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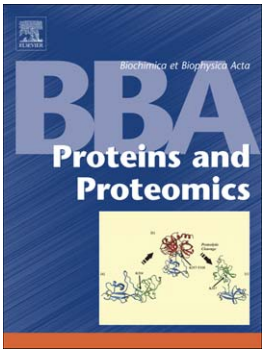

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Elucidation of a key position for acyltransfer activity in *Candida parapsilosis* lipase/acyltransferase (CpLIP2) and in *Pseudozyma antarctica* lipase A (CAL-A) by rational design

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Abstract

Performing transesterifications in aqueous media is becoming a priority challenge in lipid biotechnology in order to develop more eco-friendly and efficient biocatalytic processes in systems containing both polar and apolar substrates. In this context, our group has explored for several years the high potential of the lipase/acyltransferase CpLIP2 from *Candida parapsilosis* and of several of its homologs, that catalyze efficiently acyltransfer reactions in lipid/water media with high water activity (a_w>0.9). The discovery of a new member of this group, CduLAc from *C. dubliniensis,* with a higher acyltransferase activity than CpLIP2, has provided a new insight on structure-function relationships in this group. Indeed, the comparison of sequences and 3D models, especially of CpLIP2 and CduLAc, with those of the phylogenetically related lipase A from *Ps. antarctica* (CAL-A), allowed elucidating a key structural determinant of the acyltransferase activity: serine S369 in CpLIP2 and its equivalents E370 in CAL-A and A366 in CduLAc. Mutants obtained by rational design at this key position showed significant changes in acyltransfer activity. Whereas mutation S369E resulted in an increase in the hydrolytic activity of CpLIP2, S369A increased alcoholysis. More strikingly, the single E370A mutation in CAL-A drastically increased the acyltransferase activity of this enzyme, giving it the character of a lipase/acyltransferase. Indeed, this single mutation lowered the methanol concentration for which the initial rates of alcoholysis and hydrolysis are equal from 2 M in CAL-A down to 0.3 M in its mutant, while the exceptional stability of the parental enzyme toward alcohol and temperature was conserved.

Keywords: lipases/acyltransferases, CpLIP2, CAL-A, transesterification, rational design, biocatalysis

1. Introduction

The necessity to shift towards more environment-friendly processes is nowadays a challenge in oleochemistry. Transesterification reactions are indeed used in a lot of applications as the conversion of vegetable oils into fatty acid alkyl esters is often the starting point of oleochemical processes.

More and more biocatalysts are used to perform this reaction and particularly lipases [1, 2]. This versatile class of enzymes is commonly used for hydrolysis reactions. However, they also have the ability to catalyse esterification and acyltransfer reactions, usually under quasi-anhydrous conditions with a tight control of the thermodynamic activity of water (a_w) to avoid hydrolysis while maintaining an optimal enzyme hydration [3]. Indeed, the presence of water is generally needed for lipase activity, and it also allows to solubilize polar reactants and products [4], but it may cause the release of unwanted free fatty acids due to the hydrolysis side-reaction. Another key point in lipid biotechnology is also that, due to the high melting points of some lipid substrates, many processes must be conducted at temperatures around 50°C or more. For this, the use of thermostable lipases represents a significant advantage, especially as thermostability is often correlated with longer half-life for the enzymes [5].

More than 20 years ago, a lipase with high acyltransferase activity was discovered, secreted by the yeast *Candida parapsilosis.* This enzyme, CpLIP2, showed the exceptional ability to preferentially catalyse alcoholysis over hydrolysis even in media with a very high thermodynamic activity of water (a_w>0.95) [6-10]. Yet, at that time the mainstream trend in the biocatalysis community was to develop lipase-catalysed reactions in nearly anhydrous organic solvents. However, since a few years the development of green chemistry has evidenced the interest of developing eco-friendly reaction systems, with an increased interest for water as a green solvent and thus for enzymes able to preferentially catalyse synthesis reactions in aqueous media. Transesterification with lipases/acyltransferases is kinetically controlled due to the much higher rate of alcoholysis than hydrolysis catalysed by these enzymes, which allows the productions of new esters at concentrations above the thermodynamic equilibrium of hydrolysis/esterification, close to 100% ester. For example, CpLIP2, the first enzyme of this family studied for lipid biotechnology applications [7, 8, 10-12], catalyses transesterification by direct alcoholysis in the presence of methanol concentrations as low as 2.2 M without significant hydrolysis side-reaction. Moreover, this feature also applies to other nucleophiles but methanol, and this enzyme has been used in numerous applications including aminolysis and acylated-peptide synthesis [13, 14]. Since 2005, new lipases/acyltransferases have been identified and characterised in *Candida albicans* (CaLIP4) and *Candida tropicalis* (CtroL4) [15, 16]. All these lipases/acyltransferases belong to the same phylogenetic subgroup (> 55% of identity in their primary sequence) and are also phylogenetically related to the *Pseudozyma (Candida) antarctica* lipase A (CAL-A)-like α/β hydrolase superfamily (abH38) (≥ 30% of identity in their primary sequence with CAL-A) [16, 17]. Lipases/acyltransferases CpLIP2 and CtroL4 are characterized by a very high catalytic selectivity for acyltransfer activity over hydrolysis. However, these enzymes are much less stable than CAL-A, with a rapid decrease of activity in the presence of methanol concentrations

above 3-4 M and at temperatures above 45°C activity. Indeed, CAL-A is an exceptionally stable enzyme [18], whether in solution or in immobilized form [19-21]. On the other side, CAL-A differs from lipases/acyltransferase of the CpLIP2 sub-group by a much lower acyltransfer activity, displaying a catalytic behaviour close to that of classical lipases [16]. Therefore, CAL-A is not considered as a lipase/acyltransferase in the same way as CpLIP2 or related enzymes [16]. Ideally, the most suitable enzyme for transesterification applications would have the catalytic behaviour of CpLIP2 and the stability of CAL-A. The stability of proteins is rather a function of the overall structure and is generally not limited to a particular site [22]. The structural determinants involved in the acyltransfer specificity may thus be easier to identify and to target than trying to improve the stability of the proteins, especially as no crystal structure is available yet for CpLIP2 and its close homologs.

