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Altering the laccase functionality by *in vivo* assembly of mutant libraries with different mutational spectra

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ABSTRACT

The generation of diversity for directed protein evolution experiments shows an important bottleneck in the* in vitro *random mutagenesis protocols. Most of them are biased towards specific changes that eventually confer a predicted and conservative mutational spectrum, limiting the exploration of the vast protein space. The current work describes a simple methodology to* in vivo *recombine mutant libraries with different nucleotide bias created by* in vitro *methods. This* in vivo *assembly was based on the accurate physiology of* Saccharomyces cerevisiae*, which as host, provided its high homologous recombination frequency to shuffle the libraries in a nonmutagenic way. The fungal thermophilic laccase from* Myceliophthora thermophila *expressed in* S. cerevisiae *was submitted to this protocol under the selective pressure of high concentrations of organic solvents. Mutant 2E9 with ~3-fold better kinetics than parent type showed two consecutive amino acid changes (G614D -GGC/GAC- and E615K -GAG/AAG-) because of the* in vivo *shuffling of the mutant libraries. Both mutations are located in the C-terminal tail that is specifically processed at the Golgi during the maturation of the protein by the* Kex*2 protease. Notoriously, the oxygen consumption at the T2/T3 trinuclear copper cluster was altered and the catalytic copper at the T1 site was perturbed showing differences in its redox potential and geometry. The change in the isoelectric point of C-terminal extension upon mutations seems to affect the folding of the protein at the posttranslational processing steps providing new insights in the significance of the C-terminal tail for the functionality of the ascomycete laccases.

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Key words: in vivo assembly; laccase; C-terminal tail; protein folding.

INTRODUCTION

In the last 15 years, the idea of mimicking the darwinist concept of natural selection employing experimental protocols directed to enhance or even create novel complex functions in protein structures has opened a big window to an unexplored world.[1–5] To most of us, laboratory evolution (the generation of diversity by *in vitro* or *in vivo* methods coupled to high-throughput screening assays) represents a shortcut to tailor enzymes with several improved features.[6–9] In spite of the fact that molecular biologists and protein engineers are focusing their efforts to farther push the technology of directed enzyme evolution with the main aim of limiting the protein space, lab evolution is still in its infancy.[10,11] The requirement of more efficient high-throughput assays to screen bigger libraries—over 10^8–10^{10} variants—is one of the main hurdles.[12,13] Moreover, the generation of diversity is unequivocally limited by the nature of the genetic code.[14] In this sense, computational methods and semirational approximations (i.e. combinatorial saturation mutagenesis[15]) or more recently the introduction of new tools such as circular permutation,[16] are being used to design proteins efficiently. But, yet, we have to deal with the fact that natural evolution is somehow a sophisticated engineering process from whose efficacy we are far away. In other words, the accumulation of subtle changes at genetic level not always drive to the best enzymatic adaptation, or at least not with the molecular tools that we have currently in hand. Basically there are two ways of generating diversity: the asexual approach (i.e. the random introduction of mutations based on error-prone PCR techniques) and the sexual one (DNA recombination).[17] The latter has achieved a reasonable level of

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development enabling DNA fragments of low homology to recombine each other.[18] Thus, nowadays, the main concern in generation of diversity stems from unsatisfactory mutagenic methods[19,20] which usually display limited and predicted mutational spectra because of the intrinsic bias of each DNA-polymerase.[21] In the last few years, the use of *Saccharomyces cerevisiae* physiology as biomolecular tool is providing new alternatives to the current bottlenecks found during the construction of *in vitro* evolution libraries.[22–25] We believe that *S. cerevisiae* accurate device may be helpful to *in vivo* recombine mutant libraries created by several means, that is with different bias, and therefore, to produce an unexpected mutational spectrum. To validate this hypothesis, we have chosen as scaffold the ascomycete laccase from *Myceliophthora thermophila* (MtL) that was previously evolved for functional expression in *S. cerevisiae*.[26] Because of its biotechnological interest (with applications in the pulp-kraft bleaching, oxidation of xenobiotics, chemical synthesis…), laccase is a promising candidate to be fuelled by laboratory evolution strategies.[27–29] Laccases belong to the family of multicopper oxidases with one copper T1 where the reducing substrate binds and a trinuclear copper cluster T2/T3 in which the O_2 is reduced to two molecules of water.[28] MtL gene constitutes a suitable and complex scaffold possessing a preproleader and an exclusive C-terminal tail which codes for parts of the protein that are supposed to be cleaved during posttranslational stages in yeast.[26] The importance and processing of these polypeptides in native or heterologous hosts are not well understood yet, although they have been extensively studied in other ascomycete laccases,[30–32] and therefore have been included for mutagenesis and recombination in the current study.

This article describes the use of *S. cerevisiae* for *in vivo* recombining distinct MtL mutant libraries (from polymerases with different bias) giving rise to a variant with modifications at the C-terminal tail. Taking advantage of the eukaryotic machinery, the libraries created by several *in vitro* methodologies were *in vivo* shuffled and screened to find mutants with better fitness towards organic cosolvents generally used to enhance the solubility of many of the laccase reducing substrates. The role of the C-terminal tail and its action during protein folding is discussed in detail.

MATERIAL AND METHODS

All chemicals were of reagent-grade purity. Taq-DNA polymerase, ABTS (2,2′-azino-bis(3-ethybenzthizoline-6-sulfonic acid)) and *S. cerevisiae* transformation kit were purchased from Sigma-Aldrich (Madrid, Spain). Parent type laccase gene was engineered from MtLT2[26] through three consecutive cycles of *in vitro* evolution under the selective pressure of increasing concentrations of organic cosolvents, accumulating mutations L429V, E182K, and N552H in the mature protein (unpublished material). GeneMorph PCR mutagenesis kit and *E. coli* XL2-blue competent cells were from Stratagene (La Jolla, CA). Protease deficient *S. cerevisiae* strain BJ5465 was from LGCPromochem (Barcelona, Spain). Shuttle vector pJRoC30 with auxotrophy for uracil and with ampicillin resistance gene was from Novozymes (Davis, CA). Zymoprep yeast plasmid miniprep kit, zymoclean gel DNA recovery kit, and DNA clean and concentrator TM-5 kit were from Zymo Research (Orange, CA). QIAprep spin miniprep kit was from QIAGEN (West Sussex, UK). Restriction enzymes BamHI and XhoI were from New England Biolabs (Hertfordshire, UK).

