R18) and C) agar plates replicated onto filters with selection using 1-naphthyl palmitate. The methods used for the primary screen are given below, followed by the secondary screen methods.

*Agar plates + lipid: R8-R17.*

The agar plates were prepared with glyceryl trioctanoate in R8 and R9 and with olive oil in R10 to R23, but with detergents included in R10 and R11. As in our previous study, the LB agar plates were prepared with the lipids incorporated by sonication. For R8 and R9, the molten agar solution consisted of 0.6% TGC8 incorporated with vigorous agitation before sonication (at ≈ 60°C) for two periods of 15 min at 50% power and 50% pulse length using an Omni Sonic Ruptor® sonicator equipped with an OT-T-375 probe provided by Omni Sonic. Ampicillin was added in a final concentration of 50 μg/mL. The agar plates with olive oil had a final concentration of 0.2% (v/v) and were prepared in the same way. R10 and R11 used agar plates supplemented with an enzymatic detergent (LD2) previously heat inactivated (65 °C for one hour). The detergent was added to a final concentration of 0.1% (w/v).

There were between 300 to 500 colonies inoculated per plate. The selection was based on the quality of the halos. This usually meant that colonies with the largest haloes were selected. Not all colonies grew to the same size and so in some cases, we selected small colonies with large haloes compared to the colony size for secondary screening.

*96-well plate selection: R18*

After R17, we decided to screen large libraries for tolerance to SDS. In R18 this was done with 96-well plates. The library for R18 consisted of ~ $1.4x10^4$ variants and was plated in 40 96-well plates so that each well had, on average, between 3-4 colonies that grew in 200 μL of LB medium with 50 μg/mL ampicillin. The plates were incubated for 18 hours at 37 °C and 500 rpm in Heidolph Microplate Shaker Titramax 1000 with Incubator 1000. After incubation, 25 μL of culture were transferred to replica plates (twice to give plates A and B) and lysed with 2 μg of lysozyme.

Plate A was used to select mutants tolerant to 0.5% SDS and plate B was used to check lipolytic activity. 70 μL of SDS solution prepared in assay buffer to a final concentration of 0.5% SDS was added to plate A. After 15 minutes of incubation at room temperature, the activity was measured by adding 100 μL of pNP-C16 substrate mixture . Activity was followed for 5 minutes at 405 nm in a Spectramax M2e reader at room temperature. To test lipolytic activity, we prepared a mixture (0.02% v/v coconut oil, 0.02% olive oil v/v in 1mM Tris-HCl buffer pH 8, 0.07 mM phenol red and 0.1% Triton X-100 as emulsifier), which was sonicated for 15 minutes at medium ultrasonic power in Soniclean 160T Ultrasonic Cleaner 3 to dissolve the lipids. 175 μL of this mixture was added to plate B.

The plates were incubated at room temperature at 100 rpm for 1 hour in Heidolph Microplate Shaker Titramax 1000 and the mutants that turned yellow were selected. The mutants selected from plates A and B were streaked into LB amp plates with 0.2% olive oil and incubated overnight at 37° C. the colonies with the clearest haloes were selected and transferred to new 96-well plates with 200 μL of LB medium with 50 mg/L ampicillin (LBA) and incubated at 37 °C overnight and further tested in secondary screening.

*Agar plates – 1-Naphthyl palmitate: R19- R23.*

This screen followed a similar protocol to one that had been used to select lipases for biodiesel production (Korman et al. 2013). The screen involved replication of the colonies, their lysis, treatment of the lysate with SDS and subsequent activity determination. After preparing the library determining the cell density, around 500 colonies per plate were

inoculated on LB agar plates with Ampicillin. The plates were then incubated at 37° C overnight. The colonies were then blotted onto Whatman grade 3 filter paper (GE Healthcare), lysed with 50 mM Tris-HCl at pH 8, 0.1 M NaCl, 0.1% Triton X-100 and 1 mg/ml lysozyme (Sigma) and incubated for one hour at room temperature. The lysis buffer was decanted and the lysed cells treated with SDS for one hour, with the concentration of SDS increased in each round of evolution. The activity of the lysed cells was determined by adding a mixture of 1 mM 1-Naphthyl palmitate, 3 mM Fast Blue B, 0.5% (v/v) Triton X-100 and 0.5% (w/v) agar and 50 mM Tris-HCl pH 8. Active mutants exhibited an intense brownish colour, so the corresponding colonies on the agar plates were transferred to 96-well plates with 200 μL of LBAand incubated at 37 °C overnight. The variants were then tested in the secondary screen.

*Stage 3 Secondary screen*

The colonies of variants selected in the primary screen were grown to provide crude lysate for testing their esterase activity, and some of the rounds used other assays as well. In R18 an esterase assay was used to select variants to generate the next library. In other rounds, two assays were used to select variants. These dual assays employed an esterase to determine: A) tolerance to detergents (R8-R10), mainly SDS (R11 – R17 and R19 – R23); B) tolerance to protease (R12 – R17); and C) Thermostability (R21- R23). In these dual assays, variants that exhibited good activity in either assay were selected along with those that performed well in both assays.

Colonies were grown in 96-well plates with 200 μL of LB medium containing an antibiotic. These solutions were inoculated with colonies selected from the previous primary screen and were grown for 18 hours at 37° C in an incubated shaker (Heidolph Microplate Shaker Titramax 1000 with Incubator 1000) set at 500 rpm. Next, 25 μL (R15 – R18) or 50 μL (R11 – R14 and R19 - R23) or 100 μL (R8 – R10) aliquots of overnight culture were transferred to 96-well plates and lysed with lysozyme (final concentration 80 μg/mL) in assay buffer in in a final volume of 250 μL.

*Esterase activity*

Esterase activity was measured using *p*-nitrophenyl esters to identify the most active variants. This assay was essentially the same as used for the initial characterisation as described above as in the accompanying paper (Alfaro-Chavez et al. 2019). The reaction mixture contained 30 μL of crude lysate to which 70 μL assay buffer was added. The reaction was initiated by the addition of 100 μL of substrate mixture prepared as described under initial characterization. The formation of *p*-nitrophenolate was followed spectrophotometrically at 405 nm. The activity was taken as the initial velocity of the reaction, as described in our accompanying paper (Alfaro-Chavez et al. 2019).

*Tolerance to Detergent, SDS, or Alcalase*

With one exception, the same methods were used as those described in the Initial Characterization section given above. The exception was protein source; crude lysates were used as described in the previous paragraph. In the secondary screen for R23, the esterase activity was measured before and after treatment with 4% (w/v) SDS as described below (final characterisation). The most tolerant and active variant was characterized in Phase 3.

*Thermostability*

Lipase samples were incubated at 60-65°C for 30 minutes followed by 4 °C for 20 minutes in a Bio-Rad C1000$^{TM}$ before centrifuging in an Eppendorf 5804 Benchtop centrifuge for 5 minutes at 300 rpm. Fifty microlitres of each supernatant was added to a 96-well plate well and 50 μL of assay buffer was added before the reaction was initiated with 100 μL of pNP-C8 ester. The initial rate was measured as described above for esterase activity.

*Phase 3 - Final characterization*

We characterised several variants to gain some insights into the course of the evolution. The variants chosen were the best in their respective rounds. Variants were chosen from R13, R17, R18, R20 and R23 (R13_44C, R17_29G, R18_211H, R20_11A, R23_110A). These enzymes were purified as described in the accompanying paper (Alfaro-Chavez et al. 2019). Most of the assays were same as described above in the initial characterization. In addition, the lipolytic activity of these variants, *wt*, R7_59A and R7_82E were measured (below) and the melting temperature ($T_{50}$) was determined. We defined the $T_{50}$ as the temperature at which

the enzyme retains half of its activity after heat treatment for 45 minutes (for methods see (Alfaro-Chavez et al. 2019)). Activity as a function of SDS concentration was also determined for the R7_59A, R18_211H and R23_110A variants. All assays were done in triplicate.

*Lipolytic activity*

The method applied, detailed in the accompanying paper (Alfaro-Chavez et al. 2019), is based on the lipase assay described by (Camacho-Ruiz et al. 2015). In addition to the substrates used in the previous study, glyceryl trioleate and olive oil were studied. The method measures a shift in pH that is produced upon the release of free fatty acids in the lipase catalysed reaction. The buffer used was 1 mM Tris HCl pH=8 with 0.07 mM phenol red. Sonication was used to incorporate the following lipids into this buffer: 1 mM tributyrin-TG C4, 1 mM glyceryl trioctanoate-TG C8, 1 mM glyceryl trioleate TG C18, 0.04% coconut oil and 0.04% olive oil. The assay required 0.2% Triton X-100 for Olive oil and TG C18. The reaction was initiated by the addition of 10 μL of enzyme solution to 190 μL of suspended substrate. The reaction was followed spectrophotometrically at 560 nm. The amount of acid released as a function of pH was read from the calibration curve in the accompanying paper (Alfaro-Chavez et al. 2019). The activity / per mole of enzyme was calculated for each variant.

*Tolerance to high concentrations of SDS*

The tolerance of R7_82E, R18_211H and R23_110A to high concentrations of SDS was determined. The protein concentration used was 1 mg/ml (R7_82E) and 0.1 mg/ml (R18_211H and R23_110A) all in the assay buffer. A higher concentration of the R7 variant was needed so that its activity could be accurately measured. We added SDS stock solution (20% w/v) to 50 μL of the protein solution and made the solution to a final volume of 100 μL with final SDS concentrations of 0.1, 0.5, 2, 4, 8 and 10% . A control without SDS was also made and used to measure the initial activity of the enzymes. These solutions were left for 30 minutes at 22 $^{o}$C. The SDS was precipitated with 25 μL of assay buffer that contained 0.3 M KCl, and another 25 μL assay buffer added to give a final volume of 150 μL. The solutions were centrifuged for 30 minutes at 2235 g at room temperature and 10 μL of supernatant were transferred to fresh 96-well plates to measure esterase activity with pNP-C16. Activity was measured spectrophotometrically at 405 nm as described under initial characterization.

