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In Vitro Evolution of a Fungal Laccase in High Concentrations of Organic Cosolvents

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SUMMARY

Fungal laccases are remarkable green catalysts that have a broad substrate specificity and many potential applications in bioremediation, lignocellulose processing, organic synthesis, and more. However, most of these transformations must be carried out at high concentrations of organic cosolvents in which laccases undergo unfolding, thereby losing their activity. We have tailored a thermostable laccase that tolerates high concentrations of cosolvents, the genetic product of five rounds of directed evolution expressed in *Saccharomyces cerevisiae*. This evolved laccase—R2 variant—was capable of resisting a wide array of cosolvents at concentrations as high as 50% (v/v). Intrinsic laccase features such as the redox potential and the geometry of catalytic coppers varied slightly during the course of the molecular evolution. Some mutations at the protein surface stabilized the laccase by allowing additional electrostatic and hydrogen bonding to occur.

INTRODUCTION

Enzymatic transformation in organic cosolvents allows us to tackle many cumbersome processes, from remediation of hazardous xenobiotics to organic synthesis of pharmaceuticals, chiral intermediates, biopolymers, and many other complex molecules [1–3]. During the course of life on earth, enzymes have gradually evolved to regulate metabolic pathways in cellular environments, where water plays a pivotal role. Apart from a few cases [4], when enzymes are removed from their natural environments and introduced to organic solvents, biocatalysts are denatured or inactivated as they are not designed to tolerate these artificial media. Indeed, the equilibrium of the noncovalent interactions that determine the stability of an enzyme in aqueous media is shifted in the presence of organic solvents, causing structural perturbations and unfolding [5].

The blue multicopper oxidase laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is one of the oldest reported and most studied enzymatic systems. Its refined and well-adapted molecular architecture, with one paramagnetic blue copper at the T1 site and three coppers clustered at the T2/T3 sites, along with the particular feature of oxidizing chemical and natural redox mediators, makes it an excellent biocatalyst for dozens of biotechnological applications [6, 7]. Thus, laccases and/or the laccase mediator system (LMS) have been studied in the remediation of recalcitrant contaminants (polycyclic aromatic hydrocarbons [PAHs], chlorophenols, polychlorinated biphenyls, organophosphorus compounds, etc.) in pulp-kraft bleaching, the textile industries, and more [8–10]. Furthermore, laccases can be used in organic synthesis, as they can perform exquisite transformations ranging from the oxidation of steroid hormones to the enzymatic polymerization required for the synthesis of phenolic-based resins such as poly-α-naphthol, polypyrogallol, and polycatechol [9, 11], as well as conductive water-soluble polymers [12]. However, the flexibility among laccase substrates [13] is offset by the fact that many nonnatural substrates that are amenable to oxidation by laccases and/or LMS are barely soluble in water. This shortcoming is overcome by using high concentrations of organic cosolvents, as the K_m values for these reducing substrates (in the mM range) are greater than their solubility in aqueous solution. As expected, high concentrations of methanol, ethanol, acetonitrile, dimethylsulfoxide, and others promote laccase unfolding (above 10%–15%; v/v), limiting their enormous potential [14–16].

Here we describe the directed evolution of a fungal laccase in high concentrations of cosolvents using the MtLT2 mutant of the thermostable laccase from *Myceliophthora thermophila* as the parental enzyme [17]. The expression of this enzyme in *Saccharomyces cerevisiae* allowed us to use this organism as a DNA-recombination toolbox to generate diversity. Improved mutants were detected using a strategy that combined the effect of two different cosolvents in one screening assay. With this approach, we have engineered a highly active and

stable tolerant laccase. Additionally, we have shown that by applying laboratory evolution methods, highly conserved electrochemical characteristics can be altered without perturbing laccase function.

RESULTS AND DISCUSSION

Laboratory Evolution Approach

A laccase that is tolerant of organic cosolvents must be active and at the same time stable in the presence of high concentrations of organic media, and it should also retain its characteristics regardless of the cosolvent. These features would confer added value to these biocatalysts as, in a given transformation where solvents are necessary, thousands of turnovers could be accomplished. Thus, a laboratory evolution strategy was carefully planned based on the following premises: (1) the screening assay was carried out in the presence of two cosolvents of different chemical nature (acetonitrile and ethanol) to identify mutants active in both cosolvents and that eventually might display promiscuity in other water-solvent mixtures; (2) to improve activity and stability in organic cosolvents, only those mutants with augmented activity and that retained their stability were considered candidates for further rounds of evolution; (3) the harsh selective pressure, gradually increasing the concentrations of organic solvents from 20% to 60% (v/v) generation after generation, may further push the evolution process, as long as the parental type used in each cycle tolerates the screening conditions; and (4) spectroelectrochemistry of variants was studied to assess the influence of the evolution process on the transit of electrons through the laccase structure.

The thermostable laccase MtLT2 mutant from *M. thermophila* was considered a suitable platform for the molecular evolution protocol for several reasons. MtLT2 is highly expressed in *S. cerevisiae* [17], an indispensable requisite for directed evolution. Furthermore, it has already shown good stability in organic media and MtL is known to be an excellent scaffold to carry out evolution. Indeed, this thermostable enzyme was previously submitted to ten rounds of in vitro evolution, culminating in an MtLT2 variant harboring 12 beneficial mutations in the mature protein with a concomitant 170-fold increase in total activity above that of the wild-type. This success is clearly related to the original theory [18] that supports the direct relationship between thermostability, mutational robustness, and evolutionary capacity. Thus, in principle, thermostable enzymes should be more susceptible to evolution because they are better able to tolerate functionally beneficial but destabilizing mutations.

In the first place, the expression system in *S. cerevisiae* was tuned so that laccase production and copper uptake was not toxic to the eukaryotic host, which would limit the evolution process [14]. Sequence diversity was generated, taking advantage of the high level of homologous recombination in *S. cerevisiae* [19, 20]. Throughout the evolution protocol, the cloning and the different in vivo DNA-recombination procedures were optimized for high transformation efficiencies using homologous sequences with overlapping 30–50 bp domains that did not affect the open reading frame (the laboratory evolution scheme is summarized in Figure 1). Combinatorial saturation mutagenesis by in vivo overlap extension (IVOE) [21], in vivo DNA shuffling combined with random mutagenesis, and in vivo assembly mutagenesis (IVAM) were employed here as the DNA-recombination methods developed for this project. Both IVAM and IVOE are methods that take advantage of the eukaryotic apparatus of *S. cerevisiae*, and here they have been used in the framework of laboratory evolution experiments. In addition, conventional error-prone PCR was employed using polymerases with different biases in sequential generation or independently generated libraries (e.g., second generation). The mutational rates were adjusted to an average of one to three amino acid changes per protein.

