



Directed evolution of the PobR allosteric transcription factor to generate a biosensor for 4-hydroxymandelic acid

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Abstract

Hydroxy-mandelic acid (HMA) is widely applied in pharmaceuticals, food and cosmetics. In this study, we aimed to develop an allosteric transcription factors (aTFs) based biosensor for HMA. PobR, an aTF for HMA analog 4-hydroxybenzoic acid, was used to alter its selectivity and create novel aTFs responsive to HMA by directed evolution. We established a PobR mutant library with a capacity of 550,000 mutants using error-prone PCR and Megawhop PCR. Through our screening, two mutants were obtained with responsiveness to HMA. Analysis of each missense mutation indicating residues 122–126 were involved in its PobR ligand specificity. These results showed the effectiveness of directed evolution in switching the ligand specificity of a biosensor and improving HMA production.

Keywords Hydroxy-mandelic acid · Directed evolution · PobR · Ligand specificity

Introduction

Due to broad applications in pharmaceuticals, fragrance and industrial chemicals, natural products have been produced through various approaches including chemical synthesis, biocatalysis and extraction from natural sources (Galanie

et al. 2020). As industries are evolving toward sustainable and environmental-friendly, microbial synthesis of these compounds attracted increasing attention (Mitchler et al. 2021). Microbial metabolism has been harnessed to provide us access to natural products that are needed for the survival and reproduction of the host organisms, leading to the challenge in achieving high yields of desired products. In recent years, the iterative “design-build-test-learn” cycle of the synthetic biology has enabled rapid design and generation of highly diverse libraries (Jarboe 2018). However, screening for desirable variants remains a critical bottleneck, underlining the need for high-throughput approaches to easily identify candidate phenotypes (Cheng et al. 2018).

Ubiquitous in nature, aTFs play a vital role in response to environmental stimuli to regulate gene expression. The effector binding domain (EBD) of an aTF undergoes an allosteric conformational change induced by effector molecules, enabling its DNA binding domain (DBD) to bind or release from a specific promoter (Riaño-Pachón et al. 2007), which either activates or represses gene transcription (Hijum et al. 2009; Browning and Busby 2016). Based on this exquisite effector selectivity, aTFs have been leveraged as biosensor to detect specific compounds, or a series of compounds with structural similarity, and initiate downstream gene expression, which allows the programmable

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high-throughput screening, dynamic metabolic flux control, adaptive evolution, and selection of enzyme mutants, host strains and intracellular metabolite titer (Gupta et al. 2017; Doong et al. 2018; Chou and Keasling 2013; Li et al. 2019).

Biosensors based on aTFs have been successfully utilized in many fields. However, effector scopes of characterized aTFs known in nature are limited, and it is still challenging to develop highly efficient biosensors to detect metabolites out of their sensing repertoire. Therefore, directed evolution is applied to create biosensors for new effectors when no suitable native aTFs are available. Tang et al. engineered the AraC, a L-arabinose sensing part, to improve its specificity to ectoine, mevalonate and triacetic acid lactone by site-saturation mutagenesis on its effector binding pocket (Chen et al. 2015; Tang and Cirino 2011; Tang et al. 2013). Similarly, Dixon, et al. developed a biosensor for aromatic aldehydes, derived from an aTF PcaV for protocatechuic acid. After screening a mutant library of the PcaV effector binding pocket, a biosensor for vanillin was discovered. Mutational analysis revealed that the key mutations M113S/N114A were responsible for its gained specificity to vanillin, while I110V maintained its stable DNA binding (Machado et al. 2019). However, such straightforward aTF engineering could be much complicated if the crystal structure of a target aTF was unavailable. Ohnishi et al. built an XylS library using random mutagenesis. Through efficient selection of the library, they discovered variants with their specificity switched from *m*-toluic acid to *p*-toluic acid. Mutation analysis showed that amino acids out of its binding pocket could also contribute to the alterations of its ligand specificity (Ogawa et al. 2019).

PobR, a member of the *iclR* superfamily, is an aTF in *Acinetobacter* and consists of 271 amino acids. Its N- and C-terminal domains are responsible for DNA binding and ligand recognition, respectively. In the *Acinetobacter* sp. strain ADP1, PobR can activate the *pobA* promoter (P_{pobA}) involved in the 4-hydroxybenzoic acid (4HB) metabolism (Reyes-Ramirez et al. 2002). The *pobA* gene expression could be enhanced up to 400-fold in response to low concentrations of 4HB, while *pobA* did not express in a *pobR*-deficient host (Molina-Henares et al. 2006). Protein engineering was used to expand the ligand specificity of PobR. Jha et al. utilized structure-based rational mutagenesis combined with flow cytometry to screen for PobR mutants responsive to 3,4-dihydroxy benzoate and *p*-nitrophenol (Jha et al. 2015, 2016). In recent study, they also established a high-throughput screening method to alleviate 4HB-mediated inhibition of chorismate pyruvate-lyase (UbiC), enzyme that converts chorismate to 4HB and pyruvate. By expressing *ubiC* and *gfp* driven by the P_{pobA} , a positive feedback gene circuit was built. The UbiC variants with reduced 4HB inhibition could produce increased yields of 4HB that further activated the P_{pobA} , leading to

enhanced *ubiC* and *gfp* expression (Jha et al. 2019). These studies revealed the broad applications of the PobR- P_{pobA} in metabolite detection.

