Enhancing Thermostability by Modifying Flexible Surface Loops in an Evolved High-Redox Potential Laccase

Ana Isabel Vicente^a, Javier Viña-Gonzalez^a, Ivan Mateljak^a, Emanuele Monza^{b,c}, Fatima Lucas^{b,c}, Victor Guallar^{b,d} and Miguel Alcalde^a

^aDepartment of Biocatalysis, Institute of Catalysis, CSIC, Cantoblanco, 28094 Madrid, Spain.

^b Barcelona Supercomputing Center, Jordi Girona 29, 08034 Barcelona, Spain.

^c Current address: Zymvol, C/Almogavers 165 08018, Barcelona, Spain.

ICREA: Institució Catalana de Recerca i Estudis Avancats Passeig Lluís Companys 23, 08010 Barcelona (Spain).

*Corresponding author: Miguel Alcalde, Institute of Catalysis, CSIC, Cantoblanco, 28049 Madrid, Spain. E-mail: malcalde@icp.csic.es

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/aic.16747

Keywords: high-redox potential laccase, thermostability, flexible loops, MORPHING, molecular dynamics, *B*-factor.

Running title: Engineering flexible loops in laccase.

ABSTRACT

High-redox potential laccases (HRPLs) from white-rot fungi are versatile biocatalysts whose practical use is highly dependent on their thermostability. In this work, an evolved HRPL variant was subjected to structure-guided evolution to improve its thermostability. We first selected several surface flexible loops in the laccase structure by inspecting them through molecular dynamics and an analysis of *B*-factors. The resulting segments were grouped into three MORPHING (Mutagenic Organized Recombination Process by Homologous In vivo Grouping) blocks, which were constructed in *Saccharomyces cerevisiae* and explored at high temperatures. This evolution process gave rise to a double mutant that showed a half-life at 70°C enhanced by 31 min with an optimum temperature for activity of 75°C and similar kinetic parameters. The Ser264Lys and Ser356Asn mutations modified the contacts established between these residues and those that surround them, altering the surface loops and thereby the enzyme properties.

INTRODUCTION

Laccases (EC 1.10.3.2) are multicopper oxidases capable of catalyzing the one-electron oxidation of phenols, polyphenols, anilines and many other aromatic compounds.¹ These enzymes are triggered by O₂ from the air, releasing H₂O as the only by-product of the oxidation process. Thus, they are among the most versatile and self-sufficient green catalysts known to date.² The catalytic center of laccases is formed by four copper atoms, the T1 Cu site where reducing substrates are oxidized and a trinuclear T2/T3 Cu cluster to which oxygen binds and is reduced to two molecules of water.³ Due to the high-redox potential at the T1Cu site and their limited requirements, high-redox potential laccases (HRPLs) secreted by white-rot fungi are at the forefront of biotechnology, with possible applications in remediation, organic synthesis, pulp and paper, textile and food industries, 2nd generation biofuels and biomedical devices (*i.e.* biofuel cells and biosensors).^{4,5}

In recent years, more thermostable HRPLs are being designed to endure higher temperatures, enzymes that are also capable of withstanding mutational loads introduced through directed evolution campaigns to adapt their biochemical features to the demands of industry.^{6,7} Indeed, recent attempts to tailor more robust HRPLs have focused mainly on laccase chimeragenesis, either through SCHEMA-structure recombination *in vivo*⁸ or by swapping cupredoxin domains.⁹ While the overall outcome of these studies emphasizes the power of DNA recombination, we cannot ignore other ways to enhance the thermostability of HRPLs.^{7,10} In particular, flexible surface loops are potential targets for thermal inactivation and thus, their modification is an attractive approach to improve thermostability through less demanding screening.¹¹ This strategy is based on the assumption that most important factors to improve stability without sacrificing catalytic activity may be located at the flexible surface loops rather than at enzyme core, the former establishing fewer intramolecular contacts and therefore, their stiffening could enhance thermostability while maintaining the kinetic parameters.

We previously subjected the laccase from basidiomycete PM1, a thermostable HRPL isolated from the western Mediterranean, to directed evolution in the search for a variant heterologously expressed in yeast.¹² This evolved thermostable variant (named OB-1) was then further engineered in an ensemble of studies aimed at achieving tolerance in human blood¹³, increasing its redox potential¹⁴, and generating a diverse family of laccase chimeras with

Accepted Article

hybrid properties.⁸ Here, we have harnessed the OB-1 evolution platform to improve thermostability. We first identified potential flexible loops in the protein structure using a combination of predictive computational tools (molecular dynamics along with an analysis of *B*-factors). The segments selected were grouped into three sequence blocks and they were subjected to MORPHING, a focused evolution strategy to introduce random mutations and recombination events in defined DNA stretches.¹⁵ The final variant of this process was characterized biochemically and the mutations it carries were discussed within a structural context.

