Focused Directed Evolution of Aryl-Alcohol Oxidase in *Saccharomyces cerevisiae* by Using Chimeric Signal Peptides

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Aryl-alcohol oxidase (AAO) is an extracellular flavoprotein that supplies ligninolytic peroxidases with H₂O₂ during natural wood decay. With a broad substrate specificity and highly stereoselective reaction mechanism, AAO is an attractive candidate for studies into organic synthesis and synthetic biology, and yet the lack of suitable heterologous expression systems has precluded its engineering by directed evolution. In this study, the native signal sequence of AAO from *Pleurotus eryngii* was replaced by those of the mating α-factor and the K₁ killer toxin, as well as different chimeras of both prepro-leaders in order to drive secretion in *Saccharomyces cerevisiae*. The secretion of these AAO constructs increased in the following order: prepro-α-AAO > preproK-α-AAO > preKproα-AAO > preproK-AAO. The chimeric preproK-α-AAO was subjected to focused-directed evolution with the aid of a dual screening assay based on the Fenton reaction. Random mutagenesis and DNA recombination was concentrated on two protein segments (Met₁-Val₁₀₉ and Phe₃⁹₂-Gln₅₆₆), and an array of improved variants was identified, among which the FX7 mutant (harboring the H91N mutation) showed a dramatic 96-fold improvement in total activity with secretion levels of 2 mg/liter. Analysis of the N-terminal sequence of the FX7 variant confirmed the correct processing of the preproK hybrid peptide by the KEX2 protease. FX7 showed higher stability in terms of pH and temperature, whereas the pH activity profiles and the kinetic parameters were maintained. The Asn91 residue is in the flavin attachment loop motif, and it is a highly conserved residue in all members of the GMC superfamily, except for *P. eryngii* and *P. pulmonarius* AAO. The *in vitro* involution of the enzyme by restoring the consensus ancestor Asn91 promoted AAO expression and stability.

aryl-alcohol oxidase (AAO; EC 1.1.3.7) is a flavoenzyme of the GMC (glucose-methanol-choline) oxidoreductase superfamily, the members of which share a N-terminal FAD-binding domain containing the canonical ADP-binding motif. Secreted by several white-rot fungi, this monomeric flavoprotein plays an essential role in natural lignin degradation (1). Accordingly, AAO oxidizes lignin-derived compounds and aromatic fungal metabolites, releasing H₂O₂ that is required by ligninolytic peroxidases to attack the plant cell wall (2). Moreover, the H₂O₂ produced by AAO is an efficient vehicle to generate highly reactive hydroxyl radicals through the Fenton reaction (Fe³⁺ + H₂O₂ → OH⁻ + OH⁻ + Fe²⁺), such that OH⁻ can act as a diffusible electron carrier to depolymerize plant polymers. AAO oxidizes a variety of aromatic benzyl (and some aliphatic polysaturated) alcohols to the corresponding aldehydes. In addition, AAO participates in the oxidation of aromatic aldehydes to the corresponding acids and has activity on furfural derivatives (3).

Aspergillus nidulans (15), an unsuitable host for directed evolution experiments (16), and in *Escherichia coli* after the *in vitro* refolding of inclusion bodies, an approach incompatible with directed evolution campaigns (17).

In the present study, the native signal peptide of AAO was replaced by two different signal sequences to drive its functional expression in *Saccharomyces cerevisiae*: (i) the signal prepro-leader of the mating α-factor of *S. cerevisiae*, which has been used widely to evolve different ligninases (18–23), and (ii) the signal prepro(α)-leader and the γspacer segment of the K₁ killer toxin, which have been seen to be useful in boosting β-lactamase secretion in yeast (24, 25). For the first time, chimeric versions of these leaders were designed by combining the different pre- and pro-regions, and these constructs were subjected to conventional and focused-directed evolution using a very sensitive dual high-throughput screening (HTS) assay based on the Fenton reaction. The best mutant identified dramatically improved the total activ-

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ity and stability being readily secreted by yeast. Indeed, this active, highly stable and soluble AAO variant is a promising point of departure for new engineering goals.

MATERIALS AND METHODS

All chemicals were of reagent-grade purity. Ferrous ammonium sulfate, xylenol orange, sorbitol, benzyl alcohol, p-methoxybenzyl alcohol, veratryl (3,4-dimethoxybenzyl) alcohol, 2,4-hexadien-1-ol, ABTS [2,2’ azinobis(3-ethylbenzothiazoline-6-sulfonic acid)], horseradish peroxidase (HRP), Taq polymerase, and a yeast transformation kit were purchased from Sigma (Madrid, Spain). Zymoprep yeast plasmid miniprep, yeast plasmid miniprep kit, and a Zymoclean gel DNA recovery kit were obtained from Zymo Research (Orange, CA). Restriction enzymes BamHI and XhoI were from New England BioLabs (Hertfordshire, United Kingdom).