Culture media

Minimal medium contained 100 mL of 6.7% sterile yeast nitrogen base, 100 mL of 19.2 g/L sterile yeast synthetic drop-out medium supplement without uracil, 100 mL sterile 20% raffinose, 700 mL dd H_2O, 1 mL 25 g/L chloramphenicol. YP medium contained 10 g yeast extract, 20 g peptone, and dd H_2O to 650 mL. Expression medium contained 720 mL YP, 67 mL 1*M* KH_2PO_4 pH 6.0 buffer, 10 µL 1*M* $CuSO_4$, 111 mL 20% galactose, 1 mL 25 g/L chloramphenicol, and dd H_2O to 1000 mL. YPAD solution contained 10 g yeast extract, 20 g peptone, 100 mL 20% sterile glucose, 100 mg adenine hemisulfate, 1 mL 25 g/L chloramphenicol, and dd H_2O to 1000 mL. SC drop-out plates contained 6.7 g yeast nitrogen base, 100 mL 19.2 g/L sterile yeast synthetic drop-out medium supplement without uracil, 20 g bacto agar, 100 mL 20% sterile glucose, 1 mL 25 g/L chloramphenicol, and dd H_2O to 1000 mL.

Production of laccase in *S. cerevisiae*

One single colony from *S. cerevisiae* clone containing parent-type or mutant laccase genes was picked from a SC drop-out plate, inoculated in 3 mL of minimal medium and incubated for 48 h at 30°C and 225 rpm (Micromagmix shaker, Ovan, Spain). An aliquot of cells was removed and inoculated into a final volume of 50 mL of minimal medium (optical density, OD_{600} = 0.25) in a 500-mL flask. Incubation proceeded until two growth phases were completed (6–8 h). Thereafter, 450 mL of expression medium containing 5.6 m*M* $CuSO_4$ were inoculated with the 50 mL preculture in a 2.0-litre baffled flask (OD_{600} = 0.1). Incubation was stopped after 38–42 h at 30°C and 225 rpm (laccase activity was maximal reaching a plateau; OD_{600} = 28–30). The cells were separated by centrifugation for 20 min at 3000*g* (4°C). Supernatant was double-filtered (by both glass membrane and a nitrocellulose membrane of 0.45 µm pore size) and concentrated to 20 mL in an ultrafiltration cell (Amicon/Millipore, Barcelona, Spain) equipped with a 10 kDa cut-off membrane.

Purification of laccases

Parent and mutant laccases were purified using fast protein liquid chromatography (FPLC) equipment (LCC-500CI, Amersham Bioscience, Barcelona, Spain). Concentrated crude extract was first submitted to fractional precipitation with ammonium sulfate to 50% saturation (w/v) at 0°C and centrifuged at 17,000*g* for 20 min. The supernatant was filtered and loaded into a hydrophobic interaction column (HIC, Hiload 16/10 Phenyl Sepharose High Performance, Amersham) equilibrated with 1.8*M* $(NH_4)_2SO_4$ in 10 m*M* sodium phosphate (pH 6.1). Proteins were eluted with a linear gradient from 1.8*M* to 0*M* $(NH_4)_2SO_4$. Fractions with laccase activity were pooled, concentrated, and dialyzed against 10 m*M* sodium phosphate (pH 6.1). Semipurified laccase was applied to an anion exchange column (DEAE Sepharose CL-6B) pre-equilibrated with 10 m*M* sodium phosphate (pH 6.1). Proteins were eluted with a linear gradient from 0*M* to 0.4*M* NaCl. Fractions with laccase activity were pooled, concentrated, and dialyzed against 10 m*M* sodium phosphate (pH 6.1). Fractions throughout the purification protocol were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 8% polyacrilamide using a Miniprotean 3 Cell (BioRad, US). Proteins were stained with both Coomassie brilliant blue and silver. Protein concentration was determined using the Bio-Rad protein reagent and bovine serum albumin as a standard. Purified laccases were stored at –20°C.

In vivo assembly of mutant libraries with different mutational spectra (IvAM)

Mutagenic PCR

Mutagenic PCR was carried out using a gradient thermocycler (Mycycler, Biorad, US). Thermal cycling parameters were: 95°C for 2 min (1 cycle), 94°C for 0.45 min, 53°C for 0.45 min, 74°C for 3 min (28 cycles), 74°C for 10 min (1 cycle). The primers used for amplification were: IG88-S sense (5′-CCTCTATACTTTAACGTCAAGG-3′, binds at bp 5′-160-180-3′ of pJRoC30) and IG88-R antisense (5′-GGGAGGGCGTGAATGTAAGC-3′, binds at bp 5′-2139-2158-3′ of pJRoC30). The size of MtL gene is 1912 bp. The circular autonomous vector pJRoC30 with MtL gene has a size of 12,337 bp.

Taq/$MnCl_2$ libraries. Taq/$MnCl_2$ libraries were prepared at different concentrations of $MnCl_2$ to estimate an appropriate mutation rate. Additionally, the effect of balanced/unbalanced dNTPs and the concentration of template were analyzed.

Taq/$MnCl_2$ libraries with unbalanced dNTPs: In a 50 µL final volume, two different reactions conditions were explored (with different template concentrations). Reaction 1: 90 n*M* IG88-S; 90 n*M* IG88-R; 1.5 ng/µL laccase template; 0.2 m*M* dATP; 0.2 m*M* dGTP; 0.6 m*M* dCTP; 0.6 m*M* dTTP; 3% DMSO; 0.75 m*M* $MgCl_2$; 0.05 U/µL Taq polymerase. Reaction 2: 90 n*M* IG88-S; 90 n*M* IG88-R; 0.1 ng/µL laccase template; 0.04 m*M* dATP; 0.04 m*M* dGTP; 0.1 m*M* dCTP; 0.1 m*M* dTTP; 3% DMSO; 1.5 m*M* $MgCl_2$; 0.05 U/µL. The influence of $MnCl_2$ was studied at different final concentrations (0, 0.05 m*M*, 0.1 m*M*, 0.2 m*M*).