Selected Mutants in Cosolvents

The libraries were screened for organic cosolvent tolerance and isolated variants were purified and characterized further. To characterize the clones adequately, the organic cosolvent tolerance was defined as the ratio of the activity displayed by the laccase in the presence of organic cosolvents compared to that in their absence. In this way, the resistant enzymes selected retained a larger proportion of their activity in the presence of the cosolvents (Table 1). Indeed, not only the activity but also the stability in organic cosolvents of all variants was taken into account as a discriminatory factor, selecting only those mutants with more than 80% residual activity after incubation at different concentrations of organic cosolvents. The ultimate mutant of the evolutionary experiment, called R2, displayed a remarkable tolerance toward cosolvents, retaining nearly 20% of its activity in 50% (v/v) ethanol (with 19.2-fold overall improvement; Table 1; Figures 2A and 2B). Moreover, R2 displayed practically the same activity in 40% (v/v) ethanol or in 30% (v/v) acetonitrile (1737 and 1876 μmol product μmol laccase^{-1} min^{-1}, respectively) as the parental MtLT2 does in aqueous media (1703 μmol product μmol laccase^{-1} min^{-1}). In contrast, MtLT2 retained less than 10% of its activity under the same conditions (109 and 149 μmol product μmol laccase^{-1} min^{-1}).

The screening assay used here was based on the oxidation of the model compound ABTS in organic solvents [14] (for details, see Supplemental Experimental Procedures in Supplemental Data available with this article online). To verify that the selected variants were active in cosolvents regardless of the substrate, several genuine laccase substrates were also assessed in organic media (syringaldazine, guaiacol, and 2,6-dimethoxyphenol; Table 2). The tolerance against cosolvents was very similar for all the phenolic compounds tested, and it was even higher than with the nonphenolic compound used in the screening (e.g., 33% and 19% of retained activity in 50% ethanol [v/v] for syringaldazine and ABTS, respectively). R2 was further evaluated during the decolorization of an array of dyes of different chemical nature, displaying a tolerance

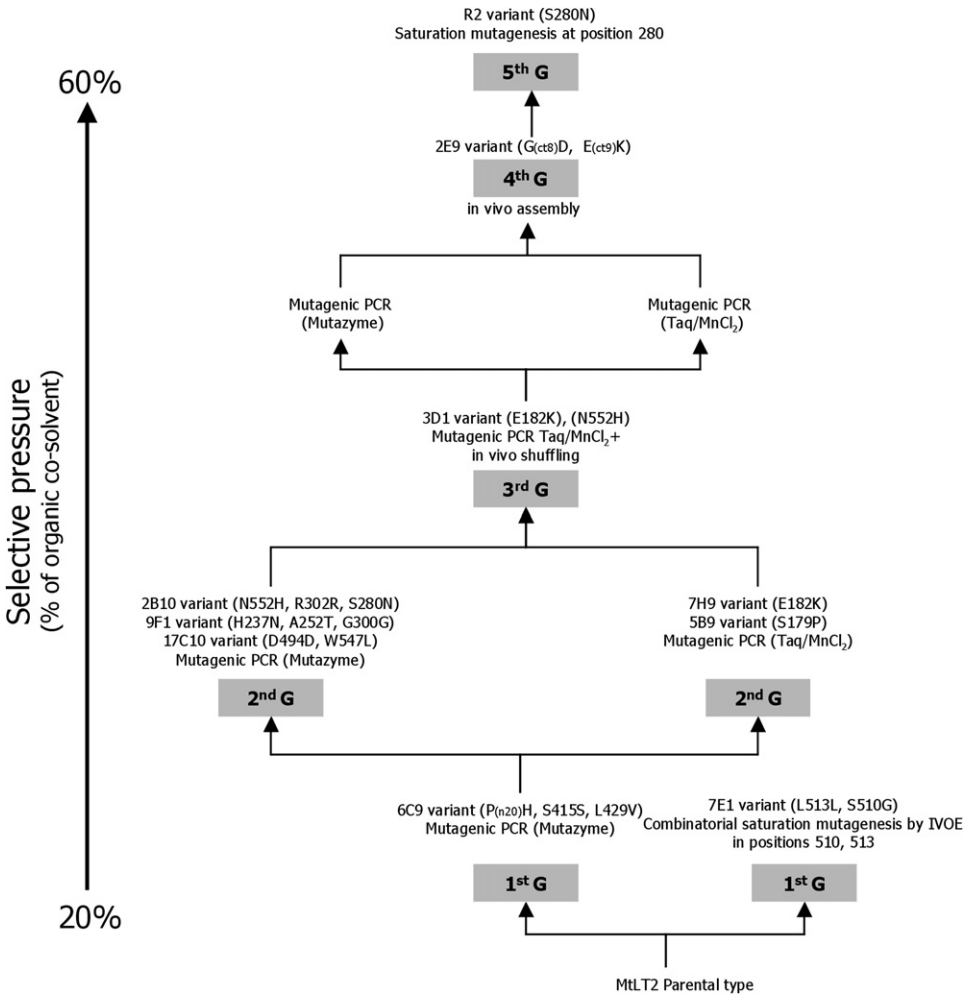


Figure 1. In Vitro Evolution Pathway: Several Methods for In Vivo DNA Recombination and Error-Prone PCR Were Used throughout the Evolution Process

Variant 7E1 from the first round was discarded as a parent for subsequent generations because of its low stability in organic cosolvents. In the third round, variants 2B10, 9F1, 17C10, 7H9, and 5B9 were submitted to amplification by Taq/$MnCl_2$ and in vivo shuffled. The fourth round was prepared by in vivo assembly of mutant libraries created by Mutazyme and Taq/$MnCl_2$, with different mutational spectra. In the last generation, position 280 was modified by site-directed mutagenesis and further explored by saturation mutagenesis.

between 15% and 23% in 50% ethanol (v/v) (Table S1). Thus, it appeared that the overall improvement detected in organic cosolvents was not substrate dependent. Additionally, R2 was tested for the oxidation of the PAH anthracene in the presence of acetonitrile (see Supplemental Experimental Procedures). The mutant completed the conversion of anthracene into anthraquinone within the first 17 hr, and it was recovered and reused with similar efficiency in the same oxidation process (data not shown), demonstrating its stability under these reaction conditions.

In order to investigate whether the evolved tolerance of the R2 variant in the presence of ethanol and acetonitrile also led to resistance to other organic solvent-water mixtures not analyzed in the screening, the mutant was evaluated with an array of biotechnologically relevant miscible cosolvents of different polarities (logP ranging from −0.23 to −1.3; Figure 2C). Promisingly, R2 behaved promiscuously and was also highly stable at elevated concentrations of dimethylformamide, dimethylsulfoxide, dimethylacetamide, acetone, and methanol. In particular, R2 retained ~85%–100% of its activity after 24 hr at solvent concentrations as high as 50% (v/v). This elevated resistance to different cosolvents can be attributed to the selection pressure established during the evolution protocol, combining and increasing the concentrations of two cosolvents generation after generation.

Finally, R2 was compared with a collection of purified fungal laccases commonly used in the presence of organic cosolvents and with redox potentials ranging from +475 mV to +790 mV (Figures 2D–2G). Our tailor-made laccase demonstrated by far the best performance of any of its counterparts, and R2 retained more than 60% of its activity in 30% ethanol (v/v) versus ~10% for all the other laccases studied.