I-Proof high-fidelity DNA polymerase was from Bio-Rad (USA). The epi- somal shuttle vector pJRc30 was from the California Institute of Technology (Caltech) and plasmids pRE1219 and pJRc30-8yN2C1 were kindly donated by S. Camarero (CIB-CSIC, Madrid, Spain).

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The Focused-directed AAO evolution. All of the PCR products were cleaned, concentrated, loaded onto a low melting-point preparative agarose gel (0.75% [wt/vol]) and purified using a Zymoclean gel DNA recovery kit. PCR products (400 ng of each) were mixed with the linearized vector (100 ng; PCR product/vector ratio of 4:1) and transformed in yeast (yeast transformation kit), promoting the recombination and cloning in vivo. Transformed cells were plated in SC (synthetic complete) dropout plates, followed by incubation for 3 days at 30°C; individual clones were fermented in 96-well plates and screened for AAO activity. For each positive construct, the plasmids were extracted and sequenced. Sequences were retransformed into yeast and fermented in 100-ml flasks while monitoring cell growth and activity (using HRP-ABTS and FOX [ferrous oxidation by xylenol orange] assays [see below]) over time.

Focused-directed AAO evolution. All of the PCR products were cleaned, concentrated, loaded onto a low melting-point preparative agarose gel (0.75% [wt/vol]) and purified using a Zymoclean gel DNA recovery kit before being cloned into pJRc30. The plasmid was linearized with BamHI and XhoI. pJRc30-preproK-AAO variant was used as DNA template for focused random mutagenesis. The preproK-AAO fusion was split into three different segments for MORPHING (Mutagenic Organized Recombination Process by Homologous IN vivo Grouping) (26). Amplified by PCR, each fragment included homologous overlapping portions of ~50 bp so that the whole gene could be reassembled in vivo by transformation into S. cerevisiae. Mutagenic regions M-I and M-II (590 bp) were amplified by PCR using the primers listed in Table S1. The amplified PCR products were then mixed and transformed into the yeast host strain, allowing for the generation of a library of random mutations. The transformed yeast cells were then plated on SC medium lacking uracil to select for the presence of the plasmid. The library was then screened for the desired activities by the method described above.
ies (~1,000 clones each) were prepared targeting segment M-I or segment M-II independently for random mutagenesis. The third library (~1,000 clones, library M-I-II) was constructed by assembling mutagenic segments (M-I and M-II) flanking a nonmutagenic amplification in the middle of the gene. Finally, the whole preexoPK-AAO fusion was subjected to Taq/MnCl₂ amplification (library M-IV), adjusting the mutational rate to 1 to 3 mutations per gene (~2,000 clones). Concentrations of 0.05 and 0.01 mM MnCl₂ were used for MOPRHG and full gene random mutagenesis, respectively.

(i) Mutagenic PCR of targeted segments. Reaction mixtures were prepared in a final volume of 50 μl containing DNA template (0.92 ng/μl), 90 nM oligonucleotide sense primer (RLMN for segment M-I and AAOMBP [5′-AATCTGTGGCATTGAGAGATC-3′] for segment M-II), 90 nM reverse primer (AAO92C [5′-GCCAGGGCAGTGAGATG AAGC-3′]) for segment M-I and RMLC [5′-GGGACGGGTGTAAGTG AAGC-3′] for segment M-II), 0.3 mM dNTPs (0.075 mM each), 3% (vol/vol) DMSO, 1.5 mM MgCl₂, increasing concentrations of MnCl₂ (0.025, 0.05, and 0.1 mM), and 0.05 U of Taq DNA polymerase/μl. Mutagenic PCRs parameters were as follows: 95°C for 2 min (1 cycle); 95°C for 45 s, 50°C for 45 s, and 74°C for 45 s for 28 cycles; and 74°C for 10 min (1 cycle).

(ii) High-fidelity PCR. Reaction mixtures were prepared in a final volume of 50 μl containing DNA template (0.2 ng/μl), 250 nM oligonucleotide sense HFF (5′-GGTGGGAACCATTGGTTG-3′) and 250 nM oligonucleotide antisense HFR (5′-GGGGCTACCGTTTGGTG AAAAA-3′). High-fidelity PCRs were performed using the following parameters: 98°C for 30 s (1 cycle); 98°C for 10 s, 55°C for 25 s, and 72°C for 45 s for 28 cycles; and 72°C for 10 min (1 cycle).