Site directed mutagenesis is an efficient approach to modify key positions and limit the number of variants to design optimised candidates for industrial purposes [23]. In 2010, a rational design strategy was successfully applied to increase the acyltransfer activity in aqueous media of the lipase B from *Ps. antarctica* (CAL-B), a lipase from a different family than CAL-A [24] . Residues supposed to be involved in the access of water through a specific tunnel were targeted for mutagenesis. In this study, the substitution of a small polar residue for a larger apolar one (S47L) drastically increased the transesterification activity. This mutation was hypothesised to block the inlet to the active site that normally keeps the concentration of water high for catalysis in the native enzyme. Very recently, similar rational design was conducted on CAL-A, also aiming at improving its acyltransfer activity in aqueous media [25]. Changes in the catalytic behaviour of CAL-A were obtained with mutations conducted to globally increase the hydrophobicity around its active site pocket, so that the alcohol would be preferred compared to water [24]. Now, advanced improvement of the acyltransfer ability of CAL-A is definitely a challenge of first priority [25], as is its alternative consisting in the improvement of the stability of lipases/acyltransferases.

Thus, to elaborate new candidates for industrial purposes, we have looked for residues possibly involved in lipase/acyltransferase activity within the diversity of the CAL-A family. First, we have identified and characterized several new lipases/acyltransferases, among which CduLAc from *C. dubliniensis* (firstly described in the present paper) has shown the highest potential to catalyse transesterification reactions so far within the family. The comparison between the elucidated crystallographic structure of CAL-A (PDB ID: 2VEO, 3GUU) [26] and homology 3D models of CpLIP2 [27] and CduLAc, concomitant with sequence analyses within the family (including a collection of CpLIP2 site-directed mutants), allowed the identification of putative key residues. These amino acids were thus modified to validate their involvement in the acyltransfer activity and to develop improved

biocatalysts. The mutants were more specifically tested for their ability to catalyse acyltransfer reactions and for their stability.

2. Experimental

2.1. Sequences analyses

Sequence visualization and multisequence alignments were generated using Seaview [28] and ClustalX [29, 30]. Blast [30, 31] searches were performed against the version of the non-redundant sequence database of NCBI [32]. Protein sequence identities were obtained using the SIM alignment tool for protein sequences program [29].

2.2. Evaluation of thermodynamic activities

Thermodynamic activity of water and methanol at 30°C in the presence of ethyl oleate in a two-phase system was determined using the UNIFAC group contribution method [33] using the LLE parameters [34, 35].

2.3. Construction of the 3D model

The 3D model of CpLIP2 was previously described by Subileau et al [27]. The 3D model of CduLAc was designed, based on the crystallographic structure of CAL-A (PDB ID: 2VEO, 3GUU) [26], using Modeller 9.14 [36-38] via UCSF Chimera 1.10 [39].

2.4. Plasmids, strains and reagents

Competent cells of *Escherichia coli* XL1-Blue MRF' (Stratagene la Jolla, CA, USA) were used for DNA propagation. *Pichia pastoris* X-33, pPICZαB vector and zeocin were from Invitrogen (Life Technologies SAS, Saint Aubin, France). SacI restriction enzyme and RNaseA were purchased from Roche Diagnostic AG (Rotkreuz, Switzerland). All lipase substrates and reagents were purchased from Sigma-Aldrich (Lyon, France) and were of analytical grade.

2.5. Lipase expression and vector production

Protein sequences of CAL-A from *Ps. antarctica* and CduLAc from *C. dubliniensis* were obtained from NCBI databases under accession numbers 2VEO_A and XP_002421466.1, respectively. The sequence of CpLIP2 from *C. parapsilosis,* accessible under number CAC86400.1, was from Neugnot et al [11], with the signal peptide suppressed. For CduLAc, PrediSi [40] was used to predict signal peptide sequences which were then suppressed in the synthesized sequences. DNA was synthesized and

subcloned by Life Technologies (Regensburg, Germany) in pPICZαB in fusion with the signal peptide of the alpha-mating factor of *Saccharomyces cerevisiae* provided in the plasmid, as described by Neang et al [16]. To produce mutants CpLIP2_S369A, CpLIP2_S369E and CAL-A_E370A, site directed mutagenesis was performed by Life Technologies on the plasmids containing the gene of CpLIP2 (for S369A and S369E) or the gene of CAL-A (for E370A).

2.6. Transformation of *P. pastoris* and selection of clones for CduLAc, CpLIP2, CAL-A and mutants expression

The clones for the expression of the different enzymes were produced as described by Neang et al [16]. Briefly, the lipase expression vectors (pPICZαB_CduLAc, pPICZαB_CpLIP2, pPICZαB_CAL-A, pPICZαB_S369A, pPICZαB_S369E and pPICZαB_E370A) were linearized with the restriction enzyme SacI. After DNA purification and concentration, the linearized plasmids were used to transform competent cells of *P. pastoris* X-33 by electroporation. Then, cells were selected by screening on plates with zeocin. The positive transformants were picked and cultured in YPD (yeast extract 5 g/L, peptone 10 g/L, glucose 10 g/L) buffered with 50 mM sodium phosphate, pH 6.5. Samples (5 µL) were dropped on YNB-Rhodamine B plates (13.4 $g.L^{-1}$ yeast nitrogen base w/o amino acids, 5 $g.L^{-1}$ rapeseed oil, 0.5% (v/v) methanol, 10 $mg.L^{-1}$ rhodamine B, 0.04 $mg.L^{-1}$ D-biotin, 0.2 $g.L^{-1}$ zeocin, 20 $g.L^{-1}$ agar, 50 mM sodium phosphate pH 6.5). Cultivations were performed at 28°C for 5 days. One clone of each lipase was selected, based on the size of the fluorescent halo on YNB-Rhodamine plates upon UV illumination. The selected clones were then grown at 28°C in YPD medium and stored at -80°C with 20% of glycerol for conservation and further use.

2.7. Heterologous production in bioreactor

Recombinant lipases were obtained from culture supernatants of transformed *P. pastoris* as described by Brunel et al [41] and modified by Neang et al [16].

2.8. Enzyme extract preparation

The final 800 mL culture medium was centrifuged at 4°C for 10 min at 10 000 x*g* to eliminate the cells. The supernatant (approximately 700 mL) was successively filtered with 5.0, 0.8 and 0.45 µm pores diameter filters (Millipore, Molheim, France) before its concentration to 90 mL by hollow-fiber tangential flow ultrafiltration using a Quixstand apparatus (GE Healthcare, Chalfont St Giles, UK) equipped with a 30 kDa cut-off module. Diafiltration with 600 mL of ultrapure water was then performed in the same ultrafiltration system. Enzymes were producted at multigram per liter scale.