Taq/$MnCl_2$ libraries with balanced dNTPs and low concentration of template: The concentrations for each reagent in 50 µL final volume were as follows: 90 n*M* IG88-S; 90 n*M* IG88-R; 0.1 ng/µL laccase template; 0.3 m*M* dNTPs (0.075 m*M* each); 3% DMSO; 1.5 m*M* $MgCl_2$; 0.05 U/µL Taq polymerase. The influence of $MnCl_2$ was studied at different final concentrations (0, 0.002 m*M*, 0.005 m*M*, 0.01 m*M*, 0.02 m*M*, 0.05 m*M*, 0.1 m*M*, and 0.2 m*M*).

Mutazyme libraries. High and low mutation rate libraries were constructed using the Genemorph kit (Stratagene, CA). The concentration of each reagent in 50 µL final volume was as follows: 370 n*M* IG88-S; 370 n*M* IG88-R; 0.8 m*M* dNTPs; 3% DMSO; 0.05 U/µL Mutazyme DNA polymerase; 13 ng/µL and 4 ng/µL laccase templates for low mutation rate libraries; and 4 pg/µL laccase template for high mutation rate library.

Preparing libraries in *S. cerevisiae*

PCR products were cleaned and concentrated (DNA clean and concentrator TM-5 kit, Zymo Research), loaded into a low melting point preparative agarose and purified using the Zymoclean gel DNA recovery kit (Zymo Research). PCR product was cloned behind the Gal 10 promoter of the expression shuttle vector pJRoC30. Cloning was performed by replacing MtLT2 gene from pJRoC30. To remove parent gene, plasmid pJRoC30 was linearized by cutting with restriction enzymes XhoI and BamHI (New England Biolabs). Linearized plasmid was concentrated (DNA clean and concentrator TM-5 kit) and loaded into a preparative low melting point agarose followed by an agarose gel extraction (Zymoclean gel DNA recovery kit). PCR products were mixed with linearized vector and transformed into competent yeast cells using the Yeast transformation kit (Sigma). Right before the thermal shock, 10% DMSO was introduced in the transformation mixture to improve the transformation efficiency (above 15,000 clones per transformation reaction).

In vivo *recombination of mutant libraries in S. cerevisiae*

Taq/$MnCl_2$ and Mutazyme libraries were equimolarly mixed. The equimolar mixture was added to 100 ng of linearized vector (ratio vector:equimolar library tested: 1:1, 1:2, 1:4, 1:6, 1:8, 1:10) and transformed into yeast competent cells as described earlier.

HTPS assays: end-point colorimetric assay in the presence of organic cosolvent

Screening protocol was performed according to Alcalde *et al.*[29] with minor modifications. Individual clones were picked into 96-well plates (Sero-well, Staffordshire, UK) containing 50 μL of minimal medium per well. In every single plate the column number 6 was inoculated with standard (parent type), and one well (H1) was not inoculated (control). Plates were wrapped with parafilm (to prevent evaporation) and incubated at 30°C, 225 rpm and 80% relative humidity in a humidity shaker (Minitron-INFORS, Biogen, Spain). After 48 h, 160 μL of expression medium were added to each well, and plates were incubated again. After 24 h, OD_{600} was recorded to determine the cell density in each well using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA). Plates (master plates) were centrifuged (Eppendorf 5810R centrifuge, Germany) for 5 min at 3000*g* at 4°C. Twenty microliter of supernatant were transferred from master plate with the help of a robot (Liquid Handler Quadra 96-320, Tomtec, Hamden, CT) to a replica plate (polypropylene solvent resistant plate). 180 μL of assay solution (final concentration in the well 3 m*M* ABTS, with/without 50% organic cosolvent, acetonitrile, or ethanol, 100 m*M* Britton and Robinson buffer pH 6.0) was added to each well of replica plate containing supernatant. Plates were stirred and the absorption at 418 nm ($\varepsilon^{+\cdot}_{ABTS}$ = 36,000 M^{-1} cm^{-1}) was recorded in the plate reader. Plates were incubated at room temperature until green color was developed, and the absorbance was measured again. Activities were calculated from the difference between absorbance after incubation and the initial absorption, divided by the OD_{600} of each well; relative activities were normalized against the parent type in the corresponding plate.

Rescreening procedures

First rescreening

Aliquots of 5 μL of the best clones were used from master plates to inoculate 50 μL of minimal media in new 96-well plates. Columns 1 and 12, rows A and H, were not used to prevent the appearance of false positives. Five wells on the microplates were inoculated with the same clone. Five wells on each plate were used for the standard. The screening procedure then was the same as earlier.

Second rescreening

An aliquot from the wells with the most active clones of first rescreening was inoculated in 3 mL of YPAD and incubated at 30°C and 225 rpm for 24 h. Plasmids from these cultures were extracted (Zymoprep yest plasmid miniprep kit, Zymo Research). As the product of the zymoprep was very impure and the concentration of extracted DNA was very low, the shuttle vectors were transformed into super-competent *E. coli* cells (XL2-Blue, Stratagene) and plated onto LB-amp plates. Single colonies were picked and used to inoculate 5 mL LB-amp media and were grown overnight at 37°C and 225 rpm. Plasmids were then extracted (QIAprep spin miniprep kit, QIAGEN). *S. cerevisiae* was transformed with plasmids from the best mutants and also with parent type. Five colonies of every single mutant were picked and rescreened as described earlier.

DNA sequencing

Plasmid-containing variants laccase genes were sequenced at the Sequencing Core Facility of the Instituto de Investigaciones Biomedicas, CSIC, Madrid, using an Applied Biosystems 377 automated fluorescent DNA sequencer. The primers used were as follows: forward: IG88-S and mtlsq2 (5′-GAAGGGCACCAACCTGC-3′, binds at bp 5′-643-659-3′ of pJRoC30); reverse: mtlsq3 (5′-CGCACGTAAAAGTCGTGG-3′, binds at bp 5′-1657-1673-3′of pJRoC30) and IG88-R.