(iii) Whole gene reassembly. The whole gene was cloned and recombined in vivo by transformation into S. cerevisiae. PC products were mixed in equimolar amounts (400 ng) and transformed with linearized plasmid (200 ng) into chemically competent cells. Transformed cells were plated on SC dropout plates and incubated for 3 days at 30°C. Colonies containing the whole autonomously replicating vector were picked and rescreened as described above.

HTS assay. Individual clones were picked and cultured in sterile 96-well plates containing 50 μl of minimal medium (SC). In each plate, column 6 was inoculated with the parental type (internal standard) and well H1 with URA3- S. cerevisiae cells (negative control). Plates were sealed 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 was added to each well, followed by culture for an additional 48 h. Finally, 20-μl portions of the supernatants were screened for activity with the FOX and HRP-ABTS assays using veratral or p-methoxybenzyl alcohol as the substrate as described below. One unit of AAO activity is defined as the amount of enzyme that converts 1 μmol of alcohol to aldehyde with the stoichiometric formation of H₂O₂ per min under the reaction conditions.

Chemical (direct) FOX assay. Aliquots of 20 μl of yeast supernatants were transferred with liquid handler robotic station Freedom EVO (Tecan, Männedorf, Switzerland) and incubated with 20 μl of substrate (2 mM p-methoxybenzyl alcohol or 10 mM veratral alcohol in 100 mM phosphate buffer [pH 6.0]) for 30 min at room temperature, and then 160 μl of FOX reagent was added with a Multidrop Combi-Reagent dispenser (Thermo Scientific, Waltham, MA) to assess the AAO H₂O₂ production [final concentration of FOX mixture in the well: 100 μM xylene orange, 250 μM Fe(NH₄)₂(SO₄)₂, and 25 mM H₂SO₄] (27). Plates were recorded in endpoint mode at 560 nm using a spectrophotometer SpectraMax 384 Plus (Molecular Devices, Sunnyvale, CA); it required ~20 min of incubation to develop an intense and stable colorimetric response. The relative activities were calculated from the difference between the absorbance value after incubation to that of the initial measurement normalized to the parental type for each plate. To enhance method sensitivity, several additives may be added to the reagent, such as organic cosolvents (DMSO), ethanol, and methanol) or sorbitol (28). In our case, the response was amplified by adding a final concentration of 100 μM sorbitol, which acts as chain amplifier generating additional ferric ions to increase the response of the method (29). The assay was validated by determining the coefficient of variance, the linearity of the response and the detection limit. The detection limit was calculated by the blank determination method on a 96-well plate with triplicate standards (0, 0.5, 1, 1.5, 2, 2.5, 3, and 4 μM H₂O₂) and several portions of supernatants from S. cerevisiae URA³- lacking plasmid (30). FOX signal stability was tested with different H₂O₂ concentrations (0, 2, 4, 6, 8, 10, 15, and 18 μM) for 300 min at 24°C.

Enzymatic (indirect) HRP-ABTS assay. Aliquots of 20 μl of yeast supernatants were added to 180 μl of HRP-ABTS reagent (final concentrations of HRP-ABTS reagent in the well: 1 mM p-methoxybenzyl alcohol or 5 mM veratral alcohol, 2.5 mM ABTS, 1 μg of HRP/ml in 100 mM phosphate buffer [pH 6.0]) dispensed with a Multidrop Combi-Reagent dispenser. The plates were incubated at room temperature and measured in endpoint or kinetic mode at 418 nm (ε₉₋₁₇ = 36,000 M⁻¹ cm⁻¹).

The dual HTS assay incorporated two consecutive rescreenings to rule out the selection of false positives.

(i) First rescreening. Aliquots of 5 μl of the best clones of the screening were transferred to new sterile 96-well plates with 50 μl of minimal medium per well. Columns 1 and 12 plus rows A and H were not used to prevent the appearance of false positives. After 24 h of incubation at 30°C and 225 rpm, 5-μl portions were transferred to the adjacent wells, followed by further incubation for 24 h. Finally, 160 μl of expression medium was added, and the plates were incubated for 48 h. Accordingly, each mutant was grown in four independent wells. The parental type was subjected to the same procedure (lane D, wells 7 to 11). Plates were assessed according to the same HTS protocol of the screening described above.