2.9. Enzymatic assays

Hydrolysis and alcoholysis (transesterification) activities were respectively determined by measuring the initial rates of fatty acid and ester production (µmol.min^{-1}.mL^{-1}), using ethyl oleate (C18:1 EE) as substrate. The transesterification ratio (%) corresponds to the relative initial rate of alcoholysis compared to the total activity (initial rates of alcoholysis plus competitive hydrolysis). Specific activity is the total activity (µmol.min^{-1}.mL^{-1}) in relation to protein concentration (mg.mL^{-1}). Results are expressed as the mean of 3 repetitions ± standard deviation.

The amount of enzyme was adapted for each biocatalyst so as to be in conditions where the initial rate of hydrolysis of ethyl oleate was proportional to protein concentration (0.002, 0.003, 0.129, 0.002, 0.004 and 0.251 mg of proteins (eq. CpLIP2) in 1 mL of reaction medium for CduLAc, CpLIP2, CAL-A, CpLIP2_S369A, CpLIP2_S369E and CAL-A_E370A respectively).

Experiments were conducted as described by Neang et al [16] with the following modifications. For reactions, 100 µL of diluted enzyme solution in 50 mM sodium phosphate buffer, pH 6.5, were added to 800 µL of the same buffer, eventually containing methanol for the transesterification assay. The reaction was started by the addition of 100 µL of ethyl oleate emulsion. The lipid substrates were prepared as emulsions consisting of 100 mM lipid emulsified by sonication (Branson Sonifier 250, 20 s, 200 watts, 145 µm amplitude) in an aqueous solution of 20 g.L^{-1} poly(vinyl alcohol). After 15 min at 30°C, the reaction was stopped with 950 µL of an ethanol/sulphuric acid (100:0.8, v/v) mixture. To study the influence of temperature on the enzymatic activity, temperatures were in the range 5°C - 80°C.

A Shimadzu GC 2010 plus gas chromatograph equipped with a flame ionization detector, an automatic sampler (injected volume 0.1 µL) and a split/splitless injector was used for analysis. The capillary column was a DB-5ht (15m x 0.25 mm, Phenomenex, Le Pecq, France). The helium carrier flow was 1 mL.min^{-1} and the split ratio was 1:50. Temperature conditions were, for reactions with individual substrates: injector 280°C, detector 290°C, oven 200 to 225°C at 10°C.min^{-1} for methyl (ME), ethyl ester (EE) and fatty acid (FA) of C18:1. Calibration curves were realized using emulsions of mixtures of FA and monoesters prepared according to the same protocol, without enzyme and alcohol.

2.10. Protein analysis

Protein concentrations in enzymatic extracts were determined by the Bradford method [42] using pure, lyophilized CpLIP2 as standard. Protein concentrations are therefore given in mg equivalent CpLIP2 protein.

3. Results and discussion

3.1. Identification of a new lipase/acyltransferase: CduLAc

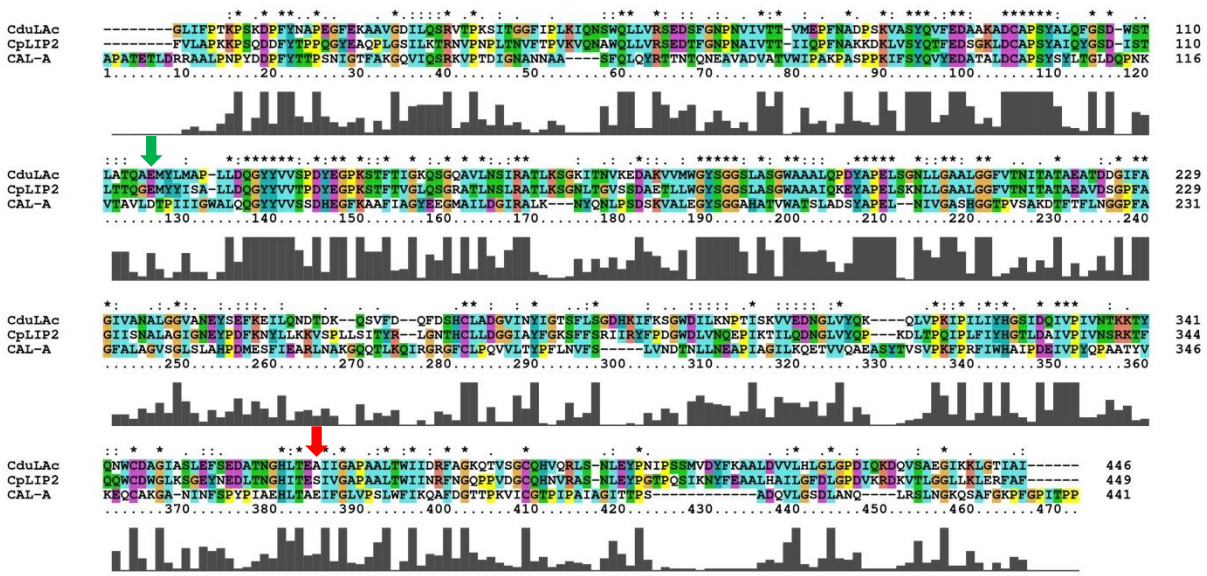

Fig.1 Multiple alignment of the protein sequences of CpLIP2, CAL-A and CduLAc, using ClustalX (red arrow: A366 in CduLAc and its equivalents S369 in CpLIP2 and E370 in CAL-A, green arrow: conserved acid residue equivalent to D122 in CAL-A)