Table 1. Activities and Stabilities of Purified Parental and Mutant Enzymes in the Presence of Organic Cosolvents

Cycle of Evolution	Variant	Activity[a] in Aqueous Media	Improvement[b] in Aqueous Media	Activity[a] in 50% (v/v) Ethanol	Improvement[b] in 50% (v/v) Ethanol	Tolerance[c] in 50% (v/v) Ethanol	Stability[d] in 50% (v/v) Ethanol	Activity[a] in 30% (v/v) Acetonitrile	Improvement[b] in 30% (v/v) Acetonitrile	Tolerance[c] in 30% (v/v) Acetonitrile	Stability[d] in 30% (v/v) Acetonitrile
Parent	MtLT2	1703 ± 11	1.0	58 ± 1	1.0	3.4	80	149 ± 3	1.0	8.8	86
1	6C9	3579 ± 14	2.1	198 ± 5	3.4	5.5	84	449 ± 11	3.0	12.6	86
2	5B9	2048 ± 5	1.2	93 ± 1	1.6	4.5	125	314 ± 9	2.1	15.3	155
2	7H9	4767 ± 32	2.8	222 ± 6	3.8	4.7	110	974 ± 13	6.5	20.4	138
2	2B10	5841 ± 9	3.4	435 ± 8	7.5	7.4	93	600 ± 12	4.0	10.3	96
3	3D1	4085 ± 15	2.4	524 ± 3	9.1	12.8	95	780 ± 23	5.4	19.6	92
4	2E9	5111 ± 7	3.0	798 ± 30	13.8	15.6	97	1486 ± 11	10.0	29.1	98
5	R2	5619 ± 18	3.3	1106 ± 5	19.2	19.7	99	1876 ± 6	12.6	33.4	100

[a] Activities are given in μmol product/μmol laccase min. Each value, including the standard deviation, comes from three independent experiments.
[b] The improvement is defined as the ratio of the activity of the corresponding mutant under the conditions specified to that of the parental MtLT2 under the same conditions.
[c] Tolerance in organic cosolvents (i.e., retained activity in cosolvents) is defined as the ratio of the activity in the presence of organic cosolvents to that in the absence of organic cosolvents, given as a percentage.
[d] Percentage of residual activity after a 24 hr incubation in organic cosolvent.

Biochemical Characterizations

The molecular mass of the mutants expressed in *S. cerevisiae* was ~130 kDa (Figure S1A), although after deglycosylation the relative molecular mass fell to ~65 kDa (Figure S1B). The doubling of the relative molecular mass by hyperglycosylation in the eukaryotic host, involving N-linked glycans, may contribute to the protection of laccase from proteolytic degradation. However, the introduction and removal of asparagine residues during evolution (S280N in the second and fifth generations and N552H in the second and third generations) did not alter protein size, indicating that those sites were not involved in the addition of carbohydrate moieties. Indeed, the sequences around these asparagines did not correspond to the recognition sequences predicted for N-terminal glycosylation.

Unlike thermostability, which was not significantly altered after evolution, the pH profile for ABTS oxidation was affected by the artificial evolution (Figures S1C and S1D). Thus, the optimal pH 4.0 for the parental MtLT2 was shifted to pH 5.0 from the first generation onward. Interestingly, a slight increase in activity at neutral pH was detected (the last variants in the evolution process retained nearly ~70% and ~30% of the residual activity at pH 6 and 7, respectively). The optimal pH of fungal laccases is typically between 3 and 5 [22, 23] and, hence, this unexpected upward shift could be of interest in engineering an alkalophilic laccase by in vitro evolution. Indeed, several applications demand fungal laccases that are active at neutral and alkaline pH (e.g., lignocellulose treatment, construction of biosensors and biofuel cells, dyestuffs processing, etc.). This assumption was corroborated by the construction of a mutant library of ~1600 clones that was explored for alkalophilic mutants (Figure S2). Consequently, several clones with improved activity were found during the screening, using the ratio of activity at pH 8/activity at pH 5 as a discriminatory factor.

Spectroelectrochemical Studies

To assess whether the mutations exerted any influence on the catalytic coppers, laboratory evolution variants were characterized spectroelectrochemically (Table 3; Figures S3A–S3E). The midpoint redox was measured and the potential ($E^{\circ\prime}$) values were obtained for the T1 site (where the reducing substrate is oxidized) and the T2/T3 cluster (where bound oxygen receives the electrons transferred from the T1 copper approximately 12 Å away along the strongly conserved His-Cys-His pathway). The $E^{\circ\prime}$ of both sites were slightly altered by in vitro evolution but, eventually, the ultimate mutant R2 displayed similar $E^{\circ\prime}$ values to the parental MtLT2 (i.e., approximately +700 mV and +400 mV for the T1 site and the T2/T3 cluster, respectively). These results are similar to previously published electrochemical studies of different laccase mutants, where direct changes of the ligands of the T1 copper or the amino acids that form the T1 substrate-binding pocket did not significantly alter the redox potential of the enzymes [24–26].

As can be seen by cyclic voltammetry (CV; Figure S3A), the electrocatalytic current at the electrodes modified by the laccases starts at a potential of about +700 mV, very close to the redox potential of the T1 site, confirming the recently proposed mechanism of laccase function