4-Hydroxymandelic acid (HMA) is an aromatic compound widely used in the pharmaceutical industry. It is a precursor of a selective β_1 -receptor antagonist atenolol and several nonribosomal peptide antibiotics (Hubbard et al. 2000). Polyhydroxy mandelic acid amide, a derivative of HMA, exhibits extremely antioxidant capacity (Ley and Bertram 2001). Due to the ability of conjugating cytotoxic chemicals and enzymic substrates, HMA also possesses an ideal prospect in targeted drug delivery systems (Gopin et al. 2003). Besides, ethyl vanillin, a flavor in foods, beverages and cosmetics, is also synthesized from HMA (Pan et al. 2013). Currently, HMA is produced by a chemical approach through the condensation of glyoxylic acid and phenol. However, this process has critical disadvantages. First, as an intermediate product in the phenol synthesis, cumene hydrogen peroxide (CHP) can explode at temperatures over 130 °C, posing a great safety hazard. Second, the reaction with sodium hydroxide is very environmentally unfriendly. Third, as a by-product in the product, 2-hydroxymannic acid (or 2-hydroxymandelic acid) is difficult to be separated from HMA (Bigi et al. 1993). Therefore, biosynthesis approach has drawn much attention in the HMA production. Recently, notable advances have been achieved in microbial production of HMA using *E. coli* and yeast. Its biosynthesis yield could be improved by introducing the HMA synthetase (HmaS) that converts 4-hydroxyphenylpyruvate (HPP) to HMA. Through enhancing the shikimate pathway, HMA production could reach 15.8 g/L and 3 g/L in *E. coli* and yeast, respectively (Li et al. 2016; Reifnath and Boles 2018).

In this study, we developed an aTF-based biosensor for HMA detection through directed evolution. A PobR random mutagenesis library was built, two reporters, enhanced green fluorescent protein gene (*eGFP*) and chloramphenicol resistance gene (*cmr*) were used to screen for mutants that could respond to HMA. Two mutants showed a response to HMA. Mutations in these two mutants were located in amino acid 122 to 126. We further characterized the obtained mutants, and evaluated the contribution of each altered amino acid in the mutants. Structural simulation showed that amino acid 122, 124, 125 and 126 may affect the amino acid in the binding pocket, thus indirectly changing the responsiveness of PobR to the ligand.

Materials and methods

Bacterial strains, media, chemicals and other materials

The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. *E. coli* DH5 α was used for plasmid construction and propagation. *E. coli* bacteria were cultured in the Luria–Bertani (LB) medium in a shaking incubator at 37 °C and 200 rpm. Each liter of LB liquid medium contained 10 g tryptone, 5 g yeast extract and 10 g NaCl dissolved in deionized water. Each liter of LB solid medium contained 15 g agar in the LB liquid medium. Regular antibiotic selection concentrations were 50 mg/L and 34 mg/L for both ampicillin and chloramphenicol. Primers used in plasmid construction and site-directed mutagenesis of *pobR* were synthesized by Ruibiotech (Harbin, China) are listed in Supplementary Table 2. All the chemicals, including HMA and other aromatic compounds, were purchased from Aladdin.

Construction of the pYB1a-PobR-eGFP-Cmr plasmid

The pYB1a-PobR-eGFP-Cmr contains the PobR coding sequence (CDS), and an engineered operon consisting of a Promoter P_{pobA} and two CDS, enhanced green fluorescent protein gene (*eGFP*) and the chloramphenicol resistance gene (*cmr*). PobR coding sequence and P_{pobA} were synthesized by Genescript. PobR coding sequence was codon optimized based on *E. coli* preference while P_{pobA} was synthesis based on its original sequence in *Acinetobacter baylyi*. The synthesized PobR CDS was amplified by PCR using the primers PobR-Gibson-F and PobR-Gibson-R. A DNA fragment containing the ampicillin resistance gene and the P15A *ori* was also amplified using the primers pYB1a-Gibson-F and pYB1a-Gibson-R with the pYB1a-eGFP plasmid as a template. The two fragments were mixed in the presence of the Exnase Multis (vazyme), followed by transformation into *E. coli* DH5 α competent cells. The generated pYB1a-PobR-eGFP was digested by XhoI and BglII, and then mixed with the XhoI-BglII digested PCR products amplified by Cmr-F and Cmr-R primers using the Cmr CDS as a template. After the ligation reaction of the DNA fragments and the transformation into *E. coli*, the pYB1a-PobR-eGFP-Cmr was constructed.