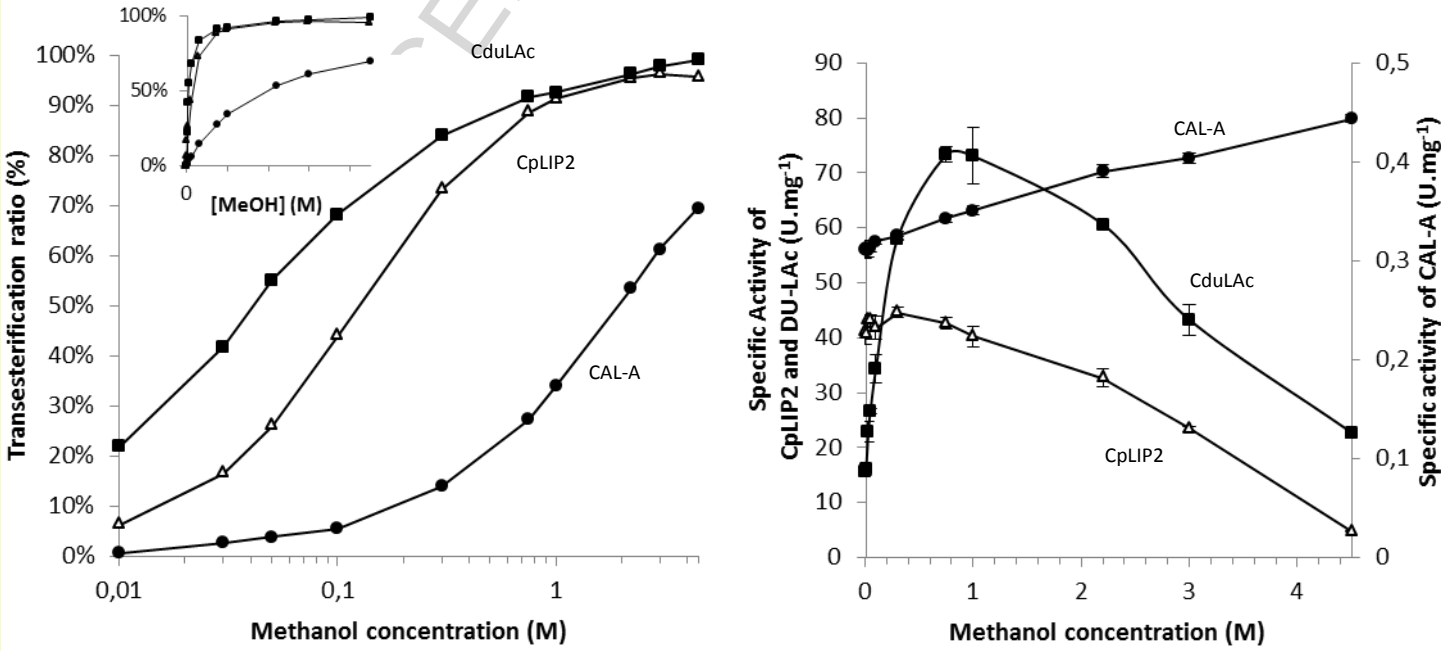

Fig.2 Effect of methanol concentration on (a) the transesterification ratio ([MeOH] in log scale) **and (b) the specific activity of CpLIP2 (Δ), CduLAc (■) and CAL-A (●)**

Among the phylogenetic subgroup of CpLIP2-like, the sequence identified as lipase 0 of *C. dubliniensis* (CduLAc) was selected on the basis of comparative sequence analysis to expand the knowledge on lipases/acyltransferases.

The sequences of CpLIP2, CduLAc and CAL-A are presented in figure 1. CduLAc exhibits nearly 60% of identity with CpLIP2 and only 32% with CAL-A. As well as in CpLIP2 and CAL-A, the conserved and common motif of lipases, GYSGG, is found in CduLAc. The alignment also evidences the two sequences CAPSY and YAPEL, which are highly conserved in the CAL-A-like superfamily. These characteristics seem to be shared by functional lipases such as LIP1 of *C. albicans* or the lipase of *Arxula adeninivorans* [43, 44]. After subcloning *CduLAc* in pPICZαB plasmid and transformation in *P. pastoris* X33, a functional CduLAc enzyme was produced at 6.97 $g.L^{-1}$ and characterized.

The acyltransfer activity of CduLAc was assessed with ethyl oleate in aqueous emulsion in the presence of various methanol concentrations between 0 and 4.5 M at 30°C, and compared with those of CpLIP2 and CAL-A (Figure 2). The transesterification ratio is characterised by the relative initial rate of alcoholysis compared to the total activity (initial rates of alcoholysis plus competitive hydrolysis). In this concentration range, the thermodynamic activity of water remains higher than 0.9. The results show that CduLAc is a lipase/acyltransferase, even better than CpLIP2, and confirms the low acyltransfer activity of CALA in these conditions. Indeed, the methanol concentration for which the initial rates of alcoholysis and hydrolysis are equal (transesterification ratio 50%) are 0.03 M for CduLAc, 0.12 M for CpLIP2 and 2 M for CAL-A. In the presence of 4.5 M methanol, the transesterification ratio is only of 69% for CAL-A while very close to 100% for CpLIP2 and CduLAc. In stoichiometric conditions (0.01 M methanol and 0.01 M ethyl oleate), the transesterification ratio is of 22% for CduLAc, 6% for CpLIP2, while no transesterification can be detected with CAL-A (only hydrolysis). These results illustrate the exceptional preference of CduLAc for the alcohol as acyl acceptor compared to water. It is also to be noted that the specific activity of CAL-A was always much lower (less than 200 times) than that of the two lipases/acyltransferases, depending on methanol concentration. Also, even if the activity of CduLAc was about 2.5 times lower than that of CpLIP2 in hydrolysis conditions (16 $U.mg^{-1}$ and 41 $U.mg^{-1}$ respectively), increasing methanol concentrations enhanced the activity of CduLAc, to reach 73 $U.mg^{-1}$ in the presence of 0.75 M methanol. Regarding the previous results obtained with other lipases/acyltransferases such as CaLIP4 and CtroL4 [15, 16], CduLAc seems to have the best potential as a wild-type acyltransferase.