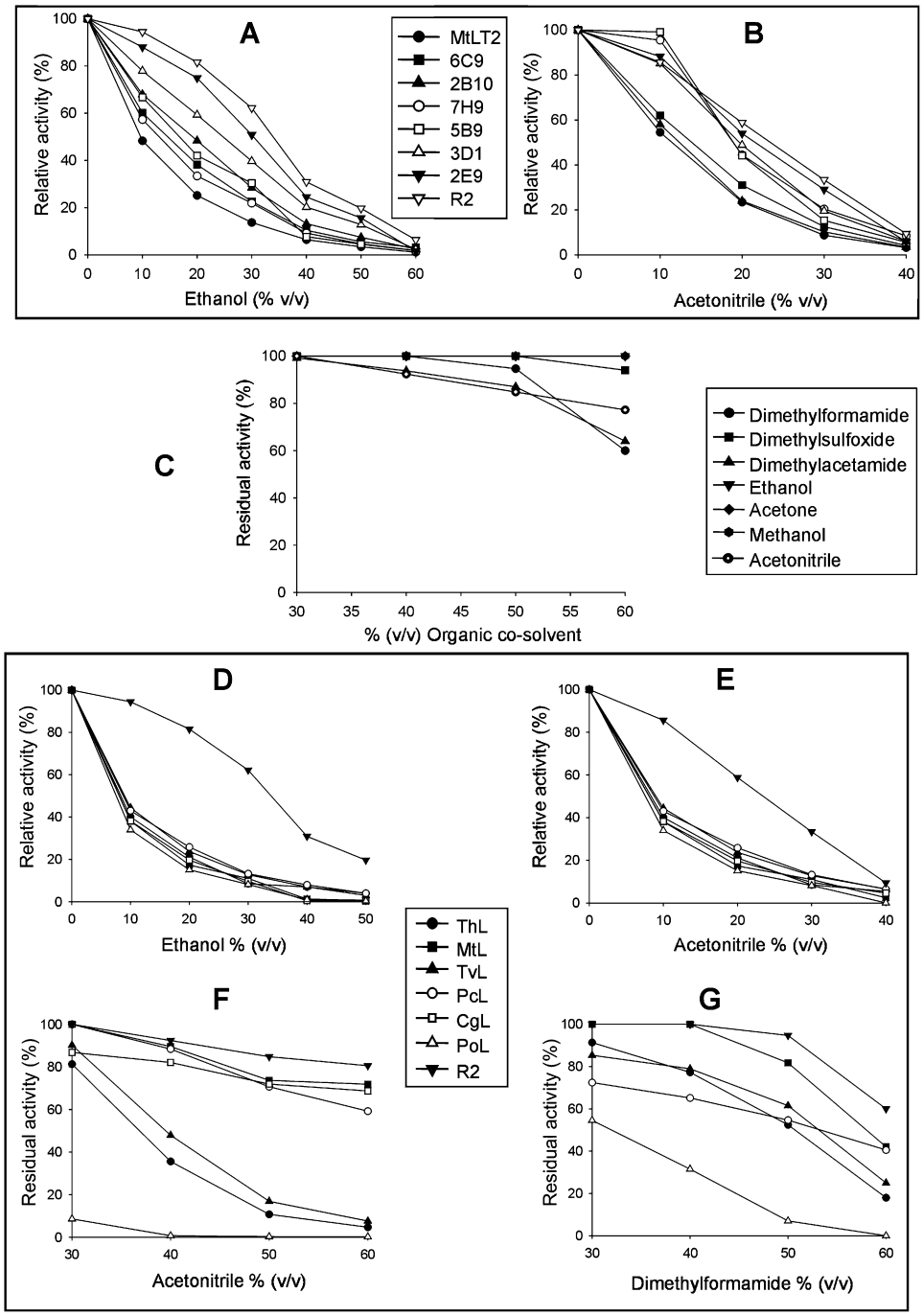


Figure 2. Organic Cosolvent Tolerance

(A and B) Relative activity profiles of parental lacasse and evolved mutants at different concentrations of ethanol and acetonitrile. Activities were assessed with 3 mM ABTS in 100 mM sodium acetate buffer (pH 4.5) containing the corresponding concentration of cosolvent. Organic cosolvent tolerance profiles of crude extracts and purified variants were in good agreement, ruling out the selection of expression mutants as parent types.

(C) Stability of variant R2 in the presence of increasing concentrations (v/v) of several organic cosolvents. Experiments were performed in screw-cap vials containing the R2 variant in cosolvent/100 mM phosphate buffer (pH 6.0) mixtures. After 24 hr at room temperature, aliquots were removed and submitted to an activity assay with 3 mM ABTS in 20 mM sodium acetate buffer (pH 4.5). Residual activity was expressed as the percentage of the original activity at the corresponding concentration of organic cosolvent.

(D and E) The initial activity of the R2 variant and several fungal laccases in the presence of increasing concentrations of ethanol and acetonitrile (v/v) is shown. ThL, *Trametes hirsuta* laccase; MtL, *Myceliophthora thermophila* laccase; TvL, *Trametes versicolor* laccase; PcL, *Pycnoporus cinnabarinus* laccase; CgL, *Coriolopsis gallica* laccase; PoL, *Pleurotus ostreatus* laccase.

(F and G) Stability after incubation of the R2 variant and several fungal laccases for 24 hr in the presence of increasing concentrations of acetonitrile and dimethylformamide (v/v). Each point represents the average of three independent measurements.

Table 2. Activities of Purified Parental and Mutant Enzymes with Typical Laccase Substrates in the Presence of Organic Cosolvents

Cycle of Evolution	Variant	Activity[a] with Syringaldazine in Aqueous Media	Tolerance[b] in 50% (v/v) Ethanol	Activity[a] with Guaiacol in Aqueous Media	Tolerance[b] in 50% (v/v) Ethanol	Activity[a] with 2,6-Dimethoxyphenol in Aqueous Media	Tolerance[b] in 50% (v/v) Ethanol
Parent	MtLT2	391 ± 4	5.5	85 ± 7	3.9	898 ± 17	5.5
1	6C9	1172 ± 12	7.3	343 ± 21	6.5	1823 ± 12	7.1
2	7H9	1315 ± 17	10.1	435 ± 15	10.1	2162 ± 25	6.9
2	2B10	1297 ± 15	11.3	750 ± 8	10.2	3149 ± 46	10.1
3	3D1	1389 ± 23	15.7	987 ± 23	15.4	2897 ± 56	14.7
4	2E9	1418 ± 8	24.3	1055 ± 9	17.1	2714 ± 13	17.5
5	R2	1843 ± 23	33.6	1180 ± 13	24.7	2755 ± 14	25.3

[a] Activities are given in μmol product/μmol laccase min. Each value, including the standard deviation, comes from three independent experiments. Syringaldazine, ε_{525} = 65,000 M^{-1} cm^{-1}; guaiacol, ε_{465} = 12,100 M^{-1} cm^{-1}; 2,6-dimethoxyphenol, ε_{469} = 27,500 M^{-1} cm^{-1}.
[b] Tolerance to organic cosolvents (i.e., retained activity in cosolvents) is defined as the ratio of the activity in the presence of organic cosolvents to that in the absence of organic cosolvents, given as a percentage.

adsorbed on a carbon electrode [27, 28]. The pronounced low-potential Faradaic processes on CVs could be attributed to the redox transformation of the laccase in contact with the electrode surface via its T2/T3 copper cluster [29–31]. In order to evaluate the effect of mutations on the geometric and electronic structure of the copper sites, spectral characterization of the purified variants was carried out (Figures S3D and S3E). Comparison of the $g_{||}$ and $A_{||}$ parameters of the parallel component of the electron paramagnetic resonance (EPR) signal from the T1 copper reveals that mutations introduced in generations 2, 4, and 5 produced subtle changes in the chemical environment of this site (Table 3). Unfortunately, only the outermost parallel hyperfine component of the T2 copper could be resolved in the spectra, which prevents extracting definitive conclusions regarding the modifications produced by the mutations at this site. Absorbance spectra recorded with the parental MtLT2 and R2 mutant are typical of "blue" laccases and contain signals that are correlated with the T1 and T3 copper centers (about 600 and 330 nm, respectively). However, differences in the shape of the spectra and the absorbance intensity in the region of 300–500 nm were observed, indicative of possible changes in the structure of both the T1 site and the T2/T3 cluster of the mutants studied. The A_{330}/A_{610} ratio for the parental MtLT2 laccase is much lower than for the R2 mutant (1.9 and 3.2, respectively), with a similar intensity in the blue absorbance band.

