Electronic Supplementary Material (ESI) for ChemComm.

Saturation mutagenesis in selected amino acids to shift *Pseudomonas* sp. acidic lipase Lip I.3 substrate specificity and activity

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Supplementary Material

Experimental Procedures

The strains used in this work, listed in Table S1, were grown in LB medium with the required antibiotics when harbouring plasmids[1].

Table S1: List of strains used in this study

Strain	Genotype or characteristics	Reference
E. coli 5K	F^- r_k^- m_k^- rpsL thr thi leu lacZ	(Godessart 1988)[2]
E coli TOP 10 (Invitrogen)	F^- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	(Invitrogen, 2012)[2]
Pseudomonas aeruginosa PAO1 Δ*lipH* PW5805	*lipH*(PA2863)-C01::ISphoA/hah	(Jacobs 2003)[3]
Pseudomonas sp. CR-611 CECT8156	High lypolitic activity strain isolated from a subtropical soil from Puerto Iguazú, Argentina	(Ruiz 2005)[4]

Standard procedures were used for DNA manipulation, amplification or sequencing[1]. Nucleic acid concentration and purity was measured using a NanoDrop® ND-1000. Mutant variants of Lip I.3 were constructed by Quikchange® (Agilent Technologies) using plasmid pBBR1MCS-Lip I.3 as a template[5]. After amplification, parental template plasmids were removed by digestion with *Dpn*I (Fermentas) for 3 h at 37ºC. DNA was ethanol precipitated and stored at -20ºC for at least 1 hour. Amplified DNAs were recovered and used to transform *E. coli* TOP10 or *P. aeruginosa* PAO1 Δ*lipH*. Two types of mutagenesis amplifications were performed: site directed mutagenesis on Lid1 variable positions I149, G152, S154 and A165 (Figure S2), including a double mutant at positions 149/152, and NNK codon degeneracy saturation mutagenesis at positions Y29, W310, N344 and F166, located at the substrate entrance tunnel. Primers are listed in Table S2, where X in Y29X, W310X, N344X, F166X corresponds to any amino acid residue substituted by the degenerated primers used for saturation mutagenesis. For sequence and mutation analysis, Vector NTI software (Vector NTI version 10.3.0; Invitrogen, Carlsbad, CA) and Blast searches were routinely performed[6]. Structural 3D homology models were generated using Swiss-Model[7-10]. Pymol software (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC) was used to visualize the 3D protein models generated. Additional analysis of calcium binding was performed using Ligand Explorer software 4.1.0[11] (RCSB PDB, www.pdb.org).

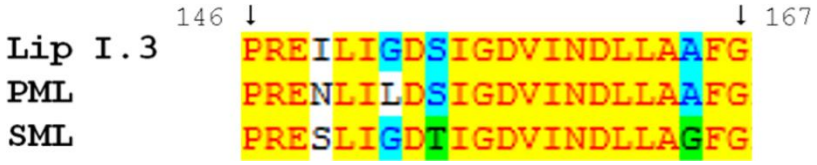


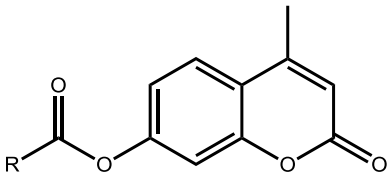
Figure S1: Lid 1 sequence alignment for Lip I.3, PML and SML. Residues at positions I149, G152, S154 y A165 are not conserved within a highly conserved region.

Table S2: List of primers used for site directed mutagenesis on Lid1 residues, and degenerate primers used for saturation mutagenesis on residues located at the substrate entrance tunnel.

Mutation	Forward primer	Reverse primer
I149N	CCAGCGGCCCACGGGAAAACCTGATTGG	CCAATCAGGTTTTCCCGTGGGCCGCTGG
I149S	CGCCAATCAGGCTTTCCCGTGGGCCGC	GCGGCCCACGGGAAAGCCTGATTGGCG
G152L	CCCACGGGAAATCCTGATTCTAGACTCCATCGGTGACGTG	CACGTCACCGATGGAGTCTAGAATCAGGATTTCCCGTGGG
I149N/G152L	GCGGCCCACGGGAAAACCTGATTCTAGACTCCATCGGTGAC	GTCACCGATGGAGTCTAGAATCAGGTTTTCCCGTGGGCCGC
S154T	CCTGATTGGCGACACCATCGGTGACGTG	CACGTCACCGATGGTGTCGCCAATCAGG
A165G	GACTTGCTCGCCGGATTCGGGCCCAAG	CTTGGGCCCGAATCCGGCGAGCAAGTC
Y29X	GCCATTACGCTGTATTCCNNKCACAACCTCGATAACGGC	GCCGTTATCGAGGTTGTGMNNGGAATACAGCGTAATGGC
F166X	CAACGACTTGCTCGCCGCANNKGGGCCCAAGGAT	ATCCTTGGGCCCMNNTGCGGCGAGCAAGTCGTTG
W310X	CGTCAACATTCCGACCNNKATCTCGCATCTGCCGACC	GGTCGGCAGATGCGAGATMNNGGTCGGAATGTTGACG
N344X	CGATCATCGTCGCCNNKCTGTCGGATCCGGC	GCCGGATCCGACAGMNNGGCGACGATGATCG