3.2. Elucidation of a key position in the lipase/acyltransferase family

Based on the previous studies on CpLIP2 [27], some regions of the 3D structure that might play a key role in the acyltranfer activity were evidenced. By comparing the sequences and the 3D models

of CduLAc and CpLIP2 with the crystallographic structure of CAL-A, more precisely in the vicinity of the active site, one position appeared to be a potential hotspot. Indeed, the polarity of the residue 369 of CpLIP2 at less than 5 Å from the catalytic histidine differs from its equivalents in the two other enzymes (Figure 1). This position carries a small polar serine (S369) in CpLIP2 (Figure 3A) while at the corresponding position an also small but apolar alanine is found in CduLAc (A366). In the more classical lipase CAL-A, the position is occupied by a large, polar and charged glutamic acid (E370) (Figure 5A). These differences in polarity, but also in steric hindrance, could play an important role in the different acyl-acceptor specificities exhibited by the biocatalysts [45]. The mechanistic model proposed by Ericsson et al. [26] suggested that the water molecule acting as the nucleophile in hydrolysis was activated by the catalytic histidine/aspartate pair before attacking the acyl enzyme. Considering its vicinity to the active site (figure 5A), the glutamic acid residue E370 in CAL-A could also participate in the activation of water and be responsible for the preference of this enzyme for hydrolysis. As shown by Larsen, et al. [24]in CAL-B, the nature and charge of neighbouring residues could also have an impact on the accessibility of water or alcohol because of the proximity of the two catalytic amino acids. Comparing the CAL-A_E370 residue and its equivalents in our lipases to the hot spots identified in CAL-B[24] is not straightforward as these enzymes sequences exhibit only about 15% identify. Yet, overall structural similarities can be observed that favour the hypothesis that this residue could play a role in the access of nucleophiles to the active site. The other strategy of rational evolution, recently conducted by Müller et al. [25] in parallel and independently to our work, also selected for mutagenesis E370 in CAL-A, among other polar residues in the vicinity of the active site. However, mutants of this position were found to catalyse lower than wild-type or no ester formation in the conditions tested and were not selected for further characterization [25]. Our rational design strategy, based on comparison of phylogenetically related sequences and structure, did not either led to the identification of the position Müller et al. [25] study pointed out (D122L in CAL-A), because this residue is highly conserved within the CAL-A superfamily, all lipases indeed carrying an acidic glutamic or aspartic residue at this position (as shown in CpLIP2, CduLAc and CAL-A in Figure 1).

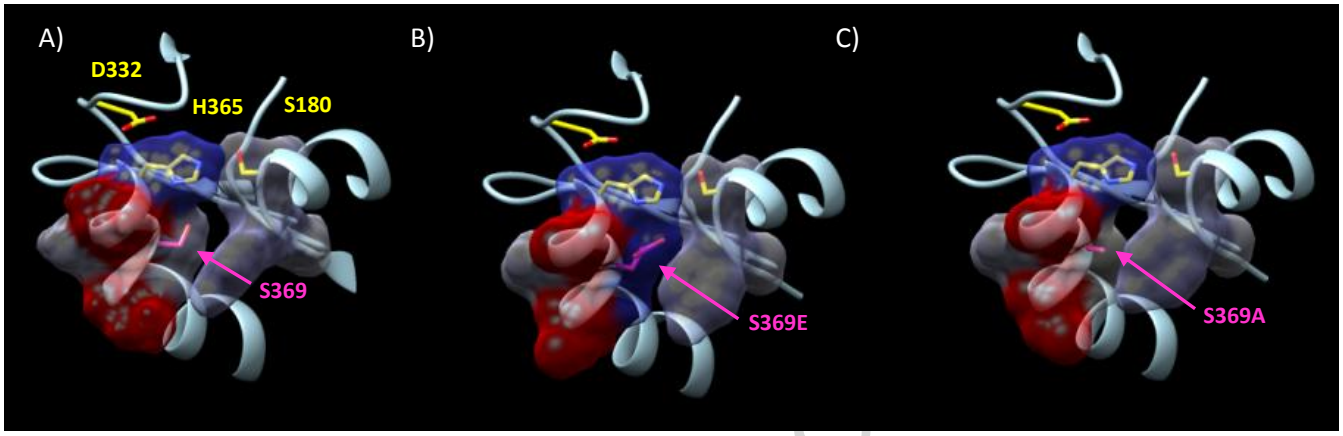

Fig.3 3D models representing the active site area of CpLIP2 with (A) the natural serine residue at position 369 and its mutants into (B) a glutamic acid like in CAL-A or (C) an alanine like in CduLAc and consequences on the hydrophobicity and steric hindrance. The catalytic triad is in yellow and the mutated amino acid in pink. Surfaces are colored in function of hydrophobicity from more hydrolphilic (blue) to more hydrophobic (red).

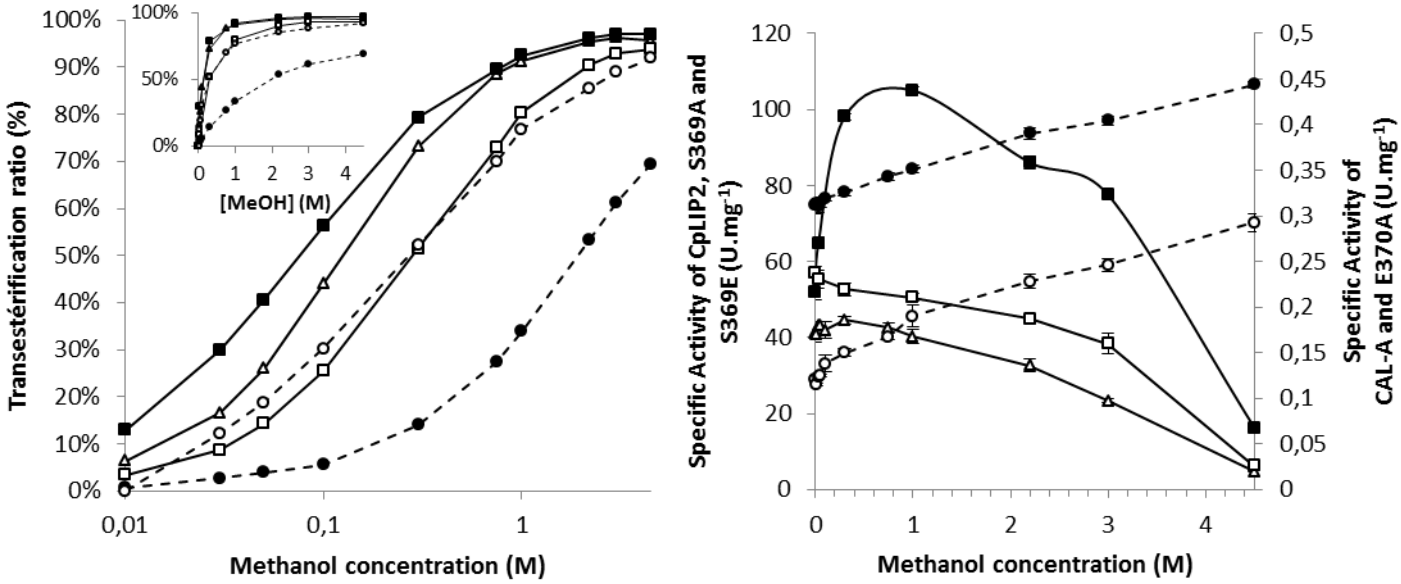

Fig.4 Effect of methanol concentration on (a) the transesterification ratio ([MeOH] in log scale) **and (b) the total activity with CpLIP2 (Δ), CpLIP2_S369A (■), CpLIP2_S369E (□), CalA (●) and CalA_E370A (○).**