Taken together, the results obtained suggest that some of the mutations which confer better tolerance against cosolvents produce slight variations in intrinsic laccase features. However, the subtle differences detected in the

Table 3. Spectroelectrochemical Characterization of the Parental and Mutant Laccases

Mutant	Mutant Description	$E^{o\prime}$ T2/T3 Cluster (mV, pH 7.0); $E^{o\prime}$, CV, mV/pH	$E^{o\prime}$ T1 Site (mV, pH 7.0)	EPR at the T1 Site	
				$g_{||}$	$A_{||}$ (cm^{-1})
Parent type MtLT2	Variant with 12 mutations accumulated from a former lab evolution experiment[a]	400; 50	690	2.212	8.0×10^{-3}
2B10	Second generation (N552H, R302R, S280N)	n.d.	700	n.d.	n.d.
7H9	Second generation (E182K)	390; 57	700	2.204	7.9×10^{-3}
3D1	Third generation (E182K) (N552H)	n.d.	680	2.204	7.9×10^{-3}
2E9	Fourth generation (Gct8D, Ect9K)	380; 60	630	2.210	7.4×10^{-3}
R2	Fifth generation (S280N)	390; 57	700	2.205	8.5×10^{-3}

n.d., not determined. The standard deviations have been calculated from five independent experiments and did not exceed 10%. All potentials are given versus NHE.
[a] See [17].

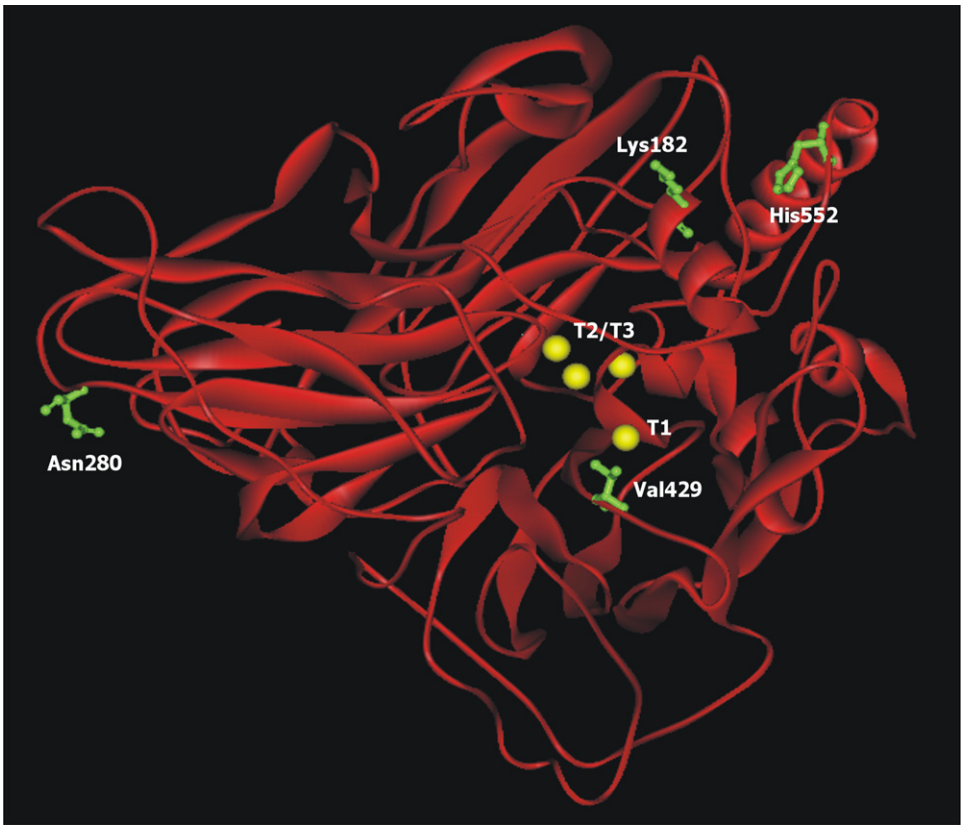


Figure 3. Amino Acid Substitutions Accumulated in the R2 Evolved Laccase
Yellow spheres represent copper atoms. Amino acid substitutions found in mature R2 are highlighted with stick-and-ball structures.

redox potentials or in the copper geometries did not negatively affect enzyme function.

Analysis of Accumulated Mutations in the Evolved Laccase

In the process of sculpting the laccase structure to favor cosolvent tolerance, R2 variants accumulated four beneficial mutations in the mature protein and two consecutive mutations in the C-terminal tail (a short polypeptide of 14 residues located at the C-terminal end that is cleaved during maturation) [17, 32]. The close resemblance between our laccase and the laccase from *Melanocarpus albomyces* (MaL; 75% identity), whose crystallographic structure has been resolved [33], allowed us to obtain a reliable model to map the locations of the mutations and to interpret their effect in the context of this structural model (Figure 3).

Mutations at the Laccase Surface

Most of the accumulated mutations in the mature R2 were situated far from the catalytic coppers (Figure 3; Table 4), at the surface of the protein, where they established new interactions and/or modified specific regions. Mutation E182K was introduced in the second generation, in the 7H9 variant, and in vivo shuffled with other beneficial mutations in the subsequent round of evolution. After changing position 182, better stability in organic media was detected (see Table 1) along with a smooth change in the EPR signal at the T1 site (Figure S3D). Residue 182 is situated in a helix at the surface of the protein, in a region far from the catalytic coppers, which seems to be one of the key-denaturing regions in the overall laccase structure (Figure 3; Table 4). The replacement of Glu182 by Lys allows the formation of a salt bridge between Lys182 and Glu55 (Figures 4A and 4B). The relevance of this bridge may reside in the fact that superficial ion pairs increase the resistance of proteins to heat and other denaturing agents, including organic solvents [5, 34].

The mutation S280N was lost during the in vivo shuffling of the third generation and was recovered by site-directed mutagenesis in the last round of evolution, giving rise to the R2 variant (Figure 1). The improved performance of this variant in cosolvents was such (Table 1) that we considered it interesting to further investigate this position in the R2 mutant by saturation mutagenesis to examine all the possible amino acid alternatives. Surprisingly, the level of fitness obtained in the second round at position 280 (in variant 2B10) was already optimal, as a similar result was obtained in the saturation experiment for R2. Although position 280 is located at the surface of the protein, far away from the catalytic coppers (Figure 3; Table 4), its introduction in the last generation increased the redox potential and gave rise to different spectral parameters

Table 4. Amino Acid Substitutions Accumulated in the R2 Evolved Laccase

Amino Acid Substitution	Nucleotide Change	Laccase Variant[a]	Location in Mature Protein	Distance to the T1 Site (Å)	Distance to the T2/T3 Cluster (Å)	Secondary Structure Motif
E182K	G685A (GAG/AAG)	7H9 (second generation)	Surface	18	20	Helix
S280N	G980A (AGC/AAC)	2B10 (second generation)	Surface	40	35	Loop
L429V	C1426G (CTA/GTA)	6C9 (first generation)	Buried	7	19	Loop
N552H	A1795C(AAC/CAC)	2B10 (second generation)	Surface	25	24	Loop

[a] Mutant in which the specified mutation was first introduced.

(Table 3; Figures S3D and S3E). Inspection of the protein model suggests that the S280N mutation allows three additional hydrogen bonds to form that stabilize the protein structure in this region (Figures 4C and 4D). The free energy from these three hydrogen bonds is equivalent to that of a salt bridge. The structural reinforcement upon mutation in this area helps resist against denaturation under the pressure of external factors (organic cosolvents, temperature, etc.).