Spectroelectrochemical characterization

Spectroelectrochemistry of purified laccases (MtL variants as well as *Trametes hirsuta* laccase as control) was carried out using a micro-spectroelectrochemical cell consisting of the gold capillary electrode. The design of the cell was described elsewhere.[33] The potential of the gold capillary of the cell was controlled by a three-electrode potentiostat BAS LC-3E from Bioanalytical Systems, BAS (West Lafayette, IN). In these measurements an Ag|AgCl|KCl reference electrode (BAS) and a platinum counter electrode were used. The absorbance spectra were monitored with PC2000-UV-VIS, a miniature fiber optic spectrometer from Ocean Optics (Dunedin, FL) with an effective range between 200 and 1100 nm. The pretreatment of the gold capillary working electrode of the spectroelectrochemical cell was carried out by washing the cell capillary with a peroxide–sulfuric acid mixture followed by rinsing with Millipore water. The redox potential of the T1 site of the enzymes was determined by mediated spectroelectrochemical redox titration, MRT, using spectroelectrochemical set-up described earlier. Complex mediator system containing four different mediators ($K_4[Fe(CN)_6]$, $K_4[W(CN)_8]$, $K_4[Os(CN)_6]$, and $K_4[Mo(CN)_8]$ with formal redox potentials of 430, 520, 640, and 780 mV vs. NHE, respectively) was used for the MRT performed in accordance with previously published methodology.[34]

Spectral investigations

EPR analysis of copper sites of purified laccases

Electron paramagnetic resonance (EPR) measurements were carried out with a Bruker ER200D instrument oper-

ating in the X-band ($\nu \approx 9.6$ GHz) using a DPPH standard (in a T-type double cavity) for frequency calibration. Portions of sample in potassium phosphate buffer were introduced into a spectroscopic quartz probe cell. The spectra were recorded at 77 K and were typically performed at 19.5-mW microwave power (no signal saturation was apparent in independent experiments up to 40 mW), 100 kHz modulation frequency, 2G modulation amplitude, 40 ms time constant, and 1×10^5 receiver gain.

Circular dichroism spectra of purified laccases

Circular dichroism (CD) spectra were recorded using a Jasco J-720 spectropolarimeter (with a scan rate of 20 nm/min and a bandwidth of 1.0 nm). The measurements were performed under N_2 atmosphere employing a 0.01-cm cell with a protein concentration of ~2 mg/mL. The parameters of the secondary structures were calculated using an in house computer program "Protein-CD v 1.5" (Moscow, Russia) as described in Refs. 35 and 36.

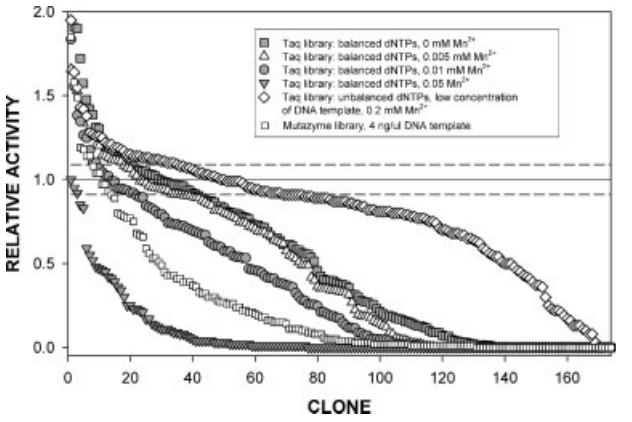

Figure 1

Landscapes for libraries made under different error prone-PCR conditions. Activity of clones is plotted in descending order. Solid horizontal line shows the activity of the parent type in the assay. Dashed lines indicate the coefficient of variation of the assay (below 10%).

O_2-reduction activity measurements

The O_2-reduction activity of laccases was measured by mass spectrometry with 3.0 m*M* ABTS as electron donor in 10 m*M* acetate buffer pH 4.5, at 27°C. The measurement cell had 10 mL volume and was separated from the mass spectrometer (Masstorr 200 DX quadrupole, VG Quadrupoles) by a teflon membrane through which gasses from the solution diffuse.[37] The response at the spectrometer at certain mass value is proportional to the partial pressure of the gas in the solution of the cell. O_2-uptake was measured at mass 16. Calibration was done with the solution saturated with pure O_2 and background signal was estimated with the solution purged with pure Ar. O_2-consumption by the mass spectrometer (measured in absence of laccase for each O_2 concentration) was subtracted to the activity measurements, in which 5–25 μg of laccase were added.

RESULTS AND DISCUSSION

Analysis of mutant libraries constructed by Taq/$MnCl_2$ and Mutazyme

Two different DNA-polymerases—Taq polymerase and Mutazyme—were used to *in vitro* create several mutant libraries of *Myceliophthora thermophila* laccase. First of all, mutation rate was tuned to introduce 1–3 amino acid changes (1–5 nucleotide substitution/laccase gene). Few exceptions aside,[38,39] higher mutations are generally not suitable in laboratory evolution experiments, especially when one wants to distinguish beneficial mutations from those that are neutral or deleterious. Mutagenic PCR using Taq DNA polymerase takes advantage from the lack of 3′-5′ proofreading exonuclease activity. Generally, the modulation of mutational rate can be accomplished by: introducing $MnCl_2$ in the reaction mixture; varying the ratios of nucleotides in the reaction; changing the initial template concentration or increasing concentration of $MgCl_2$.[14] Laccase parent gene was submitted to PCR amplification with Taq/$MnCl_2$, unbalanced dNTPs, and high concentration of template. To make a correct comparison between mutagenic PCR protocols under different conditions, small libraries (two 96 well-plates) were constructed and their landscapes provided an estimation of the mutation frequency from the fraction of inactive clones, Figure 1. Typically an error rate resulting in a library with 30–45% of clones having less than 10% of the wildtype activity is suitable for achieving improvements during a laboratory evolution process.[18] When unbalanced dNTPs were introduced, mutation rate was lower than expected (with only 4.6% mutants having less than 10% of the parent enzyme's activity under 0.2 m*M* $MnCl_2$). The concentrations of dNTPs and $MgCl_2$ were varied and the amount of template reduced to increase mutational frequency. Under these conditions—see Material and Methods section—the mutational rate was considerably enhanced (with 14% of mutants having less than 10% of the MtLT2 activity in absence of $MnCl_2$, Table I). Libraries with balanced dNTPs were further studied. The mutation frequency was smoothly tuned by just varying the $MnCl_2$ concentration, ranging from 34 to 81% of clones with less than 10% of the parents activity, for Taq libraries from 0 to 0.05 m*M* of $MnCl_2$ (Fig. 1, Table I). Mutagenic libraries were also constructed using the Mutazyme DNA polymerase. This polymerase has a high intrinsic error rate which can be simply modulated by adjusting the initial target DNA amount for the amplification reaction, the lower the concentration of

