Supplementary Material (ESI) for Molecular BioSystems


**Electronic Supplemental Information**

## Experimental Section

### Materials

The pACYCDuet expression vector, BL21(DE3) and Tuner (DE3) *E. coli* strains were obtained from Novagen (Madison, WI). *Phusion* DNA polymerase, T4 DNA ligase, and restriction endonucleases were purchased from New England Biolabs (Beverly, MA). QIAprep Spin Plasmid Mimprep Kit, QIAquick Gel Extraction Kit, QIAquick PCR purification Kit, and Ni-NTA Agarose resin were obtained from Qiagen (Valencia, CA). Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). Sterile and non-sterile 96-well plates were purchased from Rainin Instrument Company (Woburn, MA). Nylon membranes (137 mm) were purchased from GE Osmonics Labstore (Minnetonka, MN). All the other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

**Synthetic Shuffling Library Creation.** The method used to create the PhlD synthetic shuffling library was adapted from a two-step total gene synthesis method, which combines dual asymmetrical PCR and overlap extension PCR for accurate and cost-efficient gene synthesis.[1] The *phlD* gene sequence (1050 bp) was dissected into 42 forward and reverse oligonucleotides of 20-25 nt each (with 10 nt overlap at 3'-end and 15 nt overlap at 5'-end and no gap between adjacent oligonucleotides) (Supplemental Table S2). To capture the amino acid diversity from 52 PhlD homologs, degenerate oligonucleotides were designed according to the alternative amino acid compositions, and replaced the corresponding nucleotides in the original Pf-5 *phlD* sequence. The assembled *phlD* chimeric genes were cloned into the vector pACYCDuet(+), followed by transformation into the *E. coli* strain Tuner(DE3) for protein expression and library screening.

**Library Screening.** For a graphic representation of the screening system, see Supplemental Figure S1. In the two-tiered screening method, Tuner(DE3) cells electrotransformed with the library of PhlD mutants were grown on nylon membranes laid on agar plates. PhlD expression was induced for one hour at 30 °C by transferring the membrane to a second agar plate containing 1 mM IPTG. Phloroglucinol production was assayed in a pre-screen by transferring the membrane onto filter paper saturated with 0.03% Gibb's reagent in water. The active clones, indicated by the appearance of a purple halo around the colony, were picked directly from the membrane into 96-well plates for more accurate analysis. Saturated overnight culture was diluted into fresh media (v/v 1:5) containing 0.01 mM IPTG, and PhlD expression was induced for 2.5 hours at 37 °C. This low concentration of IPTG keeps the expression of PhlD at a very low level, to ensure that the amount of *E. coli* cellular malonyl-CoA does not limit the improvement in phloroglucinol production. After recording the optical density at 600 nm using a SpectraMax 340PC plate reader (Molecular Devices, Sunnyvale, CA), cell-free supernatant was assayed for phloroglucinol production by adding the cinnamaldehyde-HCl reagent (0.2% 4-hydroxy-3-methoxy-cinnamaldehyde in HCl:ethanol (v/v 1:3)). The absorbance change was monitored at 550 nm by the plate reader and normalized to optical density.

**Site-directed Mutagenesis**

Site-directed mutagenesis was carried out using the megaprimer PCR method as described elsewhere.[2] Parental PhlD variant genes were used as the template. The mutant genes were digested with restriction enzymes *Nco*I and *Kpn*I and subcloned into pACYCDuet(+). The resulting clones were sequenced to confirm that only the desired mutations were incorporated.

**Saturation Mutagenesis**

Saturation mutagenesis was carried out using an overlap extension PCR method as described elsewhere.[3] Primers pACYCfor (GGATCGGTCAT*ATG*TCTACACTTTGCCTTCCACAC, the *Nco*I restriction site is underlined and the ATG start codon is italicized) and pACYCrev (TCATTACTCGAGGGCGGTCCACTCGCCCAC, the *Kpn*I restriction site is underlined) were used as the flanking primers to amplify the mutant PhlD gene. The amplified product of correct size was purified from an agarose gel, digested with restriction enzymes *Nco*I and *Kpn*I, and subcloned into vector pACYCDuet(+).