Design and construction of the PobR mutant library

To generate PobR mutants, we developed a library through random mutagenesis of PobR using error-prone PCR amplifications. Primers PobR-mut-F and PobR-mut-R covering

the PobR CDS were used with pYB1a-PobR-eGFP-Cmr as a template. The purified PCR products containing various *pobR* mutants were used as the megaprimers in the following Megawhop PCR (Miyazaki and Takenouchi 2002) to amplify the whole plasmid using pYB1a-PobR-eGFP-Cmr as template. Then DpnI was used to digest PCR templates, and the nicks in product DNA after Megawhop PCR were sealed using T4 DNA ligase. The generated library with highly random *pobR* mutations was transformed into *E. coli* DH5 α to obtain transformants containing mutant plasmids.

Fluorescence assay and screening of the PobR mutant library

The clones of the PobR mutant library were transformed into DH5 α competent cells and cultured overnight in LB agar plates with ampicillin. 639 single colonies were picked from the plates and inoculated into 200 μ L of LB medium with ampicillin in deep-well microplates, followed by culturing in a shaking incubator at 200 rpm and 37 °C for 12 h. Then, 2 μ L of the bacteria from each well was inoculated into 200 μ L of LB medium with ampicillin and 1 g/L of HMA in deep-well microplates, and cultured on a shaking incubator for another 12 h. Finally, 100 μ L of the cultured bacteria from each well was collected to measure the optical density at 600 nm (OD₆₀₀) and green fluorescence (with 430 nm as the excitation wavelength and 510 nm as the emission wavelength).

Response to HMA analogs

The screened mutants were inoculated in 200 μ L of LB medium with ampicillin for 12 h at 37 °C. 2 μ L of each clone was added to 200 μ L of LB medium with ampicillin and 1 g/L of HMA, or each of its analogs, in deep-well microplate, and cultured for 12 h at 37 °C in a shaking incubator. Then, 100 μ L of the cultured bacteria was collected to determine green fluorescence intensity.

Site-directed mutagenesis of the PobR CDS

Five PobR mutants with single amino acid mutations (E16V, L122P, E124G, H125R, E126V) were generated by the inverse PCR (Liu and Naismith 2008) using corresponding primer pairs with the mutations present in the forward primers. Template DNAs were eliminated by DpnI digestion before transformation.

Modeling and docking

As no crystal structure of the PobR protein was available, the AlphaFold2 was employed to simulate the structure of the PobR monomer (Jumper et al. 2021). Five predictive

models were generated by the AlphaFold2 based on the confidence data, and the predicted structure with the highest score (pLDDT=91.4) was selected for the subsequent molecular docking. For this purpose, structure files of the ligand 4HB were obtained from the organic small molecule database Pubchem (<https://pubchem.ncbi.nlm.nih.gov/>). Autodock Vina was utilized as the docking engine (Trott and Olson 2010). Vina search space coordinates were set as center_x = - 0.83, center_y = - 1.292, center_z = 7.229. Dimensions of the search space were set as size_x = 33.75, size_y = 37.5, size_z = 37.5, and exhaustiveness was set at 10. The docking results listed the top 9 conformational conditions in a score based on the lowest binding energy (- 6.0 kcal/mol). Remarkably, 4HB could bind to the same pocket of the predicted protein receptor in all first four conformations, despite difference in docking postures. The interaction between the small molecule and predicted protein receptors was examined by Ligplus (Laskowski and Swindells 2011). The final three-dimensional schematic was portrayed using PyMol Version 2.2.0.

Statistical analysis

All data were derived from at least three independent experiments. Results were presented as a mean with either standard deviation (SD) or standard error of mean (SEM), and sample numbers are indicated unless otherwise noted in the figure legends. Statistical significance calculations comparing two conditions were performed using a two-tailed unpaired Student's t-test. The criterion of statistical significance level was denoted as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Results

Characterization of ligand responses of wild type Pobr

No suitable aTF has been reported to respond to HMA. However, HMA has high structural similarity to 4HB in both core structure (a hydroxyphenyl) and side chains. Therefore, we strategized to start with the 4HB-responsive aTF, Pobr, and make it directionally evolve to a potential aTF of HMA. The Pobr coding sequence (CDS) from *Acinetobacter baylyi* was synthesized based on *E. coli* codon preference. The wild type Pobr (Pobr^{WT}) activate P_{pobA} by binding to cis-acting element (Oi) (sequence was showed in Supplementary Fig. S1). The Oi and P_{pobA} were synthesized based on sequence in *Acinetobacter baylyi*. Two reporters *eGFP* and *cmr* were under the control of P_{pobA} (Fig. 1A). The generated plasmid was named as pYB1a-Pobr-eGFP-Cmr. Therefore, reporters would be expressed if the Pobr docked with ligand and

then activated P_{pobA}. Therefore, the screening by GFP signal and chloramphenicol resistance could be used to identify HMA-responsive aTFs for the P_{pobA} promoter among Pobr mutants.