This procedure was applied as a secondary screen for R23 with 4% of SDS as final concentration.

*Proteolytic digestion*

The R7 12H was subjected proteolytic attack with Alcalase®. Samples were prepared following the procedure described by Wu (Wu et al. 1999). 0.1% v/v Alcalase® was prepared with 50 mM phosphate buffer and mixed in a 1:1 ratio with 8 mg/ml of the lipase solution. Samples were incubated at 22 °C and 120 µL aliquots were removed at: 1, 2, 3, 4, 6, 8, 10, 20, 30, 40, 50 and 60 minutes. 40 µL of 10% trichloroacetic acid was added to each sample to stop the reaction. The samples were centrifuged at 12500 rpm for 5 minutes and washed twice with 200 µL diethyl ether. The sample tubes were then left open to evaporate the diethyl ether and the pellet resuspended in 100 µL of buffer (50 mM Tris-HCl pH 8) and prepared to run in a SDS-PAGE gel. The sample collected after one minute gave rise to bands in a gel that corresponded to Alcalase along with the intact variant and a collection of low molecular weight bands. It appeared that Alcalase had digested much of the enzyme after a minute. A sample collected after one minute was buffer-exchanged using an Amicon Ultra filter into 0.5 mL of 20 mM ammonium formate pH 3 using three washes at 4° C followed by centrifugation at 10000 rpm for 5 minutes. The final concentration of the sample was 2 mg/mL. The samples were analysed at the Mass Spectrometry Facility at the Research School of Chemistry (ANU) and the cleavage sites identified using the FindPept server (Gattiker et al. 2002).

## Results

The work described here can be divided (Figure 1) into three phases. In the first phase, we characterized the ability of the starting variants to degrade a variety of triacylglycerols (TAGs) and esters with long aliphatic chains, as well as their tolerance of surface active agents and proteolytic enzymes. The results of these experiments were used to design the experiments in the second phase, the evolution. The evolution was an iterative process in which three stages were repeated till the desired variants were obtained (Figure 1). In the first stage, libraries of mutated genes were generated and transformed into *E. coli* for the two-stage screening process. These libraries varied in size, the average being around $5 \times 10^4$, from which about $10^3$ variants were selected for secondary screening, and a few tens selected to generate

the next library. As will be described below, the methods used to measure activity in the secondary screen utilized variants as produced in crude lysates so that it was not clear if increases in activity were due to increased catalytic performance or a change in physical properties, such as increased expression, solubility or stability. For this reason, a selection of variants was purified and characterized; their catalytic properties were determined along with their tolerance of proteases and surface active agents.

*Phase 1 Initial Characterisation*

Our starting point was the 30 variants we had previously identified after seven rounds of directed evolution as being more stable, soluble and active than *wt* (Alfaro-Chavez et al. 2019). We tested the ability of *wt* and two selected R7 variants to degrade esters with long aliphatic chains, and their activities after being treated with solutions of three commercial laundry detergents (LD1, LD2 and LD3), SDS, an anionic detergent, and Triton X-100, a non-ionic detergent. R7_59A and R7_82E were chosen because they exhibited the best lipolytic activity with natural oils (Alfaro-Chavez et al. 2019).

Neither the *wt* nor the R7 variants exhibited significant activity in the presence of SDS, LD1 or LD2 (Table I). Although LD2 contained a protease, it was heat inactivated prior to being used, so the lack of activity is not due to proteolysis. The starting variants exhibited varying levels of tolerance to Triton X-100 and LD3. The R7_59A variant was unaffected by 2% Triton X-100, suggesting that tolerance to this reagent would not present problems for evolution. Alcalase®, a protease marketed for use in laundry detergents, almost completely inactivated the *wt* enzyme, but R7_82 exhibited some tolerance to it, suggesting that finding other variants resistant to proteases should be possible. Although the starting enzymes had reasonable levels of activity towards C3 and C8 substrates, they had low levels of activity with substrates that had long aliphatic chains (C16 and C18). For this reason, we decided to select for activity with a substrate that was mid-way between C8 and C16, so C12 esters were used in the first round (R8) of the experiments described in this paper.

The addition of TAGs to agar makes the plates cloudy, and they become clear in the presence of an active lipase, so colonies producing active lipase have clear haloes around them (Lanka and Latha 2015; Lawrence et al. 1967). Could we add detergents to the agar to test for

tolerance to these reagents? SDS lyses cells so it was pointless trying this reagent. However, low concentrations of LD1 and LD2 could be used while LD3 did not appear to be an impediment to growth of *E. coli* (Table II). We decided to try LD2 in the primary screen for R9 and R10 (Table III), but it resulted in low survival rates and so it was not used in later rounds of evolution. These results suggested that, if large libraries were to be tested for tolerance to surface active agents, the tests were best done in the secondary screen, as in R8 to R17, or by replicating the library and lysing cells, as was done in R18 to R23.

*Phase 2 Evolution*

The second phase of this work involved 16 rounds of directed evolution that can be divided into three sections based on the selection conditions (Table III). In the first section (R8-R17), selection in the primary screen was done with agar plates doped with TAGs or olive oil. As in the accompanying paper (Alfaro-Chavez et al. 2019), selection in the primary screen was done by picking colonies that gave rise to large haloes. In the second section (R18), 96 well plates were used to grow a library for primary screening and selection was done by monitoring esterase activity in the presence of SDS or natural oils. In the third section (R19-R23), the library was grown on agar plates, replicated on a filter, the cells lysed and activity monitored with 1- naphthyl palmitate. At the end of each round, the genes of variants selected in the secondary screen were sequenced (Table S2).

The genes of the best 30 variants identified at the end of the previous study (Alfaro-Chavez et al. 2019) were used, as in that paper, to generate the library screened in R8. The library size was estimated to be $3 \times 10^5$, much higher than subsequent libraries, because larger quantities of genes from more variants were used. In subsequent rounds, the library sizes varied from about $8 \times 10^3$ to $6 \times 10^4$. This was not due to the amount of DNA used to generate the library, and not because the variants were becoming lethal to the cells. The latter idea was tested in R19 by expressing the variant genes in with a plasmid that gave lower levels of expression (pET26b), which only gave a slight increase in library size. We therefore acquired a fresh stock of pETMCSIII and new primers for the epPCR (see methods), which led to relatively large libraries for the final three rounds (Table III).

*R8-R18: Broadening specificity and increasing tolerance*

The objectives of the evolution varied through the course of the experiment, and so the methods used to screen the libraries changed (Table III). In R8 through R12, the primary screen targeted rate enhancement and broader substrate specificity while exposing the variants to low levels of commercial laundry detergents. In R8, the primary screen utilised C8 TAG, with pNP-C8 in the secondary screen. This was like the conditions used to select variants in R1 through R7 of our previous study. In R12, colonies were selected after 72 hours based on haloes formed in plates containing from olive oil. In the secondary screen, good activity was obtained with pNP-C12. In these rounds, there was an increasing amount of SDS added to lysate prior to the esterase assay; in R12 the SDS concentration was 0.07%, up from the 0.02% used in the previous round.

In R13 through R17, evolution for enhanced catalytic properties continued. For instance, In R13 haloes were observed after 48 hours while in R17 haloes could be seen in just 40 hours. In the secondary screen, esterase activity was easily detected with pnp-C12 in R13 and with pNP-C16 in R17. In R13, 0.4% Alcalase was added to the crude lysate an hour prior to assaying. The same concentration of protease was used in R17, but the variants were exposed for 3 hours. Variants with good tolerance to Alcalase were easily identified in the secondary screen, but the same was not true for SDS tolerance. Attempts to increase the SDS concentration above 0.1% did not produce many variants with detectable activity. It appeared that the small secondary libraries were sufficiently large to include variants that were tolerant to protease, but not to include those with increased tolerance to SDS. Larger libraries were necessary to screen for SDS tolerance at concentrations close to the target of 2%.

In R18, the library used for the primary screen was grown in 96 well plates, replicated, cells lysed and treated with 0.5% SDS and after an hour assayed with pNP-C16 to select variants for secondary screening with pNP-C16. The residual activity of one variant was measured as a function of increasing SDS concentration (Figure 2). It was clear that the R18_211H variant was considerably more active than the R7 variants, that it retained more than half of its activity after treatment with 2% SDS for 15 minutes, and that 4% SDS was required to completely abolish activity. At this point, R18_211H was purified along with the *wt*, two R7 variants (R7_59A and R7_82E), one R13 variant (R13_44C), and one R17 variant (R17_29G). The properties of these proteins were determined, showing that directed evolution had

resulted in a significant increase in SDS tolerance (Table IV). R13_44C retained about 60% of its activity after 1 hour at 22 ºC with 2% SDS; R17_29G retained 70% whereas R18_211H retained over 80% activity in the same test. Furthermore, R18_211H was unaffected by 2% Triton X-100, 2% Alcalase, 0.5% LD2 or LD3. It appeared that selection for SDS stability was sufficient to identify variants sufficiently stable to withstand the detergent in LD2 and LD3. R18_211H lost most of its activity when treated with LD1, but was still better than the R17_29G; the increase in SDS tolerance was accompanied by an increase in thermostability. The $T_{50}$ of R18_211H was 37$^{o}$ C higher than that of the *wt*, 23$^{o}$ C higher than the best R7 variant and 18$^{o}$ C better than R17_29G (Table IV). The esterase activity of the R18 variant was similar for pNP-C3, pNP-C8, pNP-C16 and pNP-C18 substrates and its activity with the longest chain substrates was significantly better variants from previous rounds. This broadening of the substrate specificity was reflected in the lipolytic activity of the R18 variant, as it was more active than variants from previous round against long-chain TAGs.