1. L. Young and Q. Dong, *Nucleic Acids Res.*, 2004, **32**, e59.
2. G. Sarkar and S. S. Sommer, *Biotechniques*, 1990, **8**, 404.
3. R. Georgescu, G. Bandara, and L. Sun, *Methods Mol Biol*, 2003, **231**, 75.

**Table S1.** List of mutations found in 23D9 and 27B5. The mutations shared between 23D9 and 27B5 are highlighted in green. The three single mutations from mutational analysis are indicated in parentheses and highlighted in red.

| PhlD Mutant | Amino Acid Substitution | Nucleotide Substitution |
|---|---|---|
| 23D9 | I95T | ATC→ACC |
| | A123G | GCC→GGC |
| | R153S | CGG→AGC |
| | D155A | GAT→GCT |
| | L197M | CTG→ATG |
| | Q202E | CAG→GAG |
| | K210N (L) | AAG→AAC (TTG) |
| | K218N | AAG→AAC |
| | T230S | ACC→AGC |
| | Y256F (R) | TAC→TTC (AAG) |
| | S258T | AGC→ACC |
| | N262H | AAC→CAC |
| | S294A | TCG→GCG |
| | D320N | GAT→AAT |
| 27B5 | A123G | GCC→GGC |
| | P125R | CCA→CGT |
| | Q202E | CAG→GAG |
| | K210N (L) | AAG→AAC (TTG) |
| | E212G | GAG→GGG |
| | K218N | AAG→AAC |
| | T230S | ACC→AGC |
| | S258T | AGC→ACC |
| | N262H | AAC→CAC |
| | A289E (R) | GCA→GAA (AGG) |

**Table S2**. List of the forward and reverse primers used in the construction of PhlD synthetic shuffling library.

Forward primers (the *NdeI* restriction site in F_01 is italicized):

| | |
|---|---|
| F_01 | *CATATG*TCTACCCTTTGCCTTCCGC |
| F_02 | ACGTCATGTTTCCGCAACACAAGAT |
| F_03 | CACCCAGCAACAGATGGTCGATCAC |
| F_04 | CTGGAAAACCTGCACGCCGACCATC |
| F_05 | CGCGCATGGCCCTGGCCAAGCGCAT |
| F_06 | GATCGCCAACACCGAAGTCAACGAG |
| F_07 | CGCCACCTGGTGTTGCCGATCGACG |
| F_08 | AACTGGCAGTGCACACCGGTTTCAC |
| F_09 | CCACCGCAGCATCGTCTACGAGCGT |
| F_10 | GAAGCCCGCCRGATGTCGTCGGCCG |
| F_11 | CGGCGCGCCAGGCCATCGAGAATGC |
| F_12 | CGGGCTGCAGAYCAGCGACATTCGC |
| F_13 | ATGGTGATCGTCACTTCCTGCACCG |
| F_14 | GCTTCATGATGCCGTCGCTGACCGC |
| F_15 | GCACCTGATCAACGACCTGGSCCTG |
| F_16 | CSTACCTCCACCGTGCAGTTGCCGA |
| F_17 | TCGCCCAGCTGGGCTGCGTGGCCGG |
| F_18 | TGCCGCGGCCATCAACCGCGCCAAC |
| F_19 | GACTTCGCCMGCCKCGMTSCGCGCA |
| F_20 | ACCACGYACTGATCGTGTCCCTGGA |
| F_21 | ATTCTCCTCGCTGTGCTACCAGCCG |
| F_22 | GACGACACCAAGCTGCACGCCTTCA |
| F_23 | TCTCCGCGGCGCTGTTCGGCGATGC |
| F_24 | GGTATCCGCCTGCGTGMTGCGCGCC |
| F_25 | GATGACMAGGCCGGCGGCTTCAA |
| F_26 | TCAAGAASACCGRGTCGTACTTCCT |
| F_27 | GCCGAASAGCGAGCACTACATCAAG |
| F_28 | TACGACGTGAAGGACASCGGCTTTC |
| F_29 | ACTTCACCCTCGACAAGGCGGTGAT |
| F_30 | GAACTCCATCAAGGACGTGGCACCG |
| F_31 | RTGATGGAGCGCCTCAACTWCGAGA |
| F_32 | SCTTCGAACAGMACTGTGCGCASAA |
| F_33 | CGACTTCTTCATCTTCCACACCGGT |
| F_34 | GGTCGCAAGATCCTCGACGAGCTGG |
| F_35 | TGWTGCASCTGGACCTGGMAYCGAA |
| F_36 | CCGCGTCKCGCAATCGCGCAGCAGC |
| F_37 | CTGTCGGAAGCCGGCAACATTGCCA |
| F_38 | GCGTGGTGGTGTTCGACGTACTCAA |
| F_39 | GCGCCAGTTCGATTCCAACCTCAAT |
| F_40 | CGCGGCGACATCGGCCTGCTGGCAG |
| F_41 | CCTTCGGCCCGGGGTTCACCGCGGA |
| F_42 | AATGGCGGTGGGCGAGTGGACCGCCC |