The bacteria harboring pYB1a-Pobr-eGFP-Cmr were named as Pobr^{Sen} and were cultured with increasing concentrations of 4HB. The *eGFP* reporter could be activated by 4HB at a concentration as low as 0.01 g/L, and reached the highest level at 0.06 g/L of 4HB (Fig. 1B). We repeated this experiment using additional aromatic compounds with structures similar to 4HB, including HMA, mandelic acid (MA), hydroxyphenylpyruvate (HPP), phenylpyruvate (PPA), tryptophan (Trp) and phenylalanine (Phe) (Fig. 1C and D). Most compounds, including HMA, lacked the ability to promote Pobr^{WT}-mediated reporter expression, although their concentrations were increased to 1 g/L (Fig. 1C). The results indicated that the Pobr^{WT} is an aTF with both high sensitivity and decent specificity to 4HB, and its effector binding domain (EBD) does not recognize the other tested aromatic compounds, including HMA.

Creation a Pobr mutant library

We used error-prone PCR to obtain the Pobr CDS mutants and Megawhop PCR to construct the CDS mutants to the plasmids. 5.5 µg PCR products were obtained totally after Megawhop PCR and then sealed using T4 DNA ligase. Then, these products were transformed into *E. coli* DH5α and nearly 5.5×10^5 transformants were obtained (estimated as 1 ng PCR produce 55 clones showed in Supplementary Fig. S2, and totally 5.5 µg PCR products were obtained). Ten clones were randomly picked to sequence their Pobr CDS regions, which revealed diverse mutations with an average mutation rate of about 0.3%.

Screening for HMA-responsive Pobr mutants

Our screening system was based on chloramphenicol resistance. If HMA could bind to Pobr mutant and activated P_{pobA}, the strain would be able to survive on a plate with chloramphenicol. However, this activation by HMA with Pobr mutant might not be as strong as 4HB did to Pobr^{WT}. Therefore, we intentionally reduced the chloramphenicol level from its regular concentration of 34 µg/L to 20 µg/L. Our data demonstrated that 20 µg/L chloramphenicol could efficiently suppress the growth of the bacteria in the absence of functional inducer 4HB (Fig. 2, right panels). Only when the P_{pobA} was activated by 0.1 g/L 4HB, the strain could survive at 20 µg/L chloramphenicol, similar to the bacteria cultured without chloramphenicol and 4HB (Fig. 2, middle and left panels). Thus, coupling *pobR* gene transcriptional activity with chloramphenicol resistance could be used as a simple growth selection system to efficiently screen for