MATERIALS AND METHODS

Reagents and culture media

All chemicals were reagent-grade purity. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), DMP (2,6-dimethoxyphenol), Taq DNA polymerase and Yeast Transformation kit were purchased from Sigma (Spain). Pfu-ultra High-Fidelity DNA polymerase was acquired from Stratagene (USA) and iProof™ High Fidelity DNA polymerase was acquired from BIO-RAD (USA). Zymoprep Yeast Plasmid Miniprep kit and Zymoclean Gel DNA Recovery kit were from Zymoresearch (USA). The NucleoSpin plasmid® kit was purchased from Macherey Nagel (Germany). The restriction enzymes BamHI and XhoI were from New England Biolabs (UK). Expression shuttle vector pJRoC30 containing an uracil auxotrophy and ampicillin marker for selection came from California Institute of Technology (CALTECH, USA). The protease deficient

Saccharomyces cerevisiae BJ5465 yeast strain was from LGC Prochem (Spain) and *E. coli* XL-1 Blue competent cells were from Agilent technologies (USA). Sterile minimal medium contained 100 mL 67 g/L sterile yeast nitrogen base, 100 mL 19.2 g/L filtered yeast synthetic drop-out medium supplement without uracil, 100 mL 20% (w/v) filtered raffinose, 1 mL 25 g/L filtered chloramphenicol and 700 mL ddH₂O. SC drop-out plates contained 100 mL 67 g/L sterile yeast nitrogen base, 100 mL 19.2 g/L filtered yeast synthetic drop-out medium supplement without uracil, 100 mL 20% (w/v) filtered glucose, 1 mL 25 g/L chloramphenicol and 700 mL sterile ddH₂O with 20 g autoclaved bacto agar. Sterile YP medium (1.55X) contained 30.77 g bacto peptone and 15.38 g yeast extract diluted in 1000 mL sterile ddH₂O. Sterile YPD medium contained 20 g bacto peptone, 20 g yeast extract, 100 mL 20% (w/v) filtered glucose, 1 mL 25 g/L chloramphenicol and 900 mL sterile ddH₂O. Sterile laccase expression medium contained 720 mL sterile YP medium (1.55X), 67 mL filtered 1 M KH₂PO₄ buffer at pH 6.0, 110 mL 20% (w/v) filtered galactose, 30.9 absolute ethanol, 2 mL filtered 1 M CuSO₄ and 1 mL 25 g/L chloramphenicol and 69.1 mL sterile ddH₂O. For large scale flask production CuSO₄ final concentration was 4 M. Luria-Bertani (LB) medium contained 10 g bacto peptone, 5 g yeast extract and 10 g NaCl in 1000 mL sterile ddH₂O and 1 mL filtered ampicillin.

Computational prediction of surface flexible loops

Molecular Dynamics: The OB-1 model was built with the Schrodinger's homology modeling software, using the crystal structure of the *Trametes trogii*

laccase (PDB: 2HRG; 96% sequence identity vs. OB-1 laccase) as a template.^{16, 17} Protonation states of titratable residues were optimized with PROPKA simulating pH 4.0 (experimental conditions) and double-checked with the H++ server.^{18,19} Five independent molecular dynamics trajectories were run with GROMACS.²⁰ Simulations involved three 25 ns and one 80 ns simulations at 300 K, with an additional 80 ns simulations at 350 K. The enzyme was solvated with a 10 Å layer of water molecules in a dodecahedral box, adding enough ions for neutralization and for a 0.15 M NaCl buffer. The system was equilibrated as follows: i) solvent minimization; ii) system minimization; iii) 200 ps system warm up from 15 K to the target temperature at constant volume; iv) 200 ps at constant volume and target temperature; and v) 200 ps at target temperature and 1 bar. The AMBER99 force-field²¹ and the SPC explicit water model²² were used. The copper ions, modeled in their reduced (+1) state (to attenuate charge repulsion in the three-nuclear cluster), the coordinating atoms and their nearest neighbours were restrained to their initial positions with stiff 10 000 kJ mol⁻¹ nm⁻² harmonic constraints. The temperature was regulated with velocity rescaling²³ with a relaxation time of 0.1 ps, and the pressure was controlled with a Parrinello-Rahman barostat with isotropic coupling and a relaxation time of 2.0 ps. The LINCS algorithm²⁴ was employed to constrain all bond lengths, allowing a time step of 2.0 fs. A 10 Å cutoff was used for non-bonded interactions together with the particle mesh Ewald method.²⁵ Normal mode analysis was conducted with the Normal Mode Wizard for VMD (

<http://prody.csb.pitt.edu/nmwiz/>), which is based on ProDy. The ANM scheme was adopted, using the Ca as reference points. The first 10 ANM modes were linearly combined to reproduce a set of conformations. The RMSF was evaluated for the resulting ensemble.