(ii) Second rescreening. An aliquot from the best clones from the first rescreening was inoculated in 3 ml of YPD medium, followed by incubation at 30°C for 24 h at 225 rpm. The plasmids from these cultures were recovered with a Zymoprep yeast plasmid miniprep kit I. Since the product of the Zymoprep was impure and the DNA extracted was very low concentrated, the shuttle vectors were transformed into supercompetent E. coli XL2-Blue cells and plated onto LB-ampicillin (LB-amp) plates. Single colonies were selected to inoculate 5 ml of LB-amp medium and incubated overnight at 37°C and 225 rpm. The plasmids from the best mutants and the parental type were extracted (NucleoSpin plasmid kit) and transformed into S. cerevisiae. Five colonies for each mutant were picked and rescreened as described above.

AAO production and purification. (i) Production of recombinant AAO variants in S. cerevisiae. A single colony from the S. cerevisiae clone containing the AAO fusion gene was picked from a SC dropout plate, inoculated in SC medium (20 ml) and incubated for 48 h at 30°C and 220 rpm (Mintron-INFORS, Biogen, Spain). An aliquot of cells was removed and used to inoculate minimal medium (100 ml) in a 500-ml flask (optical density at 600 nm [OD₆₀₀] = 0.25). The cells completed two growth phases (6 to 8 h; OD₆₀₀ = 1), and then expression medium (900 ml) was inoculated with the preculture (100 ml; OD₆₀₀ of 0.1). After incubation for 72 h at 25°C and 220 rpm (maximal AAO activity; OD₆₀₀ = 25 to 30), the cells were recovered by centrifugation at 4,500 rpm and 4°C (Avanti J-E centrifuge; Beckman Coulter, Inc., Beckman, CA), and the supernatant was double filtered (using both a glass membrane filter and a nitrocellulose membrane [0.45-μm pore size]).

(ii) Purification of AAO mutant. AAO (FX7 variant) was purified by FPLC (AKTA purifier; GE Healthcare, United Kingdom). The crude extract was concentrated and dialyzed in 20 mM piperase buffer (buffer P [pH 5.5]) by tangential ultrafiltration (Pellecino; Millipore, Temecula, CA) through a 10-kDa-pore-size membrane (Millipore) by means of a peristaltic pump (Masterflex Easy Load; Cole-Parmer, Vernon Hills, IL). The sample was filtered and loaded onto a weak anion-exchange column (Hi-Trap Q FF; GE Healthcare) prequillibrated with buffer P and coupled to the AKTA purifier system. The proteins were eluted with a linear gradient of buffer P + 1 M NaCl in two phases at a flow rate of 1 ml/min; from 0 to 50% in 15 min and from 50% to 100% in 2 min. Fractions with AAO activity

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were pooled, dialyzed against buffer P, concentrated, and loaded onto a high-resolution resin, strong-anion-exchange column (Biosuite MonoQ 10 cm; Waters, Milford, MA) preequilibrated in buffer P. The proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in two phases at a flow rate of 1 ml/min: from 0 to 50% in 20 min and from 50 to 100% in 2 min. Fractions with AAO activity were pooled, dialyzed against buffer 20 mM phosphate buffer (pH 6.0), concentrated, and further purified by high-pressure liquid chromatography with a Superose 12 HR 10/30 molecular exclusion column (Amersham Bioscience) preequilibrated with 150 mM NaCl in phosphate buffer (pH 6.0) at a flow rate of 0.5 ml/min. The fractions with AAO activity were pooled, dialyzed against buffer (20 mM phosphate buffer [pH 6.0]), concentrated, and stored at −20°C. Throughout the purification protocol the fractions were analyzed by SDS-PAGE on 10% gels in which the proteins were stained with Protoblue Safe blue R-250, and then the enzyme band was excised and processed for N-terminal amino acid sequencing on a precise sequencer at the core facilities of the Helmholtz Centre for Infection Research, Germany.

