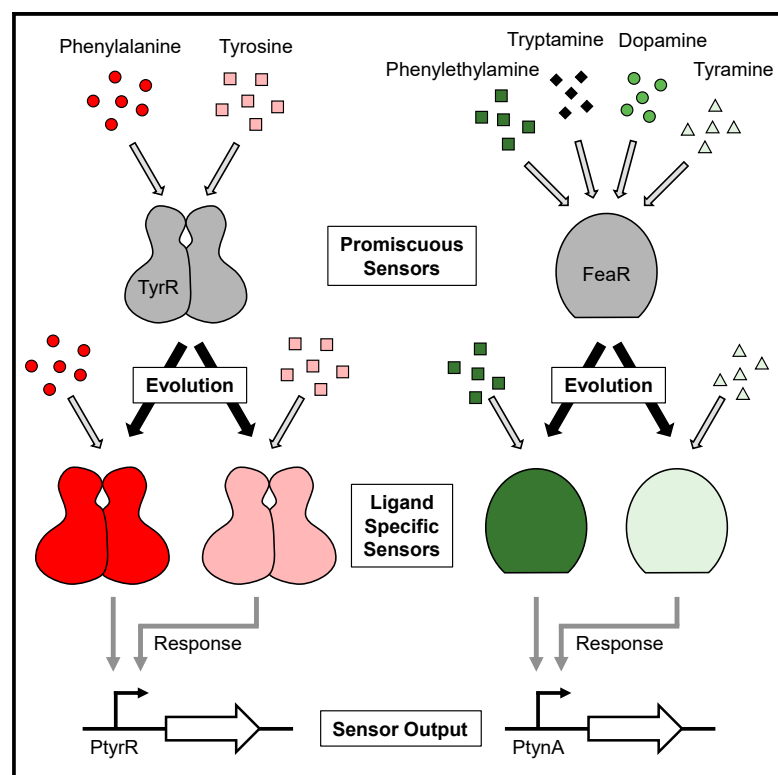


Engineering ligand-specific biosensors for aromatic amino acids and neurochemicals

Graphical abstract



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In brief

This work represents a considerable achievement in protein engineering and includes the ligand-specific sensors for aromatic amino acids such as phenylalanine and tyrosine as well as neurochemicals such as phenylethylamine and tyramine, which are structurally similar and have been implicated in a variety of medical conditions. It lays the groundwork for developing medically relevant probiotic and wearable sensors with high specificity, which is critical to differentiate metabolites with divergent functions and create smart probiotics and cell-free biosensors for accurate diagnostics.

Highlights

- Created specific sensors for structurally similar amino acids and neurochemicals
- Developed a protein engineering method requiring only a basic structural understanding
- Provided insights into the otherwise uncharacterized structure of a transcription factor



Article

Engineering ligand-specific biosensors for aromatic amino acids and neurochemicals

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SUMMARY

Microbial biosensors have diverse applications in metabolic engineering and medicine. Specific and accurate quantification of chemical concentrations allows for adaptive regulation of enzymatic pathways and temporally precise expression of diagnostic reporters. Although biosensors should differentiate structurally similar ligands with distinct biological functions, such specific sensors are rarely found in nature and challenging to create. Using *E. coli* Nissle 1917, a generally regarded as safe microbe, we characterized two biosensor systems that promiscuously recognize aromatic amino acids or neurochemicals. To improve the sensors' selectivity and sensitivity, we applied rational protein engineering by identifying and mutagenizing amino acid residues and successfully demonstrated the ligand-specific biosensors for phenylalanine, tyrosine, phenylethylamine, and tyramine. Additionally, our approach revealed insights into the uncharacterized structure of the FeaR regulator, including critical residues in ligand binding. These results lay the groundwork for developing kinetically adaptive microbes for diverse applications. A record of this paper's transparent peer review process is included in the supplemental information.

INTRODUCTION

Microbial biosensors can be utilized to kinetically regulate and quantify the products of metabolic pathways, diagnose diseases *in vivo* in probiotics, and analyze *ex vivo* samples through wearable, paper-based, and cell-free systems. Synthetic biological sensors can be created using several protein design approaches (Jones et al., 2017). However, sensors can also be obtained more efficiently by mining sensors that naturally exist in microbes (DeLorenzo et al., 2017; Hoynes-O'Connor et al., 2017). These sensors can often be directly transferred into probiotic organisms or purified for cell-free sensor applications. However, most natural sensors recognize multiple structurally similar, but functionally diverse, metabolites often found in close proximity. To accurately correlate the concentration of a chemical with biological outcomes, sensors need to differentiate between different chemicals and recognize the relevant chemical with the precise sensitivity.

Specific sensing is critical for many microbial sensor applications. A variety of protein engineering methods have been demonstrated for optimizing the selectivity and sensitivity of protein and RNA sensors. These approaches include directed evolution (Ellefson et al., 2018; Higgins et al., 2017; Meyer et al., 2019; Stainbrook and Tyo, 2019), rational design (Belsare et al., 2017), and computational *de novo* design (Taylor et al., 2016). Although each method has advantages, rational engineer-

ing can uniquely be performed using both basic knowledge of the protein structure and small library sizes, allowing for rapid construction and screening of libraries. In addition, when the structure of the protein is unknown, conserved or essential residues can also be identified through computational simulations or by aligning the sequence of the sensor with other proteins in the same family (Zvelebil et al., 1987). Despite advances in protein engineering, creating ligand-specific sense-and-respond systems remains challenging due to the need to couple subtle protein conformational changes with differential protein-DNA interactions and gene expression control (Glasgow et al., 2019; Peveler et al., 2016; Taylor et al., 2016), especially when the target ligands are structurally similar.

The aromatic amino acids phenylalanine (Phe) and tyrosine (Tyr) are common microbial metabolic engineering products and precursors derived from the same pathway (Rodriguez et al., 2014). In addition, chronically elevated levels of structurally similar Phe and Tyr are associated with the distinct disorders phenylketonuria and type 2 tyrosinemia, respectively (Table S1) (Camp et al., 2014; Cerone et al., 2002; Scriver, 2007). Sensors for both metabolites have been generated in different *E. coli* strains (Lin et al., 2018; Mahr et al., 2016). However, the sensors are based on the wild-type version of the multi-ligand responsive TyrR transcription factor (TF) from *E. coli* and have limited selectivity and low dynamic ranges. Similarly, the structurally similar amines phenylethylamine (PEA), tyramine (Tyra), and tryptamine

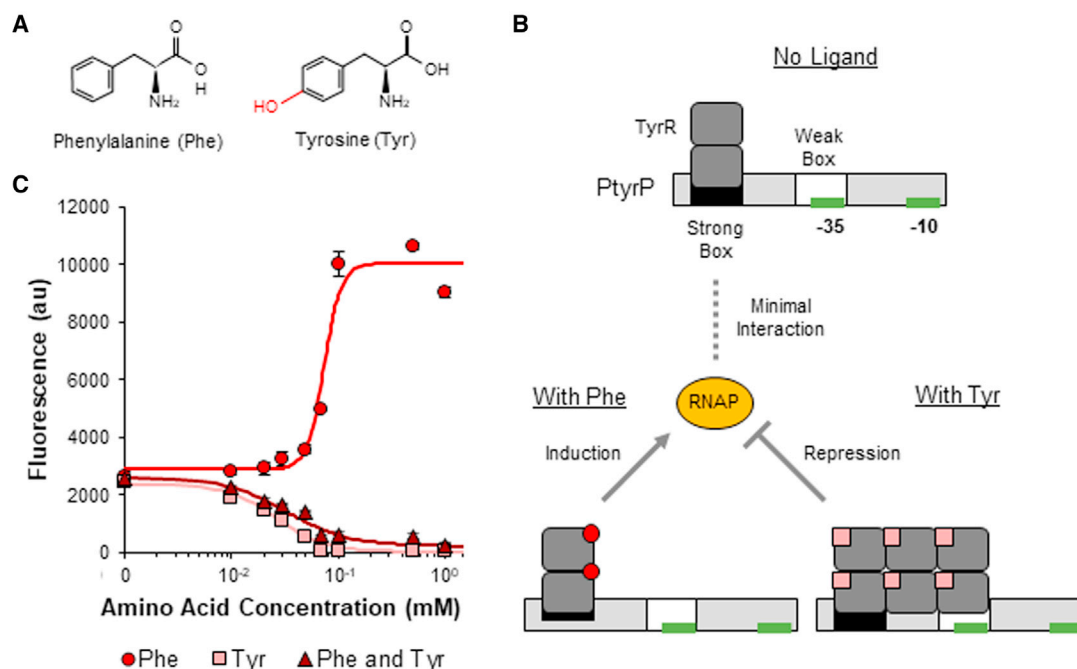


Figure 1. Phenylalanine- and tyrosine-dependent regulation of the PtryP promoter by TyrR is tyrosine-dominant

(A) Structures of the two TyrR ligands phenylalanine (Phe) and tyrosine (Tyr). Structural differences between ligands are shown in red.