Analysis of *B*-factors: The positional spread of each atom was monitored by mean-squared atomic displacements or *B*-factor, an indicator of the relative vibrational motion of each amino acid. Atoms with low *B*-factors belong to a region of the structure that is well ordered, whereas atoms with high *B*-factors generally belong to a part that is very flexible or labile with respect to binding.²⁶ The analysis of *B*-factors was performed applying the PyMOL Molecular Visualization System (version 1.3 Schrödinger, LLC) on 2HRG crystal structure.

Docking simulations

Docking simulations were performed using Glide and its default SP scoring function. The K-2SO mutant was prepared by manually introducing the Ser264Lys substitution with Maestro followed of a side chain sampling with Prime of all residues within 8 Å from Lys264. The docking grid was prepared using the 2HRG crystal bound ligand at the T1 site.

Directed evolution

Parental OB-1 laccase was obtained as described in previous work.¹² BamHI and XhoI were used to linearize the expression shuttle vector PJRoC30 and remove the parental gene. All PCR products and plasmid pJRoC30

linearized were cleaned, concentrated, loaded onto a preparative agarose gel (1%, w:v) and purified using Zymoclean Gel DNA Recovery kit. The recovered DNA fragments were cloned under the control of the GAL1 promoter of plasmid pJRoC30 linearized. Primers listed in **Supporting Table S1** were designed with Fast-PCR 6.5.37 program (University of Helsinki, Finland) and synthesized by the Sigma Aldrich (Germany). All sequences of the selected variants were sequenced by GATC Biotech (Germany) and analyzed with BioEdit Sequence Alignment Editor (Ibis Biosciences, USA).

MORPHING: MORPHING method¹⁵ was applied to construct three independent libraries named MORPHING1, MORPHING2 and MORPHING3. Primers were designed to create homologous overlapping areas of ~ 50 bp for the whole gene to be reassembled *in vivo* upon transformation in *S. cerevisiae*. i) *ep*-PCR for MORPHING blocks were carried out in a final volume of 50 μ L containing 3% DMSO, 0.3 mM dNTP mix (0.075 mM each), 1.5 mM $MgCl_2$, 0.15, 0.07 and 0.15 mM $MnCl_2$ for MORPHING1, MORPHING2 or MORPHING3, respectively, 2.5 units of Taq polymerase, 90 nM oligo sense (MOR1 FORW1 for MORPHING1, MOR2 FORW1 for MORPHING2 and MOR3 FORW1 for MORPHING3), 90 nM oligo antisense (MOR1 REV2 for MORPHING1, MOR2 REV2 for MORPHING2 and MOR3 REV2 for MORPHING3) and 46 ng of OB-1 template. The PCR program (thermal cycler MyCyclerTM (BIO-RAD, USA)) was as followed: 95°C for 120 s (1 cycle); 94°C for 45 s, 52°C, 60°C and 61°C for 30 s (for MORPHING1, MORPHING2 and MORPHING3, respectively), 74°C for 45

s (28 cycles) and 74°C for 10 min (1 cycle). ii) High fidelity PCRs for MORPHING blocks were carried out in a final volume of 50 µL containing 3% DMSO, 0.8 mM dNTP mix (0.2 mM each), 1 U IProof™ DNA polymerase, 0.5 µM oligo sense (RMLN for pre-MORPHING1, pre-MORPHING2 and pre-MORPHING3 and MOR1 FORW2 for post-MORPHING1, MOR2 FORW2 for post-MORPHING2 and MOR3 FORW2 for post-MORPHING3), 0.5 µM oligo anti sense (MOR1 REV1 for pre-MORPHING1, MOR2 REV1 for pre-MORPHING2, MOR3 REV1 for pre-MORPHING3 and RMLC for post-MORPHING1, post-MORPHING2 and post-MORPHING3) and 10 ng of OB-1 template. High-fidelity PCRs were performed with the following parameters: 98°C for 30 s (1 cycle); 98°C for 10 s, 50°C for 30 s (for pre-MORPHING1, pre-MORPHING2 and pre-MORPHING3) or 55°C for 30 s (for post-MORPHING1, post-MORPHING2 and post-MORPHING3), 72°C for 60 s (28 cycles); 72 °C for 10 min (1 cycle). PCR products were cleaned, concentrated, loaded onto a preparative agarose gel and purified. Competent *S. cerevisiae* cells were transformed in a mixture of 200 ng of each fragment with 100 ng linearized PJRoC30 plasmid. A negative control with 100 ng linearized PJRoC30 plasmid also was performed. Three libraries of 696 clones each was screened as described below.