Therefore, site-directed mutants were first designed on CpLIP2 lipase/acyltransferase to assess the role of the candidate position on the acyltransfer activity by turning CpLIP2's residue into the corresponding ones either from the more highly efficient lipase/acyltransferase CduLAc, or from CAL-A (Figure 3). Indeed, 3D model analysis showed that the substitution of serine S369 of CpLIP2 by a glutamic acid as in CAL-A (CpLIP2_S369E) should reduce the accessibility to the active site (figure 3B) and could facilitate the water activation for hydrolysis. On the contrary, the substitution of S369 into

the alanine of CduLAc (CpLIP2_S369A) releases space for more bulky nucleophiles (figure 3C), and its apolar character could also be more favourable to alcohol than water. These mutants were thus expected to present respectively a decrease and an increase of the transesterification ratio of CpLIP2.

After subcloning in pPICZαB, transformation in *P.pastoris* X33 and enzyme production, the protein concentrations at the end of the preparation of the enzyme extracts were 6.8 $g.L^{-1}$ and 7.1 $g.L^{-1}$ for S369A and S369E respectively.

The catalytic activities of CpLIP2_S369A and CpLIP2_S369E were compared to the ones of CpLIP2 and CduLAc. Experiments were conducted as described in section 3.1. The figure 4 shows that the mutations dramatically changed the catalytic behaviour of the enzymes. As expected, the substitution of the residue of CpLIP2 for that of CduLAc (CpLIP2_S369A) allowed an slight increase of the transesterification ratio, resulting in an overall behaviour closer to that of CduLAc with 50% transesterification in the presence of 0.07 M methanol (0.03 M for CduLAc and 0.12 M for CpLIP2). At 0.01 M methanol, the mutant CpLIP2_S369A presented a two fold increase of the transesterification ratio (13%) while a decrease was observed with CpLIP2_S369E (3% instead of 6% for CpLIP2). Interestingly, the substitution of the CpLIP2 residue for the one of CAL-A (CpLIP2_S369E) resulted in a general decrease of the transesterification ratios (50% transesterification ratio with 0.3 M methanol), although not reaching the low levels observed with CAL-A. In addition, the two mutants surprisingly displayed an increased specific activity compared to CpLIP2. Particularly, the CpLIP2_S369A mutant exhibited an even higher specific activity than CduLAc. Indeed, in the presence of 2.2 M methanol, the specific activity of CpLIP2_S369A was 86 $U.mg^{-1}$ compared to 61 $U.mg^{-1}$ for CduLAc. In comparison, the specific activity of CpLIP2 was 2.6 times lower than the one of CpLIP2_S369A. It appeared that the specific activity of CpLIP2_S369A was indeed enhanced by the increase of methanol concentration, as for CduLAc. The effect of S369A and S369E mutations thus supports the correlation of the acyltransferase behaviour with the hydrophobicity and the steric hindrance of the amino acids (Figure 3): the small apolar residue (alanine) is favourable to alcohol as acyl acceptor, while the bulky acid is favourable to water and hydrolysis, the medium polar serine giving intermediary behaviour. As expected, the rational choice of mutations already occurring within the set of natural homologous sequences [46] allowed the production of properly folded and active proteins. To further validate these results, the substitution with alanine was then conducted in CAL-A.

3.3. Turning CAL-A into a lipase/acyltransferase

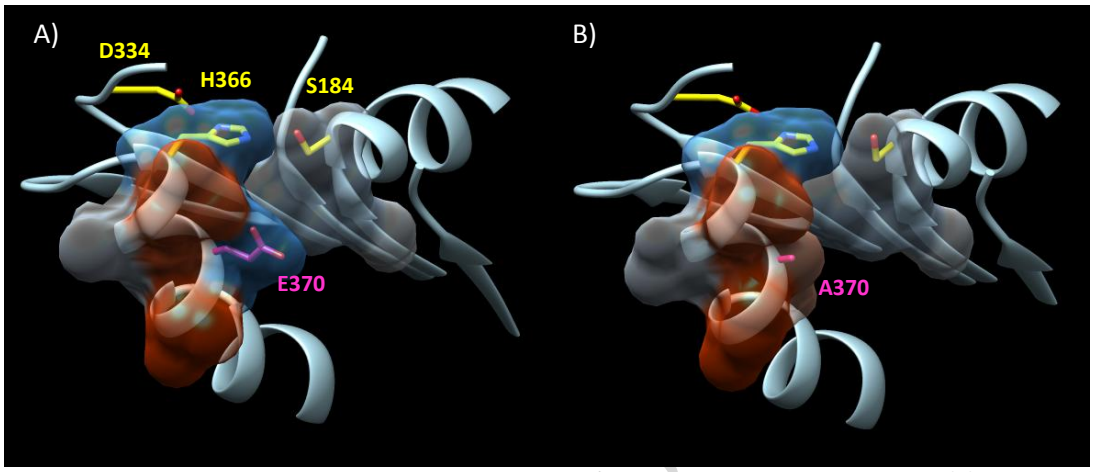

Fig.5 3D models representing the active site area of CAL-A with the natural glutamic acid residue at position 370 (A) and its mutant into an alanine like in CduLAc (B), and consequences on the hydrophobicity and steric hindrance. The catalytic triad is in yellow and the mutated amino acid in pink. Surfaces are colored in function of hydrophobicity from more hydrolphilic (blue) to more hydrophobic (red).

To figure out if the selected position indeed also plays a key role in the transesterification activity in this classical lipase, the corresponding glutamic acid in CAL-A was mutated into an alanine (CAL-A_E370A), as in the best lipase/acyltransferase CduLAc (Figure 5). Again, the replacement by an alanine reduces the steric hindrance and increases the hydrophobicity of the cavity under the active site (Figure 5B). After subcloning in pPICZαB, transformation in *P.pastoris* X33 and enzyme production, the protein concentration at the end of the enzyme extracts preparation was 1.65 $g.L^{-1}$. The results presented in Figure 4 show that the transesterification ratio obtained with CAL-A_E370A was drastically increased compared to CAL-A. Indeed, transesterification became the preferential reaction (> 50%) as soon as methanol concentration reached 0.3 M, a value comparable to the one observed for CpLIP2, instead of 2 M for the native enzyme CAL-A.