*R19 to R23: Evolution for SDS tolerance and Characterization*

Our goal was to develop a high-throughput screen to select for variants with improved tolerance to SDS. It was clear from R18 that large libraries (of the order $10^4$) were needed to improve tolerance to SDS. However, making such large libraries was laborious and expensive using 96 well plates (>600 plates needed for a $6x10^4$ library), so we decided to develop a replica agar plate assay (see Methods). In R19 through R23 variants were selected for secondary screening if they exhibited good esterase activity after treatment with SDS on a replica plate. In the secondary screen, the activity of variants was measured with an esterase assay before and after treatment with SDS so that the stability of variants could be determined. The SDS concentration was increased steadily during these rounds (Table IV). In R23 olive oil was added to the agar plates used in the primary screen, and selection for secondary screening required good lipase activity as well as esterase activity after treatment with SDS. In this round, 3% SDS was used in the primary screen while 4% was used in the secondary screen. We also selected for thermostability in the secondary screen (see Methods).

*Phase 3 Detailed characterization*

The best variants from each of R13 (R13_44C), R17 (R17_29G), R18 (R18_211H), R20 (R20_11A), and R23 (R23_110A) were purified and characterized (Table IV). In addition, for purposes of comparison, the *wt* protein and two of the better R7 variants were purified and characterized. Compared with the R7 variants, the yield of purified protein decreased. For example, the R7_82E gave 55 mg from a 250 mL culture while R18_211H grown under the same conditions gave 43 mg. Compared with R7 variants there was an increase in esterase and lipase activities (Table IV). The R23_110A variant was 2-3 times more active on C3 and C8 short chain esters compared to the R18_211H variant, but about 50% less active on C16 and C18 esters (Table IV). This was surprising as selection in the secondary screen was with a pNP-C16 substrate. Lipolytic activity dropped between R18 and R23; the activities of R23_110A with all substrates 1.5-3 times less than for R18_2117H.

SDS tolerance improved as the evolution progressed. There was no observable loss in activity in the R20 variant after treating with 2% SDS for one hour while the same treatment of R23_110A resulted in an increase in the residual activity; concentrations of SDS up to 4% activate it (Figure 2). Complete abolition of activity of the R23 variant required a one hour treatment with 10% SDS as is evident in Figure 2. Although the R23 variant exhibited extremely good tolerance to either SDS or Triton X100, it was not as tolerant of commercial powders LD1 and LD2 and Alcalase compared with the R18 variant (Table IV). For example, the R18 variant retained about 80% of its activity when treated with SDS and retained near 100% of its activity after treatment with LP2 or Alcalase whereas the activity of the R23 variant increased by a factor of 2.6 after treatment with SDS but retained only about 10% of its activity after treatment with LP2 and 40% of its activity after treatment with Alcalase. However, treatment with LD3, a product formulated to accommodate a lipase, did not diminish the activity of the R18, while it appeared to activate the R23 variant.

It was expected that increased tolerance to SDS would be accompanied by an increase in thermostability and all the post-R7 variants had increased thermal stability compared to the R7 variant. However, there did not appear to be a correlation between tolerance to SDS and thermostability. The R18 variant exhibited the best thermostability ($T_{1/2}$ = 73$^{o}$ C), but the

thermal stability of the R23 variant was lower, but still respectable ($T_{1/2}$ = 65$^{o}$ C), being close to 30 $^{o}$C higher than the *wt*.

The ability of a variant to tolerate a protease reached 100% in an R13 variant but decreased to 80% in R17_29G (Table IV). However, the ability to tolerate a protease varied within any round (Figure 3), and there were a few variants in R17 that exhibited 100% tolerance to a protease after 1 hour and maintained most of their activity after 3 hours (Table S1 in Supplementary material). An attempt was made to identify residues responsible for tolerance to Alcalase. The *wt* protein was digested and the resulting peptides sequenced. The proteolytic cut sites are shown on Figure 3. In two instances, there were changes close to the cut sites; the T185A site was 4 residues away from the cut site at 181; and the A174V site is 3 residues away from the cut site at 171. The remaining cut sites were well separated from variant changes.

*Analysis of sequence changes*

The variants often contained more than one new change (supplementary material, Table S2). Only a few persisted into subsequent rounds and some of these approached saturation (present in > 90% of sequenced variants). On average, about one change per round persisted. The initial library for this study was generated with mutated genes of Lip3 from the companion study (Alfaro-Chavez et al. 2019). This introduced four mutations that saturated the R7 final selection (V201A, N268I, P291L and M321L). By R17 there were seven more saturated mutations (M20V, M59L, A174V, T185A, S210T, D230G and L351Q) and by R23 an additional three (E46K, W107L, C202Y) and one reversion (S210T). Of these three saturations, only E46K was present in R7 (Table V). In contrast, there were mutations that reverted to wt as the selection conditions were altered. For example, the Y40F change was present in 23% of the variants sequenced in R7, all variants sequenced in R17 had reverted to Y at residue 40. E250G was present in 45% of the genes sequenced in R7 and 47% at R17 but was not present at R23.

As is evident in Figure 3, a little over half of the changes observed in R23 (55%) were found in the Cap domain, which contains just one third (35%) of the residues in the protein. Of the changes observed in R23, six were in the core domain. Four of these (M59L, W107L, M321L

and L351Q) were found in the interface with the Cap domain and two (M321L and L351Q) were in loops that contained triad residues (Figure 3). Within the Cap domain, most of the changes observed in R23 were found in the Lid portion or very close to it (V201A, C202Y and D230G). One change in R23 (A174V) was found in the region linking the Cap domain to the Core domain. The remaining changes observed in R23 (T185A, N268I and P291L) were found in helices of the Cap domain (Figure 3). Of the saturating changes observed that in R23, four of the 13 resulted in changes of sulphur containing residues. Three of the four changes occur in the core domain with one (M20V) close to the N-terminus.

## Discussion

The starting variants for the present work emerged during seven rounds of evolution (Alfaro-Chavez et al. 2019). In this evolution, crude lysate was used to select variants assessed on their ability to degrade pNP-C8 esters and C8 TAGs. These activities are essentially the same and so the evolution is one-dimensional. Large libraries, of the order $10^4$, were screened, and increases in activity occurred because the amount of active enzyme in the crude lysate increased. Directed evolution thus selected for variants with increased stability and solubility, not necessarily increased specific activity. The present work was more ambitious as selection was for four properties: increased activity, broader substrate specificity, tolerance to a protease and tolerance to detergents.

At the start of this work, the question arose: what was the best approach to improving three quite different enzyme attributes? One approach would be to evolve one attribute at a time. There were two reasons for not taking this approach. First, we were concerned that the improvements obtained in optimizing one property (say, substrate specificity) would be lost in the process of optimizing other properties (say, surfactant stability) in subsequent rounds; that is, substrate specificity would be lost as surfactant stability was enhanced. Second, we had already developed high throughput screens to improve substrate specificity (Alfaro-Chavez et al. 2019), but not to improve tolerance to proteases and surface active agents. We therefore decided to select for substrate specificity in a large primary screen followed by a smaller secondary screen where tolerance for another attribute (stability in surfactants and/or tolerance to a protease) was selected. In this way, a single cycle of directed evolution

could be used to improve all three attributes of selected variants. This approach would reduce the number of cycles required to reach the target conditions, provided that useful variants could be identified in the small libraries examined in the secondary screen and that the two properties being selected were not *completely* mutually incompatible – something we thought unlikely. This approach to evolution resulted in a slow increase in tolerance to SDS. It was thought that a more rapid increase in SDS tolerance could be achieved by screening larger libraries; this was carried out in the last six rounds of evolution.

*Phase 1 Initial characterization*

The object of these experiments was to determine the properties of the R7 variants, to select conditions for screening. The R7 variants had little tolerance of SDS while one (R7_59A) was stable in a Triton X-100 solution, and another (R7_82E) exhibited some tolerance to Alcalase (Table I). As a result, little attention was paid to Triton X-100 in the subsequent evolution, and selection for tolerance to Alcalase was delayed till the variants had acquired some level of tolerance to SDS. The R7 variants had low levels of esterase activity with substrates that had long aliphatic chains, suggesting that this property might be difficult to alter. As a result, the first two rounds of evolution selected for the ability to degrade C8 substrates. The ability to detect lipase activity in the presence of commercial laundry powders was also tested with an assay that utilized an agar plate as used in the primary screens (Table III). It appeared that activity could only be detected in very low levels of LP1 and LP2. It subsequently became apparent that this result was due to the poor tolerance of *E. coli* to these products. Cells could be grown in LP3, but this product already contained a lipase so there was little point in evolving tolerance to it in Lip3 variants.

*Phase 2 Evolution.*

The method used to generate the libraries remained the same in all the rounds of evolution, even though the library varied in size (see Results). By way of contrast, the selection conditions changed a great deal in response to the results obtained in the previous round. Selection in R8 through R17 was for altered substrate specificity along with tolerance to a protease and SDS. In the primary screen of R8 through R18, libraries of the order $10^4$ were used to identify variants with increased activity on olive oil, which has long aliphatic chains. The ability to degrade these substrates improved slowly (Table IV). For example, the best

variant in R13 exhibited lipase activity with olive oil that was enhanced by a factor of approximately 4.5 compared with the *wt*, while by R17 this had decreased to about 2.8. The secondary screens were carried out with libraries of the order $10^3$ and selection was for tolerance to SDS and proteolysis. Tolerance to proteolytic attack was achieved easily by scanning the small libraries of the secondary screen. Tolerance to SDS did not improve significantly; 0.1% SDS was used to select variants in R17. It appeared that large libraries were needed to evolve for changes in substrate specificity and improved tolerance to SDS.