Combinatorial saturation mutagenesis at positions 264 and 265 and saturation mutagenesis at position 475: For combinatorial saturation mutagenesis, two PCRs were carried out in a final volume of 50 µL containing 3% DMSO, 1 mM

dNTPs (0.25 mM each), 2.5 U Pfu-ultra polymerase and 100 ng template DNA, but each of them with different primers. For PCR1, 0.25 μ M RMLN was used as forward primer and four reversed primers in the same reaction with concentrations according to the protocol of Kille and co-workers²⁷: (i) AHN/ANH, (ii) CDB/CDB, (iii) CDB/AHN and (iv) AHN/CDB. For PCR2, four forward primers were used in same reaction with concentrations according to Kille *et al.*:²⁷ (i) NDT/NDT, (ii) VHГ/VHГ, (iii) VHГ/NDT and (iv) NDT/VHГ and 0.25 μ M RMLN as reverse primer. Codon substitutions, where N = A/T/C/G, D = no C, H = no G, B = no A, and V = no T, are shown in bold in **Supporting Table 1**. PCR reactions were performed using the following program: 95°C for 60 s (1 cycle); 95°C for 30 s, 52 or 57°C for 60 s for fragments from N-term and from C-term, respectively, 72°C for 120 s (28 cycles); 72°C for 10 min (1 cycle). PCR products were transformed together with linearized plasmid into yeast by IVOE¹⁵, and 2000 clones were screened. Saturation mutagenesis was carried out using degenerated NNK codons (N = A/T/C/G; K = T/G). Two PCRs were carried out in a final volume of 50 μ L containing 3% DMSO, 1 mM dNTPs (0.25 mM each), 2.5 U Pfu-ultra polymerase and 100 ng template DNA with 0.25 μ M RMLN and 0.25 μ M REV MNN or 0.25 μ M FORW NNK and 0.25 μ M RMLC. PCR reactions were performed as follows: for PCR1, 95°C for 60 s (1 cycle); 95°C for 30 s, 55°C for 60 s, 72 °C for 90 (28 cycles); 72°C for 10 min (1 cycle). For PCR2 95°C for 60 s (1 cycle); 95°C for 30 s, 60°C for 60 s, 72°C for 60 (28

cycles); 72°C for 10 min (1 cycle). PCR products were transformed together with linearized plasmid into yeast by IVOE¹⁵ (261 clones screened).

The high-throughput screening assay for thermostability was performed as described before²⁸ and laccase production and purification as reported elsewhere.⁸

Biochemical characterization

Thermoactivity profile: Enzyme supernatant dilutions of different laccase samples were prepared in such a way that aliquots of 20 µl gave rise to a linear response in kinetic mode. The optimum temperature for activity was estimated in prewarmed 96-well reading plates (Labnet VorTemp 56 Shaking Incubator; Labnet International, USA) with 100 mM sodium phosphate/citrate (pH 4.0) containing 3 mM of ABTS at various corresponding temperatures (25, 35, 45, 50, 55, 60, 65, 70, 75, 80, and 85°C), followed by incubation in an Eppendorf Thermomixer Comfort apparatus (Thermo Fisher Scientific). Reactions were performed in triplicate and ABTS oxidation was followed at 418 nm. Average and standard deviation are calculated from these three independent measurements.

Kinetic Thermostability: The thermostability of the different laccase samples was estimated by assessing their half-life of inactivation time ($t_{1/2}$) values using 96/384 well gradient thermocyclers. Appropriate dilutions of laccase in 20 mM bis-tris buffer pH 6.0 were incubated at 70°C. Aliquots of 50 µL were removed

after 5, 15, 25, 35, 55, 75, 95, and 120 min, chilled out on ice for 10 min and further incubated at room temperature at least for 5 min. Afterward, residual activity was determined in kinetic mode following the ABTS assay in kinetic mode (100 mM sodium phosphate/citrate buffer pH 4.0, 3 mM ABTS). Average and standard deviation are calculated from these three independent measurements. The half-life ($t_{1/2}$) was defined as the time required by the enzyme after incubation at 70°C to lose 50% of its initial activity at room temperature. To calculate $t_{1/2}$ residual activities were fitted to a single exponential decay function with SigmaPlot 10.0.