The N552H mutation from the second-generation 2B10 mutant was shuffled in vivo with the variant 7H9 during the third round of evolution. This mutation is close to a C-terminal plug [33] of four residues that closes a channel leading to the trinuclear copper center T2/T3 (Figures 4E and 4F). Our molecular model indicates that the replacement of Asn by His modifies the environment and geometry of this channel, which might affect the transit of O_2 to the active pocket. To estimate the direct consumption of oxygen by the laccase in the presence of the reducing substrate, a reaction cell connected to a mass spectrometer was set up (see Supplemental Experimental Procedures for details). Interestingly, the K_MO_2 was altered after mutation, and oxygen uptake was reduced by ~1.75-fold (from 160 μM to 280 μM). As a laccase substrate, oxygen is much more soluble in organic solvents than in water. Our results suggest that the subtle modification in oxygen affinity of the laccase after mutation may favor catalysis in cosolvents.

Mutation in the Reducing Binding Pocket

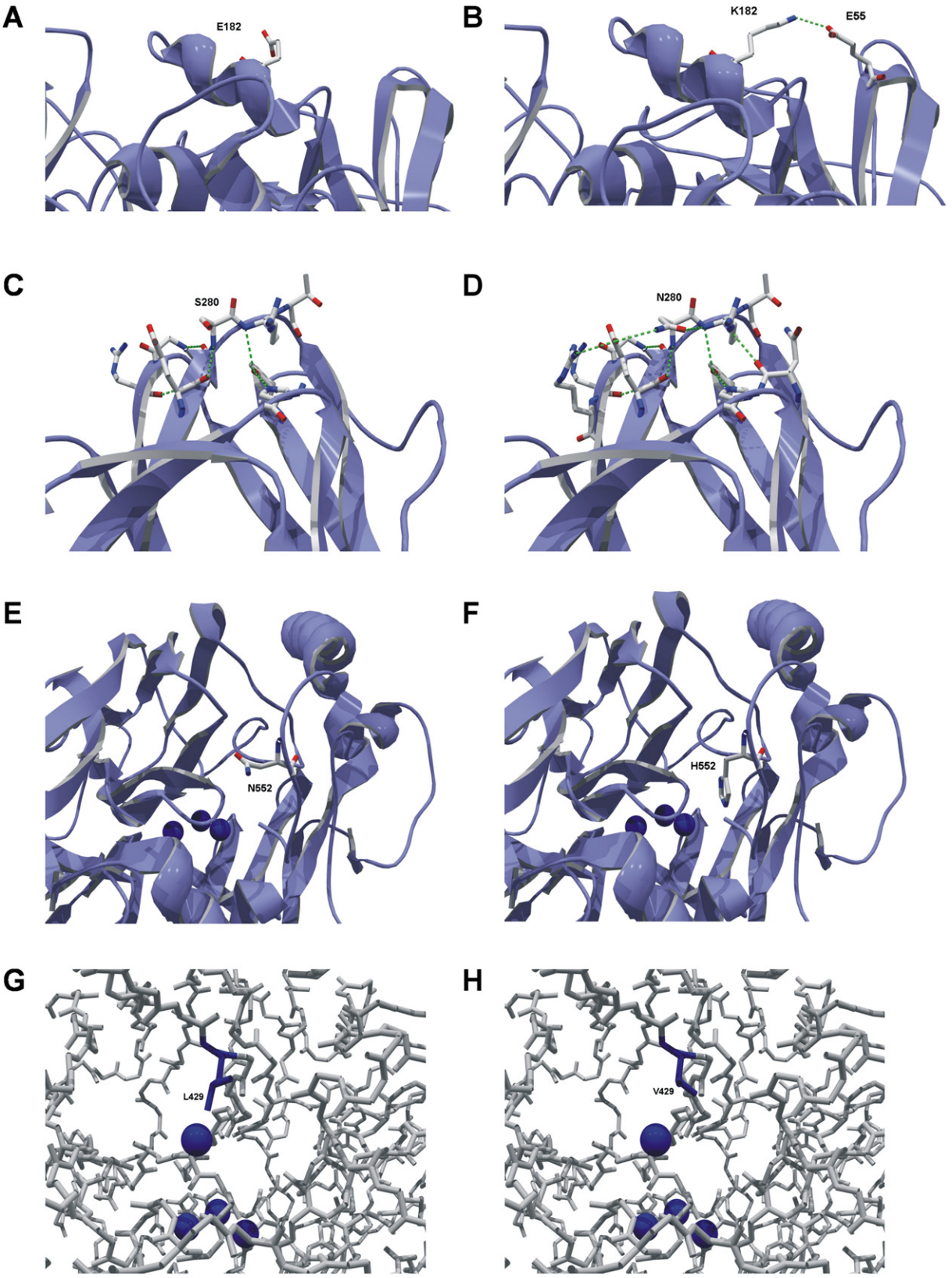
The mutation L429V was introduced into 6C9 in the first generation by random mutagenesis and was located in the vicinity of the T1 copper (Figures 3, 4G, and 4H; Table 4). Interestingly, L429 is involved in the reducing substrate binding of the homologous MaL, which characteristically has a substrate-binding pocket narrower than other laccases [33]. The change from Leu to Val at this position represents the substitution of a hydrophobic residue by another somewhat smaller hydrophobic residue. Taking into account that the activity of the 6C9 mutant against phenolic and nonphenolic substrates in aqueous solution was substantially greater (2- to 4-fold; Tables 1 and 2), it is highly likely that the mutation L429V favors substrate binding as well as tolerance to cosolvents.

Mutations in the C-Terminal Tail

The best fourth-generation mutant, 2E9, contained two beneficial mutations in consecutive codons (Gct8D and Ect9K) after the in vivo shuffling of two independent mutant libraries with different mutational spectra (for details, see Experimental Procedures). These mutations are located in the C-terminal tail that is processed by the *Kex*2 and *Kex*1 proteases in the Golgi apparatus and they do not therefore participate in the mature protein, as corroborated by C-terminal sequencing [35]. As mentioned, the EPR analysis of 2E9 and the corresponding parental type displayed some differences in the chemical environment of the T1 site (Figure S3E and Table 3). Hence, the C-terminal processing probably affects the folding of the mature protein. It is likely that the network of interactions that determine the geometry of the paramagnetic T1 copper has been affected by changes in protein folding provoked during posttranslational processing. Indeed, this could be related to the decrease in the $E^{\circ\prime}$ of the T1 site (from +680 mV to +630 mV; Table 3). It has been reported that the extremely basic character of the C-terminal tail may play an important role in protein maturation [36]. Theoretical calculations determined that the pI of the C-terminal extension shifted from 8.59 to 9.70 after the introduction of the two consecutive mutations (Table S2). We assume that these mutations contribute to the tighter binding between the C-terminal extension and the main enzymatic core (pI 5.46), establishing stronger electrostatic interactions during protein processing that would eventually affect protein folding.