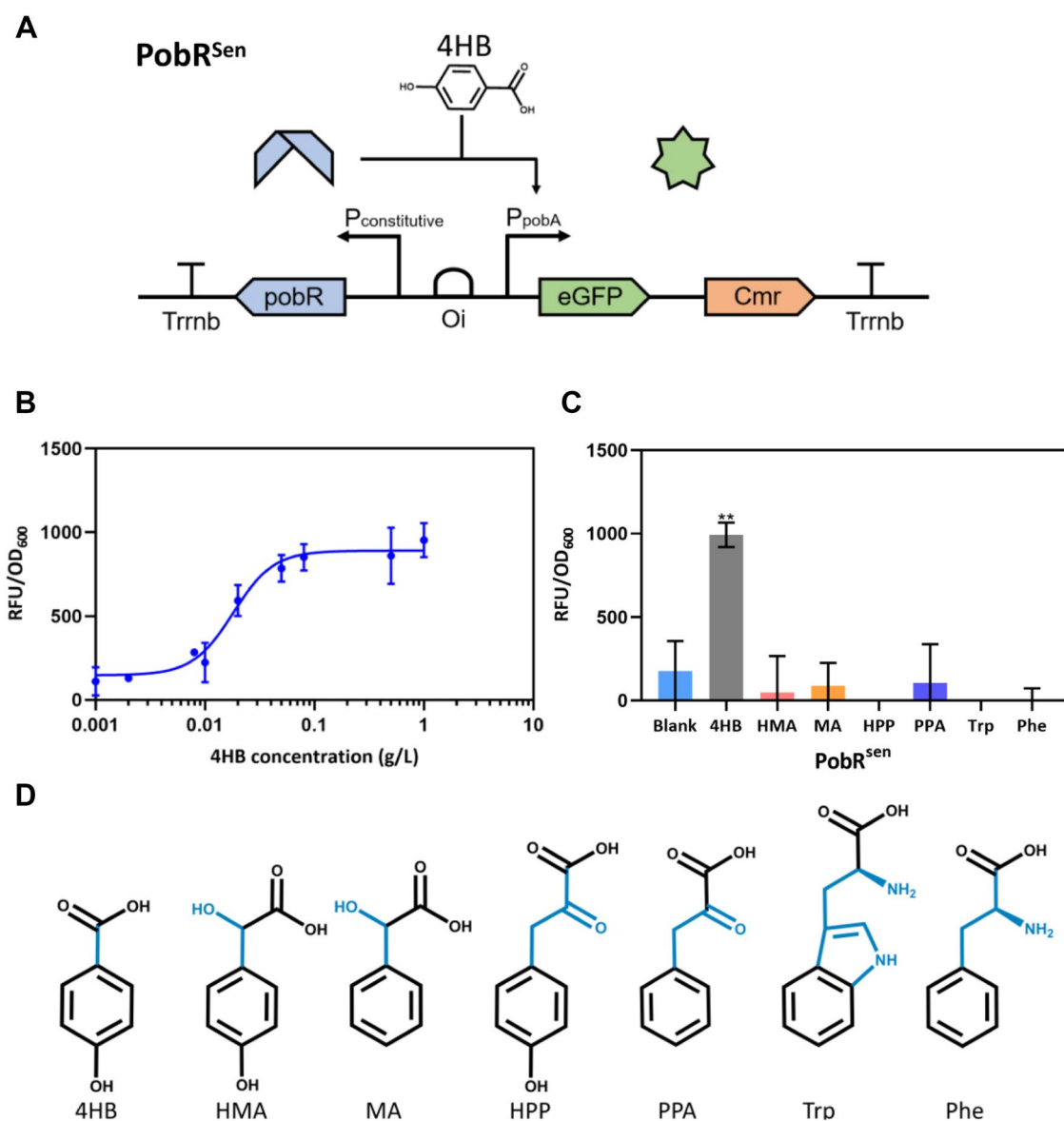


Fig. 1 **A** Schematic diagram of the biosensor to detect 4HB. **B** Dose response curves of the 4HB biosensor. Strain was treated with 0 to 0.6 g/L 4HB. **C** Responses of the biosensor treated by 4HB analogs. **D** Structures of 4HB analogs

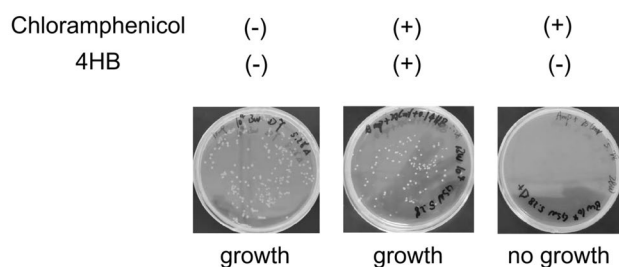
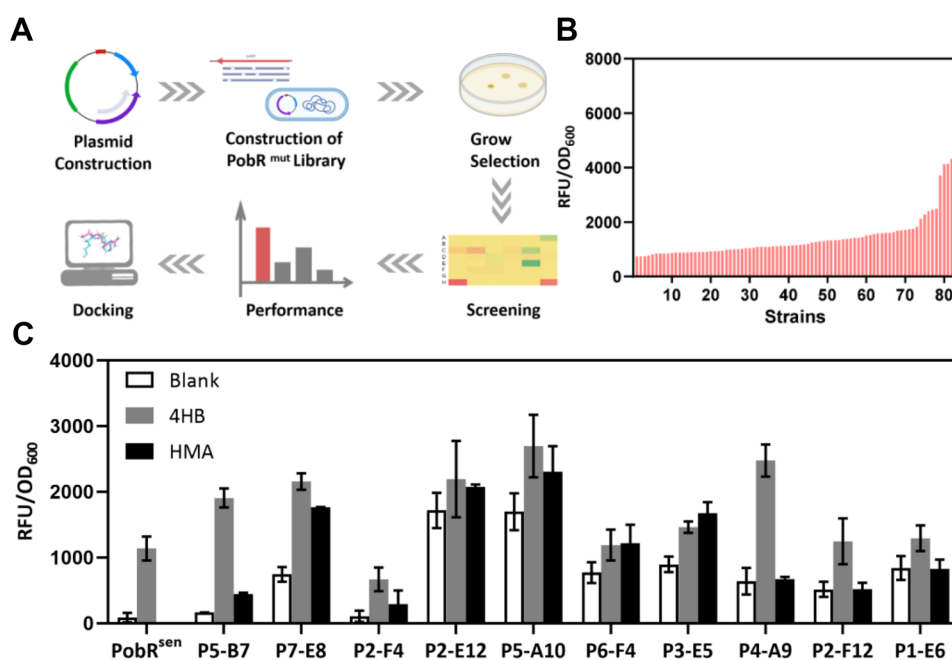


Fig. 2 The growth of strain DH5 α /pYB1a-PobR-eGFP-Cmr on the plate when treat with 0.1 g/L 4HB and 20 μ g/L chloramphenicol

HMA-responsive allosteric PobR mutants from our random mutagenesis library.