(iii) Determination of kinetic thermostability ($T_{50}$). Appropriate dilutions of purified FX7 and $g_{c}$AAO were prepared for the assay, while the samples of parental preproK-AAO were obtained from the crude supernatants. A temperature gradient scale ranging from 30 to 80°C was established as follows: 30.0, 31.4, 34.8, 39.3, 45.3, 49.9, 53, 55, 56.8, 59.9, 64.3, 70.3, 75, 78.1, and 80°C. This gradient profile was achieved using a thermal block (Mycycler). After 10 min of incubation, FX7 and $g_{c}$AAO samples were removed and chilled on ice for 10 min, followed by further incubation at room temperature for 5 min. Finally, 20-μl samples were added to 180-μl volumes of 100 mM sodium phosphate buffer (pH 6.0) containing 1 mM $p$-methoxybenzyl alcohol, and the activity was measured as anisaldehyde production by determining the absorption at 285 nm ($ε_{285} = 16,950 \text{M}^{-1}\text{cm}^{-1}$). In the case of parental preproK-AAO supernatants, the samples were subjected to an HRP-ABTS assay described above for the screening. Thermostability values were calculated from the ratio between the residual activities incubated at different temperature points and the initial activity at room temperature. The $T_{50}$ value was determined by the transition midpoint of the inactivation curve of the protein as a function of temperature, which in our case was defined as the temperature at which the enzyme lost 50% of its activity after an incubation of 10 min. All reactions were performed by triplicate.
(iv) **Thermoactivity** ($T_a$). Enzyme dilutions of purified FX7 (33 nM, final concentration) and EcAAO (18 nM, final concentration) 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 (pH 6.0) containing 1 mM $p$-methoxybenzyl alcohol at various corresponding temperatures (25, 30, 40, 50, 60, 70, 80, 90, and 99°C), followed by incubation in an Eppendorf Thermomixer Comfort apparatus (Thermo Fisher Scientific). Reactions were performed by triplicate and $p$-methoxybenzyl alcohol oxidation, followed by aldehyde production at 285 nm.

(v) **Kinetic parameters.** Kinetic constants for AAO were estimated in 100 mM sodium phosphate (pH 6.0). The final enzyme concentrations used were as follows: with $p$-methoxybenzyl alcohol, 33 and 18 nM for FX7 and EcAAO, respectively; with veratryl alcohol, 38 and 32 nM for FX7 and EcAAO, respectively; with benzyl alcohol, 62 and 47 nM for FX7 and EcAAO, respectively; and with 2,4-hexadien-1-ol, 15 and 4 nM for FX7 and EcAAO, respectively. Reactions were performed in triplicate, and substrate oxidations were monitored by measuring the absorption at 285 nm for $p$-methoxybenzyl alcohol ($ε_{285} = 16,950$ M$^{-1}$ cm$^{-1}$), 310 nm for veratryl alcohol ($ε_{310} = 9,300$ M$^{-1}$ cm$^{-1}$), 250 nm for benzyl alcohol ($ε_{250} = 13,800$ M$^{-1}$ cm$^{-1}$), and 280 nm for 2,4-hexadien-1-ol ($ε_{280} = 30,140$ M$^{-1}$ cm$^{-1}$). Steady-state kinetic parameters were determined by fitting the initial reaction rates at different substrate concentrations to the Michaelis-Menten equation for one substrate, $v/e = k_{cat}·S/(K_m + S)$, where $e$ represents the enzyme concentration, $k_{cat}$ is the maximal turnover rate, $S$ is the substrate concentration, and $K_m$ is the Michaelis constant. The data were fit using SigmaPlot 10.0 (Systat Software, Inc., Richmond, CA).

(vi) **pH activity and stability profiles.** Appropriate dilutions of enzyme samples were prepared in such a way that aliquots of 20 μl yielded a linear response in kinetic mode. The optimum pH activity was determined using 100 mM citrate-phosphate-borate buffer at different pH values (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) containing the corresponding alcohol concentration (0.3, 5, 9, and 1.2 mM for $p$-methoxybenzyl alcohol, veratryl alcohol, benzyl alcohol, and 2,4-hexadien-1-ol, respectively). To measure pH stability, enzyme samples were incubated at different times over a range of pH values. The residual activity was deduced from the activity before and after incubation with 0.3 mM $p$-methoxybenzyl alcohol in 100 mM phosphate buffer (pH 6.0).
Protein modeling. The crystal structure of the AAO from *P. eryngii* at a resolution of 2.55 Å (Protein Data Bank Europe [PDB] accession number 3FIM [31]) was used for the FX7 mutant homology model, obtained by PyMol (Schrodinger, LLC [http://www.pymol.org]).

DNA sequencing. All genes were verified by DNA sequencing (using a BigDye Terminator v3.1 cycle sequencing kit). The primers used were common to the four constructions: sense primers RMLN and AAOsec1F (5′-GTGGATCAACAGAAGATTTCGATCG-3′) and antisense primers RMLC (5′-GCTTACATTCACGCCCTCCC-3′), AAOsec2R (5′-GTGGTTAGCAATGAGCGCGG-3′), and AAOsec3R (5′-GGAGTCGAGCCTCTGCCCT-3′).