Analysis of Individual Mutations in MtLT2

In an attempt to isolate the individual effects of the mutations accumulated in the R2 variant after in vitro evolution, individual mutants were constructed by IVOE site-directed mutagenesis of the parental MtLT2 (see Figure S4 and Supplemental Experimental Procedures), and their behavior in organic cosolvents was assessed (Table S3). As expected, the L429V mutant behaved like the corresponding evolved variant (i.e., 6C9 from the first generation, which only contained an additional mutation in the signal sequence). Apart from L429V, the individual mutants did not display the improvements of the evolved variants in cosolvents. The best result was obtained with



the double mutant Gct8D-Ect9K with a 5.4-fold improvement in 50% (v/v) ethanol, but still far from the corresponding evolved mutant (i.e., the 2E9 mutant in which those mutations were first introduced) that displayed an ~14-fold improvement in 50% (v/v) ethanol.

These results are not unexpected and, in fact, it is well known that the combination of beneficial mutations during the course of natural evolution is an advantageous process that eventually produces better fitness to the environment. In a similar yet more simplified approach, the directed molecular evolution algorithm bases its success on the accumulation of beneficial mutations generation after generation, leading to the exploitation of new synergistic interactions between amino acids in the protein structure that eventually adapt proteins to the selective pressure established. Hence, the improvements generated by mutations stored during evolution must be exclusively considered in the general context of the evolved proteins, and in only a very few cases can they be extrapolated.

SIGNIFICANCE

Despite the spectacular development of laboratory evolution methods over the last 15 years, the introduction of cosolvent resistance has been almost exclusively restricted to hydrolases (proteases, lipases, and esterases) [4], with very few examples of successful tailoring of redox biocatalysts along these lines [37, 38]. Although molecular dynamics simulation of redox systems [39] and bioelectrocatalytic studies [40] are providing new insights into the role of cosolvents in protein unfolding, we are still far from understanding which factors are truly altered in a given redox biocatalyst submerged in cosolvents. It is worth noting that oxygen, the main fuel for all oxidoreductases, is far more soluble in organic solvents than in water. Therefore, this entails serious constraints derived from the tightly regulated traffic of electrons between the notoriously water-insoluble reducing substrate and the oxygen molecule. On the other hand, there are a few successful examples of attempts to engineer tolerant enzymes (i.e., those that retain both activity and stability at high concentrations of cosolvents). Although narrowly connected during natural evolution, activity and stability are enzymatic features that are modulated by different principles and, therefore, their joint improvement is not easily achieved. Indeed, it has been shown that it is easier to improve stability while maintaining high activity than to improve function while maintaining stability [41]. Interestingly, the results we achieved by directed evolution identified a laccase in organic solvents that maintained high stability while improving activity.

Fungal laccases are ideal green enzymes of huge biotechnological impact due to their few requirements (they only require air and they produce water as the only byproduct) and their broad substrate specificity, including direct bioelectrocatalysis. In the near future, the practical use of fungal laccases for troublesome transformations will expand the need for this catalyst to act in organic media, where the laccase reducing substrates are easily solubilized and further oxidized [42–46].

EXPERIMENTAL PROCEDURES

Reagents and Enzymes

The laccases from *Trametes versicolor* and *Myceliophthora thermophila*, the laccases from *Picnoporus cinnabarinus* and *Trametes hirsuta*, and the laccases from *Coriolopsis gallica* and *Pleurotus ostreatus* were kindly donated by Novozymes (Davis, CA, USA), Prof. A. Martinez (Centro de Investigaciones Biológicas, CIB-CSIC, Madrid, Spain), Prof. A. Yaropolov (Institute of Biochemistry, Moscow, Russia), and Dr. R. Vázquez-Duhaltz (UNAM, Cuernavaca, Mexico). The parental MtLT2 laccase gene was engineered as reported elsewhere [17], and $K_4(Mo[CN]_8)$ was synthesized and purified according to a previously published method [47], whereas the mediators $K_4(W[CN]_8)$ and $K_4(Os[CN]_6)$ were kindly provided by Prof. Kenji Kano (Kyoto University, Kyoto, Japan). Taq-DNA polymerase, ABTS (2,2′-azino-bis[3-ethylbenzthizoline-6-sulfonic acid]), $K_4(Fe[CN])_6$, and the *S. cerevisiae* transformation kit were purchased from Sigma-Aldrich (Madrid, Spain). The GeneMorph PCR mutagenesis kit, *Escherichia coli* XL2-blue competent cells, and the high-fidelity *Pfu*-Ultra polymerase were from Stratagene (La Jolla, CA, USA). The protease deficient *S. cerevisiae* strain BJ5465 was obtained from LGCPromochem (Barcelona, Spain), and the pJRoC30 shuttle vector with auxotrophy for uracil and with the ampicillin resistance gene was from Novozymes (Davis, CA, USA). The Zymoprep yeast plasmid miniprep kit, Zymoclean gel DNA recovery kit, and the DNA clean and concentrator TM-5 kit were from Zymo Research (Orange, CA, USA). The QIAprep spin miniprep kit was purchased from QIAGEN (West Sussex, UK) and the BamHI and XhoI restriction enzymes were from New England Biolabs (Hertfordshire, UK). All chemicals were of reagent-grade purity.

Laboratory Evolution

For each generation, PCR fragments were cleaned and concentrated (DNA clean and concentrator TM-5 kit), loaded onto a low-melting point preparative agarose gel, and purified using the Zymoclean gel DNA recovery kit. PCR products were cloned under the control of the Gal 10 promoter of the expression shuttle vector pJRoC30,

Figure 4. Location and Surroundings of Different Residues in the Parental MtLT2 Lacasse and the Mutations Introduced throughout Molecular Evolution

The blue spheres represent Cu atoms.
(A) Residue E182 in MtLT2.
(B) Mutation E182K. The new K182 residue forms an ion pair with E55.
(C) Residue S280 in MtLT2.
(D) Mutation S280N. The new N280 allows the formation of extra hydrogen bonds with neighboring residues.
(E) Residue N552 in MtLT2.
(F) The mutation N552H.
(G) Surroundings of residue L429 (blue) in MtLT2.
(H) Mutation L429V.

replacing the MtLT2 gene in pJRoC30. To remove the parental gene, the pJRoC30 plasmid was linearized with XhoI and BamHI, and the linearized plasmid was concentrated and purified as described above for the PCR fragments.