Then the PobR mutant library was cultured on LB-agar plates containing 20 μ g/L chloramphenicol and 1 g/L HMA. The majority of the variants in the PobR mutant library were nonfunctional (irresponsive to HMA), and only 639 clones survived in the chloramphenicol selection. These 639 PobR clones on the LB-agar plates were individually inoculated in 96-well plates supplemented with 1 g/L HMA. After 12 h of cultivation, both optical density at 600 nm (OD₆₀₀) and GFP signal were measured, and 83 clones with GFP/OD₆₀₀ ratios over 800 were selected for further characterization (Fig. 3A). Among them, 10 clones with relatively high GFP/OD₆₀₀

Fig. 3 The screening of the random PobR mutagenesis library. **A** The screening procedure of the PobR library. **B** Identification of 83 PobR mutants with their fluorescence intensity higher than 800 in response to HMA treatment. **C** The top ten PobR mutants with highest fluorescence signal when treated by 4HB and HMA



were tested for their response to 0.1 g/L of 4HB, the native allosteric inducer of PobR^{WT}. Compared to PobR^{WT}, several mutants, such as P7-E8 and P4-A9, exhibited increased GFP signal in the absence of any inducer, but could still be further activated by 4HB. However, the mutants P5-A10, P2-E12 and P1-E6 showed constitutive GFP expression irrespective of the presence or absence of the inducers. High background activation of these mutants helped them survive in chloramphenicol screening. Interestingly, we identified mutants P5-B7 and P7-E8 that exhibited significant activation in response of HMA (Fig. 3C). Therefore, these two mutants were selected for further studies.

Ligand specificity and detection range of PobR mutants

To determine the ligand responsiveness of the PobR mutants, the bacteria harboring the P5-B7 and P7-E8 plasmids were cultured in LB media containing HMA of concentrations from 0.01 to 4 g/L. Due to low water solubility, HMA concentration above 4 g/L was not tested. After 12 h culturing, both OD₆₀₀ and GFP signal were measured. P7-E8 clone showed higher background than P5-B7. At 2.0–3.5 g/L of HMA, the P5-B7 and P7-E8 biosensors exhibited over 3 or 2 times of maximal induction compared to the bacteria without HMA treatment (Fig. 4A and B). Between the two clones, P5-B7 displayed a lower dynamic range with its maximal induction of 840 at 3.5 g/L of HMA; the saturated detection limit of P7-E8 was about 2 g/L of HMA, indicating its higher sensitivity to HMA in a 0.05 g/L detection range (Fig. 4A and B).

To assess their specificity in detecting different aromatic compounds, *E. coli* harboring P5-B7 and P7-E8 plasmids were tested in LB medium supplemented by aromatic molecules shown in Fig. 1D. After 12 h of culturing, we observed that both 4HB and HMA could steadily activate the GFP reporter. In 1 g/L of HMA, the P5-B7 and P7-E8 mutants displayed over 2.7- and 1.7-fold induction, respectively. In addition, HPP could also trigger the eGFP expression of both mutants. Interestingly, P7-E8, but not P5-B7, could respond to PPA, which highly resembles HPP. Overall, the two selected PobR mutants had relatively low ligand specificity compared to the PobR^{WT}.

Through DNA sequence analyses, we determined that P5-B7 had substitutions at three amino acids (E16V, E124G and H125R), and P7-E8 had changes at two (L122P, E126V). Based on these mutations, we predict that the residues from 122 to 126 of the PobR protein are likely involved in ligand binding specificity.

Functional evaluation of individual mutations in PobR

To determine how each amino acid among the mutated residues in P5-B7 and P7-E8 contributed to the ligand selection, we generated five single amino acid mutants E16V, E124G, H125R, L122P and E126V of PobR, using site-directed mutagenesis, and tested them using our reporter system. Among these five mutants, only E126V showed slight induction in response to HMA, but all others did not, or even displayed decreases (Fig. 5A). All five mutants retained