From R18 to R23, large libraries were used to identify variants with tolerance to SDS while maintaining activity towards substrates with long aliphatic chains. Tolerance of SDS improved dramatically. R23_110A, for instance, exhibited activity that was increased by a factor of 2.6 upon treatment with 2% SDS for an hour; it was activated by SDS concentrations up to 4%. However, this variant exhibited reduced performance in some tests; for example, its lipolytic activity was less than the purified R18 variant. How could the screens used in R19 through R23 be modified to enable better all-round variants to be identified? This requires that the SDS tolerance be maintained and activity with long chain substrates be improved. This could be achieved by placing greater emphasis on activity. For example, the secondary screen selection could be based on activity as well as tolerance to SDS. In R23, variants were selected if they exhibited high levels of tolerance to SDS without exceptionally high levels of activity. A better all-round variant could be obtained by selecting variants that have good SDS tolerance and catalytic activity.

*Phase 3 Final Characterization*

A casual inspection of Table IV prompts the question: which is the best variant? It is difficult to answer this question as no one variant is best in all the assays. The properties of the variants changed as selection changed and the catalytic properties did not show a clear trend. The R18 variant was the most active lipase with olive oil while the R20 variant was the most active lipase with glyceryl trioleate. The catalytic properties of the R23 variant (Table IV) were inferior to variants obtained in previous rounds, but it was much more stable in SDS. Similarly, Alcalase tolerance shows no clear trend as the evolution progresses. One R7 variant shows some tolerance of Alcalase; by R13 Alcalase tolerance is near 100%; in R17, it dropped to 80%; in R18 and R20, it increased to 100%; and in R23, it dropped again to 41%. It appears that

tolerance to Alcalase is easily lost and acquired. It should be noted that our use of the word "tolerance" implies stability in solution for a few hours (as was the case in our assays). We did not test for longer time periods.

The one property that steadily improves during evolution is the tolerance to SDS. This reagent easily denatures the *wt* and R7 variants. The R13 variant retains 59% of its activity after treatment with SDS while the R20 retains 100% after the same treatment. The R23 variant (R23_110A) is activated by SDS. If a variant was required to catalyse reactions in the presence of SDS, the R23_110A activities (Table IV) should be multiplied by 2.6. Such a correction would make the R23 variant the best choice for inclusion in a detergent – except that R23_110A is susceptible to proteolysis. As noted above, tolerance to proteolytic attack was readily acquired through selection with small libraries produced in secondary screens. The R23 variant used to generate the data in Table IV was selected because of its ability to tolerate SDS and no consideration was given to its tolerance of Alcalase. It is very probable that other R23 variants were tolerant of Alcalase and, if this were not the case, tolerance could easily be regenerated by screening small libraries.

In the early rounds of evolution, commercial detergents were used in selection. These products were not ideal for evolution because their composition is not always publically available and they can change at the discretion of the manufacturer, so we decided to use selection with SDS instead. Nevertheless, purified variants were tested for tolerance to commercial products. Interestingly, the R23 variant was not the variant most tolerant of commercial powders. In going from R18 to 23 the tolerance to SDS increased, but the tolerance to LD1 and LD2 decreased. It is probable that SDS is not present in any of the commercial product and that in concentrating on SDS stability in R18 through R23 we weakened the ability of variants to tolerate the detergents present in LD2. The R18_21H variant showed the best overall tolerance to commercial powders, but its tolerance to LD1 of only 16% could be described as marginal. However, the R18_21H variant was unaffected by the detergents in LD2 that had been formulated to contain a protease. It is not clear that the protease in LD2 is Alcalase, but if it were then R18_21H could be added to this product so that enzymes could act on both proteins and lipids.

*Comparison with other studies*

Directed evolution has been used to identify detergent stable variants of other proteins, but the methods used for selection were quite different to that used here (Scott and Plückthun 2013; Yong and Scott 2015). In these studies, the Cellular High-Throughput Encapsulation Solubilisation and Screening (CHESS) method was used (Yong and Scott 2015). In one study, very large libraries ($10^{10}$) were screened with an instrument (FACS) that detected a fluorescence signal. The variant proteins were tested with 2% SDS in a buffered 10% glycerol solution. One variant that exhibited enhanced tolerance to SDS, had only two changes from the starting protein. By way of contrast, our libraries were much smaller and required multiple rounds of evolution to give enhanced stability, but the selection in our experiments was for multiple properties, not just SDS tolerance. When our experiments were focused on enhancing SDS tolerance (R18-R23), the tolerance quickly rose and selection involved treating the enzyme with 4% SDS, with no glycerol present to enhance stability. We argue that our methods are more versatile and more than adequate to identify the small number of changes required to give enhanced tolerance to SDS.

To the best of our knowledge, directed evolution has not previously used to enhance protease tolerance in lipases, though other approaches have been. To enhance protease resistance, Grbavčić and coworkers used a lipase from the same organism as a protease (Grbavčić et al. 2011). This assumes that the lipase has evolved to be tolerant of the protease: even if true, it is unlikely that both enzymes will be suitable in all other respects for use in a laundry detergent. Traditional protein engineering approaches have also been used (Frenken et al. 1993; Markert et al. 2001). In one such approach, protease cleavage sites were identified and site specific mutagenesis used to alter the protein sequence near the cleavage site. This approach is labour intensive and assumes that the changes will not degrade the activity of the enzyme. Finally, previous studies (Ece et al. 2015) have shown that increased thermal stability can give rise to increased tolerance to surface active agents, but evolution with selection for tolerance to surface active agents has not been described in the literature. Overall, we expected that directed evolution would be a more efficient way to generate tolerance to both proteases and surface active agents. Directed evolution has been used to generate protease tolerance for small peptides using a different method (Fiacco et al. 2016) to that used in the present study.

Directed evolution has been used in lipases to identify enzyme variants with altered substrate specificity (Bornscheuer 2008; Klaus Liebeton et al. 2000b; Prasad et al. 2011; Reetz et al. 1997). Our study differs from those cited in that substrate specificity was altered along with other properties.

*Sequence analysis*

Studies involving directed evolution often give some insight into the relationship between sequence and various physical properties. The present study is unusual in that selection was made for three attributes, but there are many other properties that might be affected by the evolution. For example: enantioselectivity (Engström et al. 2010; K. Liebeton et al. 2000a; Reetz et al. 1997), stability in organic compounds (Dror et al. 2014; Korman et al. 2013), thermostability (Augustyniak et al. 2012; Liu et al. 2017; Yu et al. 2012; Zhang et al. 2003), substrate specificity (Fujii et al. 2005).

The changes in sequence that occur during evolution could provide information about the role of specific residues in catalysis as well as the physical properties of the enzymes. Unfortunately, changes in the sequence were not made singly against a constant background so it is difficult to know the effects of a single mutation on the properties of a variant. In R1 to R7 (Alfaro-Chavez et al. 2019), selection for increased activity resulted in enhanced stability and solubility with modest gains in activity. From R8 onwards, selection was based on more than one property and only a small subset of variants was thoroughly characterized, making the task of assigning a role to specific changes more difficult. For example, it has been suggested that surfactant stability correlates with thermal stability (Ece et al. 2015; Salameh and Wiegel 2010). In this study, the SDS stability increased as evolution progressed, but the same cannot be said for thermos-stability. The R23 variant exhibited greatly enhanced tolerance to a heat shock that the wt or the R7 variants, but it was less stable than the R18 variant. Our results give some support to the proposition that surfactant and thermal stability, however, they also suggest that this proposition may breakdown as SDS concentration increases; more data are required to confidently answer this question.

Understanding the relationship between sequence and function is usually done with the aid of one or more structures. We were unable to obtain useable crystals of Lip3 or of variants identified in R7 or R17. We therefore adopted a homology modelling approach (Alfaro-Chavez et al. 2019) and mapped the locations of the changes onto a schematic of the structure (Figure 3). Three of the changes fixed in the R7 variants (Alfaro-Chavez et al. 2019) reverted to *wt* by R17. This is best explained by the shuffling process in library generation, rather than sequence changes introduced by the error prone polymerase. Some of the genes for the R7 variants would have had wild-type sequences at these positions, and so shuffling gave *wt* fragments that were more viable under the new selection conditions than the R7 variant sequences. One of these reversions (green squares in Figure 3) occurred in the cap domain that covers the active site: we posit that it is important for substrate specificity. Up to R7, selection was for C8 substrates while in subsequent rounds it was for substrates with longer chains. The two other changes occurred at the start and end of the sequence, at positions 40 and 347 (green squares in Figure 3). In these cases, the changes occurred in a piece of strand that linked two elements of secondary structure and were solvent accessible. We speculate that these two changes enhanced stability in the absence of detergents, but did not stabilise – or were destabilising – in their presence. Only four changes observed in R7 (Table V and green circles in Figure 3) were preserved in R17; these were residues 201, 268, 291 and 321. As noted above and explained more fully in our previous paper, three of these four changes presumably enhance the stability and solubility of the enzyme.

At the end of R17 (Table V), we sequenced 11 variants. 11 changes were saturated, of which five were from R7 (four saturated and one not saturated in R7). The M20V and M59L changes may reflect oxygen sensitivity (see below). D230 is in the lid of the cap domain, suggesting a role in catalytic enhancement. Other changes are in surface accessible loops that connect secondary structure elements and are possibly responsible for enhancing tolerance to surface active agents. There were two changes that saturated in R17 (T185A and L351Q) were close to protease cleavage sites (see Figure 3). Both changes could have altered protease tolerance. As expected, there were no changes in the interior of the molecule core.