First Generation

Two independent libraries were prepared. A mutagenic library (~1500 mutants) was constructed with a Genemorph kit (Stratagene), adjusting the mutation rate to 1.1–3.5 mutations per kb. Error-prone PCR was carried out on a gradient thermocycler (Mycycler; Bio-Rad, Hercules, CA, USA) using the following parameters: 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); and 74°C for 10 min (1 cycle). The primers used for amplification were: IG88-S sense (5′-CCTCTATACTTTAACGTCAAGG-3′) and IG88-R antisense (5′-GGGAGGGCGTGAATGTAAGC-3′). To promote in vivo ligation, overhangs of 40 bp and 66 bp that were homologous to the linearized vector were designed. PCR products (400 ng) were mixed with the linearized vector (100 ng) and transformed into competent cells using the yeast transformation kit (Sigma). For the second library (~1700 mutants), combinatorial saturation mutagenesis by in vivo overlap extension (IVOE) was carried out [21]. Positions 510 and 513 were targeted because of their implication in the overall biocatalyst activity [24]. Two separate PCR reactions were simultaneously prepared to amplify the two DNA fragments that overlapped at specific positions corresponding to amino acids 510 and 513 in the MtLT2 sequence. PCR reactions were performed in a final volume of 50 μl containing each primer (0.25 μM), 100 ng of template (MtLT2), dNTPs (0.25 mM each), 3% dimethyl sulfoxide (DMSO), and 2.5 units of Pfu-Ultra DNA polymerase. PCR1 was carried out using the following oligonucleotides: IG88-S sense and 3CPOantisense (5′-GGTAGAC GACGCC*SNN*GCCGCC*SNN*GACGTGCCAGGCGAT-3′). The primers for PCR2 were: 3CPOsense (5′-ATCGCCTGGCACGTC*NNS*GGC GGC*NNS*GGCGTCGTCTACC-3′) and IG88-R antisense. The codons in italics were submitted to saturation mutagenesis, where N is (A + T + C + G) and S is (G + C). The PCR conditions were as follows: 95°C for 2 min (1 cycle); 94°C for 0.45 min, 55°C for 0.45 min, 74°C for 2 min (28 cycles); and 74°C for 10 min (1 cycle). PCR fragments (200 ng each) were mixed with the linearized vector (100 ng; ratio PCR product:vector, 4:1) and transformed in yeast as described above.

Second Generation

The second round was performed by mutagenic PCR construction, exploring two independent libraries using both Mutazyme and Taq-DNA polymerases. The Mutazyme library (~1500 mutants) was made as described above for the first round. The Taq/$MnCl_2$ library (~1500 mutants) was prepared in a final volume of 50 μl containing 90 nM IG88-S, 90 nM IG88-R, 0.1 ng/μl parental laccase, 0.3 mM dNTPs, 3% DMSO, 1.5 mM $MgCl_2$, 0.05 U/μl Taq polymerase. Different concentrations of $MnCl_2$ (0, 0.05, 0.1, and 0.2 mM) were tested to estimate the appropriate mutation rate, and the PCR conditions were as used in the first generation.

Third Generation

The best variants of the second round (2B10, 9F1, 17C10, 7H9, and 5B9) were submitted to Taq/$MnCl_2$ amplification and recombined by in vivo DNA shuffling (~1000 mutants). PCR mutated products were mixed equimolarly (80 ng of each product) and transformed along with linearized vector into yeast (ratio PCR product:vector, 4:1).

Fourth Generation

A library of ~1000 mutants was built by in vivo assembly of two independent mutant libraries with different mutational spectra (IVAM). An equimolar mixture of Taq/$MnCl_2$ and Mutazyme libraries was added to 100 ng of linearized vector and transformed into yeast competent cells (ratio equimolar library:vector, 8:1).

Fifth Generation

The last variant was submitted to site-directed mutagenesis at position 280 and further explored by IVOE saturation mutagenesis (~400 mutants) [21]. For site-directed mutagenesis, the oligos used in PCR1 were: IG88-S sense and JAR-antisense (5′-CCAGTAGTTCCCG GGCGTTCGGTTGGCTTCGATGACG-3′). For PCR2, the oligos were: IG88-R antisense and JAR-sense (5′- GTCATCGAAGCCAACCGAAC GCCCGGGAACTACTGG-3′). For saturation mutagenesis, the oligos used in PCR1 were: IG88-S sense and bobaf5 antisense (5′-CCAGTA GTTCCCGGGCGTTCG*SNN*GGCTTCGATGACG-3′). For PCR2, the oligos were: bobaf5 sense (5′-GTCATCGAAGCC*NNS*CGAACGCC CGGGAACTACTGG-3′) and IG88-R antisense. The codons submitted to saturation mutagenesis are in italics, where N is (A + T + C + G) and S is (G + C).

Screening Protocol

Screening was performed according to Alcalde et al. [14], with minor modifications (see Supplemental Experimental Procedures). Parent type and selected mutants were produced and thoroughly purified as described elsewhere [21].

Electrochemical Characterization

Spectroelectrochemical Studies

Spectroelectrochemistry was carried out on the different MtLT2 mutants, as well as on *Trametes hirsuta* laccase, using a microspectroelectrochemical cell consisting of a gold capillary electrode as described elsewhere [48]. The potential of the gold capillary in the cell was controlled by three-electrode potentiostat BAS LC-3E from Bioanalytical Systems (BAS, West Lafayette, IN, USA). In these measurements, an Ag|AgCl|KCl reference electrode (BAS) and a platinum counter electrode were used. The absorbance spectra were monitored with a PC2000-UV-vis miniature fiber optic spectrometer from Ocean Optics (Dunedin, FL, USA) with an effective range between 200 and 1100 nm.

The redox potential of the T1 site of the enzymes was determined by mediated spectroelectrochemical redox titration (MRT) using the spectroelectrochemical setup described above. A 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 mV, 520 mV, 640 mV, and 780 mV versus the normal hydrogen electrode [NHE], respectively) was used for the MRT in accordance with previously published methods [49].

Cyclic Voltammetry Measurements

Laccase-modified spectrographic graphite electrodes (SPGE) served as the working electrodes. The surface of the SPGE (type RW001, 3.05 mm diameter, 13% porosity) from Ringsdorff Werke GmbH (Bonn, Germany) was prepared by polishing on wet fine emery paper (Tufback Durite, P1200; Allar, Sterling Heights, MI, USA). It was then rinsed thoroughly with Millipore water (Millipore, Billerica, MA, USA) and allowed to dry. A volume of 10 μl of laccase solution was placed on the electrode surface and allowed to adsorb, and after 15 min the electrode was rinsed again with water. Cyclic voltammograms of the laccase-modified electrode were recorded using three-electrode potentiostat CV-50W (BAS). The reference electrode was an $Hg_2|Hg_2Cl_2|KCl_{sat}$ electrode (SCE, 242 mV versus NHE) and the counter electrode was a platinum wire. The supporting electrolytes were 0.1 M citrate-phosphate buffers (pH 4.0 and 7.0).

Electron Paramagnetic Resonance Analysis of Copper Sites of Purified Laccases

EPR measurements were carried out with a Bruker ER200D instrument (Bruker, Bremen, Germany) operating in the X-band ($\nu \approx 9.6$ GHz) using a DPPH (α,α'-diphenyl-β-picrylhydrazyl) standard for frequency calibration (in a T-type double cavity). Portions of sample in potassium phosphate buffer were introduced into a spectroscopic quartz probe cell. The spectra were recorded at 77K and were typically performed at 19.5 milliwatt microwave power (no signal saturation was apparent in independent experiments up to 40 milliwatts), 100 kHz modulation frequency, 2G modulation amplitude, 40 ms time constant, and 1×10^5 receiver gain.

Supplemental Data

Supplemental Data include four figures, three tables, and Supplemental Experimental Procedures and are available at http://www.chembiol.com/cgi/content/full/14/9/1052/DC1/.

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