Table I
Estimation of Mutation Rate in Taq and Mutazyme Libraries

Library	DNTPs	$MnCl_2$ concentration [m*M*]	DNA template concentration [ng/μL]	% of mutants with less than 10% of parent enzymes activity[a]	% of inactive mutants[a]
TAQ-I	Unbalanced	0.2	1.5	4.6	2.3
TAQ-II	Unbalanced	0	0.1	14.2	8.1
TAQ-III	Balanced	0	0.1	33.9	21.8
TAQ-IV	Balanced	0.002	0.1	35.2	29.9
TAQ-V	Balanced	0.005	0.1	41.3	32.2
TAQ-VI	Balanced	0.01	0.1	47.1	34.5
TAQ-VII	Balanced	0.05	0.1	81.1	56.3
TAQ-VIII	Balanced	0.1	0.1	92.5	81.0
MUTAZYME-I	Balanced	—	13	46.2	36.8
MUTAZYME-II	Balanced	—	4	56.3	47.1
MUTAZYME-III	Balanced	—	0.004	96.9	92.4

[a]Calculations based on the profile landscapes (Fig. 1).

template the higher the mutational rate. When DNA template concentration was between 13 and 4 ng/μL, libraries landscapes were appropriate (from 36.8 to 47.1% of inactive clones).

In vivo *assembly of mutant libraries (IvAM) in* S. cerevisiae

The Transition/Transversion ratio (Ts/Tv) is commonly used to calculate the bias in the mutational spectra. Both Mutazyme and Taq DNA polymerases favor transitions (purine to purine changes and pyrimidine to pyrimidine changes) over transversions (purine to pyrimidine changes and vice versa) (Ts/Tv ranged from 2.9 to 0.8).[40–42] Apart from this similarity, both polymerases behave in different manner. First, they do not share the same AT→GC/GC→AT ratio. Taq is biased to AT→GC changes whereas Mutazyme is biased to GC→AT changes. Moreover, Mutazyme is highly likely to incorporate A→N and T→N changes whereas Taq DNA is biased to G→N and C→N mutations.

Generally, it is advisable to combine the mutation biases of these or other polymerases by alternating between them in successive rounds of evolution, or in a parallel approach just creating independent mutant libraries with different polymerases in one single generation.[18] We consider that the recombination of mutant libraries with different mutational spectra can also constitute an interesting alternative during a laboratory laccase evolution process. To this end we tackled the *in vivo* construction of an unique library in *S. cerevisiae*, from the two *in vitro* independent libraries (Taq/$MnCl_2$ and Mutazyme). Owing to the high homologous recombination frequency of *S. cerevisiae*, *in vivo* DNA-shuffling of mutant libraries takes place generating a mutational spectra which is derived from the own features of both polymerases. Libraries Taq-V and Mutazyme-I were selected to validate this procedure. Both libraries showed a suitable mutational rate (Table I) to reasonably build a generation where beneficial mutations could be identified and at the same time evaluated the *in vivo* recombination process.

Taq-V and Mutazyme-I libraries were prepared using a set of primers which allowed to amplify extrahomologous sequences at the 5′and 3′ ends (40 bp and 66 bp, respectively). These big overhangs recombined with the ends of the linearized vector forming an autonomously replicating plasmid without altering the open reading frame. Both libraries were equimolarly mixed and transformed along with the linearized vector in *S. cerevisiae* (see details in Material and Methods). To guarantee the recombination/transformation efficiency, six different linearized vector:equimolar library were investigated. Ratio 1:8 provided the best result with over 15,000 clones per transformation reaction, which is quite similar to that obtained with the circular replicating plasmid.

Screening the library constructed by IvAM in organic cosolvents

The screening was done in the presence of high concentrations of acetonitrile and ethanol to find variants with resistance towards these cosolvents of biotechnological significance and commonly employed in multiple biotransformations catalyzed by laccases.[11,29] Results were very consistent in both media showing 38.0% and 36.4% of clones with less of 10% wildtypes activity for ethanol and acetonitrile, respectively. Over 1000 clones were screened, finding several mutants with around 2-fold better rates than parent type both in acetonitrile and ethanol, Figure 2. Several variants were sequenced and in all cases a mutation frequency of 1–4 nucleotide/laccase gene was detected. All sequenced mutants displayed transitions over transversions. This result is not unexpected keeping in mind that both polymerases are biased towards these sort of changes. As can be appreciated in the landscapes, one variant, 2E9, highlighted among remaining positive mutants. 2E9 was purified and further characterized showing ~3-fold better kinetics than parent