At the end of R23, there were 13 saturated changes; three were new, but present in R17 variants (E46K, W107L and C202Y, blue circles in Figure 3A) and one that was saturated in R17

and lost by R23 (S210T, red square in Figure 3A). Three mutations suggest that changes in charged residues can improve SDS tolerance (E46K, reversion of E347 to K and D230G). The reverse change, K347E, occurred in 30% of variants in R7, but disappeared when selection involved SDS. Similarly, the D230G change that saturated in R17 removed a negatively charged residue from the surface of the protein. The W107L change could give increased stability by replacing an exposed large aromatic sidechain with a smaller one.
The C202Y is discussed below.

At the end of R23 there were three changes in which methionine residues were altered. The M21L change saturated in R7. The changes that were introduced between R7 and R17 include three changes to methionine and cysteine residues: M20V, M59L and C202Y, with the C202Y change saturating in R23. We suspect that the oxidation sensitivity of Met and Cys (Means and Feeney 1971) may explain these changes. M20 and M321 are located on the surface of our model structure, but there are other sulphur containing residues that are accessible to solvent and not modified, so oxidation sensitivity cannot be the sole explanation. The M59L and C202Y changes affect buried residues. M59L may stabilize by giving better packing, but this is probably not true of C202Y, as this residue is in the cap domain close to the hinge point of the lid, suggesting that the change may enhance catalysis.

*Concluding Remarks*

Perhaps the most important aspect of this work has been the development of screens to select for variants with enhanced properties. It may be possible to adapt these screens for tolerance to surface-active agents other than SDS and proteases other than Alcalase. The primary goal of the work was to produce variants with enhancements in three, potentially orthogonal, properties and to develop methods to achieve this end. In this aim, we have succeeded. Variants have been identified that degrade oils and that tolerate surface active agents and proteases. Our protocols can be used in the future to evolve other enzymes for applications in laundry detergents. However, the present work also illustrates how proteins can evolve to survive what was initially an extremely hostile environment. In two studies, a relatively small number of changes were required to convert an enzyme that had poor solubility and weak activity in water to one that was not only soluble, but exhibited enhanced

tolerance to heat treatment and that showed preferential activity in detergent solutions, conditions not likely to be encountered by the organism from which the enzyme was found.

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**References**


Adrio, Jose L. and Demain, Arnold L. (2014), 'Microbial Enzymes: Tools for Biotechnological Processes', *Biomolecules,* 4 (1), 117-39.

Alfaro-Chavez, Ana, et al. (2019), 'Improving on nature's shortcomings: evolving a lipase for increased lipolytic activity, expression and thermostability', *Protein Engineering, Design and Selection*.

Arnold, Frances H. (1998), 'Design by Directed Evolution', *Accounts of Chemical Research,* 31 (3), 125-31.

--- (2018), 'Directed Evolution: Bringing New Chemistry to Life', *Angewandte Chemie (International ed. in English),* 57 (16), 4143-48.

Arnold, Frances H. and Volkov, Alexander A. (1999), 'Directed evolution of biocatalysts', *Current Opinion in Chemical Biology,* 3 (1), 54-59.

Augustyniak, Wojciech, et al. (2012), 'Biophysical characterization of mutants of Bacillus subtilis lipase evolved for thermostability: Factors contributing to increased activity retention', *Protein Science,* 21 (4), 487-97.

Bezard, J., Bugaut, M., and Clement, G. (1971), 'Triglyceride composition of coconut oil', *Journal of the American Oil Chemists' Society,* 48 (3), 134-39.

Bornscheuer, Uwe T. (2008), 'Alteration of lipase properties by protein engineering methods', *OCL,* 15 (3), 184-88.

Camacho-Ruiz, María de los Angeles, et al. (2015), 'A broad pH range indicator-based spectrophotometric assay for true lipases using tributyrin and tricaprylin', *Journal of Lipid Research,* 56 (5), 1057-67.

Celniker, Gershon, et al. (2013), 'ConSurf: Using Evolutionary Data to Raise Testable Hypotheses about Protein Function', *Israel Journal of Chemistry,* 53 (3-4), 199-206.

Dror, Adi, et al. (2014), 'Protein engineering by random mutagenesis and structure-guided consensus of Geobacillus stearothermophilus Lipase T6 for enhanced stability in methanol', *Applied and environmental microbiology,* 80 (4), 1515-27.

Ece, Selin, et al. (2015), 'Improving thermal and detergent stability of Bacillus stearothermophilus neopullulanase by rational enzyme design', *Protein Engineering, Design and Selection,* 28 (6), 147-51.

Engström, Karin, et al. (2010), 'Directed Evolution of an Enantioselective Lipase with Broad Substrate Scope for Hydrolysis of α-Substituted Esters', *Journal of the American Chemical Society,* 132 (20), 7038-42.

Fiacco, Stephen V., et al. (2016), 'Directed Evolution of Scanning Unnatural-Protease-Resistant (SUPR) Peptides for in Vivo Applications', *ChemBioChem,* 17 (17), 1643-51.

Frenken, L. G., et al. (1993), 'Pseudomonas glumae lipase: increased proteolytic stability by protein engineering', *Protein Eng,* 6 (6), 637-42.

Fujii, Ryota, et al. (2005), 'Directed evolution of Pseudomonas aeruginosa lipase for improved amide-hydrolyzing activity', *Protein Engineering, Design and Selection,* 18 (2), 93-101.

Gattiker, Alexandre, et al. (2002), 'FindPept, a tool to identify unmatched masses in peptide mass fingerprinting protein identification', *PROTEOMICS,* 2 (10), 1435-44.

Grbavčić, Sanja, et al. (2011), 'Production of lipase and protease from an indigenous Pseudomonas aeruginosa strain and their evaluation as detergent additives: Compatibility study with detergent ingredients and washing performance', *Bioresource Technology,* 102 (24), 11226-33.

Gupta, Namita, Rathi, Pooja, and Gupta, Rani (2002), 'Simplified para-nitrophenyl palmitate assay for lipases and esterases', *Analytical Biochemistry,* 311 (1), 98-99.

Hasan, Fariha, et al. (2010), 'Enzymes used in detergents: Lipases', *African Journal of Biotechnology,* 9 (31), 4836-44.

Korman, Tyler P., et al. (2013), 'Dieselzymes: Development of a stable and methanol tolerant lipase for biodiesel production by directed evolution', *Biotechnology for Biofuels,* 6 (1), 70-70.

Kumar, Dinesh, et al. (2008), 'Microbial proteases and application as laundry detergent additive', *Research Journal of Microbiology,* 3 (12), 661-72.

Lanka, Suseela and Latha, J. Naveena Lavanya (2015), 'A short review on various screening methods to isolate potential lipase producers: Lipases-the present and future enzymes of biotech industry', *International Journal of Biological Chemistry,* 9 (5), 207-19.

Lawrence, R. C., Fryer, T. F., and Reiter, B. (1967), 'Rapid Method for the Quantitative Estimation of Microbial Lipases', *Nature,* 213 (5082), 1264-65.

Liebeton, K., et al. (2000a), 'Directed evolution of an enantioselective lipase', *Chem Biol,* 7 (9), 709-18.

Liebeton, Klaus, et al. (2000b), 'Directed evolution of an enantioselective lipase', *Chemistry & Biology,* 7 (9), 709-18.

Liu, Yihan, et al. (2017), 'Improvement in thermostability of an alkaline lipase I from Penicillium cyclopium by directed evolution', *RSC Advances,* 7 (61), 38538-48.

Markert, Yvonne, et al. (2001), 'Increased proteolytic resistance of ribonuclease A by protein engineering', *Protein Engineering, Design and Selection,* 14 (10), 791-96.

Maurer, Karl-Heinz (2004), 'Detergent proteases', *Current Opinion in Biotechnology,* 15 (4), 330-34.

Means, Gary and Feeney, Robert (1971), *Chemical Modification of Proteins* (USA).

Niyonzima, F. N. and More, S. S. (2015), 'Microbial detergent compatible lipases', *JOURNAL OF SCIENTIFIC & INDUSTRIAL RESEARCH,* 74 (2), 105-13.

Porter, Joanne L., Rusli, Rukhairul A., and Ollis, David L. (2016), 'Directed Evolution of Enzymes for Industrial Biocatalysis', *ChemBioChem,* 17 (3), 197-203.

Powell, Keith A., et al. (2001), 'Directed Evolution and Biocatalysis', *Angewandte Chemie International Edition,* 40 (21), 3948-59.

Prasad, S., Bocola, M., and Reetz, M. T. (2011), 'Revisiting the lipase from Pseudomonas aeruginosa: directed evolution of substrate acceptance and enantioselectivity using iterative saturation mutagenesis', *Chemphyschem,* 12 (8), 1550-7.

Quintero-Flórez, Angélica, et al. (2015), 'The Fatty Acid Composition of Virgin Olive Oil from Different Cultivars Is Determinant for Foam Cell Formation by Macrophages', *Journal of Agricultural and Food Chemistry,* 63 (30), 6731-38.

Reetz, Manfred T., et al. (1997), 'Creation of Enantioselective Biocatalysts for Organic Chemistry by In Vitro Evolution', *Angewandte Chemie International Edition in English,* 36 (24), 2830-32.

Saeki, Katsuhisa, et al. (2007), 'Detergent alkaline proteases: enzymatic properties, genes, and crystal structures', *Journal of Bioscience and Bioengineering,* 103 (6), 501-08.

Salameh, Moh'd A. and Wiegel, Juergen (2010), 'Effects of Detergents on Activity, Thermostability and Aggregation of Two Alkalithermophilic Lipases from Thermosyntropha lipolytica', *The open biochemistry journal,* 4, 22-28.