Steady-state kinetic constants: Kinetic parameters for ABTS and DMP were estimated in 100 mM sodium phosphate/citrate buffer, pH 4.0. Reactions were performed in triplicate and substrate oxidations were followed through spectrophotometric changes ($\epsilon_{418}\text{ABTS}^{\cdot+} = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$; $\epsilon_{469}\text{DMP} = 27,500 \text{ M}^{-1} \text{ cm}^{-1}$). To calculate the K_m and k_{cat} values, the average V_{max} was represented against substrate concentration and fitted to a single rectangular hyperbolic function with SigmaPlot 10.0.

RESULTS AND DISCUSSION

Computational analysis of flexible surface loops and the construction of MORPHING blocks

Given their value as predictive tools, we combined molecular dynamic simulations with an analysis of B -factors to examine the flexible surface loops of

OB-1 laccase.^{11,26} We first ran an approximated normal mode analysis (anisotropic network model, ANM) using ProDy²⁹ and five independent molecular dynamics simulations under different conditions (see Methods for details), **Supporting Figure S1**. From these experiments, the root mean square fluctuation (RMSF) for each residue belonging to a surface loop was adopted to single out stability hot-spot regions. As a result, three specific segments were found by molecular dynamic simulations: (a) Lys157-Thr164; (b) Asp250-Asp254; and (c) Ser264-Arg267, **Figure 1**. By contrast, an analysis of the *B*-factors identified six potential flexible regions in the loops: (i) Tyr137-Ser143; (ii) Ser176-Val186; (iii) Glu281-Val300; (iv) Leu304-Asp322; (v) Ser356-Ser360; and (vi) Asn475-Pro478, **Figure 1**. Despite an overall match between *B*-factors and RMSF could be expected, (large) deviations would not be surprising given that molecular simulations represent the protein dynamics in water at room temperature, with no agglomeration/oligomerization effects, which is radically different from the data directly extracted from the crystal structure model. Given that all the residues in these different segments represented ~16% of the protein sequence, their exploration by saturation mutagenesis was beyond our capacity. Thus, to achieve a reasonable compromise in the screening of these regions, we turned to MORPHING.¹⁵ This method is a simple and reliable protocol to create libraries that harnesses the high frequency of homologous recombination in *S. cerevisiae*, such that small protein segments can be subjected to random mutagenesis and DNA recombination in “one-pot”.

Accordingly, the three segments identified by molecular dynamics, together with five of the six segments from the *B*-factor analysis, were grouped into three independent MORPHING blocks, (with each block comprising 2 or three predicted segments), **Figure 2**. The segment (vi) was not included within any MORPHING block as it was located too far from the other segments.

Modifying the surface loops

The three MORPHING blocks were constructed from the OB-1 laccase, cloned in *S. cerevisiae*, and their mutant libraries (each containing 700 clones, 2100 in total) were quickly screened for thermostability using a previously developed high-throughput assay.²⁸ The Ser356Asn mutation in the best mutant identified (the 7A11 variant from MORPHING3-segment-v) showed a 4-fold improvement in thermostability. Using 7A11 as a template, we explored the Asn475 residue by saturation mutagenesis. We selected this residue because it belongs to the excluded segment (vi), and it is the most thermolabile residue within this region. Given that the molecular dynamics identified Ser264 and Gly265 as the most flexible region of the protein, they were also subjected to combinatorial saturation mutagenesis. After screening 2300 clones, the K-2SO variant was identified that incorporated the single Ser264Lys substitution and a silent mutation at Gly265, while no improved variants were identified in the Asn475 saturation mutagenesis library.

Biochemical characterization

The parental OB-1 laccase, and the 7A11 and K-2SO variants were characterized biochemically. We first measured their kinetic thermostability by assessing the half-life of thermal inactivation at 70°C ($t_{1/2}$). We observed a notable increase in the $t_{1/2}$ of K-2SO, 31 min longer than that of the parental OB-1 laccase (**Figure 3A**), whereas the thermoactivity profiles³⁰ were similar in the three variants, with K-2SO showing the optimum temperature of activity at 75°C, **Figure 3B**. Kinetic constants were measured using the classical laccase substrates, ABTS and DMP. The 7A11 variant showed similar catalytic efficiencies for ABTS as the parental OB-1 due to a roughly 3-fold enhancement in the k_{cat} that was compensated for by an increase in the K_{m} . However, the K-2SO mutant recovered the affinity for ABTS, which along with the improved k_{cat} gave rise to a ~1.7-fold higher catalytic efficiency than that of the parental OB-1 laccase, **Table 1**. For the phenolic substrate DMP, the $k_{\text{cat}}/K_{\text{m}}$ of the 7A11 variant increased 2-fold, mostly due to the enhanced k_{cat} , whereas the catalytic efficiency decreased almost 2.3-fold for K-2SO as a consequence of the lower substrate affinity in conjunction with the decrease in the k_{cat} .