RESULTS AND DISCUSSION

Construction of chimeric signal prepro-leaders. In terms of yeast expression, the replacement of native signal peptides to foster foreign protein secretion has been used widely for years. Recently, the directed evolution of the α-factor prepro-leader permitted the functional expression of antibodies (32) and different classes of ligninases (including high-redox potential laccases, versatile peroxidases, and unspecific peroxygenases) (18–22). In the present study, the prepro-leaders from the mating α-factor and the K1 killer toxin, along with their chimeric combinations, were tailored and attached to the native AAO for functional expression and directed evolution. The mating α-factor prepro-leader is formed by 19 and 64 amino acids from the pre- and pro-leaders, respectively (33–35) (Fig. 1A). The pre-leader initiates endoplasmic reticulum translocation, and it is finally removed by the action of a signal peptidase that cuts between residues 19 and 20. The pro-leader contains three Asn-linked glycosylation sites, and it is thought to be involved in the folding and maturation of the protein before it is packed into vesicles for exocytosis. The pro-leader is processed in the Golgi compartment by the action of the KEX2, STE13, and KEX1 proteases, although the latter is not needed for heterologous protein secretion. The K1 killer prepro-toxin is preceded by a prepro-sequence of 44 residues, prepro(*α*), that undergoes similar processing as the α-factor prepro-leader, albeit without the requirement of STE13 and KEX1 activity (24, 25, 36) (Fig. 1B). The prepro-toxin contains an internal γ-segment of 85 residues with three extra KEX2 recognition sites for processing in the Golgi compartment. Bearing in mind the common features of these two prepro-leaders in terms of processing and secretion, the following four fusion constructs were attached to the mature AAO (Fig. 1C): (i) the prepro-*α*-AAO containing the full α-factor prepro-leader, (ii) the preproK-AAO formed by the prepro(δ) of K1 toxin connected to a truncated version (64 residues) of the γ-segment known to be important for correct processing (the truncated segment stretched from positions 169 to 233 and preserved the three N-glycosylation sites: N181, N203, and N216 [24]), (iii) the chimeric preproK-AAO comprising the α-factor pre-leader fused to the truncated γ-segment, and (iv) the chimeric preKpro-*α*-AAO formed by the prepro(δ) of the K1 toxin linked to the α-factor pro-leader. In addition, we modified the prepro(δ)-containing constructs, fusions ii and iv, by site-directed mutagen-
We tried to enhance the membrane permeability of the pre-AAO (0.35 U/liter), and preproK-AAO (0.06 U/liter) constructs did not incorporate mutations in the mature protein or in the prepro-sequence (apart from the aforementioned P43K substitution) and that all of the elements were properly assembled in vivo as intended. Fermentations were translated from the high-throughput format to larger volumes (10 ml) for each construct studied. Irrespective of the substrate (p-methoxybenzyl or veratryl alcohol), the hierarchy of activity of the fusion genes was maintained: prepro-AAO (1.5 U/liter), preproK-AAO (0.5 U/liter), preKpro-AAO (0.35 U/liter), and preproK-AAO (0.06 U/liter) (Fig. 2). We tried to enhance the membrane permeability of the yeast by adding ethanol to the expression medium, and yet the activity detected was 5-fold lower, which was probably due to growth inhibition as a consequence of changes in yeast physiology and the redox balance of the medium (39).

### TABLE 1 Selected mutants of preproK-AAO libraries

<table>
<thead>
<tr>
<th>Variant</th>
<th>Library</th>
<th>Mutation Location</th>
<th>Secondary motif</th>
<th>Total activity improvement (fold)</th>
<th>$T_{50}$ (°C)</th>
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<td>FX7</td>
<td>M-I</td>
<td>cAC[H91N]AAC</td>
<td>FAD-binding domain</td>
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<tr>
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<tr>
<td>12G12</td>
<td>M-I</td>
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<td>Signal peptide; FAD-binding domain</td>
<td>Loop 1.9</td>
<td>61.8</td>
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The specific activity for $p$-methoxybenzyl alcohol was assayed in microscale fermentations (96-well plates), and the four fusions produced detectable AAO activity in the culture broth, which was consistent in the two colorimetric assays used (see below). The secretion driven by the corresponding constructs was as intended. Fermentations were translated from the high-throughput format to larger volumes (10 ml) for each construct studied. Irrespective of the substrate ($p$-methoxybenzyl or veratryl alcohol), the hierarchy of activity of the fusion genes was maintained: prepro-AAO (1.5 U/liter), preproK-AAO (0.5 U/liter), preKpro-AAO (0.35 U/liter), and preproK-AAO (0.06 U/liter) (Fig. 2). We tried to enhance the membrane permeability of the yeast by adding ethanol to the expression medium, and yet the activity detected was 5-fold lower, which was probably due to growth inhibition as a consequence of changes in yeast physiology and the redox balance of the medium (39).