(B) Schematic of how TyrR regulates the PtryP promoter in a Phe- and Tyr-dependent manner.

(C) Transfer curves for PtryP with plasmid-based overexpression of wild-type TyrR in response to Phe, Tyr, and the specified concentrations of both Phe and Tyr. Points represent experimental data while lines represent the fitted curves (Table S2). Values and error bars are the average and standard deviation of biological triplicate, respectively. See also Figure S1.

(Trypta) are all commonly found in food and in the gut (Bhattarai et al., 2018; Chen et al., 2019; Marcobal et al., 2012; Shalaby, 1996) but contribute to distinct biological outcomes (Table S1). For example, extreme levels of PEA have been associated with a variety of psychological disorders (Irsfeld et al., 2013; Myojin et al., 1989), the presence of Tyra leads to catecholamine release and an increase in blood pressure (Bianchetti et al., 1982; Rafehi et al., 2019), and the presence of Trypta causes serotonin release and the stimulation of gastrointestinal motility (Takaki et al., 1985). Additionally, the presence of PEA, Tyra, and Trypta in food are indicators of microbial contamination (Marcobal et al., 2012), and eating foods with high levels of Tyra can lead to poisoning (Marcobal et al., 2012). Currently, there are no biosensors with high ligand specificity for these chemicals.

In this work, we generate sensors for the aromatic metabolites Phe, Tyr, PEA, and Trypta using *Escherichia coli* Nissle 1917 (EcN) as a host. To generate sensors for these aromatic metabolites, we identify and engineer two sensor systems: the TyrR (Phe and Tyr) sensor and the TynA-FeaR (aromatic amine) sensor system. We engineer the ligand selectivity of the TyrR and TynA-FeaR sensor systems and tune their sensitivity by rationally selecting and individually mutating amino acids in TyrR, TynA, and FeaR. This method of rational protein engineering quickly generates multiple small libraries that can be efficiently screened, making it an attractive approach for specificity engineering. Altogether, we generate the sensors specific for Phe, Tyr, PEA, or Tyra. In engineering FeaR, we also provide insights into the uncharacterized structure of FeaR and identify residues

important for ligand binding. This work provides sensors with diverse applications in microbial biosensing as well as a generalizable approach to modulating the specificity of ligand-protein binding while maintaining protein-DNA interactions and thus downstream gene expression control.

RESULTS

Developing specific phenylalanine and tyrosine sensors

Phe and Tyr are structurally similar metabolites utilized throughout the body for many processes as additives in food and animal feed and as precursors for many chemicals and pharmaceuticals (Figure 1A) (Liu et al., 2019). Notably, two distinct disorders (phenylketonuria and tyrosinemia) cause a buildup of dietary Phe or Tyr (Mitchell et al., 1990; Scriver, 2007), necessitating sensors that can differentiate between the two amino acids. Microbial TyrR regulates its target promoters in both a Phe- and Tyr-dependent manner (Figures 1B and S1A). In the absence of either amino acid, TyrR forms homodimers that bind to high-affinity recognition sites, or strong boxes (Pittard et al., 2005). When bound to Phe, the dimers interact with RNA polymerase to influence transcription initiation. When bound to Tyr, TyrR forms homohexamers that bind to both the strong box and the adjacent low-affinity weak box and interact with RNA polymerase. When the binding sites are located upstream of the -35 site of the promoter, these interactions increase expression of the downstream gene. When the binding sites are overlapping or downstream of the -35 site, TyrR instead

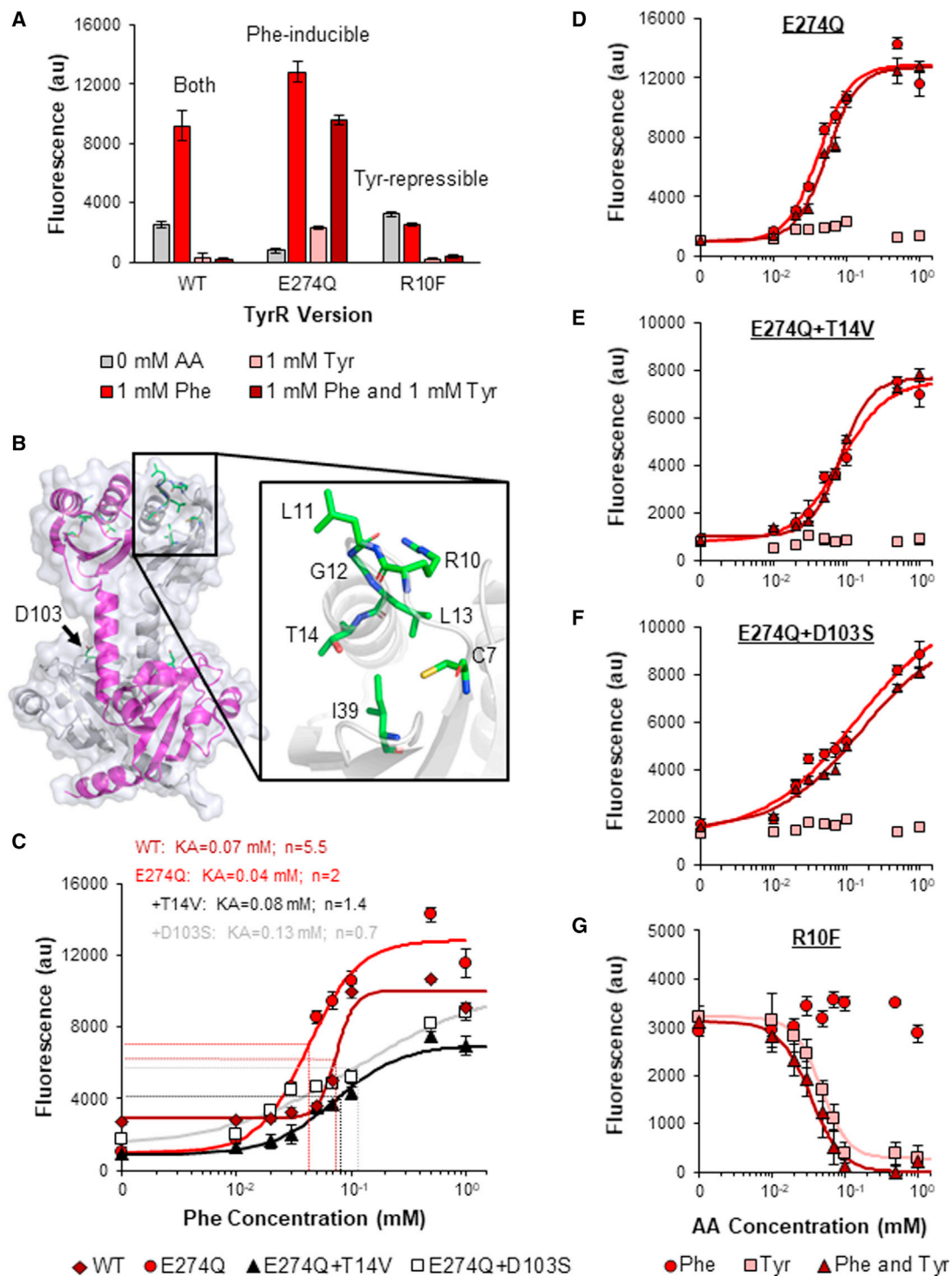


Figure 2. Development of phenylalanine- and tyrosine-specific sensors

(A) Response of the P_{tyrP} promoter to 1 mM phenylalanine (Phe), 1 mM tyrosine (Tyr), and both 1-mM Phe and 1-mM Tyr with plasmid-based overexpression of wild type (WT), E274Q mutant, and R10F mutant TyrR.

(B) Structure of the Phe-binding pocket in the N-terminal regions of the TyrR homodimer. TyrR monomers are in white and magenta. Selected amino acids with known roles in Phe-dependent modulation of RNA polymerase are shown in elemental coloring: carbon atoms are shown in green, nitrogen in blue, oxygen in red, and sulfur in yellow. The structure was obtained from RCSB protein data bank (Reference ID 2JHE), and the image was generated using PyMOL.