Reverse primers (the *BamHI* restriction site in R_42 is in italicized):

| | |
|---|---|
| R_42 | *GGATCC*TCAGGCGGTCCAC |
| R_41 | TCGCCCACCGCCATTTCCGCGGTGA |
| R_40 | ACCCCGGGCCGAAGGCTGCCAGCAG |
| R_39 | GCCGATGTCGCCGCGATTGAGGTTG |
| R_38 | GAATCGAACTGGCGCTTGAGTACGT |
| R_37 | CGAACACCACCACGCTGGCAATGTT |
| R_36 | GCCGGCTTCCGACAGGCTGCTGCGC |
| R_35 | GATTGCGMGACGCGGTTCGRTKCCA |
| R_34 | GGTCCAGSTGCAWCACCAGCTCGTC |
| R_33 | GAGGATCTTGCGACCACCGGTGTGG |
| R_32 | AAGATGAAGAAGTCGTTSTGCGCAC |
| R_31 | AGTKCTGTTCGAAGSTCTCGWAGTT |
| R_30 | GAGGCGCTCCATCAYCGGTGCCACG |
| R_29 | TCCTTGATGGAGTTCATCACCGCCT |
| R_28 | TGTCGAGGGTGAAGTGAAAGCCGST |
| R_27 | GTCCTTCACGTCGTACTTGATGTAG |
| R_26 | TGCTCGCTSTTCGGCAGGAAGTACG |
| R_25 | ACYCGGTSTTCTTGATCTTGAAGCC |
| R_24 | GCCGGCCTKGTCATCGGCGCGCAKC |
| R_23 | ACGCAGGCGGATACCGCATCGCCGA |
| R_22 | ACAGCGCCGCGGAGATGAAGGCGTG |
| R_21 | CAGCTTGGTGTCGTCCGGCTGGTAG |
| R_20 | CACAGCGAGGAGAATTCCAGGGACA |
| R_19 | CGATCAGTRCGTGGTTGCGCGSAKC |
| R_18 | GMGGCKGGCGAAGTCGTTGGCGCGG |
| R_17 | TTGATGGCCGCGGCACCGGCCACGC |
| R_16 | AGCCCAGCTGGGCGATCGGCAACTG |
| R_15 | CACGGTGGAGGTASGCAGGSCCAGG |
| R_14 | TCGTTGATCAGGTGCGCGGTCAGCG |
| R_13 | ACGGCATCATGAAGCCGGTGCAGGA |
| R_12 | AGTGACGATCACCATGCGAATGTCG |
| R_11 | CTGRTCTGCAGCCCGGCATTCTCGA |
| R_10 | TGGCCTGGCGCGCCGCGGCCGACGA |
| R_09 | CATCYGGCGGGCTTCACGCTCGTAG |
| R_08 | ACGATGCTGCGGTGGGTGAAACCGG |
| R_07 | TGTGCACTGCCAGTTCGTCGATCGG |
| R_06 | CAACACCAGGTGGCGCTCGTTGACT |
| R_05 | TCGGTGTTGGCGATCATGCGCTTGG |
| R_04 | CCAGGGCCATGCGCGGATGGTCGGC |
| R_03 | GTGCAGGTTTTCCAGGTGATCGACC |
| R_02 | ATCTGTTGCTGGGTGATCTTGTGTT |
| R_01 | GCGGAAACATGACGTGCGGAAGGCA |

**Figure S1**. The two-tiered high-throughput screening method for identifying PhlD mutants with improved phloroglucinol production.