### TABLE 2 Biochemical properties of recombinant native $\varepsilon_AAO$ and evolved AAO (FX7 mutant)

<table>
<thead>
<tr>
<th>Biochemical properties</th>
<th>$\varepsilon_AAO$</th>
<th>FX7 mutant</th>
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<tr>
<td>Molecular mass (Da)$^a$</td>
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<td>3.9</td>
<td>4.3</td>
</tr>
<tr>
<td>N-terminal end sequence</td>
<td>MADFDYVVGD$^d$</td>
<td>ADFDYVVGD</td>
</tr>
<tr>
<td>Sp act (U/mg)$^b$</td>
<td>74</td>
<td>24</td>
</tr>
<tr>
<td>Secretion level (mg/liter)</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Masses were estimated based on SDS-PAGE, MALDI-TOF-MS after deglycosylation with PNGase F, or the amino acid composition.

$^b$That is, the specific activity for $p$-methoxybenzyl alcohol.

$^c$This information was obtained from reference 17.

$^d$Met1 (M) was added to the N-terminal end for cloning in E. coli.
constructed (single M-I and M-II, as well as M-I-II combined), in addition to one conventional mutant library (targeting the full preproK-AAO by random mutation and DNA recombination; M-IV). Mutational loads were adjusted to introduce 1 to 3 amino acid changes per protein, and the four libraries were explored with a highly sensitive dual HTS system to detect AAO activity irrespective of the substrate. This method coupled a standard HRP-ABTS indirect colorimetric assay to a direct chemical method (FOX) based on the Fenton reaction in order to detect H$_2$O$_2$. The latter is typically used to measure H$_2$O$_2$ in biological fluids, and more recently to determine L-amino acid oxidase and lipoxygenase activities (27,28, 43, 44), but to our best knowledge it has never been used to evolve H$_2$O$_2$-producing enzymes. The limit of sensitivity of the FOX assay was 2 nM H$_2$O$_2$, and it was further shifted to 0.4 nM H$_2$O$_2$ through the inclusion of sorbitol to propagate the response (see Materials and Methods for details). Two consecutive rescreenings were carried out to avoid the selection of false positives. After exploring $\approx$5,000 clones, we identified five mutants with a total activity improvement over the parental type ranging from 2- to 5-fold and, significantly, the FX7 variant (H91N) from the combined M-I-II MORPHING displayed a dramatic 96-fold improvement in total activity with respect to the parental type (Table 1). The remaining mutations found in these variants (i.e., T[K50]A, S88T, L170M, I194V, D341N, and R481S) are located at a distance of $\approx$20 residues from one another, making them suitable candidates for future DNA shuffling studies or to be evaluated by site-directed recombination (see Fig. S1 in the supplemental material).

**Biochemical characterization.** The FX7 variant was purified to homogeneity and characterized biochemically. Since the weak secretion of parental preproK-AAO (0.5 U/liter) in *S. cerevisiae* hindered its purification, the properties of purified FX7 were compared to that of the native AAO heterologous expressed in *E. coli* after *in vitro* refolding (eAAO). FX7 was secreted at 2 mg/liter with a specific activity for p-methoxybenzyl alcohol of 24 U/mg (Table 2). Both FX7 and eAAO enzymes showed similar kinetic constants, as well as pH activity profiles, with all of the substrates tested (Fig. 4A and B), although turnover rates ($k_{cat}$) were lower if the enzyme is expressed in *S. cerevisiae* instead of *E. coli* (Table 3). The N-terminal sequencing of FX7 confirmed the correct cleavage of the chimeric preproK-leader, avoiding unwanted modifications at the N terminus. FX7 was heavily glycosylated (~50% gly-
TABLE 3 Kinetic parameters of native Ec-AAO and evolved AAO (FX7 mutant)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kinetic constant</th>
<th>(e_c)AAO</th>
<th>FX7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m) (mM)</td>
<td>Mean SEM</td>
<td>Mean SEM</td>
</tr>
<tr>
<td>(p)-Methoxybenzyl alcohol</td>
<td>0.035 0.001</td>
<td>0.034 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(k_{cat}) (s(^{-1}))</td>
<td>105 6 54 4</td>
<td>136 3 52 1</td>
</tr>
<tr>
<td></td>
<td>(k_{cat}/K_m) (s(^{-1}) mM(^{-1}))</td>
<td>2979 66 1562 44</td>
<td>1555 67 866 53</td>
</tr>
<tr>
<td>Veratryl alcohol</td>
<td>0.504 0.043</td>
<td>0.388 0.023</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(k_{cat}) (s(^{-1}))</td>
<td>66 2 28 1</td>
<td>91 2 51 3</td>
</tr>
<tr>
<td></td>
<td>(k_{cat}/K_m) (s(^{-1}) mM(^{-1}))</td>
<td>131 8 71 3</td>
<td>124 6 70 3</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>0.770 0.011</td>
<td>0.510 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(k_{cat}) (s(^{-1}))</td>
<td>22 1 19 1</td>
<td>91 2 51 3</td>
</tr>
<tr>
<td></td>
<td>(k_{cat}/K_m) (s(^{-1}) mM(^{-1}))</td>
<td>28 2 36 3</td>
<td>124 6 70 3</td>
</tr>
<tr>
<td>2,4-Hexadien-1-ol</td>
<td>0.087 0.001</td>
<td>0.059 0.004</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(k_{cat}) (s(^{-1}))</td>
<td>136 3 52 1</td>
<td>1555 67 866 53</td>
</tr>
<tr>
<td></td>
<td>(k_{cat}/K_m) (s(^{-1}) mM(^{-1}))</td>
<td>1555 67 866 53</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Steady-state constants were estimated in 100 mM sodium phosphate buffer (pH 6) at 24°C. All reactions were performed in triplicate.