(C) Modulation of the Phe-induction threshold for the P_{tyrP} promoter by saturation mutagenesis of the TyrR's Phe-binding pocket. Two TyrR mutants, E274Q+T14V and E274Q+D103S, were obtained with increased K_A values (2-fold and 3-fold higher than the E274Q mutant, respectively).

(legend continued on next page)

represses expression (Pittard et al., 2005). Given the complexity of DNA-TyrR binding and ligand-TyrR binding modes, we decided to take a stepwise approach to develop ligand-specific sensors with the hypothesis that targeted engineering of TyrR would enable its differential ligand binding without affecting DNA-binding interactions.

We first characterized six *E. coli*-native promoters using genomic expression or plasmid-based overexpression of TyrR to identify the best promoter in EcN (Figures S1B and S1C). We selected P_{tyrP} as our sensor as it displayed an activating response to Phe and a repressing response to Tyr, allowing for clear differentiation between the presence of each amino acid, especially with overexpression of TyrR (Figures 1B and 1C). The P_{tyrP} promoter has a strong box upstream of the -35 site and a weak box overlapping the -35 site, allowing TyrR to induce expression in the presence of Phe and repress expression in the presence of Tyr. In addition, P_{tyrP} showed the highest fold induction in the presence of Phe (4-fold) compared with all tested promoters and strong repression in the presence of Tyr (59-fold). Notably, high expression levels of TyrR are required for Tyr-dependent repression of P_{tyrP}, as shown by the lack of repression when only controlled by genomic TyrR expression (Figure S1B). This lack of response gives P_{tyrP} a false appearance of Phe-specificity when paired with low TyrR expression levels. However, we found that TyrR regulated the promoter in a Tyr-dominant manner. In the presence of Tyr and overexpression of TyrR, TyrR repressed P_{tyrP} independent of the presence of Phe (Figure 1C).

The E274Q TyrR mutation was previously shown to prevent TyrR from forming hexamers, which occurs when TyrR is bound to Tyr (Kwok et al., 1995). Since hexamerization is required for Tyr-mediated repression of P_{tyrP}, this mutation should render TyrR nonresponsive to Tyr but maintain Phe-dependent induction. When we inserted the mutation into the plasmid-expressed TyrR, Tyr-dependent repression of P_{tyrP} was mitigated and the Tyr-dominant response of the TF was eliminated (Figure 2A). In addition, the mutant had an improved fold induction in response to Phe (16-fold). This is the highest dynamic range demonstrated by a Phe sensor to date.

We next tuned the Phe-sensitivity of the E274Q mutant to create sensors that better align with variable physiological concentrations and improve the utility of the sensors for therapeutic and diagnostic applications. We selected eight amino acid positions with known roles in TyrR-mediated promoter induction (Figure 2B) (Verger et al., 2007; Yang et al., 1996). Each amino acid has been shown to be important, but potentially non-essential, for Phe binding. When we obtained the transfer curve of the parent E274Q sensor, half-maximal induction was observed at 0.04 mM Phe (Figures 2C and 2D), lower than the Phe concentrations in human intestines, serum, and urine (Table S1, 0.05–0.3 mM) (Adibi and Mercer, 1973; Kouchiwa et al., 2012; Lin et al., 2018). Thus, we performed saturation mutagenesis on each amino acid individually and screened the small, targeted libraries for a reduced sensitivity to Phe (Figure S2). Three variants

displayed reproducibly lower sensitivities: E274Q + T14V, E274Q + D103H, and E274Q + D103S with K_A values of 0.08, 0.11, and 0.13 mM, respectively (Figures 2C and S3). The E274Q + T14V and E274Q + D103S variants were selected for further characterization as they displayed the widest range of sensitivities and maintained the highest fold-inductions. Both mutants displayed consistent sensitivities to Phe in the presence and absence of Tyr and had no apparent response to Tyr alone (Figures 2E and 2F). When we combined both mutations in E274Q TyrR, the response to Phe was eliminated. These Phe-specific sensors have the potential to be applied to kinetically diagnose and treat disorders that cause Phe-dysregulation without interference from intestinal Tyr.

We also engineered TyrR for Tyr specificity. Because TyrR binds to Phe and Tyr using two separate binding pockets, the libraries designed to identify less sensitive Phe sensors were used to identify variants completely devoid of Phe-activity, while maintaining Tyr activity (Figure S4). The most selective mutant, R10F, demonstrated 15-fold repression in the presence of Tyr independent of the presence of Phe (Figure 2A). The positively charged R10 residue is positioned near the region where the ligand's carboxyl group could stabilize Phe binding. However, conversion to a hydrophobic F10 may instead prevent this stabilization. In addition, no significant response to Phe alone was observed. When we characterized the transfer curve of the mutant, we identified K_A of 0.05 mM in response to Tyr (Figure 2G). In addition, we demonstrated how rational saturation mutagenesis can effectively generate functionally diverse, specific sensors from one promiscuous TF. As expected for all chemical sensors, one caveat of these Phe- and Tyr-specific sensors is that they cannot differentiate elevated concentrations of the two ligands in rich medium, which has saturating concentrations of the two amino acids (Figure S5) (Sezonov et al., 2007).

Engineering ligand-specific aromatic amine sensors

The body harbors a variety of aromatic amines with diverse neurological functions. Many of these metabolites, including dopamine (DA), PEA, Tyra, and Trypta, can be found in the intestines (Bhattarai et al., 2018; Chen et al., 2019). Many commensal microbes can convert these amines into aldehydes through monoamine oxidases (Figure 3A) (Zeng and Spiro, 2013). These aldehydes can be further catabolized to carboxylic acids by aldehyde dehydrogenases (Zeng and Spiro, 2013). In some *E. coli* strains, expression of the monoamine oxidase TynA and aldehyde dehydrogenase FeaB are regulated by the AraC-type FeaR TF, which was shown to induce expression of TynA and FeaB in the presence of multiple aldehydes formed from the corresponding amines (Zeng and Spiro, 2013). We found this *tynA-feaB-feaR* gene cluster to be present in select commensal *E. coli* strains, but not in EcN (Figure S6). This promiscuous sensing system provides an opportunity to sense a variety of important aromatic amines using engineered microbes. However, since each amine has a different function, it is essential that the microbe can differentiate the metabolites.

(D–G) Transfer curves for P_{tyrP} with plasmid-based overexpression of E274Q (D), E274Q+T14V (E), E274Q+D103S (F), and R10F (G) TyrR variants in response to Phe, Tyr, and the specified concentrations of both Phe and Tyr. Points represent experimental data while lines represent the fitted curves (Table S2). The responses to Tyr (D–F) or Phe (G) were not fit due to a lack of response. Values and error bars are the average and standard deviation of biological triplicate, respectively. AA, amino acid. See also Figures S2–S5.

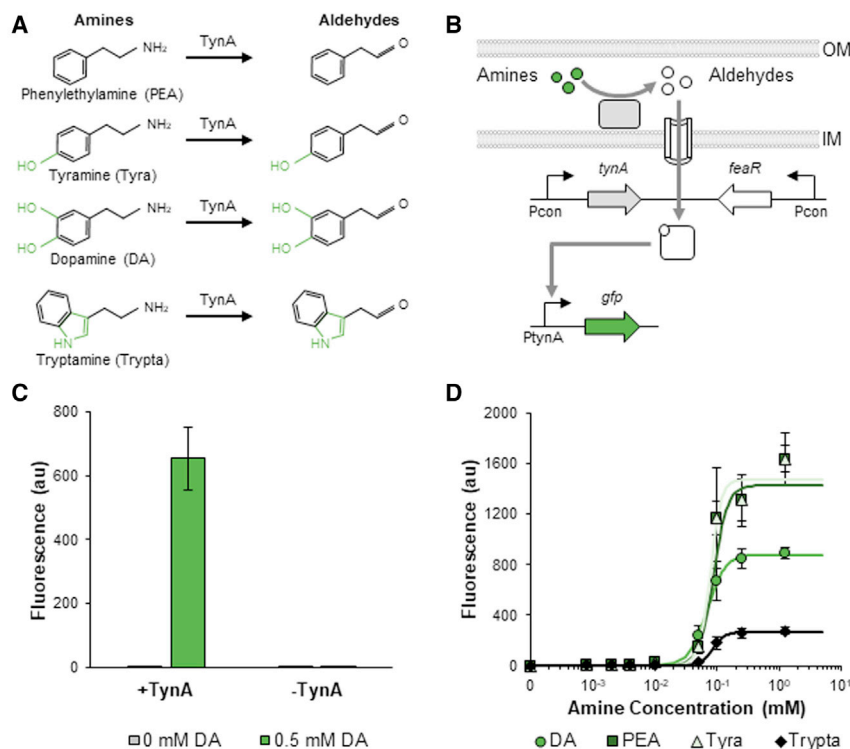


Figure 3. Development and characterization of sensors for aromatic amines

(A) Structures of the four TynA substrates phenylethylamine (PEA), tyramine (Tyra), dopamine (DA), and tryptamine (Trypta) as well as the corresponding aldehyde ligands for FeaR. Structural differences between ligands are shown in green.