The crystal structure of the *Trametes trogii* laccase was used as a template to map the mutations, sharing 96% sequence identity with OB-1.³¹ According to our model, the Ser264Lys substitution might generate a new salt bridge with Asp205, structurally reinforcing what is considered the labile segment (c) predicted by molecular dynamics, which is tightly connected with structural beta sheets, **Figure 4A, B**. Interestingly, this change was slightly

detrimental to the oxidation of DMP but beneficial to that of ABTS, which could be explained by the stabilization of the sulfonate groups with the extra positive charge. Inspection of our model indicated that Ser264 is one of the amino acids that conform the entrance to the catalytic site and it was recently identified as a key determinant for the recognition of substrates at the T1Cu site.³² Docking calculations were performed to correlate the drastic change in K_m for DMP. As seen in **Supplementary Figure 2**, the substrate position is very similar in both variants. Nevertheless, the presence of a lysine at position 264 introduces a change in the local electrostatic environment contributing, as predicted by the protein preparation wizard of Maestro, to the deprotonation of Asp205 at the pH assayed (4.0). While such a change is important to establish the new salt bridge with adjacent Lys264, it weakens the interaction of DMP with Asp205 and reduces slightly the docking score by 0.3 units (from -5.2 to -4.9).

The Ser356Asn mutation lies in segment (v) and it is located in the cupredoxin domain III of the laccase. Thus, our data seems to indicate the possible reorganization of this region due to the loss of a H-bond with the neighboring Leu352, **Fig 4C, D**. While the rigidification of flexible loops is beneficial for thermostability, only a few recent studies are addressing that the gain in flexibility of loose loops, largely exposed to the solvent, may increase the enzyme capability to adapt to high temperatures.³³ From the few data available, it seems like these flexible loops should be partly independent. That is, loops that do not interact with heavy structural secondary structure motifs

and that are partly protruding from the protein. This is the case of myoglobin³³ and it fits well also with the Ser356Asn mutation of the present study.

CONCLUSIONS

Assisted by computational tools and with a minor screening effort, we have successfully modified flexible loops at the surface of a HRPL. As in other case studies, the modification of such labile regions was accompanied by enhanced thermostability without a drastic drop in activity. In the near future, we will seek to combine these and other stabilizing mutations discovered by different approaches, such as classical adaptive evolution¹², consensus design³⁴ and laccase chimeragenesis⁸ in a drive towards designing more robust HRPLs.

ACKNOWLEDGMENTS

This work was supported by the Spanish Government project BIO2016-79106-R-Lignolution, CTQ2016-79138-R, and the European Union projects FP7-KBBE-2013-7-613549-INDOX and COST-ActionCM1303.

REFERENCES

1. Alcalde M. Laccases: Biological Functions, Molecular Structure and Industrial Applications, In Polaina J, MacCabe AP. Industrial enzymes: Structure, function and applications. New York: Springer, 2007:459-474.

2. Riva S. Laccases: Blue Enzymes for Green Chemistry. *Trends Biotechnol.* 2006; 24:219-226.
3. Jones SM, Solomon EI. Electron transfer and reaction mechanism of laccases. *Cell. Mol. Life Sci.* 2015;72:869-883.
4. Alcalde M. Engineering the ligninolytic enzyme consortium. *Trends Biotechnol.* 2015;33:155-162.
5. Mate DM, Alcalde M. Laccase: a Multi-Purpose Biocatalyst at the Forefront of Biotechnology, *Microb. Biotechnol.* 2017;10:1457-1467.
6. Rodgers CJ, Blanford CF, Giddens SR, Skamnioti P, Armstrong FA, Gurr SJ. Designer Laccases: a Vogue for High-Potential Fungal Enzymes? *Trends Biotechnol.* 2010;28:63-72.
7. Mate DM, Alcalde M. Laccase Engineering: From Rational Design to Directed Evolution. *Biotechnol. Adv.* 2015;33:25-40.
8. Mateljak I, Rice A, Yang K, Tron T, Alcalde M. The generation of thermostable fungal laccase chimeras by SCHEMA-RASPP structure-guided recombination in vivo. *ACS Synth. Biol* 2019 In press.
9. Pardo I, Rodríguez-Escribano D, Aza P, de Salas F, Martínez AT, Camarero S. A highly stable laccase obtained by swapping the second cupredoxin domain. *Scientific Reports* 2018;8:5669.
10. Hilden K, Hakala TK, Lundell T. Thermotolerant and thermostable laccases. *Biotechnol. Lett.* 2009;31:1117.