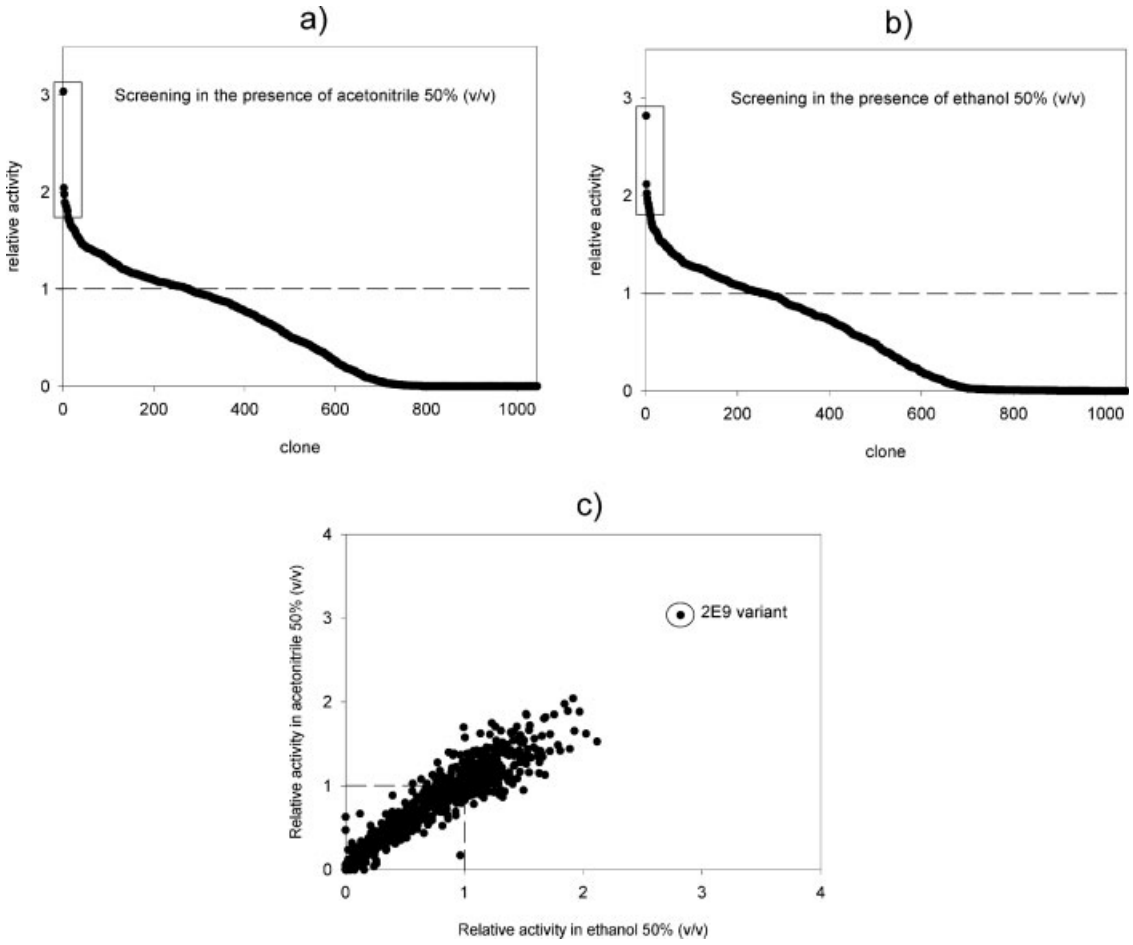

Figure 2

*Landscapes of generation created by in vivo assembly of Mutazyme/Taq libraries. Activity of clones is plotted in descending order. Dashes line indicates the activity of parent type. (**a**) Screening in the presence of 50% acetonitrile; (**b**) screening in the presence of 50% of ethanol; (**c**) activities of clones in acetonitrile and ethanol. Framed clones were submitted to rescreening procedures (see Method section).*

type along with significant changes in the redox potentials at the T1 site and in the oxygen uptake at the T2/T3 copper cluster (Table II). 2E9 variant showed a good tolerance towards organic solvents keeping 51% and 30% of its initial activity under the presence of ethanol 30% (v/v) and acetonitrile 30% (v/v), respectively (Table III, Fig. 3). DNA sequencing revealed an unexpected result. 2E9 presented two mutations in consecutives codons (G614D -GGC/GAC- and E615K -GAG/AAG-). In both cases two transitions G→A were produced. Although these kind of changes are more biased for Mutazyme than for Taq/$MnCl_2$ (43.7% vs. 13.6%) the participation of the Taq/

Table II

Kinetics Parameters, Redox Potential, and Molecular Oxygen Consumption for Parent Type and 2E9

	Catalytic constants with ABTS[a]					
Variant	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($s^{-1}M^{-1}$)	Improvement (folds)	$E^{o\prime}$ T1 site (mV, pH 7.0)[b]	K_MO_2 (μM)[c]
Parent type	68.2	55	1.24×10^6	1	680	280
2E9	85	24	3.55×10^6	2.9	630	410

[a]The activities were assayed in acetate buffer 100 mM pH 4.5 at 22°C. $\varepsilon^{*+}_{418\ nm\ for\ ABTS} = 36{,}000\ M^{-1}cm^{-1}$.
[b]Values calculated from spectroelectrochemical tritations (see further details in Material and Methods section).
[c]A reaction cell connected to a mass spectrometer was constructed to estimate the direct consumption of oxygen in the presence of the ABTS (see further details in Material and Methods section).

Table III
Activities of Parent Type and 2E9 Variant in the Presence of Organic Solvents

	Initial turnover rates in absence of organic solvent (μmol product/μmol laccase min)	Improvement	Initial turnover rates in ethanol 30% (v/v) (μmol product/μmol laccase min)	Improvement	% of relative activity in ethanol 30% (v/v)[a]	Initial turnover rates in acetonitrile 30% (v/v) (μmol product/μmol laccase min)	Improvement	% of relative activity in acetonitrile 30% (v/v) *
Parent type	4085 ± 15	1	1620 ± 34	1	39.7	800 ± 13	1	19.5
2E9 variant	5110 ± 7	1.25	2600 ± 23	1.6	50.8	1485 ± 21	1.9	29.1

Each value was obtained from three independent experiments.
[a]Percent of relative activity comes from the ratio of initial rates in the presence of organic cosolvent to that in the absence of organic cosolvent.

$MnCl_2$ in these mutations can not be ruled out either. The likelihood of introducing two positive mutations consecutively by using conventional error prone PCR is hardly expected and to our best knowledge there are no reports of this phenomena in other protein evolution investigations. Rather than serendipity, this result suggests that the approach of *in vivo* recombining mutant libraries with different bias in *S. cerevisiae* can provide other alternatives to the standard *in vitro* protocols.