CAL-A_E370A thus appears as a lipase/acyltransferase which catalyses the production of esters at higher rate than the native enzyme. For example, in the presence of 2.2 M methanol the mutation E370A allowed an increase of the transesterification ratio of CAL-A from 53% to 86%. Interestingly, even with the change of catalytic properties, the CAL-A_E370A mutant enzyme was resistant to high methanol concentrations like CAL-A.

This single position appears therefore to be central for the ability of the enzyme to catalyze the acyltransfer reaction (Figure 5). A small apolar residue at this position is confirmed to be more favourable to methanol than to water as nucleophile. Compared to the structural role of the CAL-B mutations proposed by Larsen, et al. [24], the CAL-A_E370 residue (and equivalents) may not be directly involved in a tunnel leading the nucleophile to the active site. In our case, the substitution for a small alanine increased the hydrophobicity and released space, but not access, close to it. It

resulted in an overall environment more favourable to alcohol than water. The effect of the mutation on hydrolysis alone (increased with CpLIP2-S369A and decreased with CAL-A_E370A compared to the respective wild-types) could neither be related to an obvious opening effect.

The fact that, surprisingly, Müller et al [25] did not identify the CAL-A_E370A mutant as having a much increased acyltransfer activity compared to the wild type may be explained by the reaction conditions used. In our study, low methanol concentrations were used (0.01-4.5 M) and activities were initial rates over 15 min in order to evidence differences of affinity of the enzymes toward alcohol vs water. On the contrary, the ethanol/water mixture (14.1 µL ethanol + 14.1 µL water) used by Müller et al. [25] corresponded to a very high alcohol concentration (8.6 M), for which the thermodynamic equilibrium of ester formation would be above 90% even with classical catalysts. In methanolysis with immobilized biocatalysts, methanol concentration even reached 18 M (67.5 µL methanol + 25 µL aqueous phase), yielding an observed ~99% transesterification ratio for the best mutant (D122L) vs ~97% for the wild type [25]. Moreover, very long reaction times (24h) were used by Müller et al [25]. It is thus difficult to compare our results obtained with CAL-A_E370A with the data published on the variants described by Müller et al. [25].

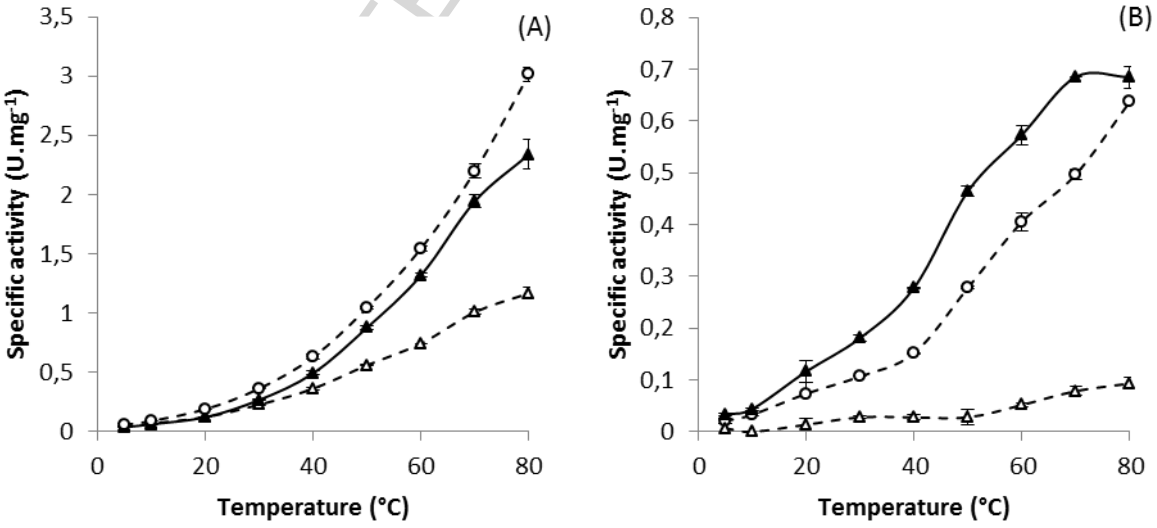

Fig.6 Influence of temperature on the catalytic activity of enzymes CAL-A (A) and CAL-A_E370A (B) in the absence (empty circles (○), dotted line: hydrolysis activity) **and in the presence of MeOH** (black-filled triangle (▲): alcoholysis activity; empty triangle(△), dotted line: competitive hydrolysis activity). Reactions were performed at the desired temperatures, pH 6.5, in the presence of 10 mM of ethyl oleate in PVA emulsion, and eventually 2.2 M methanol.

Because the operational stability of the enzymes is crucial for industrial enzymatic development, the thermostability of the new mutant of CAL-A, CAL-A_E370A was studied. The catalytic activities of CAL-A and its mutant CAL-A_E370A in a temperature range of 5 to 80°C were evaluated in parallel for both hydrolysis and alcoholysis of ethyl oleate (C18:1 EE). Reactions were performed in buffered aqueous emulsions at pH 6.5 with or without 2.2 M methanol, with the pH adjusted to 6.5 for each temperature. The CAL-A_E370A mutant presented the same activity profile vs temperature as CAL-A (Figure 6). Thus, although this mutation induced a radical change in ability of the enzyme to catalyse preferentially acyltransfer over hydrolysis, it allowed the conservation of the remarkable behaviour of the wild enzyme toward temperature.