11. Nestl BM, Hauer B. Engineering of flexible loops in enzymes. *ACS Catal.* 2014;4:3201-3211.
12. Mate D, Garcia-Burgos C, Garcia-Ruiz E, Ballesteros AO, Camarero S, Alcalde M. Laboratory Evolution of High-Redox Potential Laccases, *Chem. Biol.* 2010;17:1030-1041.
13. Mate D, Gonzalez-Perez D, Falk M, Kittl R, Pita M, De Lacey AL, Ludwig R, Shleev S, Alcalde M. Blood tolerant laccase by directed evolution. *Chem. Biol.* 2013;20:223-231.
14. Mateljak I, Monza E, Lucas MF, Guallar V, Aleksejeva O, Ludwig R, Leech D, Shleev S, Alcalde M. Increasing redox potential, redox mediator activity and stability in a fungal laccase by computer-guided mutagenesis and directed evolution. *ACS Catal.* 2019. In press.
15. Gonzalez-Perez D, Molina-Espeja P, Garcia-Ruiz E, Alcalde M. Mutagenic Organized Recombination Process by Homologous In Vivo Grouping (MORPHING) for Directed Enzyme Evolution. *PLoS One*, 2014,9:e90919.
16. Jacobson MP, Friesner RA, Xiang Z, Honig B. On the Role of the Crystal Environment in Determining Protein Side-Chain Conformations. *J. Mol. Biol.* 2002;320:97–608.
17. Jacobson MP, Pincus DL, Rapp CS, Day TJ, Honig B, Shaw DE, Friesner RA. A Hierarchical Approach to all-atom Protein Loop Prediction. *Proteins: Struct. Funct. Genet.* 2004;55:1070 351–367.

18. Gordon JC, Myers JB, Folta T, Shoja, V, Heath LS, Onufriev AH. ++: a Server for Estimating pK as and Adding Missing Hydrogens to Macromolecules. *Nucleic Acids Res.* 2005;33:W368–W371.
19. Olsson MH, SØndergaard CR, Rostkowski M, Jensen JH. PROPKA3: Consistent Treatment of Internal and Surface Residues in Empirical pK a Predictions. *J. Chem. Theory Comput.* 2011; 7:525–537.
20. Pronk S, Páll S, Schulz R, Larsson P, Bjelkmar P, Apostolov R, Shirts MR, Smith JC, Kasson PM, van der Spoel, Hess B, Lindahl E. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. *Bioinformatics* 2013;29:845–854.
21. Duan Y, Wu C, Chowdhury S, Lee MC, Xiong G, Zhang W, Yang R, Cieplak P, Luo R, Lee T, Caldwell J, Wang J, Kollman P. J. A point-charge force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations. *Comput. Chem.* 2003; 24:1999–2012.
22. Toukan K, Kahled T, Aneesur R. Molecular-dynamics study of atomic motions in water. *Phys. Rev. B: Condens. Matter.* 1985;31:2643–2648
23. Bussi G, Donadio D, Parrinello M. Canonical sampling through velocity rescaling *J. Chem. Phys.* 2007;126:014101.
24. Hess B, Berk H, Henk B, Berendsen HJC, Johannes GE. LINCS: A linear constraint solver for molecular simulations *J. Comput. Chem.* 1997;18:1463–1472.