(B) Schematic of aromatic amine sensing using the TynA-FeaR system. The TynA enzyme converts periplasmic amines to aldehydes, which are imported into the cytoplasm. In the presence of the aldehydes, FeaR induces expression from the P_{tynA} promoter.

(C) Validation that FeaR regulates P_{tynA} in response to aldehydes and not amines. DA induces P_{tynA} expression in the presence, but not absence of TynA.

(D) Transfer curves of P_{tynA} after 24 h of induction with DA, PEA, Tyra, and Trypta. Points represent experimental data while lines represent the fitted curves (Table S2). Values and error bars are the average and standard deviation of biological triplicate, respectively. See also Figures S6 and S7.

To develop sensors for aromatic amines, we first characterized the TynA-FeaR system in EcN. We constructed a sensor plasmid, which consisted of constitutively expressed TynA and FeaR from *E. coli* MG1655, and a reporter plasmid, which consisted of GFP under the control of P_{tynA} (Figure 3B). We next confirmed that the system induces expression of P_{tynA} in response to aromatic aldehydes, not amines (Figure 3C). In the presence of TynA, the addition of 0.5 mM DA resulted in a 410-fold increase in GFP expression. In contrast, the induction was eliminated when TynA was inactivated. We next characterized the sensitivity and selectivity to different ligands. The system displayed the strongest response to the addition of PEA or Tyra, inducing expression of the reporter by more than 1,500-fold with the addition of 1.25 mM of each amine (Figures 3D and S7). The addition of DA resulted in the next highest response, with approximately 900-fold induction. Trypta had the weakest response, inducing expression by approximately 280-fold. The system showed a similar sensitivity to each ligand, with half-maximal induction occurring at 0.07–0.09 mM (Table S2).

Due to their superior induction and roles in numerous processes, we selected PEA and Tyra as targets for engineering selectivity in the TynA-FeaR system. This system presents the interesting opportunity to optimize ligand selectivity using two different knobs of control. Either TynA or FeaR can be engineered for selectivity. However, the protein structure and mode of ligand binding of FeaR have not been elucidated. In contrast, the structure of TynA has been characterized. Thus, we first focused on improving the amine selectivity of TynA. Several residues in TynA have been identified as catalytically essential, including D413 and Y496 (Figures 4A and S8) (Saysell et al., 2002; Wilmot et al., 1997). To optimize the selectivity of TynA to PEA and Tyra without fully eliminating enzymatic activity,

we elected to individually mutate the amino acids adjacent to these two essential residues, including positions 411–412, 414–416, 493–495, and 497–498.

To quickly screen variants from the libraries for a response to each ligand, we developed a growth-based assay by incorporating the carbenicillin resistance gene, *bla*, and the sucrose counterselection gene, *sacB*, onto the reporter plasmid under the control of P_{tynA} (Figure S9). When the constitutively expressed TynA produces an aldehyde, both Bla and SacB are expressed. To isolate variants with improved selectivity to a specific amine, rounds of positive selection (with the amine of interest and carbenicillin) and negative selection (with all other amines and sucrose) can be performed. In positive selection, cells that fail to respond to the amine of interest will be killed by carbenicillin. In negative selection, cells that remain promiscuous and respond to one of the undesired amines will be killed by sucrose. After multiple rounds of these dual selections, the activities of variants were validated by fluorescence measurements.

We successfully identified two PEA-specific variants (Figure 4B). Both variants possessed a G494S mutation in TynA. However, one variant displayed a stronger fluorescence output in response to PEA. Upon further investigation, we identified an A81T mutation in the FeaR protein of the stronger sensor. Under the pressure of carbenicillin in our dual-selection system, a cell bearing the weak PEA-specific G494S TynA mutant additionally acquired the A81T FeaR mutation to improve *bla* expression and cell survival. This double mutant sensor (G494S*) displayed 140-fold induction in response to PEA with an insignificant response to other tested amines (Figures 4C and S10A), while the single mutant sensor (G494S) displayed 50-fold induction by PEA (Figure S11).

We also identified two variants with improved selectivity to Tyra (Figure S12). The best sensor, G415H, induced expression of the reporter by 200-fold and 79-fold in response to Tyra and

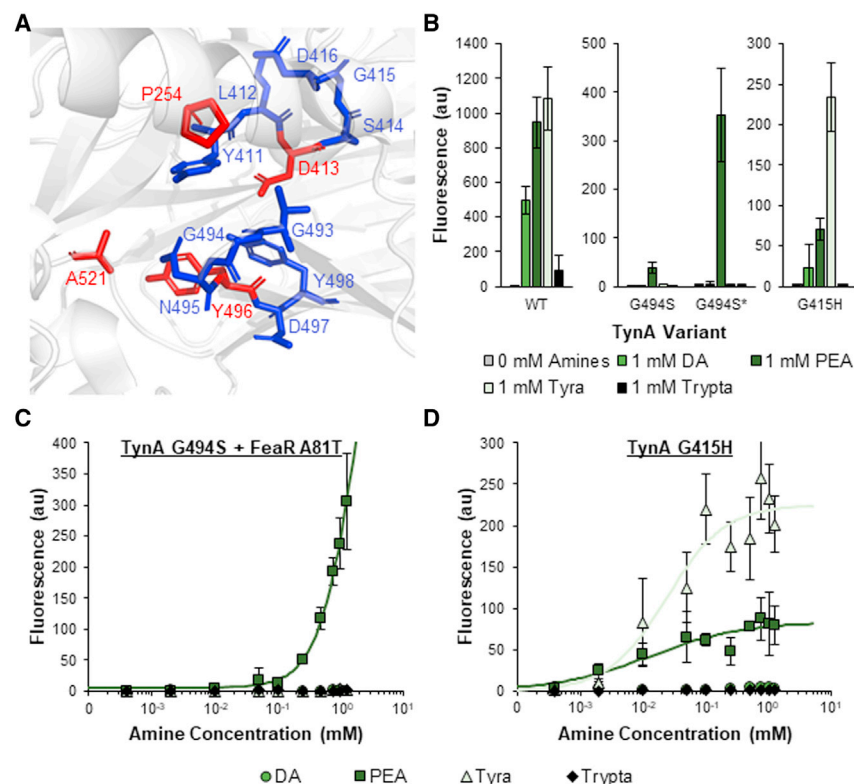


Figure 4. Identification of phenylethylamine- and tyramine-selective sensors through TynA engineering

(A) Structure of the TynA catalytic domain. Un-mutagenized amino acids with known catalytic roles are in red. Amino acids selected for mutagenesis are in blue. The structure was obtained from RCSB protein data bank (Reference ID 6EZZ), and the image was generated using PyMOL.

(B) GFP fluorescence from PtynA when induced for 24 h with 0 mM and 1 mM dopamine (DA), phenylethylamine (PEA), tyramine (Tyra), and tryptamine (Trypta). PtynA was regulated by wild-type TynA (left), G494S TynA (middle), or G415H TynA (right) and either wild-type or A81T (*) FeaR.

(C and D) Transfer curves of PtynA after 24 h of induction with DA, PEA, Tyra, and Trypta. PtynA was regulated by the G494S TynA mutant with the A81T FeaR mutant (C) or the G415H TynA mutant with wild-type FeaR (D). Points represent experimental data while lines represent the fitted curves (Table S2). The responses of G494S* to DA, Tyra, and Trypta and the responses of G415H to DA and Trypta were not fit due to a lack of response. Values and error bars are the average and standard deviation of biological triplicate, respectively. See also Figures S8–S12.

PEA, respectively, with minimal response to DA and Trypta (Figures 4B, 4D, and S10B). The G494S and G494S* variants are the PEA-specific biosensors and the G415H variant is the Tyra-selective biosensor. In addition, this work further demonstrates how the ligand specificity of a protein can be quickly and effectively optimized through targeted mutagenesis of catalytically adjacent residues.