Scott, Daniel J. and Plückthun, Andreas (2013), 'Direct Molecular Evolution of Detergent-Stable G Protein-Coupled Receptors Using Polymer Encapsulated Cells', *Journal of Molecular Biology,* 425 (3), 662-77.

Stemmer, W. P. (1994), 'Rapid evolution of a protein in vitro by DNA shuffling', *Nature,* 370 (6488), 389-91.

Stevenson, Bradley J., Liu, Jian-Wei, and Ollis, David L. (2008), 'Directed Evolution of Yeast Pyruvate Decarboxylase 1 for Attenuated Regulation and Increased Stability', *Biochemistry,* 47 (9), 3013-25.

Turner, Nicholas J. (2009), 'Directed evolution drives the next generation of biocatalysts', *Nature Chemical Biology,* 5, 567.

Wu, Chenyi, et al. (1999), 'Proteolysis of native proteins. Trapping of a reaction intermediate', *Journal of Biological Chemistry,* 274 (2), 1108-15.

Yong, K. J. and Scott, D. J. (2015), 'Rapid directed evolution of stabilized proteins with cellular high-throughput encapsulation solubilization and screening (CHESS)', *Biotechnology and Bioengineering,* 112 (3), 438-46.

Yu, Xiao-Wei, et al. (2012), 'Enhanced thermostability of a Rhizopus chinensis lipase by in vivo recombination in Pichia pastoris', *Microbial Cell Factories,* 11 (1), 102.

Zhang, Ningyan, et al. (2003), 'Improving tolerance of Candida antarctica lipase B towards irreversible thermal inactivation through directed evolution', *Protein Engineering, Design and Selection,* 16 (8), 599-605.

Zhao, H., et al. (1998), 'Molecular evolution by staggered extension process (StEP) in vitro recombination', *Nat Biotechnol,* 16 (3), 258-61.

**List of figures:**

**Fig 1.** Schematic diagram to illustrate the stages in evolution

**Fig 2.** Tolerance of variants of lipase Lip3 to SDS after several rounds of evolution

**Fig 3.** A. Schematic diagram of lipase Lip3. Arrows represent β sheets, barrels α-helices. Catalytic triad S145, D318 and H350 are in green and residues of the oxyanion hole in orange. Mutations saturated in R7 and retained throughout evolution in green circles. Mutations saturated in R17 and retained until final evolution in red circles. Mutations saturated in R23 in blue circles. Mutations that were saturated but disappeared are in squares. Alcalase® hydrolysis sites are indicated by asterisks (*). B. Model of Lip3. In the left the complete structure, core domain in blue, cap domain in brown, lid in red. In the right green spheres show mutations saturated in R7, red spheres saturations in R17, blue spheres saturations in R23 and black spheres cut sites of protease.

**Table I.** Initial characterization of wild-type and variants from R7.

| | wt | R7 59A | R7 82E |
|---|---|---|---|
| Detergent Effect | | | |
| % remaining activity after 60 minutes of incubation | | | |
| | % | % | % |
| SDS 2% | 1±2 | 1±1 | 2.3±2.5 |
| Triton X-100 2% | 20±3 | 99±1 | 37±1 |
| LD1 0.5% | 5±1 | 2±1 | 1.9±0.1 |
| LD2 0.5% | 2.2±0.3 | 1.9±0.2 | 2.5±0.2 |
| LD3 0.5% | 30.7±0.3 | 64.7±0.2 | 36.6±0.4 |
| Alcalase® 2% | 4.0±0.4 | 6.5±0.5 | 48.8±0.4 |
| Specific Activity | | | |
| μM *p*-nitrophenol/min/μg protein | | | |
| pNP-C3 | 15.9±0.02 | 99.9±0.1 | 74.1±0.1 |
| pNP-C8 | 96.6±0.1 | 147.56±0.02 | 148.14±0.01 |
| pNP-C16 | 1.635±0.002 | 3.06±0.02 | 0.782±0.004 |
| pNP-C18 | 1.566±0.003 | 4.16±0.01 | 0.89±0.01 |

**Table II.** Test of growth in agar plates with detergents

| Plates | CG 8823 wt | R9 library |
|---|---|---|
| LB amp | ++ | ++ |
| LB amp TG C8 | ++ | ++ |
| LB amp TG C4 | ++ | ++ |
| 0.05% LD2 | ++ | ++ |
| 0.1% | + | + |
| 0.5% | - | - |
| 1% | - | - |
| 2% | - | - |
| 0.05% LD1 | ++ | ++ |
| 0.1% | + | + |
| 0.5% | - | - |
| 1% | - | - |
| 2% | - | - |
| 0.5% v/v LD3 | ++ | ++ |
| 1% | + | ++ |
| 1.5% | + | ++ |
| 2% | + | + |

(++) >2000 colonies

(+) ~500 colonies

(-) no growth

**Table III.** Experimental details of the continuous generation during directed evolution

| Round | Primary Screening | | | Secondary Screening | |
|---|---|---|---|---|---|
| | Library | Variants[1] | Selection[2] | Selection[3] | Variants[4] |
| 8 | $2.72*10^5$ | 820 | TG C8 / 18 hours | pNP-C12/ Detergent tolerance | 10 |
| 9 | $2.4*10^4$ | 620 | TG C8 / olive oil / 18 hours/72 hours | pNP-C12/ Detergent LD1 | 19 |
| 10 | $6*10^4$ | 620 | olive oil and detergent / 72 hours | pNP-C12 / 0.01% SDS | 14 |
| 11 | $6*10^4$ | 880 | olive oil and detergent / 72 hours | pNPC12 / 0.02% SDS | 20 |
| 12 | $2.4*10^4$ | 350 | olive oil/ 72 hours | pNP-C12 / 0.07% SDS | 15 |
| 13 | $6.4*10^4$ | 530 | olive oil / 48 hours | pNP-C12 / 0.1% SDS / 0.4% protease 1 hour | 12 |
| 14 | $1.4*10^4$ | 260 | olive oil / 48 hours | pNP-C12 / 0.1% SDS / 0.4% protease 3 hour | 20 |
| 15 | $8*10^3$ | 180 | olive oil /48 hours | pNP-C12 / 0.1% SDS / 0.4% protease 1 and 3 hours | 10 |
| 16 | $2.1*10^4$ | 260 | olive oil / 40 hours | pNP-C16 / 0.1% SDS / 0.4% protease 1 and 3hours / 0.02% triton X-100 / oils | 20 |
| 17 | $2.4*10^4$ | 260 | olive oil / 40 hours | pNP-C16 / 0.1% SDS / 0.4% protease 1 and 3 hours / 0.1% triton X-100 / oils mixture + phenol red | 17 |
| 18 | $1.4*10^4$ | 350 | 96-well plate pNP-C16/SDS 0.5%/oils mixture | pNP-C16 | 14 |
| 19 | $3.3*10^4$ | 440 | LB kan plates (pET26b)/1%SDS-naphthyl palmitate | pNP-C16/0.5% SDS | 6 |
| 20 | $3.3*10^4$ | 180 | LB amp plate (new primer)/2%SDS-naphthyl palmitate | pNP-C16/0.5% SDS | 3 |
| 21 | $6*10^4$ | 450 | LB amp plate (new primer)/2%SDS-naphthyl palmitate | pNP-C16/0.5% SDS/ thermo 65°C 30' | 11 |
| 22 | $6*10^4$ | 720 | LB amp plate (new primer)/2.5%SDS-naphthyl palmitate | pNP-C16/0.5% SDS/ thermo 65°C 30' | 9 |
| 23 | $6*10^4$ (150 col/plat) | 540 | LB amp plate +olive oil/3%SDS-naphthyl palmitate | pNP-C16/4% SDS removed with KCl/ thermo 65°C 30' | 10 |

[1] The number of variants selected in the primary screen.
[2] Selection was done for lipolytic activity in agar plates. For R9, two sets of agar plates were used, one was prepared with TG C8 incubated for 18 hours and the other was with olive oil and incubated for 72 hours; time required for halo formation decreased from 72 to 40 hours for olive oil and the incubation temperature was 37°C in all rounds.
[3] Selection was interactive in the secondary screening. The temperature at which the selected colonies were grown in 96-well plates for a later assay for esterase activity
[4] The number of variants selected in the secondary screen.