.......

FIG 5 Thermostability and thermoactivity of AAO variants. (A) Thermostability \(T_{50}\) of FX7 (○), preproK-AAO parental type (●), and \(e_c\)AAO (□). (B) Thermotactivity \(T_a\) of FX7 (○), and \(e_c\)AAO (□). Each point represents the means and standard deviations of three independent experiments.
ulation of AAO expression to reduce the inhibition of ligninolytic peroxidases by H$_2$O$_2$ (51).

Conclusions and outlook. In this study, the AAO from 

P. eryngii

was functionally expressed in 

S. cerevisiae

by engineering chimeric prepro-leaders that allowed us to construct and screen mutant libraries in yeast. The particular design of an ad hoc chimeric prepro-leader, combined with a focused-directed evolution strategy and a sensitive dual screening assay, has led to obtain an active, highly stable AAO variant that is secreted by yeast as a correctly processed enzyme.

Although hardly used, the biotechnological potential of AAO should not be underestimated. In nature, the gradual release of H$_2$O$_2$ by AAO supplies peroxidases with a continuous source of cooxidant for lignin degradation. In vitro, this effect can be mimicked by controlling the addition of H$_2$O$_2$ with sensors and peristaltic pumps, although with limited success (52). Very recently, we introduced the FX7 variant into episomal bidirectional vectors to coexpress versatile peroxidase-AAO and/or unspecific peroxygenase-AAO (unpublished data). These self-sufficient expression systems could be used to evolve efficient enzymatic cascade reactions (e.g., for the oxidative conversion of 5-hydroxymethylfurans into value-added chemicals (53)). Significantly, the FX7 variant and its future offspring could also be included to design an autonomously consolidated bioprocessing yeast, with a full artificial secretome that includes the most important elements of the ligninolytic enzyme consortium. Such a microbe would have a number of potential applications in lignocellulose biorefineries for the production of fuels and commodities (22, 54). Finally, the directed evolution platform presented here is an invaluable tool for protein engineering, which can be applied from the design of efficient stereoselective aryl secondary alcohol oxidases to the synthesis of natural flavors (1).

ACKNOWLEDGMENTS This study was supported by the European Commission projects Indox FP7- KBBE-2013-7-613549 and Cost-Action CM1303-Systems Biocatalysis and by the National Projects Dewry (BIO201343407-R) and Cambios (RTC-2014-1777-3).

REFERENCES


FIG 6 (A) Consensus Asn91 in GMC superfamily. Sequence logo of the GMC signature 1 (prosite PS00623) in 329 GMC sequences from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/), JGI (http://jgi.doc.gov/), and Prosite Expasy (http://prosite.expasy.org/PS00623), including cholesterol oxidase, choline oxidase, aryl-alcohol oxidase, pyridoxine oxidase, methanol oxidase, glucose oxidase and dehydrogenase, pyranose oxidase and dehydrogenase, cellobiose dehydrogenase, 1-sorbose-1-dehydrogenase, and hydroxynitrile lyase proteins. (B) N91H mutation in FX7 variant. A molecular model using as the template the P. eryngii AAO crystal structure (PDB code 3FIM) was prepared to map the mutation. The residues of the active site His502, His546, and R206 are depicted in blue, FAD is depicted in yellow, and the H91N mutation is depicted in red.