Lypolitic activity of the mutants was determined in duplicate using MUF-heptanoate and MUF-oleate (Figure S2) as substrates (Sigma), as previously described[5, 12]. Control and mutant strains were inoculated on agar plates and incubated at 37 ºC overnight. Grown cultures were used to inoculate 1 mL LB supplemented with 400 µg/mL chloramphenicol (LB-Cam400), in 96-well, 2 mL deep well plates (ABgene). In each plate, 8 replicates of *P. aeruginosa* PAO1 Δ*lipH* bearing empty vector pBBR1MCS or containing recombinant plasmid pBBR1MCS-Lip I.3 were included as negative and positive controls, respectively. Plates were incubated overnight at 30 ºC with agitation at 180 rpm, and 100 µL grown cultures were added to 900 µL fresh LB-Cam400 in deep well plates. New plates were incubated at 30 ºC with agitation at 180 rpm for 48 h, and cultures were centrifuged in a Jouan CR422 centrifuge using rotor CR/GR 4.22 at 2830 ×*g* for 45 min. Supernatants were recovered and stored at 4ºC for further activity analysis. All activity assays were done on microtiter plates, performing two repetitions for each replicate and at least two replicates for assay. Three blanks were always set up for each set of conditions (reaction mixture without enzyme, without substrate, or without neither enzyme nor substrate).

A catalytic unit was defined as the amount of enzyme that releases 1 µmol of MUF in 1 minute under the conditions used. Activity measurements were performed as previously described[5].



R = (CH_2)$_5$$CH_3$
R = (CH_2)$_7$CH=CH(CH_2)$_7$$CH_3$

Figure S2 – 4-methylumbelliferyl (MUF) heptanoate and oleate

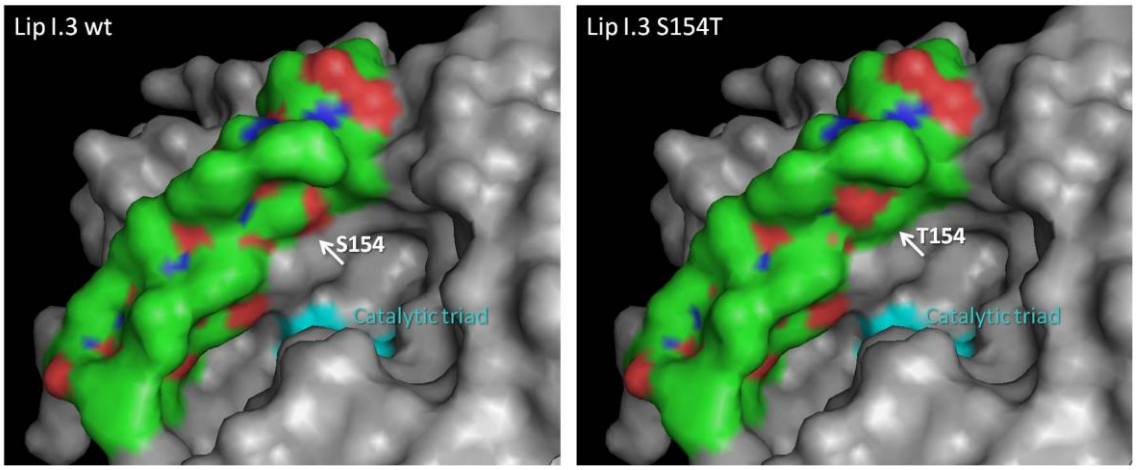
Analysis of the shift on substrate specificity

For each mutant, lypolitic activity was measured on MUF-heptanoate and MUF-oleate as described earlier. The ratio of activity on MUF-heptanoate over MUF-oleate was calculated (Table S3). A shift on substrate specificity can be seen for several mutants when compared to wild type Lip I.3, with G152L and S154T presenting the larger relative activity on MUF-oleate.

Table S2: Shift on substrate specificity for some of the obtained mutants.

	Lip I.3 wt	W310Q	W310F	W310M	S154T	G152L
MUF-heptanoate/ MUF-oleate	5.9	4.4	4.3	4.2	2.8	2.6

Figure S3 – Modelling of the S154T mutation. The substitution could provide a wider opening of Lid 1 and additional hydrophobicity gain resulting from substitution of the OH group from S154 by a methyl group when replacing the residue by threonine (T154).



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