Characterization and engineering of FeaR

Intrigued by the appearance of the A81T FeaR mutation in the G494S* sensor, we next sought to understand the importance of the 81st residue for ligand binding. A protein motif search indicated that the position 12–185 of FeaR is a ligand-binding domain of the AraC-like TF, and the position 218–298 is its DNA-binding domain with a helix-turn-helix motif at positions 258–298. Since no experimentally derived structures of FeaR exist, we applied computational simulations based on comparative modeling to predict the structure. The algorithm utilized the moderate homology of FeaR to CuxR, another AraC-like TF with a previously solved structure, to comparatively predict the structure of FeaR (Schäper et al., 2017). Visualization of the predicted structure revealed that A81 is part of a solvent-accessible beta-barrel, and the alanine sidechain is oriented toward the interior of the barrel (Figure S13). Based on this information, we hypothesized that the beta-barrel is likely the aromatic aldehyde ligand-binding site. In agreement with this theory, our ligand docking simulations using wild-type FeaR found the beta-barrel to be the most thermodynamically favorable ligand-binding site within the protein, with A81 oriented in close proximity to the bound ligand (Figure 5A).

To further elucidate the role of the A81T mutation in ligand selectivity, we inserted the A81T mutation into the FeaR-TynA sensor plasmid with wild-type TynA. The mutation had an insignificant impact on the maximum response of the sensor to PEA and Tyra but significantly reduced the responses to DA and Trypta (Figure S14). However, when we inserted the same mutation into the sensor plasmids with the Tyra-selective S414M and G415H TynA variants, both sensors became PEA-selective (Figure S14). The fluorescence output of each variant in response to PEA was also improved by the A81T mutation. We hypothesize that the improved PEA-dependent output and PEA selectivity created by the A81T mutation may be a result of an altered sensitivity profile, where A81T FeaR is more sensitive to PEA-aldehyde and less sensitive to the remaining aldehydes. While the S414M and G415H TynA mutations weaken the catalytic activity of the enzyme, unequally reducing the amount of each aldehyde produced (as shown by the lower fluorescence output of the two variants relative to wild-type TynA; Figure S12), the A81T FeaR mutation may improve the sensitivity, and thereby selectivity, of FeaR to PEA-aldehyde. However, the high catalytic activity of wild-type TynA toward Tyra may be sufficient to overcome the elevated barrier of activation required by A81T FeaR to induce Tyra-dependent expression. Indeed, the A81T FeaR mutant demonstrated a lower K_A value than wild-type FeaR when paired with both wild-type and G494S TynA (Figure S15). The A81S FeaR mutant paired with G494S TynA showed a very similar transfer function to that of A81T FeaR/G494S TynA, possibly due to the structural similarity of the side chains (Figures S15B and S16).

Noting the PEA selectivity given by the A81T mutation and the smallest size of the PEA-aldehyde (no OH group), followed by

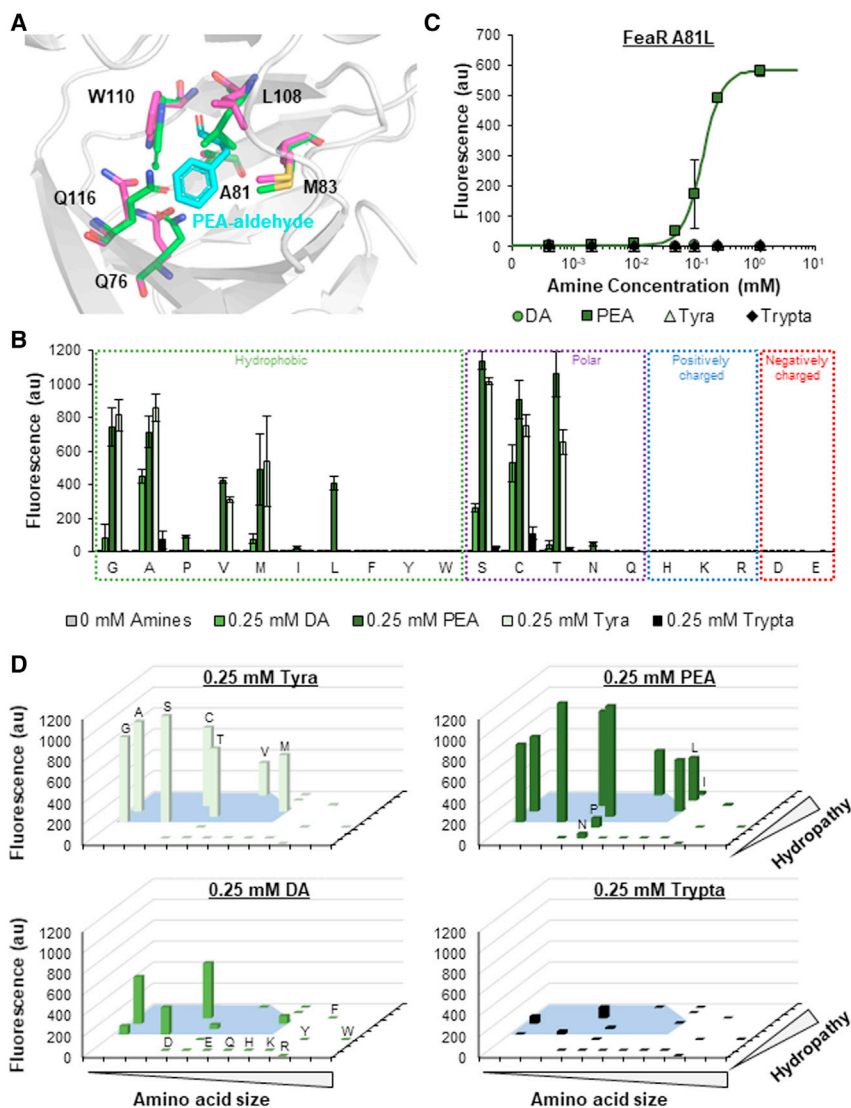


Figure 5. Engineering FeaR to identify improved phenylethylamine-specific sensors

(A) Computationally predicted ligand-binding region of FeaR. Key residues are shown with (magenta) and without (green) phenylethylamine-aldehyde (PEA-aldehyde; cyan) complexed. The structure was predicted using the Robetta web server. Ligand docking was modeled using the Rosetta Ligand Docking Server hosted by ROSIE. The image was generated using PyMOL.

(B) Fluorescence from PtynA when induced for 24 h with 0.25 mM dopamine (DA), phenylethylamine (PEA), tyramine (Tyra), and tryptamine (Trypta). PtynA was regulated by wild-type TynA and 20 different FeaR variants with the specified amino acid in the 81st position. Residues for each group are listed from left to right in order of increasing size. Non-zero values and error bars are the average and standard deviation of biological triplicate, respectively. Variants with significant expression in response to PEA were determined by unpaired t tests ($p < 0.05$).

(C) Transfer curve of PtynA after 24 h of induction with DA, PEA, Tyra, and Trypta. PtynA was regulated by wild-type TynA and the A81L FeaR mutant. Points represent experimental data while the line represents the fitted curve (Table S2). The responses to DA, Tyr, and Trypta were not fit due to a lack of response. Values and error bars are the average and standard deviation of biological triplicate, respectively.

(D) Reorganization of Figure 6B with respect to the size and hydrophobicity index of the 81st residue (Table S3). The blue area represents characteristic values that permit binding of Tyra. See also Figures S13–S18.

that of Tyra- (1 OH), DA- (2 OHs), and Trypta- (additional 5-membered ring) aldehyde (Figure 3A), we hypothesized that small residues, including alanine as in wild-type FeaR, would cause ligand promiscuity, with ligand size-dependent response as shown in Figure 3D. Slightly larger residues such as threonine would have improved selectivity toward the smallest PEA-aldehyde, but much larger or charged residues may fully eliminate ligand binding. We also hypothesized that the hydrophobicity index of the 81st residue may also play a role in the sensor's performance due to differences in ligand polarities caused by the different number of hydroxyl groups. Notably, amines are positively charged in physiological pH, but the true ligands are uncharged aldehydes.