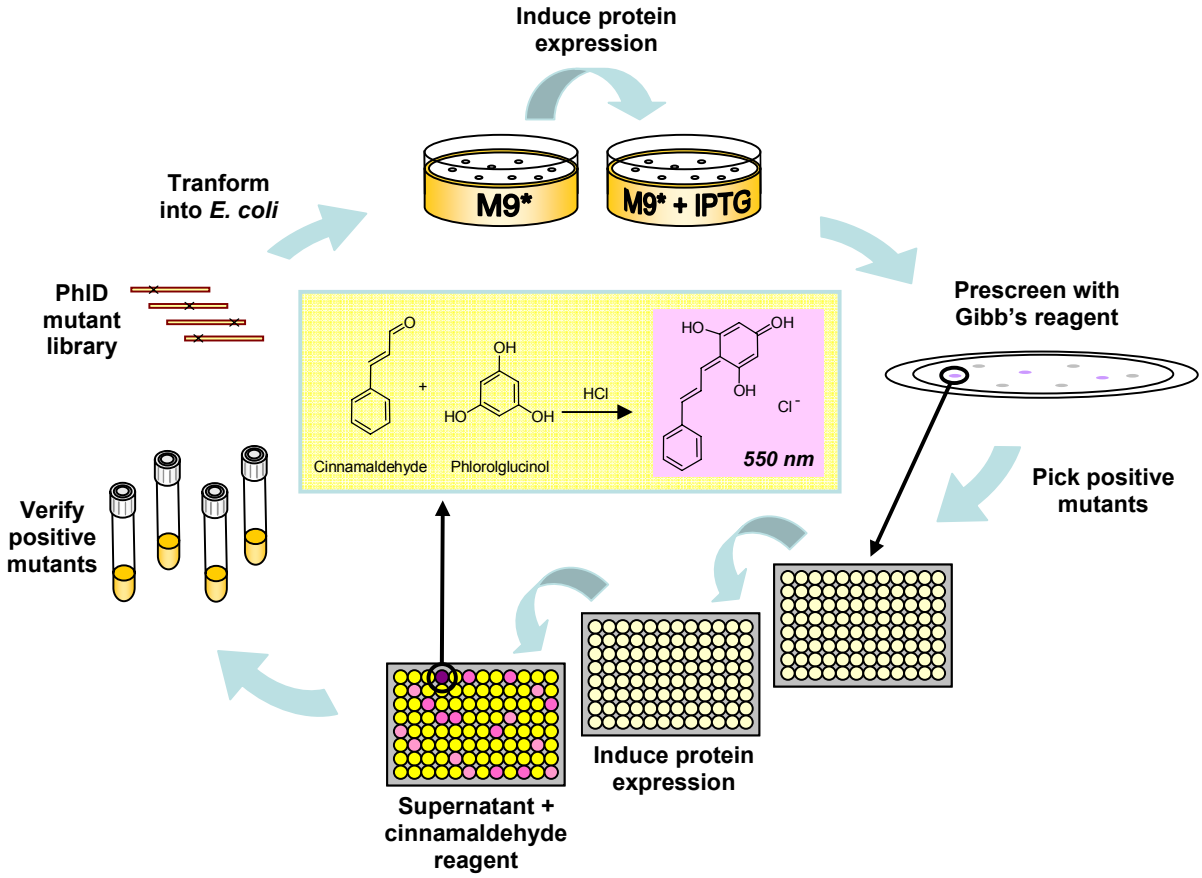

**Figure S2**. Locations of the mutations from 23D9 and 27B5, mapped onto the PhlD homology structural model. The three beneficial single mutations confirmed by saturation mutagenesis are labeled and colored in green, while all other mutations are colored in yellow. The catalytic triad is colored in red.