4. Conclusion

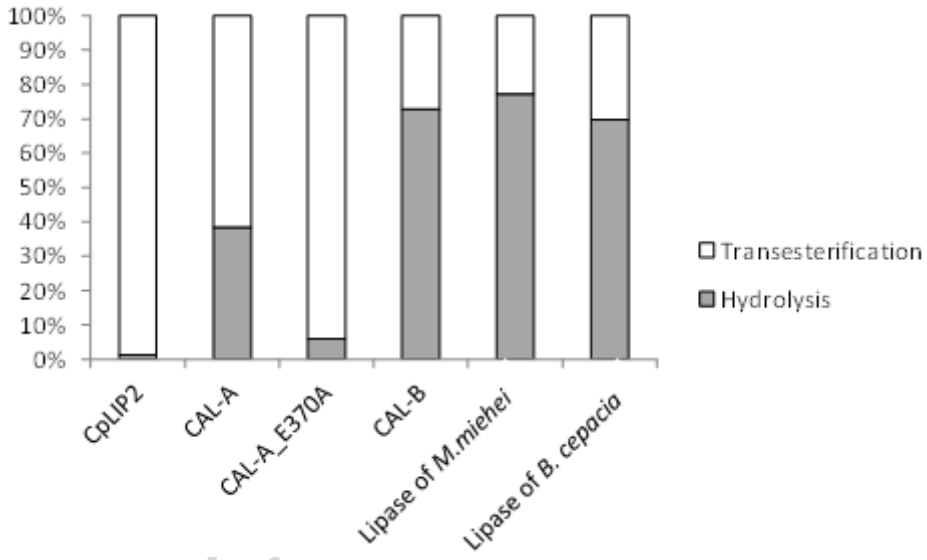

Fig.7 Relative initial rates of hydrolysis and transesterification of ethyl oleate in emulsion catalyzed by various lipases at 50°C in the presence of 2.2M methanol (a_w 0.96)

The study of CpLIP2 homologs allowed the identification of a new lipase/acyltransferase, CduLAc. Compared to CpLIP2, this enzyme requires a lower methanol concentration to favour transesterification as the main reaction. Indeed, in the presence of 0.05 M methanol, methanolysis already represents 55% of the total specific activity of CduLAc, while it only represents 26% with CpLIP2 and 4% with lipase CAL-A. Moreover, unlike CpLIP2, the activity of CduLAc is enhanced by the presence of methanol in the reaction medium.

Within the superfamily of CAL-A, the existence of a phylogenetic sub-group of lipases/acyltransferases is confirmed, as all the currently known wild-type lipases/acyltransferases (CpLIP2, CaLIP4, CtroL4 and CduLAc) belong to it. Further investigations of the sequences in this

group could permit the identification of new lipases/acyltransferases with other properties and to extend the range of potential industrial applications.

The high level of identity between the sequences of CduLAc and CpLIP2 (60%) was of peculiar interest to limit the number of potential amino acids involved in the differences of transesterification activity. The strategy of rational comparison of phylogenetically related enzymes (comparison of sequences and of 3D structures or models) allowed the successful identification of one key position in the vicinity of the active site: S369 in CpLIP2 and the corresponding E370 in CAL-A. The substitution of these amino acids for the alanine of CduLAc at this position (A366) allowed a straightforward increase of the transesterification activity both in CpLIP2 and in CAL-A alanine mutants, in which the transesterification ratio was increased respectively 1.8 - 1.1 times and 4 - 5 times in the presence of 0.03 - 0.1 M methanol. Besides, this single mutation in the core of the proteins did not affect the stability of the enzymes. The mutated residues, located inside the protein, do not seem to be involved in structural bonds that could be responsible for the thermostability of the protein. The most interesting result was the impact of the mutation E370A on CAL-A, as illustrated on figure 7, by comparison of the percentages of transesterification and hydrolysis obtained with the enzymes presented in this study and with other classical lipases. Indeed, this single mutation induced a radical change in the catalytic behaviour of the enzyme, as it became a lipase/acyltransferase according to the criterion of high acyltransfer activity in the presence of low alcohol concentration and high a_w, with a 50% alcoholysis being observed in the presence of 0.3 M methanol versus 2 M in the wild type. The fact that the CAL-A_E370A mutant was discarded in a parallel study shows that the choice of screening conditions is crucial for the identification of improved enzymes. The conservation of the properties of stability in the presence of methanol and at high temperatures makes of the mutant CAL-A_E370A a great candidate for applications. However, this single mutation in CAL-A was not able to achieve as high a transesterification ratio as with CpLIP2 and CduLAc, and the reverse mutation in CpLIP2 only resulted in a partial loss of its acyltransferase character, indicating that other structural determinants are involved in the much better affinity of lipases/acyltransferases for other nucleophiles than water.

5. Acknowledgments

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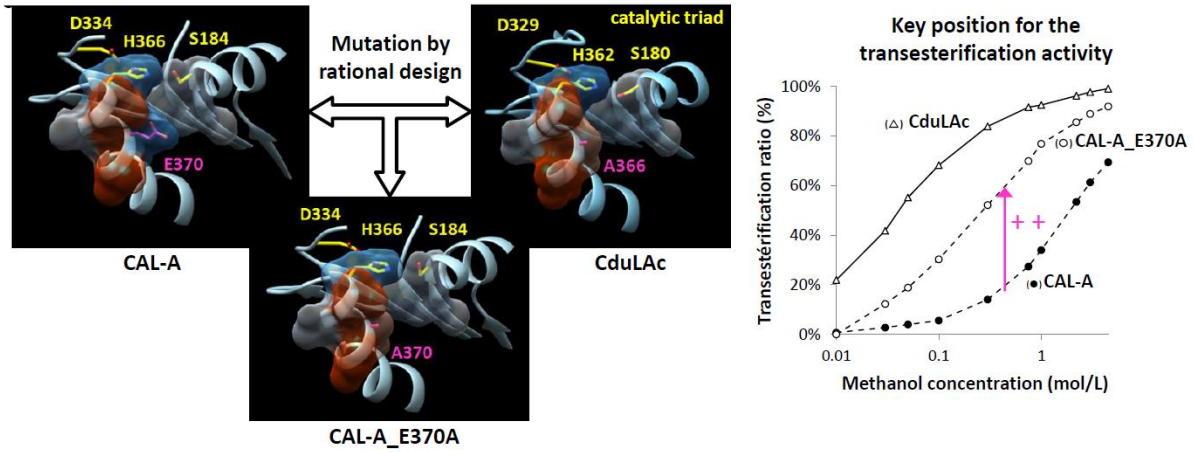

Graphical abstract

Highlights

Lipases/acyltransferases CpLIP2 and the newly characterized CduLAc from *Candida dubliniensis* display a very high selectivity for acyltransfer reaction

A key structural determinant of acyltransfer activity was identified by comparing the sequences and 3D models of CpLIP2 and CduLAc with that of their structural homolog lipase CAL-A.

A single mutation allowed a dramatic increase of the acyltransfer activity of CAL-A while keeping its thermostability.