To test this prediction, we mutated A81 and paired each mutant with the promiscuous wild-type TynA (Figure 5B). Consistent with our predictions, A81L, A81P, A81I, and A81N were identified as PEA-specific variants, with 580-fold induction for the A81L sensor (Figure 5C). The A81L and A81P variants also demonstrated improved sensitivities to PEA relative to the G494S* PEA-specific sensor, with half-maximal expression at

0.14 and 0.05 mM PEA, respectively (Figures 5C, S17, and S18; Table S2). As predicted, a clear pattern between the identity of residue 81 and ligand selectivity was observed (Figure 5D; Table S3). FeaR recognized Tyra-aldehyde at a range of size and hydrophobicity index values (Figure 5D; blue area). The ranges with Tyra-dependent activity were non-discrete (as shown by the shape of the blue area). This suggests that the size and polarity of the 81st residue collaboratively dictate ligand selectivity. Small deviations outside of this space allowed FeaR to maintain PEA-aldehyde activity, but not Tyra activity. Similarly, the ranges that allowed binding of FeaR to the larger DA-aldehyde and Trypta-aldehyde ligands were smaller. Large deviations in either residue size or hydrophobicity index caused a complete loss of activity in response to all ligands.

We performed structural simulations of the wild-type (promiscuous), A81T (Tyra- and PEA-responsive), A81L (PEA-specific), and A81H (non-functional) variants to understand how the mutations may alter the structure and thus ligand binding of FeaR. Residues of increasing size ($A < T < L$) protruded further into and occupied more space in the ligand-binding pocket, which is consistent with the observed size effect. All three mutations also shifted the positions of several side chains within the beta-barrel relative to the wild-type structure (Figure S19),

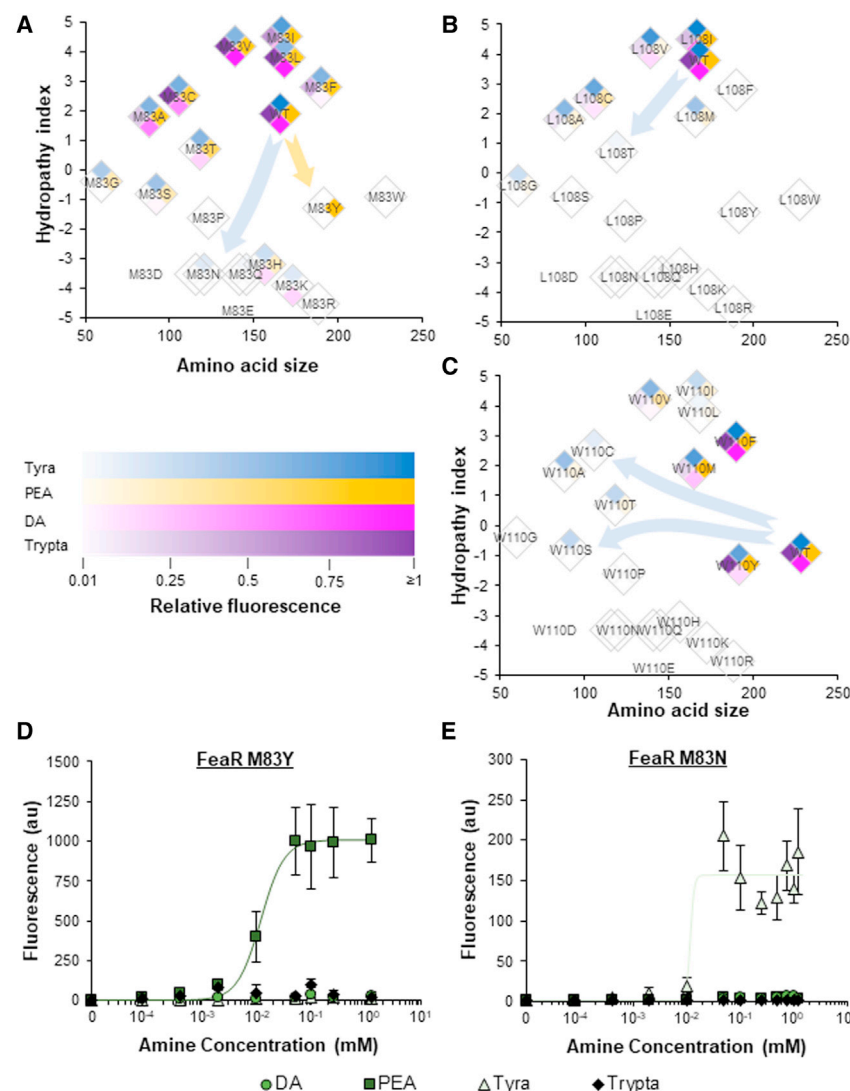


Figure 6. Engineering additional FeaR amino acids to identify amine-specific sensors

(A–C) Fluorescence from PtnA when induced for 24 h with 0.25 mM dopamine (DA), phenylethylamine (PEA), tyramine (Tyra), and tryptamine (Trypta). PtnA was regulated by wild-type TynA and 20 different FeaR variants with the specified amino acid in the (A) 83rd, (B) 108th, or (C) 110th position. Residues for each group are listed with their size and hydropathy index (Table S3). Values are the average of biological triplicate. To obtain the relative fluorescence, fluorescence values for each variant were normalized to the fluorescence of the wild-type sensor (see STAR Methods; Equation 3).

(D and E) Transfer curves of PtnA after 24 h of induction with DA, PEA, Tyra, and Trypta. The expression of PtnA was regulated by wild-type TynA with (D) M83Y or (E) M83N FeaR variants. Points represent experimental data while lines represent the fitted curves (see STAR Methods and Table S2). Values and error bars are the average and standard deviation of biological triplicate, respectively. See also Figures S19–S22.

and S21). The M83Y FeaR variant displayed the most sensitive response of all PEA-specific sensors, with half-maximal expression at 0.012 mM PEA. The best Tyra-specific variant, M83N, recognized its ligand with a comparable sensitivity.

To further confirm the specificity of the best performing sensors, we next tested the response of each to the four amines and the four respective carboxylic acids, 3,4-dihydroxyphenylacetic acid (DOPAC), phenylacetic acid (PAA), 4-hydroxyphenylacetic acid (HPAA), and indole-3-acetic acid (IAA) in minimal medium with casamino acids and LB medium (Figure S22). No sensors significantly responded to the

contributing to the contrasting ligand selectivity. Of particular interest are the differences in the three residues forming the top of the pocket, M83, L108, and W110, as each has severe differences in rotation angles. However, no consistent trends exist between the size of the 81st residue and the orientation of these positions.

Given their significant rotations, we hypothesized that residues M83, L108, and W110 may also play important roles in ligand binding, potentially by making contacts with alternative functional groups in the ligand. We hypothesized that mutagenizing these residues may reveal a sensor for DA, Tyra, or Trypta. We individually mutated each residue and successfully identified multiple ligand-specific sensors for PEA or Tyra (Figures 6A–6C and S20). Randomizing the amino acids in positions 108 and 110 was only able to generate Tyra-specific variants, while randomizing the amino acid in position 83 was able to generate both Tyra- and PEA-specific variants. Each amino acid position demonstrated unique size and hydropathy index profiles for ligand specificity. We further characterized these ligand-specific sensors to assess their ligand sensitivity (Figures 6D, 6E

carboxylic acids in either medium, confirming the specificity of FeaR to only aldehydes. All mutant sensors remained specific to PEA or Tyra in minimal medium with casamino acids. However, all sensors displayed no output in LB medium, suggesting that the system may be regulated by catabolite repression.

Together, this work provides insights into the structure and activity of FeaR. In addition, by engineering FeaR, we provide the best performing PEA- and Tyra-specific sensors for future applications. Future work could include random approaches of protein engineering such as error-prone PCR-based directed evolution to develop additional ligand-specific sensors for the larger DA and Trypta amines. In addition, further exploration of the relevant regulatory pathways is required to uncouple the activity of the sensors with resource availability.

DISCUSSION

The ligand-specific sensors developed here have the potential for diverse applications, including (1) monitoring food quality, (2) diagnosing or treating metabolic, digestive, and neurological

disorders in probiotics or *ex vivo* wearable, paper-based and cell-free systems, and (3) dynamically regulating enzymatic pathways for microbial metabolic engineering. The high degree of ligand specificity shown by the engineered sensors allows them to effectively differentiate between diverse structurally similar aromatic metabolites. We demonstrate an efficient and effective method of rational protein engineering by individually performing saturation mutagenesis on logically selected amino acid residues. We show the generalizability of this method by applying it to three protein systems, including both enzymes and TFs. The simplicity of the approach and small library sizes make it an attractive first step in sensor engineering. Although this protein engineering method requires basic structural knowledge of the protein of interest, the scope of the required information is less than fully computation-based engineering approaches. However, we also show how protein simulations can be used to identify important residues from uncharacterized proteins.