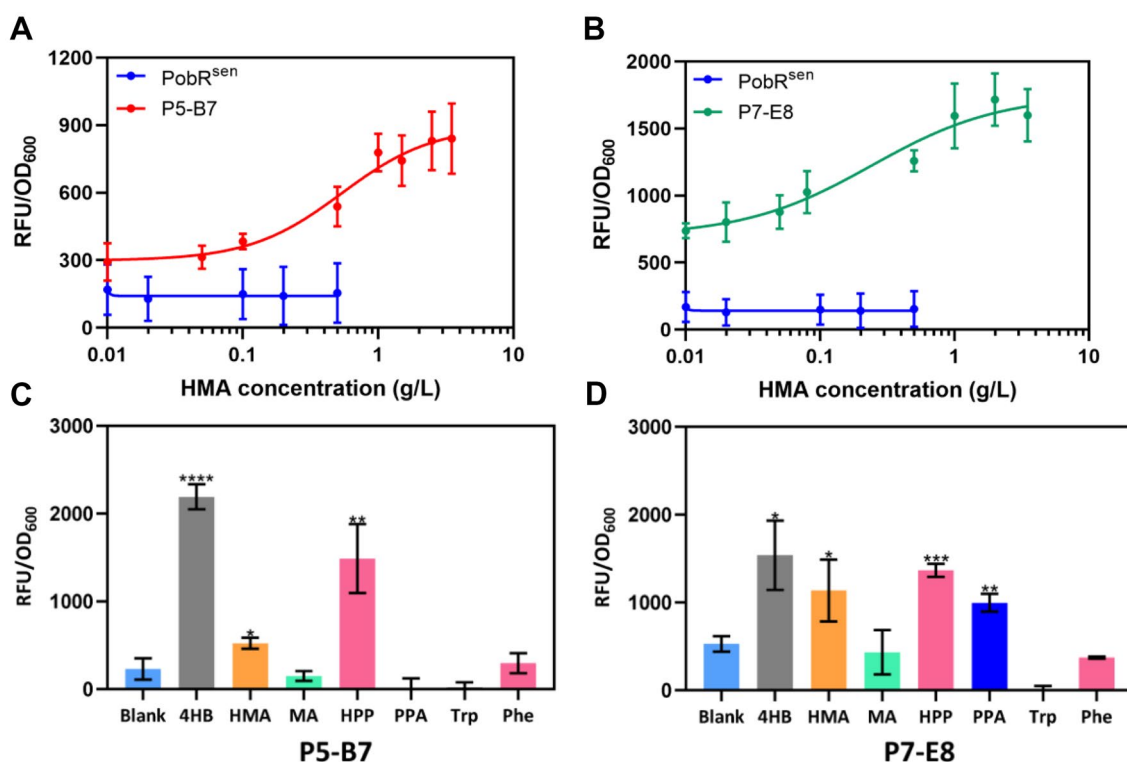


Fig. 4 Ligand sensitivity and specificity of the two selected mutants. **A** and **B** Analyses of fluorescence changes of PobR^{Sen} and P5-B7 (**A**) or P7-E8 (**B**) in response to HMA treatment. **C** and **D** Fluorescence

changes of the P5-B7 (**C**) and P7-E8 (**D**) to the treatments of different aromatic compounds with structural similarity to HMA

responses to 4HB, while E126V was better than the rest on this aspect.

Importantly, all five single amino acid mutants showed decreased response to HMA, compared to the original mutants P5-B7 and P7-E8, and reduced induction by 4HB compared to P5-B7 (Fig. 5A). Based on these data, we concluded that the synergy or collaboration of multiple amino acid mutations, especially by L122P, E126V, E124G and H125R, could alter or extend the ligand binding capacity of the PobR protein towards a direction of increased response to HMA-mediated allosteric induction.

Modeling and docking

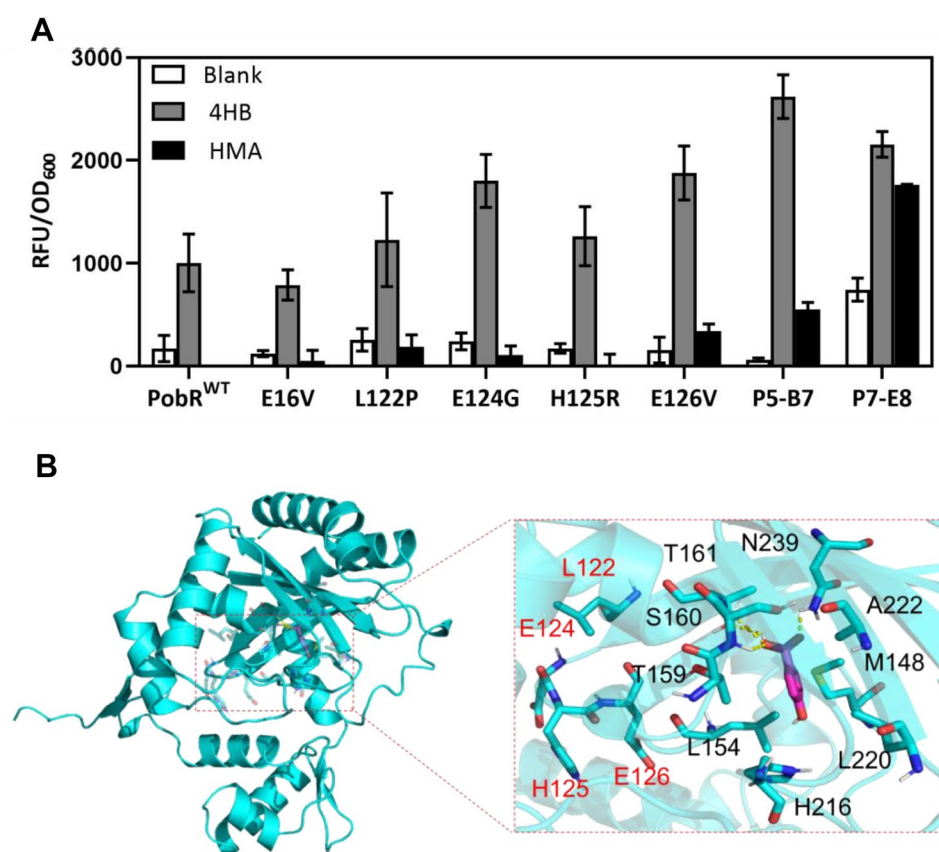
To better understand the effects of amino acid substitutions on the response of the PobR protein, the AlphaFold2 was employed to simulate the protein structures of the PobR mutants. Autodock Vina was used to dock the 4HB and HMA, to the proteins. Interactions between the small molecule ligands and proteins, as a result of the docking, were visualized by LigPlus (Laskowski and Swindells 2011).