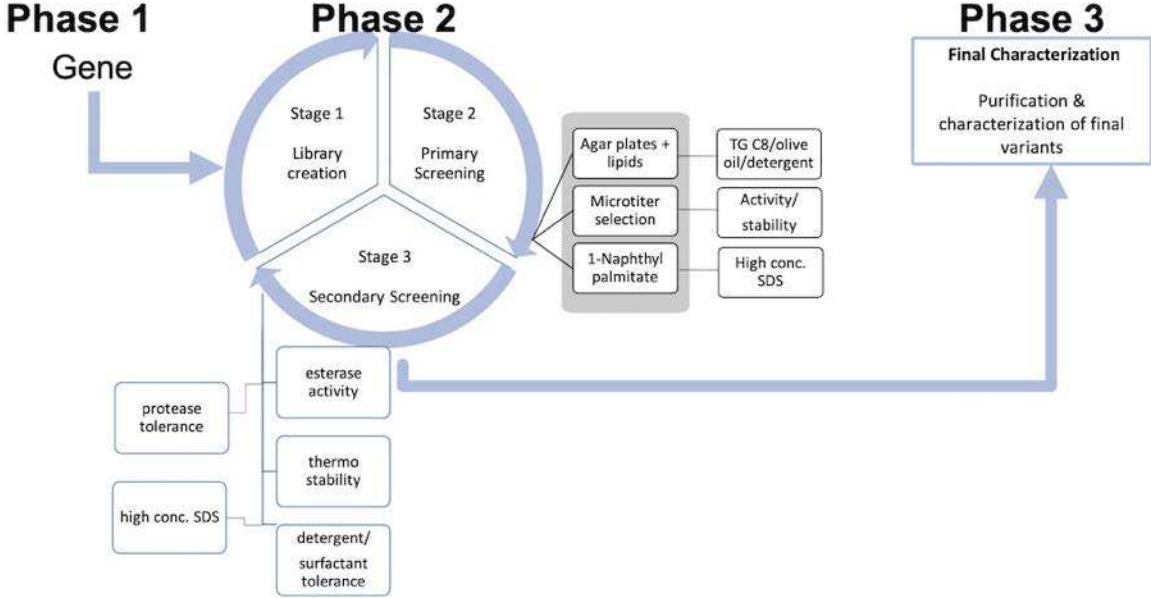
**Table IV.** Characterization of mutants for library of surfactant tolerance.

| | wt | R7_59A | R7_82E | R13_44C | R17_29G | R18_211H | R20_11A | R23_110A |
|---|---|---|---|---|---|---|---|---|
| **Detergent Effect** | | | | | | | | |
| **% remaining activity after 60 minutes of incubation** | | | | | | | | |
| **SDS 2%** | 1±2 | 1±1 | 2.3±2.5 | 59±1.5 | 69±1.5 | 84±10 | 102±2 | 260±3 |
| **Triton X-100 2%** | 20±3 | 99±1 | 37±1 | 101.5±0.4 | 95.1±1.4 | 98±2 | 98±1 | 118±4 |
| **LD1 0.5%** | 5±1 | 2±1 | 1.9±0.1 | 1.8±0.1 | 7.2±3.2 | 16.9±0.2 | 1.2±0.2 | 11±1 |
| **LD2 0.5%** | 2.2±0.3 | 1.9±0.2 | 2.5±0.2 | 98±3 | 72.7±3.1 | 98±1 | 44±6 | 11±1 |
| **LD3 0.5%** | 30.7±0.3 | 64.7±0.2 | 36.6±0.4 | 98±2 | 122.3±5.2 | 97±2 | 102±1 | 140±5 |
| **Alcalase® 2%** | 4.0±0.4 | 6.5±0.5 | 48.8±0.4 | 96±4 | 80.3±2.3 | 101±2 | 100±3 | 41±1 |
| **Specific Activity** | | | | | | | | |
| µM *p*-nitrophenol/min/µg protein | | | | | | | | |
| **pNP-C3** | 15.9±0.02 | 99.9±0.1 | 74.1±0.1 | 106.7±0.3 | 320.1±5 | 131.6±0.4 | 476.7±0.1 | 366.7±0.2 |
| **pNP-C8** | 96.6±0.1 | 147.56±0.02 | 148.14±0.01 | 145.61±0.03 | 857.6±6 | 146.7±0.02 | 460.81±0.03 | 697.6±0.1 |
| **pNP-C16** | 1.635±0.002 | 3.06±0.02 | 0.782±0.004 | 56.6±0.1 | 21.3±2.2 | 139.09±0.05 | 295.32±0.01 | 95.56±0.01 |
| **pNP-C18** | 1.566±0.003 | 4.16±0.01 | 0.89±0.01 | 65.98±0.12 | 29.7±1 | 160.2±0.1 | 152.66±0.01 | 94.2±0.1 |
| **Lipolytic activity** | | | | | | | | |
| µM acid released/min/ µM protein | | | | | | | | |
| **Tributyrin** | 4.5±0.3 | 23±1 | 16±3 | 65±1 | 30±3 | 66±1 | 71±8 | 40.2±0.3 |
| **glyceryl trioctanoate** | 9.5±0.2 | 60±1 | 57±1 | 59.4±0.1 | 78±1 | 55±1 | 36.4±4.1 | 21.7±0.4 |
| **coconut oil** | 3.1±0.2 | 14.5±3.5 | 14±2 | 13±1 | 12.4±2 | 20±2 | 19±2 | 10±1 |
| **glyceryl trioleate** | 0.2±0.2 | 2.4±0.4 | 2±1 | 17.3±0.3 | 14±0.2 | 21±1 | 23±2.0 | 13.3±0.4 |
| **olive oil** | 0.08±0.01 | 1.3±0.3 | 1.2±0.5 | 3.7±0.3 | 2.2±0.3 | 16.9±0.4 | 15±1 | 5.1±0.3 |
| **Thermostability** | | | | | | | | |
| [°C] | | | | | | | | |
| **$T_{50}$, 45 min** | 37.3±0.1 | 45±0.4 | 49.6±0.7 | 57.1±0.2 | 55.8±0.7 | 73.8±0.3 | 58.2±0.4 | 65.1±0.8 |

**Table V.** Alterations in amino acid sequences

| ID | 20 | 40 | 46 | 59 | 107 | 174 | 185 | 186 | 201 | 202 | 210 | 211 | 225 | 230 | 234 | 242 | 250 | 268 | 291 | 305 | 321 | 347 | 351 | act | Subs (pNP ) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WT | M→ V | Y→ F | E→ K | M→ L | W→ L | A→ V | T→ A | R→ P | V→ A | C→ Y | S→ T | N→ H | T→ A | D→ G | N→ S | Y→ H | E→ G | N→ I | P→ L | K→ G | M→ L | K→ E | L→ Q | 1 0.0 5 | C8 C16 |
| R7 (30) % | 10 | 23 | 3 | 10 | 0% | 10 | 3 | 0 | 100 | 0 | 30 | 0 | 23 | 7 | 0 | 0 | 47 | 93 | 100 | 3 | 93 | 30 | 3 | 104-222 | C8 |
| R17 (11) % | 91 | 0 | 55 | 100 | 27 | 100 | 100 | 0 | 100 | 9 | 91 | 18 | 9 | 100 | 18 | 18 | 45 | 100 | 100 | 27 | 100 | 0 | 100 | 0.94-1.15 | C16 |
| R18_211H | V |  | K | L | L | V | A |  | A | Y |  |  |  | G |  |  |  | I | L |  | L |  | Q | 1 | C16 |
| R23 (8) % | 100 | 0 | 100 | 100 | 100 | 100 | 100 | 63 | 100 | 100 | 0 | 0 | 0 | 100 | 13 | 0 | 0 | 100 | 100 | 0 | 100 | 0 | 100 | 0.2-1.2 | C16 |
| Consurf* | E | E | E | B | E | B | B | E | B | B | E | E | E | E | e/f | b | e/f | B | E | E | B | E | B |  |  |
| Solv. Access. (model) | 28.9 | 131.4 | 135.4 | 13 | 136.8 | 2.7 | 23.5 | 205.9 | 93.6 | 32.6 | 59.8 | 80.3 | 120.5 | 72.1 | 61 | 129.5 | 92 | 0.03 | 3.6 | 141 | 10.2 | 127.8 | 16.1 |  |  |