Functionality of the C-terminal tail

Mutant libraries were built throughout the whole laccase gene, including several processing sites, because of their significance in the laccase total activity.[26] Thus, the sequence targeted for random mutagenesis included: signal leader (22 amino acids) + pro-leader (25 amino acids) + mature protein (559 amino acids) + C-terminal tail (14 amino acids). Signal leader, pro-leader, and C-terminal tail code for parts of the laccase that are cleaved during maturation. The signal leader is necessary for the secretion of the laccase, which is firstly glycosylated upon translocation to the endoplasmic reticulum. Afterwards, the yeast secretion machinery operates through the Golgi in processing, further glycosylating and finally secreting the enzyme. Pro-leader peptide may act as a chaperone (as happening with the α-factor mating pheromone) although its function in laccase processing is not well understood yet.[43] The function of C-terminal tail in the ascomycete laccases is also under the shadow of controversy.[26,32] Kiiskinen and Saloheimo[32] studied the expression of the *Melanocarpus albomyces* laccase, MaL, with 75% of homology to MtL in *S. cerevisiae*, suggesting that yeast apparatus was not able to process the C-terminus correctly. By contrast, we reported the correct processing of the C-terminus of MtL in *S. cerevisiae* after the introduction of a *Kex*2 protease site[26] which was further corroborated by C-terminal sequencing. *Kex*2 protease is placed at the Golgi, where it cleaves fusion proteins with Lys-Arg processing sites (as also happens with the α-factor prepro leader). At this point, it is worth noting that the two consecutives amino acid changes of 2E9 are located in the middle of the C-terminal tail (the eighth and ninth amino acid of the C-terminal tail). In the current study, parent type already possessed a *Kex*2 protease recognition site at the C-terminal processing site of the protein, adjusting therefore the protein sequence to the different protease specificities of *S. cerevisiae*.

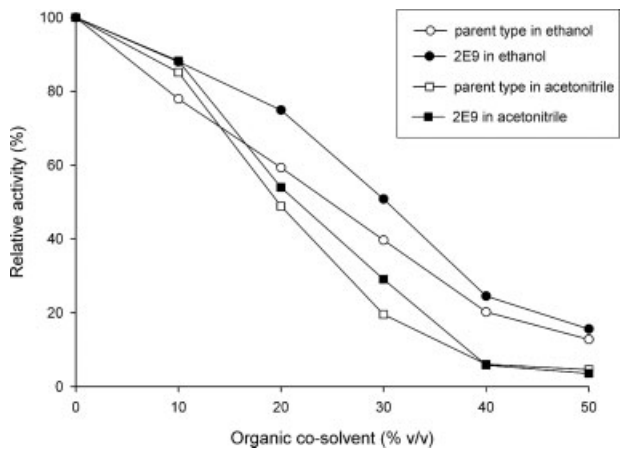

Figure 3
Relative activities of parent type and 2E9 variant at different concentrations of ethanol and acetonitrile. Each point represents the average of three independent experiments.

Since the C-terminal polypeptide is processed and therefore is not part of the mature protein, it is not straightforward to find out how those changes can modulate the function of the mature 2E9. To evaluate whether or not the mutations at the C-terminal tail might affect the geometric and electronic structure of catalytic coppers in the mature protein, spectral investigations of both parent type and 2E9 variant were performed. Comparison of the $g_{||}$ and $A_{||}$ parameters of the parallel component of the T1 copper signal [Fig. 4(a)] from EPR spectroscopy reveals that the mutations induced small but significant changes in the chemical environment of this site ($g_{||} = 2.204$ and $A_{||} = 7.9 \times 10^{-3}$ cm^{-1} for the parent laccase, and $g_{||} = 2.210$ and $A_{||}$

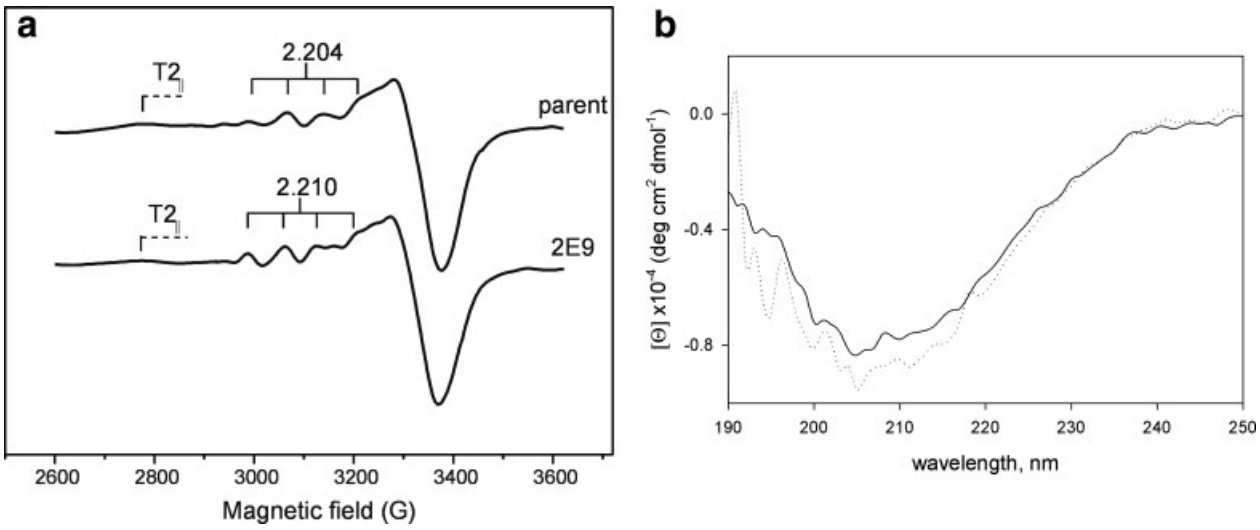

Figure 4

*Spectral investigation of parent type and 2E9 variant. (**a**) EPR spectra (**b**) CD spectra. Dotted line, parent type; solid line, 2E9 variant.*

$= 7.4 \times 10^{-3}$ cm^{-1} for 2E9). Hence, the redox potential value at the T1 site (Table I) was also perturbed upon mutations. Similar alterations have been reported when several residues in the neighborhood of the T1 site were submitted to site directed mutagenesis.[44–46] The geometry of the paramagnetic copper T1 in the overall architecture of MtL comes from a complex network of interactions[47] (van der Waals forces, electrostatic interactions, hydrogen bonds, and covalent linkages) throughout the laccase structure. This network is building during protein folding at the posttranslational processing steps. CD spectra of the parent type and 2E9 mutant in the far UV region were recorded in order to compare the secondary structure of the enzymes. As shown in Figure 4(b), spectra of both laccases were very similar and had a single broad negative band with minimum at about 205 nm. The theoretical quantitative comparison of the content of different structure motifs (see Materials and Methods section) addressed that the secondary structure of the 2E9 mutant was not significantly altered by the mutations (data not shown).