In the docking of PobR^{WT} to its natural inducer 4HB, we selected the second-best binding configuration with binding energy of -6.0 kcal/mol for subsequent analysis,

although the first-best conformation displayed the lowest binding energy (-6.2 kcal/mol). The reason was that the second-best one showed the most consistency of its ligand binding posture to a previously reported conformation (Jha et al. 2016). In this conformation, the carboxylic acid group of 4HB forms hydrogen bonds with S160, T161 and N239, and the aromatic ring of the ligand makes hydrophobic interaction with T159, M148, H216, L220 and A222 (Supplementary Fig. S3).

From a spatial point of view, the amino acids corresponding to the mutated sites of P5-B7 (E16V, E124G, H125R) and P7-E8 (L122P, E126V) are located in the vicinity of the proposed effector binding site. However, the influence of these sites on ligand-receptor binding was not recognized by LigPlus (Fig. 5B). It is possible that mutations of residues 122, 124, 125 and 126 affect the key sites in the binding pocket, thus indirectly changing the responsiveness of PobR to the ligand. The scenarios that substitutions of the residues without direct contact to the ligands could impact the overall response specificity of the proteins have been previously reported (Scholz et al. 2003). Nevertheless, the contributions of the residues in the vicinity of the binding pocket to the allosteric changes of the proteins deserve further attention.

Fig. 5 Characterization of single amino acid PobR mutants and prediction of 4HB docking to the PobR protein. **A** Fluorescence changes of the five PobR mutants with single amino acid alterations in comparison with the PobR^{WT} and two multi-site mutants (P5-B7 and P7-E8) when treated by 4HB (0.1 g/L) and HMA (1 g/L). **B** Predicted 4HB docking to the PobR protein. Ribbon structure representation of the PobR^{WT} protein predicted by Autodock Vina is shown left. The enlarged docking site of 4HB (Lightmagenta) is shown right with exhibition of key amino acid residues



Discussion

Recently, some ingenious selection methods have been developed for aTF directed evolution to alter its compound binding specificity (Flachbart et al. 2021; Jia et al. 2020). PobR is an aTF responding to 4HB which has been engineered to biosensor for other aromatic compounds. Previous studies employed homology modeling and ligand docking approaches to identify PobR's ligand binding pocket. Through conservative mutations in the binding pocket, the authors successfully altered PobR's effector specificity to 3,4-dihydroxy benzoate and p-nitrophenol (Jha et al. 2015, 2016). As 4HB and HMA shared a high structural similarity, HMA-responsive PobR mutants could be possibly discovered by screening a random PobR mutant library using a rational and convenient reporter system. Therefore, these conceptions inspired us to develop HMA-responsive aTFs through screening a PobR mutant library.

Antibiotic treatment is considered as an easy and economic method to enrich required variants with limited laborious efforts. When combined with quantitative selection approaches, such as bioluminescence and fluorescence assays, highly efficient systems can be established to screen for desirable clones (Ogawa et al. 2019; Jia et al. 2020; Leavitt et al. 2017). In this study, we used the combination of

eGFP and *cmr* regulated by the promoter P_{pobA} activated by the PobR^{WT} or its mutants to screen for HMA responsive aTFs. In this system, if HMA could bind a PobR mutant and allosterically trigger its transcriptional activity on the promoter, the bacteria would survive on a plate supplied with both chloramphenicol and HMA. Therefore, our reporter system can greatly facilitate the screening process to obtain HMA-responsive PobR mutants.

Previous studies suggested that the 118–135 residues of PobR constituted a conserved domain. Thus, alterations in this region reduced the 4HB dependence of PobR, but allowed the binding of other effector molecules (Kok et al. 1998). The single amino acid substitutions of E124 and E126, which have also been identified in the P5-B7 and P7-E8 mutants, respectively, of the current study, could generate PobR mutants responsive to quinone acid or protocatechuic acid, respectively (Kok et al. 1998).

Our studies revealed high basal *eGFP* expression of the P7-E8 clone, and low ligand specificity of both P5-B7 and P7-E8 clones (Fig. 4C and D). These could be due to the attenuated allostery and/or distorted ligand binding pocket caused by the multiple-site mutations. The P7-E8 clone showed decent activation in response to PPA and HPP, which are also key compounds in aromatic amino acid synthesis (Li et al. 2020). To the best of our knowledge,

no aTF for PPA and HPP has been reported. Therefore, the P7-E8 clone may be potentially used in the directed evolution of aromatic amino acids and their derivatives.

By regulating an actuator such as a growth-essential protein or fluorescent protein, aTFs-based biosensor was widely applied as a high throughput screening method in enzyme directed evolution, dynamic control of gene expression and adaptive laboratory evolution. However, there are a limited number of characterized aTFs known in nature (Godara and Kao 2020; Snoek et al. 2020). In this study, we exhibited the strategy of directed evolution to tailor the ligand selectivity of aTFs continued to provide biosensors with extended detection capabilities.

Overall, we succeeded in generating HMA-responsive biosensors using directed evolution approaches, and laid the foundation for the high yield biosynthesis of HMA. Methods to improve their sensitivity and specificity will be further investigated.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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