25. Essmann U, Ulrich E, Lalith P, Berkowitz ML, Tom D, Hsing L, Pedersen LG. A smooth particle mesh Ewald method. *J. Chem. Phys.* 1995;103:8577.
26. Reetz MT, Carballeira JD, Vogel A. Iterative saturation mutagenesis on the basis of B factors as a strategy for increasing protein thermostability. *Angew. Chem. Int. Ed.* 2006;45:7745–7751.
27. Kille S, Acevedo-Rocha CG, Parra LP, Zhang ZG, Opperman DJ, Reetz MT, Acevedo JP. Reducing codon redundancy and screening effort of combinatorial protein libraries created by saturation mutagenesis. *ACS Synth. Biol.* 2013;2:83–92.
28. Garcia-Ruiz E, Mate D, Ballesteros A, Martinez AT, Alcalde M. Evolving thermostability in mutant libraries of ligninolytic oxidoreductases expressed in yeast. *Microb. Cell Fact.* 2010;9:17.
29. Bakan A, Meireles LM, Bahar I. ProDy: Protein Dynamics Inferred from Theory and Experiments. *Bioinformatics* 2011;27:1575-1577.
30. Cirino PC, Georgescu R. Screening for thermostability. *Methods Mol. Biol.* 2003;230:117–125.
31. Matera I, Gullotto A, Tilli S, Ferraroni M, Scozzafava A, Briganti F. Crystal structure of the blue multicopper oxidase from the white-rot fungus *Trametes trogii* complexed with p-toluate. *Inorg. Chim. Acta* 2008;361: 4129-4137.

32. Pardo I, Santiago G, Gentili P, Lucas F, Monza E, Medrano FJ, Galli C, Martinez AT, Guallar V, Camarero S. Re-designing the substrate binding pocket of laccase for enhanced oxidation of sinapic acid. *Catal. Sci. Technol.* 2016;6:3900-3910.
33. Julió Plana L, Nadra AD, Estrin DA, Luque FJ, Capece L. Thermal Stability of Globins: Implications of Flexibility and Heme Coordination Studied by Molecular Dynamics Simulations. *J Chem Inf Model* 2019;59:441–52.
34. Gomez-Fernandez B, Alcalde M. Consensus mutagenesis of a high-redox potential laccase. *BBA Prot. and Proteom.* 2019. Submitted.

Table 1. Kinetic parameters of the evolved HRPLs.

Substrate		OB-1	7A11	K-2SO
ABTS	K_m (mM)	$0.006 \pm 0,001$	$0.017 \pm 0,002$	$0.006 \pm 0,001$
	k_{cat} (s^{-1})	179 ± 4	566 ± 24	298 ± 9
	k_{cat}/K_m ($mM^{-1}s^{-1}$)	29,833	33,294	49,666
DMP	K_m (mM)	$0.150 \pm 0,008$	$0.140 \pm 0,005$	$0.440 \pm 0,001$
	k_{cat} (s^{-1})	85 ± 1	160 ± 6	108 ± 1
	k_{cat}/K_m ($mM^{-1}s^{-1}$)	566	1143	245

Reactions were performed in triplicate and substrate oxidation was followed by spectrophotometry. To calculate the K_m and k_{cat} values, the average V_{max} was plotted against the substrate concentration and fitted to a single rectangular hyperbolic function using SigmaPlot 10.0.

FIGURE LEGENDS:

Figure 1. Segments selected by *B*-factors analysis and molecular dynamics (A) *B*-factor representation generated with the PyMOL Molecular Graphics System (Version 1.6 Schrödinger, LLC) using the crystal structure of the *Trametes trogii* laccase (PDB: 2HRG). The flexibility increases as the thickness of the line representing the region augments and according to the following color code: lowest flexibility -blue/green/yellow/red- highest flexibility. (B) Front and (C) back views of the laccase structure highlighting the segments selected (in red from *B*-factor analysis and in cyan from molecular dynamic simulations). The protein surface and the protein's secondary structures are shown in grey.

Figure 2. MORPHING blocks. Front and back views of the laccase model highlighting the MORPHING blocks in light blue, orange and yellow, with

segment (vi) shown in green and the non-mutagenized region in grey. The protein surface and the protein's secondary structures are shown using the same color pattern as described above. Blue spheres represent the Cu atoms. The model was generated with the PyMOL Molecular Graphics System (Version 1.6 Schrödinger, LLC) and based on the *Trametes trogii* laccase crystal structure (PDB: 2HRG).

Figure 3. Kinetic thermostability of evolved HRPLs. (A) The $t_{1/2}$ of thermal inactivation at 70°C and (B) the thermoactivity (optimum temperature for activity) of the parental type OB-1 (black circles), and the 7A11 (white circles) and K-2SO variants (black triangles). Each point represents the mean and standard deviation of three independent measurements.

Figure 4. Location of the mutations. Representation of the parental OB-1 (A, C) and K-2SO mutant (B, D) in cartoon mode (light blue), with the residues shown in stick mode and interactions as broken yellow lines. Blue spheres represent the Cu atoms. The model was generated using the PyMOL Molecular Graphics System (Version 1.6 Schrödinger, LLC) and based on the crystal structure of the *Trametes trogii* laccase (PDB: 2HRG).

Figure 1

Figure 3

Figure 4