Protein engineering for specific ligand-protein interactions has been extensively performed (Meyer et al., 2019), especially for facilitating chemical drug screening. However, coupling ligand-protein binding with an output response has remained challenging (Peveler et al., 2016). Current approaches include designing proteins that fluoresce upon ligand binding (Dou et al., 2018) or employing ligand-binding TFs (Taylor et al., 2016). While the former can be used to develop sensitive sensors, the latter can generate sensors as well as controllers for downstream functions such as gene expression. Because this TF-based system requires the maintenance or engineering of DNA-protein interaction in addition to engineering ligand-protein binding, developing ligand-specific sensors using this system has been challenging, especially when the target ligands are structurally similar. To address this challenging issue, we demonstrated two approaches: optimizing ligand-TF binding specificity and sensitivity by leveraging differential multimerization patterns of TyrR without affecting DNA-TF binding interactions (Figures 1 and 2) and employing the dual-knob modulation of the specificity and sensitivity of substrate-enzyme and ligand-TF interactions while maintaining DNA-TF-binding interactions (Figures 3, 4, 5, and 6).

The *feaR-tynA* system represents a unique way to develop sensors using two different control knobs. As shown in Figures 4 and S14–S16, Tyra-selective sensors (S414M or G415H TynA) can be transformed into PEA-specific sensors by introducing A81T FeaR, sensor sensitivity (G494S TynA) can be improved by A81T or A81S FeaR mutations, and PEA-Tyra-selective sensors (A81T or A81S FeaR) can become PEA-specific sensors when combined with G494S TynA. One caveat is that TynA and FeaR mutagenesis often led to reduced output signals, sometimes making a PEA-specific sensor (FeaR-A81P) not responsive to any ligand due to lowered aldehyde concentrations (e.g., TynA-G494S + FeaR-A81P). The output of the sensors could be further improved by optimization of the ribosome binding site of the reporter or mutagenesis of the P_{tynA} reporter promoter. Furthermore, for engineered TynA variants, increasing the expression of the enzyme through optimization of its promoter or ribosome binding site may improve signal output by increasing the amine to aldehyde conversion rate. Notably, the intermediate aldehydes are rarely found in natural environments,

eliminating the possibility of extracellular aldehydes contributing to sensor outputs. However, until the sensor activity is uncoupled from catabolite repression, applications may be limited to environments lacking a preferred carbon or nitrogen source (Zeng and Spiro, 2013).

Altogether, we generated the specific microbial sensors for Phe, Tyr, PEA, and Tyra. This work provides ligand-specific sensors that can be applied to create probiotics for diverse applications. In addition, the generalizable protein engineering techniques demonstrated here can be used to quickly and effectively engineer enzymes and TFs for ligand specificity and sensitivity. Although we specifically developed sensors with potential applications primarily in medicine and food quality, these enzymes and sensors can be similarly engineered to produce fuels, pharmaceuticals, and commodity chemicals in response to the levels of metabolites or the products in a dynamic way. This work represents a considerable achievement in the challenging goal of engineering ligand-specific sense-and-respond systems for medically relevant chemicals through coupling protein conformational changes caused by ligand binding with DNA interactions.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cels.2021.10.006>.

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AUTHOR CONTRIBUTIONS

T.S.M. conceived the project. A.G.R., C.X., M.B.A., H.Y., and T.S.M. designed experiments and analyzed the data. A.G.R., C.X., and H.Y. performed the

experiments. M.B.A. performed the protein structure and ligand docking simulations. A.G.R., M.B.A., and T.S.M. wrote the manuscript.

DECLARATION OF INTERESTS

The authors have filed a provisional application with the US Patent and Trademark Office on this work.

INCLUSION AND DIVERSITY

One of the authors is a female scientist.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>E. coli</i> DH10B	Invitrogen	EC0113
<i>E. coli</i> Nissle 1917 (plasmid free)	DSMZ	DSM 16700
Chemicals, peptides, and recombinant proteins		
Agar	Sigma	A1296
L-Alanine	Sigma	05129
Anhydrotetracycline HCl	Sigma	37919
L-Arginine monohydrochloride	Sigma	A6969
L-Asparagine	Sigma	A4159
L-Aspartic acid	Sigma	A9256
Bacto casamino acids	ThermoFisher	223050
CaCl ₂ 2H ₂ O	Sigma	C5080
carbenicillin disodium salt	Sigma	C1389
Chloramphenicol	Gold Biotechnology	C-105
L-Cysteine hydrochloride monohydrate	Sigma	C6852
Deoxynucleotide Set (dNTPs)	G-Biosciences	786-460
3,4-Dihydroxyphenylacetic acid	Sigma	850217
1,4-Dithiothreitol (DTT)	Sigma	3483-12-3
Dopamine hydrochloride	Sigma	H8502
DpnI	New England BioLabs	R0176
D-(+)-Glucose	Sigma	G8270
L-Glutamine	Sigma	G3126
L-Glutamic acid	Sigma	G1251
Glycerol	Fisher Scientific	BP229-1
Glycine	Sigma	410225
Histidine	Sigma	H8000
4-Hydroxyphenylacetic acid	Sigma	H50004
Indole-3-acetic acid	Sigma	I5148
L-Isoleucine	Sigma	I7403
Kanamycin monosulfate	Gold Biotechnology	K-120
KCl	Sigma	P5405
KH ₂ PO ₄	Sigma	P9791
LB broth, Miller	VWR	J106
L-Leucine	Sigma	61819
L-Lysine	Sigma	L5501
M9 minimal salts, 5X	Sigma	M6030
L-Methionine	Sigma	M9625
MgCl ₂	Sigma	7786-30-3
MgSO ₄ 7H ₂ O	Sigma	63138
Na ₂ HPO ₄	Sigma	S5136
NaCl	Sigma	S7653
NAD ⁺	Sigma	53-84-9
PEG-8000	Sigma	1546605
Phenylacetic acid	Sigma	P16621
L-Phenylalanine	Sigma	P5482
Phenylethylamine hydrochloride	Sigma	P6513

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Phusion high-fidelity DNA polymerase	New England BioLabs	M0530
L-Proline	Sigma	P5607
SapI	New England BioLabs	R0569
L-Serine	Sigma	S4311
Spectinomycin dihydrochloride pentahydrate	Gold Biotechnology	S-140
T4 DNA ligase	New England BioLabs	M0202
T5 exonuclease	New England BioLabs	M0663
Taq DNA Ligase	New England BioLabs	M0208
L-Threonine	Sigma	T8441
Tris base	Sigma	77-86-1
Tryptamine	Sigma	193747
L-Tryptophan	Sigma	T0254
Tyramine hydrochloride	Sigma	T2879
L-Tyrosine	Sigma	T8566
L-Valine	Sigma	V0513

Recombinant DNASee [Tables S4](#) and [S6](#)**Software and algorithms**

Chem3D 16.0	PerkinElmer	N/A
MOTIF Search	Genome.jp	N/A
Rosetta Ligand Docking Server		N/A
Robetta web server	Song et al., 2013	N/A

Other

14 mL round bottom tubes	Fisher Scientific	14-959-11B
96-deep well plates	Fisher Scientific	E951032808
96-well black assay microplates	Fisher Scientific	07-000-088
96-well clear round bottom assay microplates	Corning	353910
PureLink Quick Plasmid Miniprep Kit	Invitrogen	K210011
Zymoclean Gel DNA Recovery Kit	ZYMO Research	D4008

RESOURCE AVAILABILITY**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Tae Seok Moon (tmoon@wustl.edu).

Materials availability

Plasmids generated in this paper are available upon request from the Lead Contact. This study did not generate additional new unique reagents.

Data and code availability

The data that support the findings of this study are provided in the main text, [supplemental information](#), or [Data S1](#).

This paper does not report original code.

METHOD DETAILS**Plasmids, strains, and reagents**

All plasmids, strains, and genetic parts used in this study are summarized in [Tables S4](#), [S5](#), and [S6](#), respectively. All plasmids were assembled in *E. coli* DH10B using the Gibson Assembly or Golden Gate Assembly methods. EcN was transformed with the purified and sequence-verified plasmids for testing. The EcN strain used in this work lacks its native plasmids (obtained from DSMZ). The *tynA*, *feaR*, and *tyrR* genes and P_{tynA}, P_{tyrP}, P_{tyrR}, P_{mtR}, P_{tyrB}, P_{aroF}, and P_{aroP} promoters were obtained from *E. coli*

MG1655 genomic DNA. pHJY23 containing the inactivated *tynA* gene (Figure 3C) was constructed by removing the internal region (973–2230th bases) of *tynA* from plasmid pHJY22 by digesting with EaeI and ligating the linear product. The pHJY028 P_{tynA} reporter plasmid was optimized by randomizing the ribosome binding site for GFP and assaying variants for a response to DA.