In one of our previous work, we created a C-truncated version of *Myceliophthora thermophila* laccase by introducing a stop codon at the processing site.[26] The catalytic efficiency of the resulting enzyme, which was processed without the C-terminal extension, was reduced 10-fold although the expression level was kept. Thus, it seems to be clear that somehow the C-terminal tail exerts a strong influence during processing steps which eventually is affecting in how mature enzyme behaves. Similar C-terminal processing has also been described for laccases from the ascomycetes *Neurospora crassa*,[31] *Podospora anserine*,[30] and *Melanocarpus albomyces*.[32] It has been suggested that carboxyl-terminal processing may play a role in the activation of the enzyme. This hypothesis is based on the inherent basic character of the C-terminal tail (in our parent type, 4 of 14 residues are basic ones, two Lys and two Arg conferring to the tail of an isolectric point (pI) of 8.59). This value is considerably higher than that of the mature protein that is 5.29. According to this theory,[31] the C-terminal tail forms a loop interacting with the active site to prevent binding of copper ions during processing. Interestingly, throughout the directed evolution process the pI of mature protein practically kept unalterable but not that of the C-terminal tail, Table IV. In particular, the two mutations located in 2E9 variant shifted the pI up to 1.1 units to the basic side from 8.59 to 9.70 while keeping constant the mature protein pI at 5.46, the pH activity profile of 2E9 did not significantly change (Fig. 5). We assume that these mutations might contribute to a higher grade of tightness between

Table IV

C-Terminal Tail Sequences of MtL and Mutants

Laccase variant	C-terminal tail sequence	C-terminal tail pI	Mature laccase pI
MtLwt[a]	KHRWVEEGEWLVKA	6.76	5.13
MtLL1[a]	KRRWVEEGEWLVKA	8.59	5.13
MtLT2[a]	KRRWVEEGEWLVKA	8.59	5.29
MtL3D1 (parent type)	KRRWVEEGEWLVKA	8.59	5.46
MtL2E9	KRRWVEEDKWLVKA	9.70	5.46

In grey the aminoacidic changes through the lab evolution process. Underlined the *Kex2* processing site. pI was calculated using the Expasy Proteomics Server (www.expasy.org), Primary Structure analysis, with the tools for theoretical calculations of pI and MW.

[a]Reference 26.

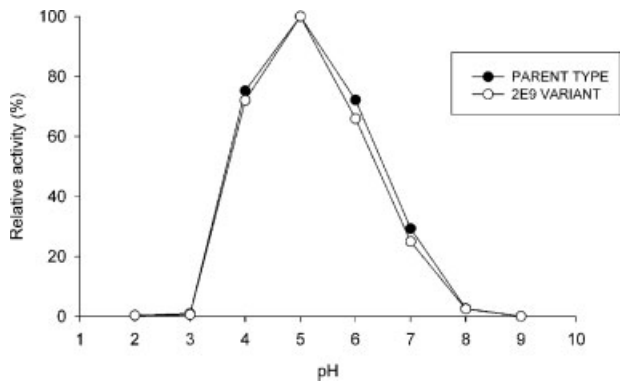

Figure 5

pH activities profiles of parent type laccase and 2E9 variant. Activities were measured in B&R buffer at different pHs with ABTS as substrate. Laccase activity is normalized to the optimum activity value. Each point represents the average of three independent experiments.

the C-terminal extension and the main enzymatic core, which would affect to the protein folding and therefore the final mature enzyme. Hence, the differences in the EPR T1 signals and in the estimated redox potentials [Fig. 4(a), Table II], whereas similarity of secondary structures was kept [Fig. 4(b)]. Our theory is further supported by the fact that also the K_MO_2 was altered in 2E9 -oxygen uptake was reduced by ~1.46-fold (Table II). Taking into account that the oxygen molecules access to the trinuclear copper cluster through a broad channel,[48] any subtle modification during the protein folding might have affected the entrance and exit of O_2 with the concomitant increase in the K_MO_2.

Although to validate these hypothesis only crystallographic comparative analysis of parent type and 2E9 could give us new insights (currently under investigation), in view of the reported data it seems highly likely that the broad differences in pI between mature protein and C-terminal tail might help to establish stronger electrostatic interactions between both polypeptides during protein processing altering the eventual laccase functionality.

CONCLUSIONS

In this work we have taken advantage of the cellular mechanism of *S. cerevisiae*, which unlike *Escherichia coli*, displays a high homologous recombination frequency.[23,49] This virtue, along with its ability for glycosylation/secretion make *S. cerevisiae* a suitable host for laboratory evolution of eukaryotic proteins. *S. cerevisiae* possesses a high transformation efficiency, and using an appropriate episomal vector does not integrate the foreigner plasmid into the chromosome (as *Pichia pastoris* does), enabling its further manipulation. All these features have been extensively exploited to evolve enzymes by *in vivo* shuffling without introducing additional mutations (*in vivo* shuffling is a nonmutagenic method, unlike most of *in vitro* DNA recombination protocols generally employed in bacteria).[18,50] Here, we have used these qualities to assemble mutant libraries with different bias using a protocol that we have named IvAM. With this approach, the laccase functionality in cosolvents was explored, pointing out the significance of C-terminal extension in the function of ascomycete laccases. IvAM constitutes a robust methodology that can be straightforwardly used to simplify the process of enzyme engineering by laboratory evolution. The future use of *S. cerevisiae* machinery in the laboratory evolution picture can be useful to further push laccases and other oxidoreductases towards technological applications (bioremediation schemes, design of biofuel cells).[51]

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