*(Celniker et al. 2013)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Phase 2
Stage 1
Library creation
Stage 2
Primary Screening
Stage 3
Secondary Screening
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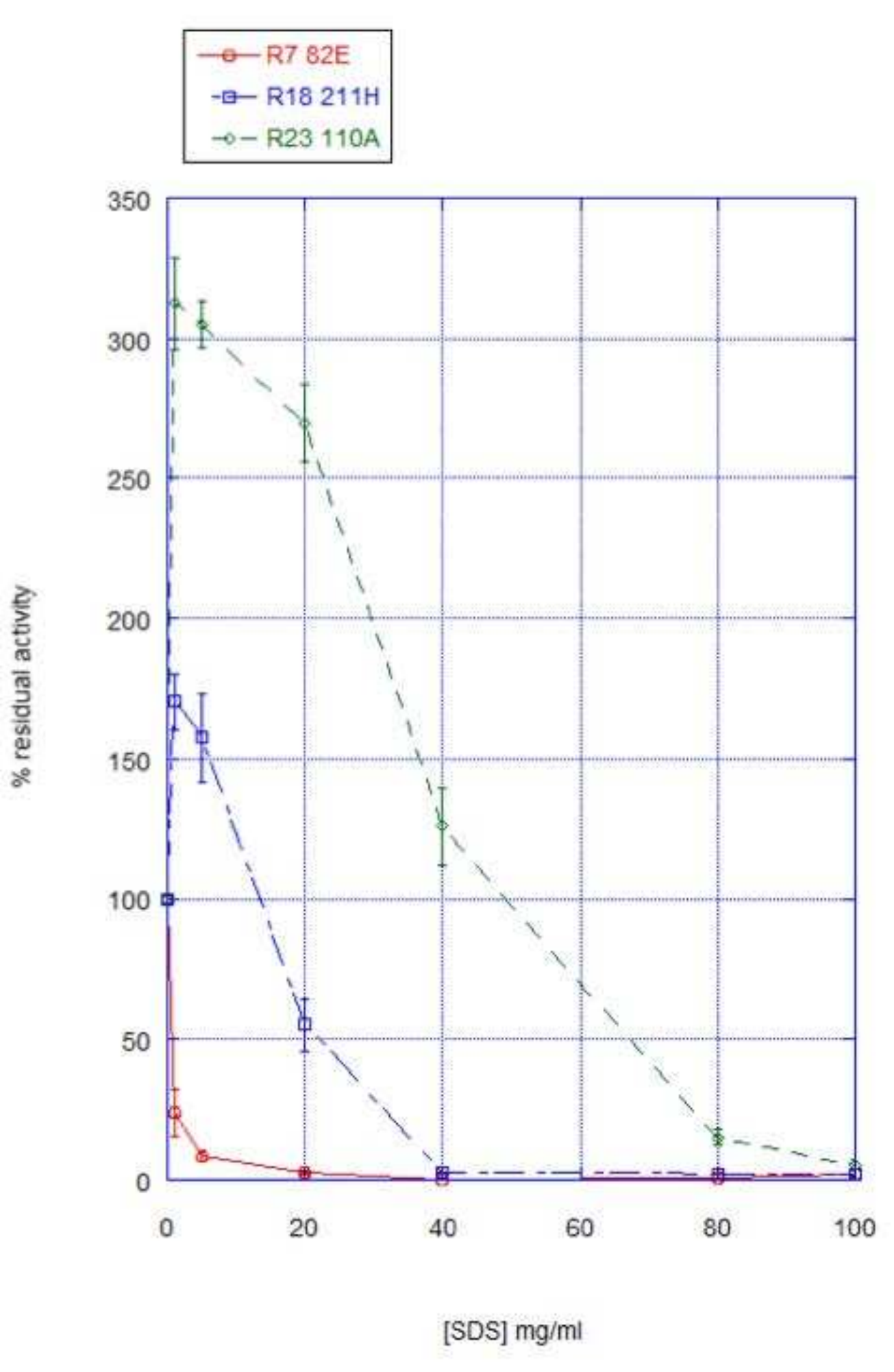
TG C8/olive oil/detergent
Microtiter selection
Activity/ stability
1-Naphthyl palmitate
High conc. SDS
Phase 3
Final Characterization
Purification & characterization of final variants
esterase activity
protease tolerance
thermo stability
high conc. SDS
detergent/ surfactant tolerance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R18 211H
R23 110A
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250
200
150
100
50
0
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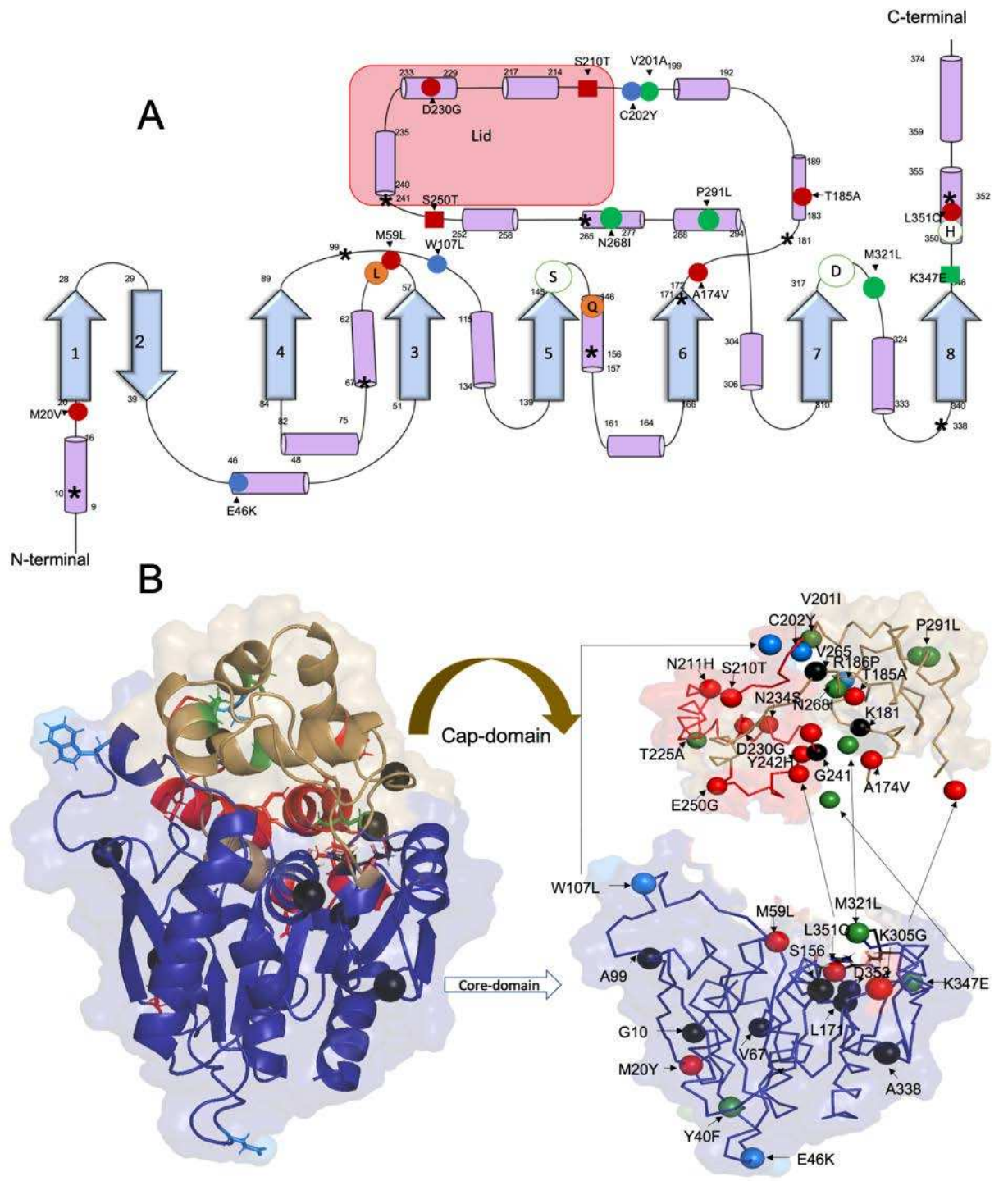
0
20
40
60
80
100
[SDS] mg/ml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N234S
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D352
K347E
G10
L171
V67
M20Y
A338
Y40F
E46K

**Supplementary Material**

Table S1. Characterization of variants as indicated by residual activity for tolerance to surfactants, detergents and Alcalase ®

| | Time [min] | wt | R7_9A | R7_82E | R13_44C | R18_211H | R20_11A |
|---|---|---|---|---|---|---|---|
| | | % | % | % | % | % | % |
| **SDS 1%** | **30** | 7.98±0.29 | 29.5±0.1 | 57.3±0.1 | 85.02±0.05 | 101.63±0.02 | 100.3±0.1 |
| | **60** | 1.98±0.05 | 31.3±0.1 | 58.83±0.02 | 86.1±0.1 | 98.94±0.03 | 102.8±0.1 |
| | **90** | 0.9±0.1 | 30.1±0.1 | 59.78±0.01 | 82.82±0.03 | 99±3 | 98±5 |
| | **120** | 0.6±0.5 | 28.9±0.4 | 54±1 | 78±2 | 97±2 | 100±1 |
| **SDS 2%** | **30** | 4.39±0.02 | 2.3±0.1 | 3.8±0.4 | 19±1 | 21±6 | 92±5 |
| | **60** | 1±2 | 1±1 | 2.3±2.5 | 59±17 | 84±13 | 102±2 |
| | **90** | 1.1±0.4 | 0.3±0.5 | 6±6 | 0.5±0.1 | 5±1 | 58±8 |
| | **120** | 0 | 1±1 | 2.5±0.5 | 3±2 | 2±1 | 0.4±0.2 |
| **Triton X-100 2%** | **30** | 80 ±1 | 128±2 | 87±1 | 100±3 | 100±1 | 98.3±0.2 |
| | **60** | 20±3 | 99±1 | 37±1 | 101.6±0.4 | 98±2 | 98±1 |
| | **90** | 18.2±0.4 | 89±1 | 34±1 | 100±3 | 99±1 | 99.02±0.39 |
| | **120** | 16.5±0.6 | 75.8±0.5 | 25±2 | 101±1 | 98±2 | 101±2 |
| **LD1 0.5%** | **30** | 5.95±0.42 | 1.7±0.1 | 0.1±0.4 | 25.9±0.4 | 74±5 | 50±2 |
| | **60** | 5±1 | 2±1 | 1.9±0.1 | 1.8±0.1 | 16.9±0.2 | 1.2±0.2 |
| | **90** | 1.1±0.2 | 0.2±0.1 | 0.5±0.2 | 0.9±0.1 | 6.6±0.1 | 0.18±0.01 |
| | **120** | 0.5±0.3 | 0.03±0.02 | 0.2±0.2 | 1.2±0.2 | 5±1 | 2±2 |
| **LD2 0.5%** | **30** | 13±3 | 0.8±0.5 | 15±1 | 98.1±0.4 | 102±2 | 97±1 |
| | **60** | 2.2±0.3 | 1.9±0.2 | 2.5±0.2 | 98±3 | 98±1 | 44±6 |
| | **90** | 1.5±0.2 | 0.6±0.1 | 2.6±0.1 | 97±1 | 76±1 | 9±3 |
| | **120** | 2.5±0.4 | 0.6±0.2 | 1.5±0.1 | 95±1 | 45±1 | 2.2±0.1 |
| **LD3 0.5%** | **30** | 83±2 | 125±42 | 85±1 | 101±3 | 100±1 | 100±2 |
| | **60** | 30.7±0.3 | 64.7±0.2 | 36.6±0.4 | 98±2 | 97±2 | 102±1 |
| | **90** | 21.1±0.3 | 52.3±0.4 | 30.6±0.1 | 99±2 | 100±1 | 101±2 |
| | **120** | 21±1 | 45.9±0.4 | 24±1 | 98.4±0.5 | 99±3 | 102±1 |
| **Alcalase® 2%** | **30** | 2.±3 | 5±1 | 39±2 | 99.7±0.2 | 100±2 | 99±1 |
| | **60** | 3.97±0.41 | 6±1 | 48.8±0.4 | 96±4 | 100.5±2 | 100±3 |
| | **90** | 7±2 | 5±1 | 50±1 | 86±2 | 95±2 | 99±3 |
| | **120** | 6±1 | 4.6±0.3 | 41±1 | 59.3±0.4 | 76±2 | 66±1 |