Plasmid DNA was isolated using the PureLink Quick Plasmid Miniprep Kit (Invitrogen, Waltham, MA, USA), and PCR products were extracted from electrophoresis gels using the Zymoclean Gel DNA Recovery Kit (ZYMO research, Irvine, CA, USA). Enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Gold Biotechnology (Olivette, MO, USA). All sequencing was performed by Genewiz (South Plainfield, NJ, USA). Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA).

Aromatic amino acid sensing assay

Everything but (Eb) medium was prepared by supplementing M9 minimal medium with 1 mM MgSO₄, 100 μM CaCl₂, 0.4% w/v glucose, and all non-aromatic amino acids (0.8 mM alanine, 5 mM arginine, 0.4 mM asparagine, 0.4 mM aspartate, 0.1 mM cysteine, 0.6 mM glutamate, 0.6 mM glutamine, 0.8 mM glycine, 0.2 mM histidine, 0.4 mM isoleucine, 0.8 mM leucine, 0.4 mM lysine, 0.2 mM methionine, 0.4 mM proline, 10 mM serine, 0.4 mM threonine, and 0.6 mM valine). Single colonies of EcN containing the relevant sensor plasmids were transferred to 5 mL Eb medium in 14 mL round bottom tubes and incubated overnight at 250 rpm and 37°C. Experimental cultures were prepared by diluting overnight cultures 200x into 0.6 mL fresh Eb medium supplemented with the respective ligands (Phe and Tyr) in 2 mL 96-deep well plates (Eppendorf, Hamburg, Germany). Cultures were grown for 8 h at 37°C and 250 rpm before sampled for fluorimetry or flow cytometry analysis. All medium was supplemented with the relevant antibiotics for plasmid maintenance (34 μg/ml chloramphenicol and 100 μg/ml spectinomycin).

Aromatic amine sensing assays

For fluorescence-based quantification, single colonies were transferred to 5 mL LB medium (VWR, Radnor, PA, USA) in 14 mL round bottom tubes and incubated overnight at 250 rpm and 37°C. Unless otherwise specified, experimental cultures were prepared by diluting overnight cultures 100x into 0.6 mL M9 minimal medium supplemented with 1 mM MgSO₄, 100 μM CaCl₂, and 2% w/v glycerol as well as the respective ligands (DA, PEA, Tyra, and Trypta) in 2 mL 96-deep well plates. Cultures were grown for 24 h at 37°C and 250 rpm. After 5 h and 24 h of incubation, samples were obtained from the cultures for flow cytometry analysis.

For growth-based library screening, *bla* (encoding β-lactamase) and *sacB* (encoding levansucrase) were incorporated downstream of *gfp* in the pHJY028 reporter plasmid (Table S4). For optimization, ribosome binding site libraries were designed for *bla* and *sacB* using the RBS Calculator (Table S6) (Salis et al., 2009). The optimization resulted in reporter pCX008. To test sensor variants using the pCX008 reporter, single colonies were transferred to 0.6 mL LB medium in 2 mL 96-deep well plates and incubated overnight at 250 rpm and 37°C. The overnight cultures were diluted 50x into 0.6 mL M9 minimal medium supplemented with 1 mM MgSO₄, 100 μM CaCl₂, and 2% w/v glycerol in 2 mL 96-deep well plates. For positive selection, cultures were supplemented with 300 μg/mL carbenicillin. Cultures were then incubated for 2 h at 250 rpm and 37°C. After the 2 h incubation, 2 μL of cells were plated onto M9 agar plates supplemented with 2% glycerol and either 300 μg/mL carbenicillin and 1 mM of the amine of interest (positive selection) or 5% (w/v) sucrose and 1 mM of the non-desired amines (negative selection). Plates were incubated overnight at 37°C.

Fluorimetry

200 μL culture samples were collected and transferred to 96-well assay microplates (clear, flat bottom black, Greiner Bio-One,). The fluorescence and culture absorbance (Abs) were measured using a Tecan microplate reader (Infinite M200 Pro) as previously described (Lee et al., 2016). The fluorescence of GFP was measured with an excitation at 483 nm and emission at 530 nm. The Abs of the samples was measured at 600 nm. The measured fluorescence was normalized by dividing by the Abs and subtracting the same ratio obtained from non-fluorescent wild-type cells (Equation 1).

$$Fluorescence (au) = \frac{Fluorescence_{sample}}{Abs(600nm)_{sample}} - \frac{Fluorescence_{wild-type}}{Abs(600nm)_{wild-type}} \quad (\text{Equation 1})$$

Flow cytometry

Flow cytometry was performed as previously described (Shopera et al., 2017). Culture samples were collected and diluted to a final OD₆₀₀ of ~0.005–0.01 in 200 μL filtered phosphate-buffered saline supplemented with 2 mg/ml kanamycin in 96-well assay microplates (U-bottom, REF-353910 from BD Biosciences, San Jose, CA, USA). The fluorescence of the samples was measured using a Millipore Guava EasyCyte High Throughput flow cytometer with a 488 nm excitation laser and 512/18 nm emission filter. Cytometry data was gated by forward and side scatter. FlowJo (TreeStar Inc.) was used to obtain the arithmetic mean of the fluorescence distribution. The averages of the arithmetic means were calculated from three biological replicates. The average fluorescence of the non-fluorescent wild-type cell was subtracted from each sample to obtain the final fluorescence (au) values (Equation 2). To obtain the relative fluorescence in Figures 6A–6C, fluorescence values for each variant were normalized to the fluorescence of the wild-type sensor using Equation 3.

$$Fluorescence (au) = Fluorescence_{sample} - Fluorescence_{wild-type} \quad (\text{Equation 2})$$

$$\text{Relative Fluorescence} = \frac{\text{Fluorescence}_{\text{Variant}}^{\text{Amine}} - \text{Fluorescence}_{\text{Variant}}^{\text{No Amine}}}{\text{Fluorescence}_{\text{WT FeaR}}^{\text{Amine}} - \text{Fluorescence}_{\text{WT FeaR}}^{\text{No Amine}}} \quad (\text{Equation 3})$$

Hill equation fitting

The Hill equation (Equations 4 and 5) was used to fit lines to the fluorescence data. The model was fit to the experimentally collected data by minimizing the root mean square error (RMSE; Equation 6). Fitted values are listed in Table S2.

For repressible constructs(DeLorenzo et al., 2018):

$$F = F_{\max} - \frac{(F_{\max} - F_{\min})}{1 + \left(\frac{K_A}{[L]}\right)^n} \quad (\text{Equation 4})$$

For inducible constructs(DeLorenzo et al., 2017):

$$F = F_{\min} + \frac{(F_{\max} - F_{\min})}{1 + \left(\frac{K_A}{[L]}\right)^n} \quad (\text{Equation 5})$$

where

F = Calculated fluorescence

$F_{\max}$ = Maximum fluorescence

$F_{\min}$ = Minimum fluorescence

K_A = Half maximal constant

n = Hill coefficient

$[L]$ = Ligand concentration

$$\text{RMSE} = \sqrt{\frac{\sum_{N=1}^N (F - F_{\text{exp}})^2}{N}} \quad (\text{Equation 6})$$

where

F = Calculated fluorescence

F_{exp} = Actual experimental fluorescence

N = Number of data points

FeaR protein modeling

FeaR structural motifs were annotated using the MOTIF Search web tool (genome.jp). Structural predictions were made using the comparative modeling function of the Robetta web server with the wild-type or mutant FeaR amino acid sequences as inputs(Song et al., 2013). Predicted structures were used as the basis for ligand docking simulations. Three-dimensional conformers of the aldehydes of DA, PEA, Tyra, and Trypta were generated using Chem3D 16.0 (PerkinElmer, Waltham, MA). Ligand conformer and protein structure.pdb files were uploaded to the Rosetta Ligand Docking Server hosted by ROSIE, with the ligand of interest initially centered at the coordinates of the 81st residue's side chain(Combs et al., 2013; Lyskov et al., 2013). All protein structures were visualized using PyMOL 2.4.1 (Schrödinger, Inc., New York, NY). Values for amino acid size and hydropathy index were obtained from literature for the analysis in Figure 5D and are shown in Table S3 (Kyte and Doolittle, 1982; Perkins, 1986).

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical details of experiments, including significance criteria and sample size can be found in the figure legends. No sample size calculations were performed during the design of experiments. No samples were excluded.